EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS IN THE COMMON MARMOSET: A NOVEL MODEL FOR MULTIPLE SCLEROSIS

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IN DE PENSEELAAP:
EEN NIEUW MODEL VOOR MULTIPELE SCLEROSE

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Voor mijn verleden
Aan onze toekomst

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Chapter I

General introduction

1. Multiple Sclerosis

1.1 General features

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) with presumed autoimmune pathogenesis. First described in detail in 1866 by Charcot and Vulpian as "sclerose en plaques disséminées", MS is the most common neurological disease of young adults in the Western world, with a prevalence of 1 in every 1,000 individuals. MS is, next to trauma, the main neurological disorder causing disability in humans of young age (*Compston, 1998*).

The majority of MS patients are diagnosed between the age of 20 and 40 years. The first clinical symptoms can include headache, sensory loss, vision abnormalities (including double vision, disturbed nystagmus reflex and optic neuritis), limb weakness, impairment of bladder and bowel function, spasms (*Thompson, 1999; Wingerchuk, 2000*). Recent advances in magnetic resonance imaging (MRI) techniques have improved the diagnosis of "clinically definite MS" since lesions can be directly "visualized" and to some extent characterized (*Miller, 1998; Filippi, 1998; Fazekas, 1999; Rovaris, 1999; Poser, 2001; Bitsch, 2001; Brex, 2002*).

MS is a heterogeneous disease in which three main clinical subgroups can be distinguished. The majority of MS patients (±70%) develop a relapsing-remitting disease course in which intervals of clinical exacerbations (relapse) alternated by complete or partial recovery (remission). After a variable period of time approximately half of these patients subsequently develop progressive worsening of the disease, so-called secondary progressive MS. Another subset of patients (15-20%) develop primary progressive MS in which clinical

neurological deficit accumulates from the onset in the absence of relapses or clear remissions. In a small subset of progressive relapsing MS (±5%) the neurological deficit is progressive from onset with the occurrence of superimposed exacerbations (*Lublin, 1996; Thompson, 2000; Poser, 2001; Barkhof, 2001*).

1.2 Histopathological features

The lesion, the pathological hallmark of MS, is predominantly located in the white matter of the CNS, in both the brain and spinal cord. The lesion is characterized by a focal area of infiltrated mononuclear cells with a variable degree of demyelination, axonal damage, oligo-dendrocyte loss and gliosis. The target of the autoimmune attack in MS is thought to be the myelin sheath, a multilamellar insulating layer wrapping around the axons that is produced by oligodendrocytes (*Bologa, 1985; Keirstead, 1999*). Immune cells are believed to enter the CNS through the blood-brain-barrier (BBB) which in MS has lost its impermeability for cells and molecules (*Hickey, 1991; Cserr, 1992; Hohlfeld, 1997*) and/or via the choroid plexus (*Engelhardt, 2001*).

Although the pathological picture in MS is highly heterogeneous, at least 4 fundamentally different patterns of demyelination can be discerned. In two patterns the autoreactive T-cells (type I) or T-cells with antibodies (type II) are thought to play a central role in the disease process, while the pathological characteristics of the patterns III and IV are suggestive of virus- or toxin-induced oligodendrocyte death (*Lucchinetti, 1996; 2000*). It remains to be established whether the different pathological patterns are formed by distinct immunopathogenic processes, or are associated with a specific clinical course and/or progression of disease, or indeed may benefit from different treatment

strategies. In this thesis the main focus will be on chronic MS as this type of disease is particularly modeled by experimental autoimmune encephalomyelitis (EAE) in the common marmoset.

The perivascular cuff is considered to be the earliest clearly identifiable histological alteration in the CNS of MS patients. Based on the histological characteristics of the macrophages four different lesional stages have been distinguished. 1: Early active lesions are characterized by the presence of myelin oligodendrocyte glycoprotein (MOG) within $MRP14^+$ macrophages (Mϕ) together with massive leukocyte infiltration. 2: In late active lesions Mϕ stain positive for proteolipid protein (PLP) and myelin basic protein (MBP), but not for MOG. Furthermore, these Mϕ are $27E10^+$, but $MRP14^-$. 3: Very few lymphocytes are present within inactive lesions. The Mϕ are PLP^- but still express the late Mϕ antigen detected by 25F9. 4: Remyelinating lesions are characterized by thin myelin layers and oligodendrocytes containing PLP mRNA (*Lucchinetti, 2000*).

The myelin sheath of the CNS white matter has long been regarded as the main target of the destructive process. However, lesions have also been found in the cerebral cortex (*Peterson, 2001*), hypothalamus (*Huitinga, 2001*), optic nerve and optic tract (*Evangelou, 2001*), and brain stem (*Bjartmar, 2001a*). Remyelination with relative preservation of axons is commonly observed in MS lesions (*Keirstead, 1999*). Until recently it was generally thought that the clinical signs in MS were the result of demyelination, although there is no clear correlation between the extent of demyelination and the severity of clinical signs. However, in the last years attention has returned to axonal injury (*Ferguson, 1997; Trapp, 1998; Bitsch, 2000*) and axonal loss (*Barnes, 1991; Trapp, 1998; Bjartmar, 2001b*) that represent major pathological features in more advanced stages of

chronic MS. While the initiating factors of MS are still elusive and the exact target of the immune response is unknown, the pathological processes ultimately result in an impairment of the electrical impulse, which is the likely cause of the neurological dysfunction as observed in MS cases (*Matthews, 1998; De Stefano, 1998; Bjartmar, 2000; 2001b De Stefano, 2001; Kalkers, 2001; Brex, 2002*).

1.3 Therapies

No definite cure of MS is presently available and the outcome of recent MS therapeutic trials is debated (*Bryant, 2001; Pryse-Phillips, 2001*). Of all the new therapies evaluated in MS, interferon (IFN)-β has exerted the best clinical effects until now. Treatment with different formulations of IFN-β has reduced the clinical relapse rate in relapsing-remitting and secondary progressive forms of MS as well as the development of new active lesions which can be visualized by contrast-enhancement on MRI using Gadolinium diethylene-triamine-pentaacetic acid (Gd-DTPA) (*PRIMS, 1998; Kappos, 1998; Jacobs, 2000; Brex, 2001*). Copolymer-1 (Copaxone) has been reported to exert comparable effects as IFN-β (*Johnson, 1995; 1998; Miller, 1998; Gran, 2000*). More recently, the immune-modulating synthetic drug mitoxantrone (Novantrone) has been approved for treatment of secondary progressive and progressive relapsing MS (*Miller, 2000; Weinstock-Guttman, 2000; Rudick, 2001*).

In recent years a number of new therapies have been developed based on data in animal models. While highly effective in the autoimmune model of MS, EAE, intervention strategies mediated by IFN-γ (*Panitch, 1987*), altered peptide ligand (*Bielekova, 2000; Kappos, 2000*), anti-CD4 (*Van Oosten, 1997*) and antibodies to tumor necrosis factor (TNF)-α (*Van Oosten, 1996; Lenersept study group, 1999; Selmaj, 2000*) were shown to be inactive and in some cases even detrimental

when applied in MS patients. Recent trials with oral feeding of myelin (*Martin, 2001*), administration of Linomide (*Noseworthy, 2000b; Schwid, 2000; Hedlund, 2001*) or transforming growth factor (TGF)-β (*Calabresi, 1998*) were discontinued because of high toxicity or limited therapeutic success. Some MS patients seem to benefit from plasma exchange (*Keegan, 2002*) or intravenous injection of immunoglobulins (*Achiron, 2000*). While still controversial, symptomatic treatment with cannabis (marihuana) can relieve many clinical symptoms like pain, sleeping disorders or spasticity, but it has no proven effect yet on the progression of disease (*Williamson, 2000; Baker, 2000; 2001*).

Several phase I clinical trials with new therapeutic strategies are currently underway or will be initiated in the near future. Such therapies target immunological effector pathways that are thought to be involved in MS pathogenesis. These include co-stimulation pathways, immune deviation based strategies (including gene therapy and vitamin D3 analogues), inhibitors of matrix-metalloproteinases, induction of peripheral tolerance, stem cell and bone marrow transplantation (reviewed in *Noseworthy, 1998; 1999; 2000a; Wiendl, 2000*).

2. Etiology of multiple slcerosis

The current concept on the initiation of MS holds that in genetically susceptible individuals a bacterial or viral infection leads to activation of a pre-existing autoreactive repertoire of T- and B-cells. The combined cellular and humoral autoimmune attack to components of the CNS leads to myelin damage and neurological deficit. The initiation of MS involves the interplay of genetic factors and infectious pathogens, but other factors such as diet and stress, are also thought to be involved in the regulation of the course of the disease.

2.1 Genetic factors

Epidemiological and demographic studies point at a significant influence of genetic factors in the initiation and/or perpetuation of MS (*Weinshecker, 1994; Kurtzke, 1995; Ebers, 1995; Sadovnick, 1997; Chataway, 2001*). The absolute risk of a first-degree relative of an MS patient to develop the disease is 20 to 40 times higher than in the general population. Moreover, in approximately 5% of dizygotic twins, and in over 25% of monozygotic twins, both individuals are affected. The fact that in approximately 75% of identical twins only one sibling develops clinical MS points to an additional role of environmental factors (*Ebers. 1986; Sadovnick, 1993a; 1993b; Mumford, 1994*).

Among the candidate genes that have been identified, certain haplotypes of human leukocyte antigen (HLA) class II genes reveal the strongest association, this being the HLA-DR2/Dw2-DQ6 haplotype for people in Northern Europe of Caucasoid origin (*Hillert, 1994*). No association of HLA-DR and -DQ polymorphisms with the clinical course has been found thus far

(*Liblau, 2000*; *MSGC, 2001*). HLA-DP gene polymorphisms possibly exert a regulatory influence on the perpetuation of the disease (*Yu, 1998*). Despite the generally assumed role of viral antigens in MS, HLA class I genes are only weakly associated with disease (*Fogdell-Hahn, 2000*). To date no clear association of MS susceptibility with polymorphisms in non-major histocompatibility complex (MHC) genes has been found (*Ebers, 1996; Bell, 1996; Haines, 1996; Sawcer, 1996; MSGC, 2001*). However, genes encoding for cytokines and their receptors (*Schrijver, 1999; Owens, 2001*), Fc receptors (*Myhr, 1999*) and chemokines and their receptors (*Huang, 2000; Arimilli, 2000*) likely contribute to disease expression as well as progression.

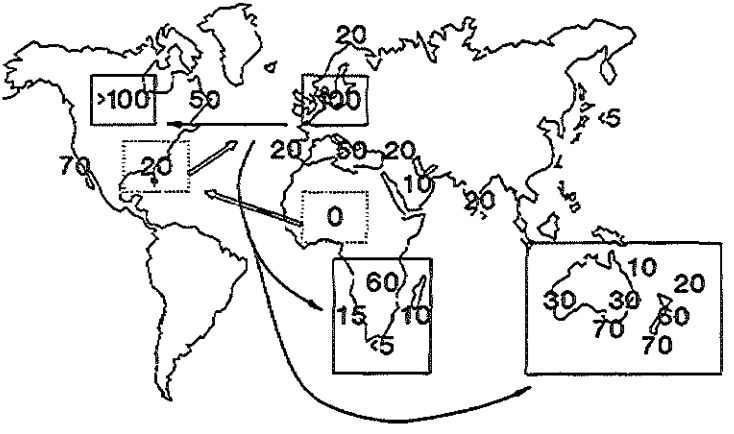

Figure 1: Summary of epidemiological patterns in multiple sclerosis. Numbers are estimates for prevalence per 100,000 individuals. Solid lines with arrows represent migration vectors of northern Europeans. Open lines with arrows represent migration routes of Africans to the Caribbean and Mississippi delta and to the United Kingdom. In South Africa the number refer to English-speaking whites migrating as adults (6), English-speaking whites migrating as children (15), Afrikaners (10) and Cape Coloureds (<5), as estimated in the mid 1980s (Figure kindly provided by Prof. Dr. A. Compston, University of Cambridge, Cambridge, United Kingdom).

2.2 Environmental factors

Since MS remains absent in a high proportion of genetically identical individuals, it is generally assumed that other factors significantly contribute to disease susceptibility as well as resistance. The prevalence of MS varies with latitude and is 1 in 1,000 in the northern countries of Europe, the United States of America, Canada and southern Australia. The disease is relatively rare in Asian countries like China and Japan, and is only incidentally diagnosed among native black Africans (Figure 1) (*Weinshencker, 1994; Kurtzke 1995; Wingerchuk, 2000*). Although the reason for this variation is not understood, the geographical differences may be explained in part by differences in genetic make-up, while demographic studies indicate that epigenetic factors are involved as well (*Alter, 1966; Dean, 1967; Elian, 1990*).

Exposure to (an) infectious agent(s) during adolescence is regarded as an important predetermining risk factor for disease initiation (*Kurtzke, 1993*). During the last decades, many viruses have been put forward as possible cause of MS (*Rasmussen, 1993; Monteye, 1998; Cermelli, 2000; Atkins, 2000; Ascherio, 2001*). Although thus far none of the viruses have stood the test of time, it is intriguing that all of the demyelinating diseases in humans and animals in which the ethiological agent is known, that agent is a virus. Members of the herpesvirus family are one class of possible candidates of relevance for MS. Most of these are neurotropic, have a life long persistence and are periodically reactivated (see for reviews: *Lampert, 1978; Major, 1992; Bilzer, 1996; Cohen, 2001; Stohlman, 2001*). It has been postulated, however, that the absence rather than the presence of a primary viral infection early in life may play a major role (*Haahr, 1994; 1995*). The observation that a history of a delayed Epstein-Barr virus (EBV) infection, resulting in infectious mononucleosis, is associated

with increased risk of developing MS later in life supports this hypothesis (*Munch, 1997; Ascherio, 2001*). The strongest argument for the involvement of an infectious agent thus far reported is the recent introduction of MS on the Faroe islands. While no cases of MS were reported before 1940, three epidemics occurred after the invasion by the British army during World War II and the outbreak of MS was specifically localized to those towns occupied by the British. The identity of this mysterious agent has remained elusive (*Kurtzke, 2001*).

2.3 Myelin proteins

The myelin sheath of the CNS is a highly complex structure that likely harbors the potential autoantigens to which the autoimmune responses in MS are directed (*Voskuhl, 1998; Schmidt, 1999*). Already in 1966 it became clear that serum components derived from EAE affected animals were able to induce alterations of cultured CNS tissues (*Ross, 1966*). Although there was already experimental evidence that clinical disease can be transferred with autoreactive lymph node cells (*Paterson, 1960*), direct evidence that these mediate demyelination *in vitro* as well was published only decades later (*Lyman, 1986*). Thus far, no evidence has been found in humans that a genetic variation of CNS-proteins is a risk factor for MS (*Rose, 1993; Roth, 1995; Rodriguez, 1997; Seboun, 1999*).

Roughly two fractions can be identified in CNS myelin: the lipid fraction (75%) and the protein fraction (25%). Notably, only a few myelin proteins have been identified and purified thus far (Figure 2). It goes beyond the scope of this introduction to give an overview on the myelin proteins studied in EAE and their relevance in MS. However, several excellent reviews describing the current views on MBP, PLP, galactocerebroside C and other

proteins, including their role in MS and EAE, have been published (*Martin, 1992; Martin, 1995; Stuerzebecher, 2000; Kerlero de Rosbo, 2000; Iglesias, 2001; O'Connor; 2001*). As the work described in this thesis is mainly focussed on the role of MOG, the characteristics of this CNS-specific protein are described in more detail.

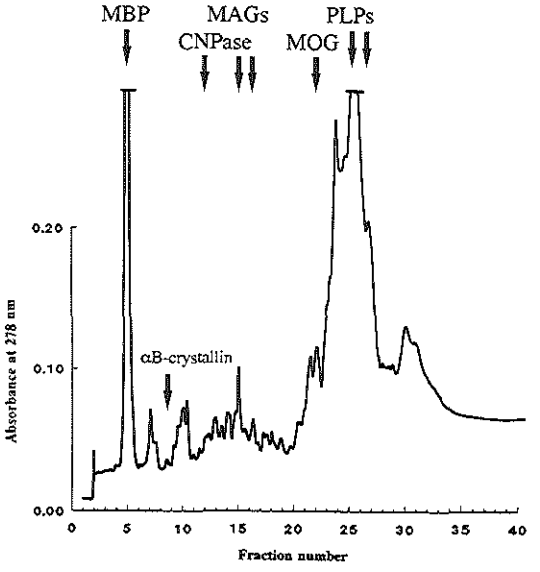

Figure 2: RP-HPLC fractionation of the complete collection of CNS myelin proteins derived from human white matter. As shown, many myelin proteins elute as sharp single protein peaks, like MBP (fractions 5/6) and MOG (fraction 22). Others such as PLP or myelin-associated glycoprotein (MAG) exist as differentially modified species with varying hydrophobicities and elute over a series of adjacent fractions. In this way MAG is recovered in fractions 10-15 and PLP in fractions 22-32 (Figure kindly provided by Dr. J.M. van Noort, TNO-PG, Leiden, The Netherlands).

2.4 Myelin oligodendrocyte glycoprotein

MOG was first described as myelin glycoprotein M2 which is exclusively expressed in the CNS where it is produced by oligodendrocytes (*Lebar, 1981; Lebar 1986*). After purification of the protein (*Abo, 1993*), the cloning (*Pham-Dinh,*

1994; Hilton, 1995) and sequencing of the gene (*Roth, 1995*), MOG was further characterized in detail (Figure 3). Being a member of the immunoglobulin superfamily, it is encoded within the MHC region and is strongly conserved throughout evolution (*Pham-Dinh, 1993; Burger, 1993; Pham-Dinh, 1995b; Lambracht, 1995*).

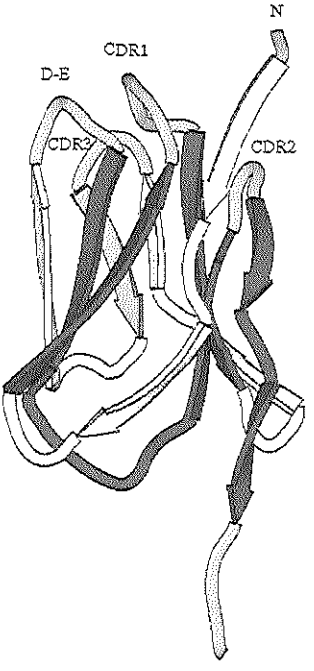

Figure 3a: Myelin oligodendrocyte glycoprotein. This figure shows a schematic representation of the extracellular region of murine MOG shaded according to structure prediction confidence levels. Regions of high prediction confidence are light gray, and regions with lower confidence are black. The β-strand and the complementarity-determining regions (CDR1, CDR2, and CDR3) are labeled, as is the fourth loop that joins β-strands D-E. The D-E loop is analogous to the HV4 loop in T-cell receptors. The conserved Trp^{39} residue and Cys^{24}-Cys^{98} disulfide bridge common to the IgSF motif are shown in the hydrophobic core of the protein. The putative *N*-glycosylation site, Asn^{31}, is shown in CDR1. The figure was produced using the program Molscript and kindly provided by Dr. P. Hjelmström, Yale University school of medicine, New Haven, CT).

MOG becomes expressed late in myelinogenesis and is a quantitatively minor constituent of CNS myelin. MOG knockout mice have a structurally and functionally normal myelin sheath (*Pham-Dinh, personal communication*). Three possible functions of MOG have been proposed: MOG might function as a cellular adhesion molecule, a regulator of oligodendrocyte stability and/or a mediator of interactions between myelin and the immune system (*Hjelmstrom, 1998; Johns, 1999*). MOG was first shown to be encephalitogenic in mice (*Amor, 1994*). Although autoimmunity to MOG is most likely not the exclusive trigger of disease for all chronic MS cases, MOG has unique antigenic properties and has emerged as one of the most interesting candidates for being a primary target of the autoimmune reactions in MS. By its exposure on the surface of the oligodendrocyte plasma membrane and the outermost surface of mature myelin, MOG is directly accessible to infiltrating T-cells and antibodies (*Pham-Dinh, 1995a; Della Gaspera, 1998*).

Although there are conflicting data on the relevance of T-cell reactivity towards MOG in MS (*Kerlero de Rosbo 1997; 1998; Wallstrom, 1998; Diaz-Villoslada, 1999; Hellings, 2001*) an important role of anti-MOG antibodies (Ab) is generally accepted. The number of anti-MOG Ab producing cells as well as the anti-MOG Ab levels are increased in the CSF of MS patients (*Sun, 1991; Xiao, 1991*). Moreover, several groups have reported an increased incidence and more persistent anti-MOG Ab reactivity in sera of MS patients than in patients with other inflammatory neurological diseases or healthy controls (*Lindert, 1999; Reindl, 1999*). Similar patterns of Ab localization were found in MS patients and marmosets in areas where pathological changes of white matter occurred as well as in demyelinated lesions (*Genain, 1999; Raine, 1999*).

All mammalian species tested thus far are susceptible to MOG-induced EAE. In most species autoimmune reactions towards MOG give rise to similar

clinical and neuropathological features as found in MS (*Storch, 1998; Iglesias, 2001; Abdul-Majid, 2000; Von Büdingen, 2001b; Genain, 2001; Brok, 2001b*). Direct injection of immune serum from EAE-affected rats into the CSF of healthy animals resulted in demyelination (*Lassmann, 1983*). The demyelinating properties of anti-MOG Ab have been documented *in vitro* using brain cell cultures (*Kerlero de Rosbo, 1990*) and anti-MOG Ab were shown to facilitate myelin uptake by macrophages *in vitro* (*Van der Goes, 1999*). Numerous *in vivo* studies have demonstrated the contribution of anti-MOG Ab to EAE in mice (*Schluesener, 1989; Piddlesden, 1993; Litzenburger, 1998; Tsunoda, 2000; Morris-Downes, 2002*), rats (*Linington, 1987; 1988; Lassmann, 1987; 1988*) and marmosets (*Genain, 1995a; 1995b; 1996a; 1996b*). Recent studies have stressed the importance of the Ig subclass (*Ichikawa, 1999*) and epitope specificity of MOG Ab for disease pathogenesis (*Brehm, 1999*). Further experimental evidence on the importance of MOG is provided by the findings that T- and B-cells specific for mimicry motifs shared by viral antigens and MOG can cause demyelinating disease in mice (*Mokhtarian, 1999; Miller, 2001a; 2001b*) and rhesus monkeys (*'t Hart, submitted*).

2.5 Other modulating factors

Females are approximately twice as much affected as males, a feature that has been described in other autoimmune diseases as well (*Vyse, 1996; Whitacre, 2001*). Gender-dependent differences in the functioning of the hypothalamus-pituitary-adrenal (HPA) axis may contribute to this phenomenon (*Dowdell, 1999; Morale, 2001; Voskuhl, 2001*). The relapse rate of MS is significantly reduced during pregnancy while shortly after delivery an exacerbation occurs in a significant proportion of MS patients (*Confavreux, 1999*). One of the important factors next to estrogen and progesterone (*Ito, 2001*) seems to be early pregnancy factor (*Zhang, 2000*). There is also experimental evidence that stress

factors have an impact on the disease course (*Mason, 1991; Morale, 2001; Heneka,2001*). Of all studied heat shock proteins, αB-crystallin seems to fulfil most criteria for having a relevant role in MS (*Van Noort, 1995; Van Sechel, 1999; Bajramovic, 2000*).

Thus far evidence is lacking that major differences exist between MS patients and normal individuals with regard to their immunological potential. However, it has been postulated that there is a disturbed immune regulation after clinical manifestation of the disease (*Wilkin, 1989*). Although several factors have been characterized that may play a role in the initiation and/or perpetuation of the disease it will remain difficult to prove retrospectively whether these abnormalities are a cause or a result of the disease. Taken together, the literature data discussed thus far warrant the conclusion that our understanding of the disease mechanisms in MS is still limited and that adequate therapies for the disease are lacking. It can be envisaged that recently developed techniques such as single nucleotide polymorphism technology and cDNA microarray as well as the present knowledge of the human genome will accelerate the identification of (potentially) interesting factors (*Encrinas, 2001; Ibrahim, 2001; Whitney, 2001; Martin, 2001; The human genome, 2001*). In addition, by the development of new and more valid animal models for the disease we hope to improve our insights into the mechanisms of MS and thus develop more effective therapies for the disease.

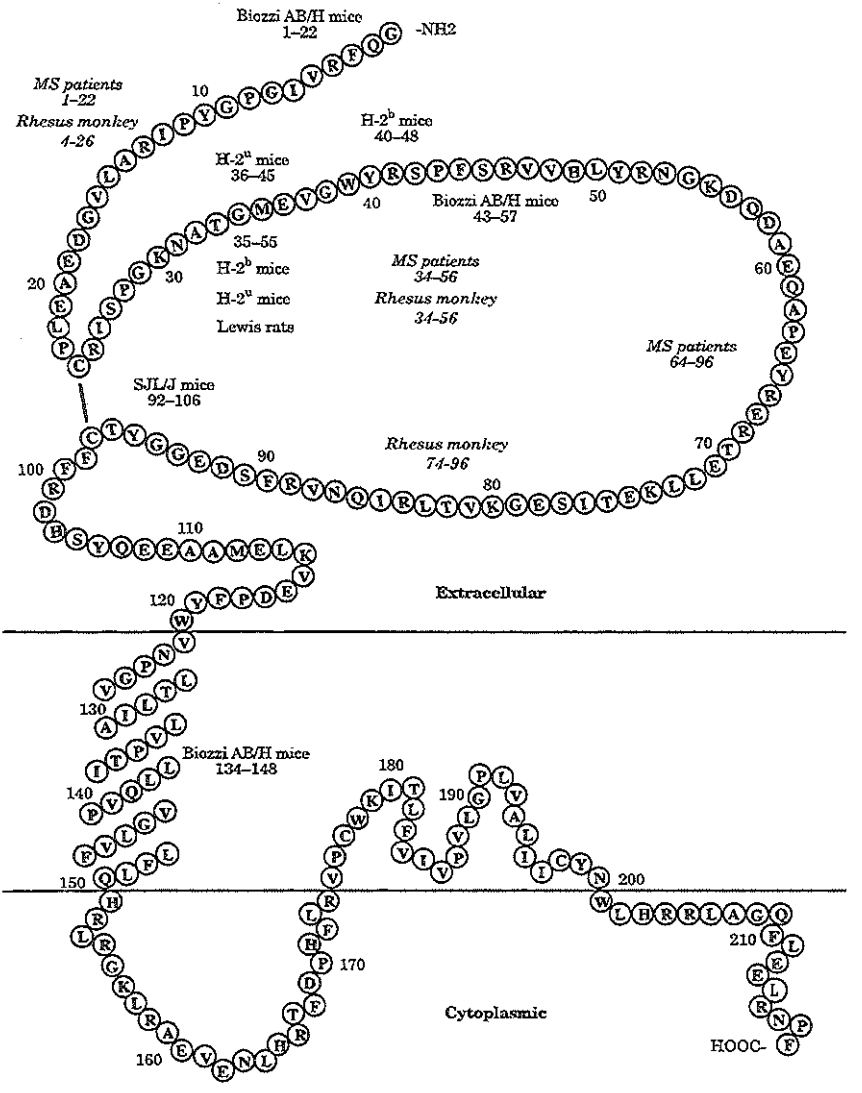

Figure 3b: Location of the encephalitogenic epitopes of rodents and of epitopes recognized by PBLs from MS patients and rhesus monkeys. The model represents the mouse MOG sequence and the epitopes recognized by MS patients and rhesus monkeys were demonstrated using synthetic peptides according to the human sequence, which differs from mouse MOG by about 10%. Figure was kindly provided by Dr. Kerlero de Rosbo, The Weizmann Institute of Science, Rehovot, Israël.

3. Experimental models of multiple sclerosis

3.1 Rodent models

The attractive aspect of EAE models in inbred strains of rats and mice is the high reproducibility, the relative genetic simplicity, the high number of identical offspring and the low costs. In some models the disease is acute while others display an MS-like relapsing remitting disease in which, after several disease episodes, irreversible neurological deficit is observed. Of significant importance are the numerous knock-out and knock-in strains in which the contribution of single factors to the disease process can be investigated (*Wong, 1999; Goverman, 1999; Eynon, 1999; Fazekas, 2000; Iglesias, 2001; Owens, 2001*). Although toxin-induced demyelination and cryo-induced lesions most likely do not reflect the pathological processes in the majority of chronic MS patients, these models are useful in studying processes like remyelination and regeneration of various cell types (*Phillips, 1997; Jeffery, 1999a; 1999b; Blakemore, 2000*). The two most prevailing rodent models are autoimmune-based or caused by viral infections.

3.1.1 Virus models

The presence in MS patients of immune reactivity to CNS proteins does not necessarily imply that this response is the primary cause of the disease. Autoreactive T-cells and Ab may also be formed as a reaction to the (massive) myelin release caused by viral infection of the CNS or oligodendrocyte dystrophy. Many viruses have been implicated in the ethiology of MS although this has not been formally proven. Also in animals many viruses are known to induce demyelination in the CNS and are used to investigate the

possible mechanisms of CNS damage as observed in MS. These include Visna virus of sheep (*Clements, 1996*), caprine virus of goats (*Norman, 1983*) and canine distemper of dogs (*Summers, 1994*).

The best investigated virus-based murine models of MS are mouse hepatitis virus, Theiler's murine encephalomyelitis virus (TMEV) and Semliki Forest virus (SFV) infection models. Virus-induced models of inflammatory demyelination are complex in terms of the immuno-pathogenesis (reviewed by *Kielian, 1990; Atkins, 1994; Garoff, 1994; Fazekerley, 1993; Monteyne, 1997; Kim, 2000; Allsopp, 2000*).

Viral infection may cause direct damage to the oligodendrocyte or the myelin-sheath as is for instance observed after infection with mouse hepatitis virus, leading to oligodendrocyte damage and the subsequent release of myelin antigens (*Weiner, 1973; Houtman, 1996*). As a general reaction to tissue damage, including cytolytic viral infection, myelin may be phagocytosed and processed by local APC (*Katz-Levy, 1999; 2000*) or transported to the cervical lymph nodes (cLN), which drain the cerebrospinal and interstitial fluids of the brain, where myelin-reactive T- and B-cells may be activated *de novo* (*Weller, 1996; Phillips, 1997*). Another mechanism by which virus infection may induce tissue damage is by the phenomenon of molecular mimicry. This was first described by *Fujinami (1985)* in which a viral peptide with an identical sequence to an encephalitogenic region of MBP induced inflammation in the CNS of rabbits. More recent studies have examined the cross reactivity of viral and myelin proteins in more detail (*Wucherpfenning, 1995; Grann, 1999; Mokhtarian, 1999; Liblau, 2000; Olson, 2001; Miller, 2001a; 2001b*). Yet a further mechanism by which viruses may induce CNS damage is by the incorporation of host cell antigens such as myelin proteins or glycolipids into the viral envelope of budding viruses, thereby allowing the peripheral immune

recognition of CNS restricted antigens (*Webb, 1984; Dalgliesh, 1987; Pathak, 1990*). The role of $CD8^+$ cells in MS and EAE has been long ignored, but recent reports on MS show a regained interest in this cell type (*Koh, 1992; Jiang, 1992; Tsuchida, 1994; Vizler, 1999; Babbe, 2000; Huseby, 2001; Steinman, 2001*). The encephalitogenic capacity of $CD8^+$ T-cells was recently confirmed by Jiang and colleagues who showed that $CD8^+$ T-cells control the Th-phenotype of myelin-reactive $CD4^+$ T-cells in EAE-affected mice (*Jiang, 2001*). Furthermore, $CD8^+$ cells are able to transfer severe EAE in mice (*Sun, 2001*). Finally, viral antigen specific T-cells can induce (*Ufret-Vincenty, 1998*), or alternatively, trigger CNS disease (*Theil, 2001*). Importantly, unraveling the mechanisms and target cells in these models will possibly prove its value in the near future when used as expression vectors for MS therapy (*Smerdou, 1999; Schlesinger, 2001*).

3.1.2 Autoimmune models

After the demonstration that post-vaccinal encephalomyelitis develops in monkeys by the injection of CNS homogenate (*Rivers, 1933*), the immunopathogenesis of EAE using inbred rodent strains have been widely studied. The disease patterns that can be obtained depend on the immunization protocol (*Tsunoda, 1998; Storch, 1998; Rabchevsky, 1999; Maatta, 2000; Heeger, 2000*), animal species (*Burgess, 1978*), gender (*Voskuhl, 1996; Bebo, 1996; Ding, 1997; Bebo, 1998; Butterfield, 1999*) and the genetic background (*Abdul-Majid, 2000; Tsunoda, 2000; Sobel, 2000; Constantinescu, 2001; Kjellen, 2001*). In addition, the age of the animals (*Endoh, 1990; Smith, 1999*), the number of injected T-cells (*Lassmann, 1988*) and the source of myelin or protein preparation that is used for disease induction (*Sun, 1995; Storch, 1998; Tsunoda, 1999; 2000*) were shown to influence the course of the disease as well. Following immunization with

myelin proteins in adjuvant, neurological disease is initiated by CNS inflammation due to migration of myelin-reactive proinflammatory $CD4^+$ Th_1 cells across the BBB. Alternatively, EAE may be induced by transfer of autoreactive myelin-specific $CD4^+$ T-cells into naïve recipients. The interaction of these T-cells with local APC, presenting the myelin antigen to which the infiltrating T-cells were originally sensitized in the periphery, induces a sequence of events that leads to inflammation and damage of myelin and axonal structures. Based on the data obtained in viral and autoimmune MS models in rodents a general concept has been put forward for the immunological pathways that are thought to play a significant role in MS (see for review: *Noseworthy, 1999; 2000a*). Therapeutic strategies for MS were thus far mainly based on proven efficacy in rodent EAE models. These studies, however, have not lead to an absolute therapy or cure for MS thus far. In our search for effective drugs in MS, monkey models may be of help as an important intermediate between rodents and humans (*Bontrop, 2001*).

3.2 Non-human primate models

Rodent models have been of undisputed significance to our present view on the immunopathogenesis of MS. However, in order to be able to model the disease in all its aspects, a lab animal model is needed of comparable genetic complexity as humans. Such models can also be useful for the safety and efficacy testing of potential therapeutics, like engineered or humanized Ig molecules as well as functionally intact human Ab produced by transgenic mice. As a result of the species specificity, rodent models are invalid and non-human primate models are the only ethical alternative left. Regulatory authorities such as the American Food and Drug Administration (FDA) have

a clear standpoint on the importance of preclinical research in non-human primates (FDA guidance S6: Preclinical safety evaluation of biotechnology-derived pharmaceuticals; http://www.fda.gov/cber/ publications.htm).

3.2.1 EAE in Old World monkeys

Spontaneous cases of MS-like disease have rarely been documented in captive colonies of non-human primates (*Marrazi, 2001*), although natural infection and resultant encephalomyelitis are widely described (*Kalter, 1997*). Rivers and colleagues published the first report on EAE in non-human primates. Rhesus monkeys (*Macaca mulatta*) were found to develop disease after injection with healthy CNS tissue or when inoculated with a rabies strain that was cultured on brain cells (*Rivers, 1933; 1935*). Later, an MS-like syndrome was evoked in chimpanzees (*Pan troglodytes*) by inoculation of MS-brain material (*Lief, 1976; Rorke, 1979; Wroblewska, 1979*). However, this research has not received any follow-up, probably due to the high costs and the obvious ethical constraints.

In the past decades, three macaque species have been used for investigating the immunological mechanisms in MS-like disease. EAE was induced using whole brain homogenate, myelin, or MBP emulsified in complete Freund's adjuvant (CFA). The rhesus monkey appeared to be more susceptible to MBP-induced disease than the cynomolgus monkey (*Macaca fascicularis*) while the African green monkey (*Chlorocebus aethiops*) was relatively resistant to the disease (*Stewart, 1991; Gallo, 1991; Massacesi, 1992; Rose, 1987; 1994*). An important issue for the use of rhesus monkeys is that this species is susceptible to natural infections similar to those observed in man. Importantly, infections follow a similar course as in humans, such as in the case of Herpes Simplex virus type 1, EBV and cytomegalovirus (CMV) (*Hunt,*

1993; Kalter, 1997; Lockridge, 1999; Cohen, 2000). The rhesus monkey likely represents an excellent outbred animal model to unravel how viruses are involved in the induction of inflammatory demyelination of the CNS leading to demyelination such as is observed in MS patients (*manuscript in preparation*).

The genetic make-up of the MHC class II region in rhesus monkeys is at least as complex as in humans. Both species share not only *Mhc-DP*, *-DR* and *-DQ* loci, but also allelic lineages (*Bontrop 1999; Doxiadis, 2001*). Moreover, the diversity of the T-cell receptor (TCR) in rhesus monkeys is comparable with humans and antigens processed by rhesus monkey APC are presented in the correct conformation to human specific T cell clones (*Bontrop, 1995; Meinl, 1995; Geluk, 1999*). Interestingly, epitope selection from MBP or MOG is highly comparable with humans (*Alvord, 1984; Meinl, 1995; Kerlero de Rosbo, 2000*). Although some animals seem to be resistant to the disease evoked by immunization with MBP, 100 % of the animals are highly susceptible to EAE induced with myelin or whole brain homogenate. The lesions, which are exclusively located in the brain, are characterized by strong inflammation and necrosis, thus resembling pattern I of MS lesions (*Lucchinetti, 2000*). Several observations indeed point at a central role of $CD4^+$ T-cells. Disease susceptibility to MBP was found to be linked to the MHC class II allele *Mamu-DPB1*01* (*Slierendregt, 1995*) although other *Mamu* class II loci may be involved as well. Furthermore, Ab therapies directed to CD4 or MHC class II have a protective effect on myelin-induced EAE in Old World monkeys (*Van Lambalgen, 1987; Rose, 1988*). Finally, autologous transfer of an MBP-specific Th1-like cell line was found to induce a mild form of encephalomyelitis (*Meinl, 1997*).

Recent studies using MOG revealed that rhesus and cynomolgus macaques are equally susceptible to MOG-induced disease (*Kerlero de Rosbo, 2000*; *'t Hart, unpublished observation*). Also in this case EAE is characterized by destructive lesions in the brain. Thus, the disease in rhesus monkeys usually follows an acute course, irrespective of the antigen used for immunization. However, studies from our laboratory have recently shown that a form of EAE more closely resembling MS can be induced in rhesus monkeys by immunization with a synthetic peptide representing amino acids 34–56 ($phMOG_{34-56}$) of the extracellular domain of *Escherichia coli* derived recombinant human MOG ($rhMOG^{Igd}$). It was found that in a randomly selected group of 16 animals a variable disease patterns develops, ranging from a hyperacute to a chronic relapsing/remitting course. While intermolecular epitope spreading was present in animals with chronic disease, cellular as well as humoral responses remained confined to the disease-inducing peptide in acute responders. Although the number of monkeys in this study is still too small to draw firm conclusions, inclusion of more animals might reveal an association of certain MHC haplotypes and/or environmental factors with particular disease patterns (*manuscript in preparation*).

3.2.2 EAE in New World monkeys

The first EAE models making use of New World monkeys were described in the early 1970's. The EAE models in the owl monkey and the squirrel monkey seem to resemble the early macaque models in many aspects (*Murray, 1992*). However, at the time the work described in this thesis was started, the first publications appeared on a challenging new EAE model, namely in the common marmoset (*Callithrix jacchus jacchus*). For a better understanding of

this model, the basic characteristics of this animal species are reviewed on the following pages.

3.2.2.1 The common marmoset

About 70 million years ago, the South American continent became invaded by mammals. The present fauna of this continent harbors the descendants from three waves of colonization in which the New World monkeys are surviving for more than 58 million years. New World monkeys are placed in the infraorder *Platyrrhini* or New World monkeys. Apes, Old World monkeys (*Catarrhini*) and New World monkeys are thought to share a common ancestor, the *Omomydae* who lived about 55 million years ago in Europe and North America. Common marmosets belong to the *Callitrichidae* family of *Platyrrhini* comprising marmosets and tamarins (Figure 4) that have diverged from each other around 10 million years ago (*Schneider, 1993*).

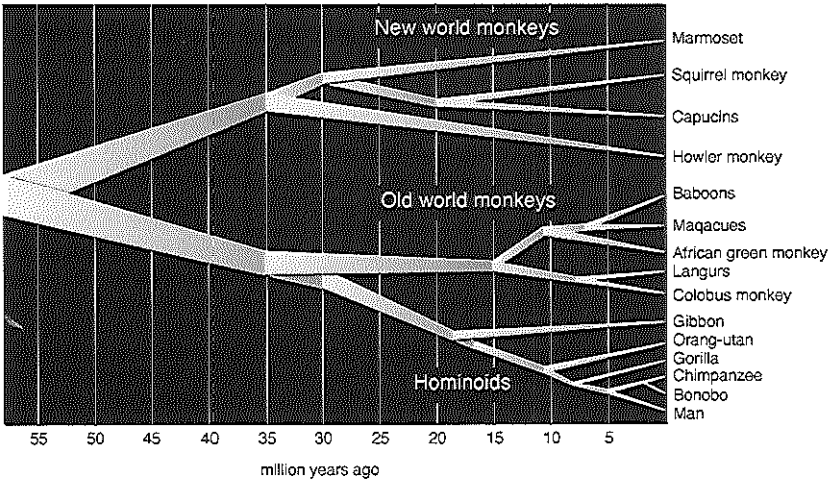

Figure 4: Evolutionary tree (Figure kindly provided by Dr. R.E. Bontrop, BPRC, Rijswijk, The Netherlands)

Four genera and about 36 (sub)species have been described thus far. The genus *Callithrix* contains at least 15 species, the largest number of all neotropical primate genera (*Mittermeier, 1992*). Based on their geographical distribution (*Rylands, 1996*), cytogenetical (*Canavez, 1996; Nagamichi, 1996; Nagamichi, 1997; Nagamichi, 1999*) and biochemical characteristics (*Alves, 1995; Von Dornum, 1999*), two major groups are recognized. The Amazonian *Callithrix* species, the *argentate* group and *Cebuella*, have 44 chromosomes while *Callithrix* species of the Atlantic coast, the *jacchus* group, have 46 chromosomes (*Canavez, 1996*). The species *jacchus* contains subspecies like *C. aurita, C. geoffroyi, C. kuhli, C. penicillata* and *C. jacchus*. It is currently believed that *C. penicillata* is the closest living relative of *C. jacchus* (*Rylands, personal communication*). *Callitrichidae* are smaller than the 'true' New World monkeys the *Cebidae*. The apparently different features of marmosets compared to tamarins, including the smaller body size, are in fact secondary specializations to their natural habitat (*Rylands, 1996*). Because the placental blood circulation is fused during development, *Callithrichidae* are usually born as non-identical twins with a natural bone marrow chimerism, an unique feature among monkeys. This causes a permanent state of tolerance for alloantigens between chimeric twins and the sharing of cell systems of hematopoietic origin (*Niblack, 1977; Gengozian, 1978*). This feature is of fundamental importance in EAE research since cell transfer studies between individuals can be performed otherwise limited to inbred strains or genetical identical animals (*Genain, 1994*).

Nowadays the *C. jacchus jacchus* or common marmoset, also known as white-tufted titi or in Dutch "penseelaap", is one of the few New World

monkey species that is not on the list of endangered species of the World Wildlife Foundation (Figure 5).

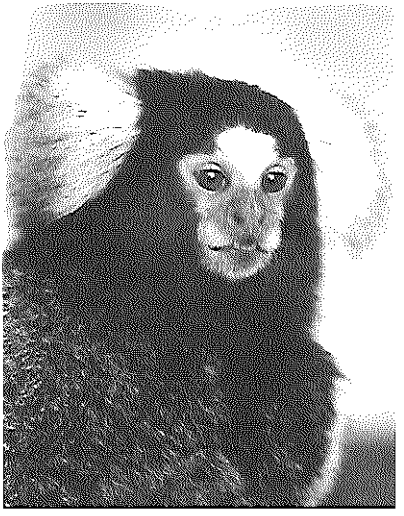

Figure 5: The common marmoset

Common marmosets are social animals, which live in a group of 3-15 individuals, and have their natural habitat in the Atlantic rain forest in the North and North-East of Brazil. *C. jacchus* has extended its range into gallery forests and forest patches in the, nowadays, dry north-east (caatinga), and the bush savanna (cerrado) of central Brazil (*Rylands, 1996*) (Figure 6). In their natural habitat, marmosets feed on insects, fruits, nectar, birds' eggs, and even small lizards, frogs and birds. However, these animals are able to occupy relatively hostile habitats, in terms of extreme seasonal variation or even lack of fruit supply, through their extreme specialization for tree-gouging to obtain gums. Adult animals weigh 350-450 grams.

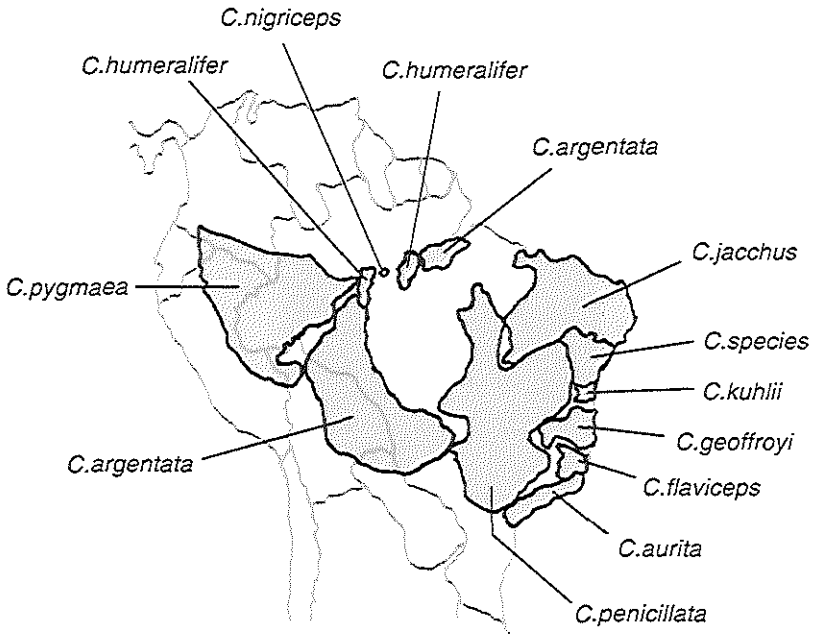

Figure 6: The natural habitat of *Callithrix* species

Their length of about 20 cm with a tail of approximately 30 cm makes the animals relatively easy to handle in captivity. Moreover, breeding costs are low in comparison to Old World monkeys, while the small size keeps test material requirements to a minimum (*Haig, 1999*). Common marmosets are relatively resistant to virally induced disease but susceptibility to various bacterial strains has been described (*Potkay, 1992*).

Common marmosets have been extensively used in biomedical research. Valid experimental models have been described for leishmaniasis (*Cuba-Cuba, 1993*), hepatitis A (*Vitral, 1995*), atherosclerosis (*Crook, 1990*), Parkinson's disease

(*Allen, 1986*) and malaria (*Mitchell, 1988*). In addition, marmosets are frequently used in toxicology research (*Siddall, 1978; Woolley, 1994*). The marmoset is often the species of choice to study reproductive biology (*Hearn, 1978; Nievergelt, 1996; Abbott, 1997; Einspanier, 1999*), the physiological effects of stress (*Costall, 1988; Elliott, 1990; Norcross, 1999*) and behavioral science (*Dettling, 1997; Box, 1997*).

Several reports appeared in the literature showing the close immunological relationship of common marmosets with humans. Cytokine mediated responses of marmoset T- and B-cells were reported to be comparable with man (*Quint, 1990*). In addition, the IgE system in marmosets was shown to be similar to that of humans (*Schmidt, 1996*). Furthermore, cross-reactivity of peripheral blood mononuclear cells (PBMC) surface antigens with mAb raised against human CD molecules had already been documented (*Neubert, 1996; Foerster, 1997*). The high similarity between human and marmoset V-D-J-CB chain sequences as well as a comparable length of their CDR3 region was document somewhat later (*Uccelli, 1997*).

3.2.2.2 The common marmoset EAE model.

In the initial protocol described in 1995 by Massacesi and colleagues, inflammatory demyelination was induced by intradermal inoculation of human whole brain homogenate emulsified in "enriched" CFA, containing 3 mg *Mycobacterium tuberculosis* per ml oil. Following the immunization two intravenous injections of 10^{10} heat-killed *Bordetella pertussis* were given, at 0 and 48 h after disease induction, respectively. The common marmoset EAE model as described was found to approximate the relapsing-remitting / chronic progressive form of MS (*Massacesi, 1995*).

The new EAE model in common marmosets was unique in a number of respects:

(1) Immunization with human brain homogenate mimics MS in many aspects.
Immunization with human white matter results in comparable clinical presentation of the disease as seen in humans, while clear MS pattern II-like lesion pathology is present at necropsy (*Lucchinetti, 2000*). Immunization with MBP or PLP induces clinical disease, but result in minor or no demyelination (*Genain, 1997*). MOG was identified as a major target antigen in the demyelinating process, since immunization with the extracellular domain of recombinant rat MOG resulted in pronounced CNS-specific demyelination (*Genain, 1995a*).

A major drawback of the original model in our hands was that animals experienced serious discomfort from the immunization protocol. In order to diminish the large ulcerative skin lesions developing at the inoculation sites, we reduced the concentration of *M. tuberculosis* in our inoculum. Furthermore, we omitted the *B. pertussis* with the objective to obtain a milder form of EAE. Notably, in some of the animals receiving *B. pertussis* next to the myelin/CFA inoculum we observed a destructive inflammatory CNS pathology rather than specific demyelination (*'t Hart, 1998*). In all experiments described in this thesis, commercially available CFA containing *M. butyricum* was used instead of *M. tuberculosis* for further reduction of skin lesions and standardization of the immunization procedure. These modifications resulted in a milder form of myelin-induced EAE with substantially delayed onset of disease as well when compared to the original publication.

(2) Marmosets provide an unique system to directly test the effect of different APC-T cell interactions by T cell transfer between (non)-identical fraternal siblings.

Auto-reactive MBP-specific T-cells with different specificity were shown to be part of the normal immune repertoire of healthy, non-immunized marmosets (*Genain, 1994*). As in rodents, MBP-reactive T-cells can be transferred between chimeric siblings and produce inflammatory CNS disease in the absence of demyelination (*Genain, 1995a; Massacesi, 1995*). By contrast, induction of CNS demyelination required co-transfer of purified IgG derived from animals immunized with myelin or MOG (*Genain, 1995b*).

(3) The complexity of the brain of the common marmoset is considerably higher than that of rodents (Figure 7).

These features make the common marmoset EAE model particularly useful to study the histopathological aspects of lesions that are visualized in the intact animal with MRI. In our first published study we showed a good correlation between the radiological alterations in the brains as detected by *in vivo* MRI and the lesion characteristics as determined by histopathology (*'t Hart, 1998*). Such information is instrumental for preclinical evaluation of new therapies that use MRI to assess their effect on lesions. Among primates, however, the organization and the specialization of the common marmoset brain is thought to be less complex than that of higher order species such as the macaques. This is among others reflected by the less extensive gyration of the cortex (*Prado Reis, 1979a; 1979b; Stephan, 1980*).

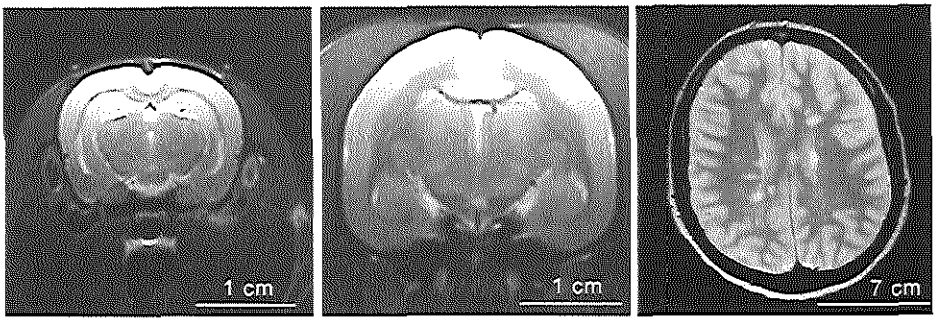

Figure 7: T2-weighted *in vivo* magnetic resonance imaging of rat (left), common marmoset (middle) and human brain (right). As shown, the brain white matter content (vizualized as grey) in common marmosets is substantially higher despite the comparable size of the brain. MR images of rat and marmoset were kindly provided by Dr. E. Blezer (Image Science Institute, University of Utrecht, The Netherlands) and the scan of the human brain was kindly provided by Prof. Dr. F. Barkhof (Free Univerisity Hospital, Amsterdam, The Netherlands).

(4) The marmoset EAE model can be used to evaluate new therapeutic MS strategies with human specificity.

Genain and colleagues first documented the potential of this new EAE model to test and evaluate new therapeutic approaches (*Genain, 1995c, 1997*). The relevance of this non-human primate model was further increased by the finding that similar patterns of accessory molecules as well as pro- and anti-inflammatory cytokines are detectable in brains of EAE-affected marmosets as has been described for chronic MS (*Laman, 1998*).

4. Outline of this thesis

The experimental work that forms the core of this thesis has been grouped in three chapters (II.1; II.2; II.3):

$CD4^+$ T-cells are thought to play a central role in EAE and MS. In general, these cells become activated by recognition of peptides that are presented in the context of MHC class II molecules, expressed by antigen presenting cells (APC). Therefore, the Mhc class II region of the common marmoset was characterized by nucleotide sequence analysis of the polymorphic exon 2 segments. The results of this study are presented in ***Chapter II.1.1***.

To study the immunological processes in more detail, a panel of antibodies for phenotyping of peripheral blood mononuclear cell populations was identified in ***Chapter II.1.2***.

In ***Chapter II.2.1***, evidence is provided that the monomorphic Caja-DR molecule plays an essential role in the initiation of EAE in the common marmoset and can be held responsible for the 100% disease incidence.

Since T- and B-cell reactivities synergize in the initiation, the development and the perpetuation of EAE, detailed analysis of these responses were performed in both myelin- as well as in MOG-induced EAE. The results of this study are described in ***Chapter II.2.2***.

The data presented in ***Chapter II.2.3*** provide evidence that the drainage of myelin to cLN is an important feature in the progressive broadening of the anti-MOG T-cell and Ab response in chronic EAE.

Blocking of co-stimulatory pathways is thought to be a potentially successful approach for the treatment of autoimmune diseases. This concept was investigated using a mouse derived human-specific anti-CD40 molecule

(***Chapter II.3.1***) as well as a chimeric antagonist anti-human CD40 mAb (***Chapter II.3.2***) in the myelin- and the MOG-induced EAE model in the common marmoset, respectively.

Recent data suggest that the beneficial effect of IFN-β on MS may be exerted via suppression of IL-12 production. Therefore the therapeutic potential of a human-anti-human IL-12p40 mAb was evaluated as described in ***Chapter II.3.3***. In ***Chapter III.1*** the relevant features of the marmoset EAE model to MS have been reviewed. The characteristics of the model have been compared to those of the more classical models in rhesus macaques (see also ***Chapter III.2***). Finally, on basis of the new data obtained in our non-human primate models of EAE we have formulated a hypothesis on the genetic and immunopathogenic mechanisms in MS (***Chapter III.3***).

Chapter IV contains a summary of this thesis and suggestions for further research, including a schematic view of the immunological pathways in the pathogenesis in marmoset EAE based on the results described in this thesis.

Chapter II

Experimental chapters

1.
Immunological characterization of the common marmoset

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Evolution

The common marmoset: A new world primate species with limited *Mhc* class II variability

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ABSTRACT **The common marmoset (*Callithrix jacchus*) is a New World primate species that is highly susceptible to fatal infections caused by various strains of bacteria. We present here a first step in the molecular characterization of the common marmoset's *Mhc* class II genes by nucleotide sequence analysis of the polymorphic exon 2 segments. For this study, genetic material was obtained from animals bred in captivity as well as in the wild. The results demonstrate that the common marmoset has, like other primates, apparently functional *Mhc-DR* and *-DQ* regions, but the *Mhc-DP* region has been inactivated. At the *-DR* and *-DQ* loci, only a limited number of lineages were detected. On the basis of the number of alleles found, the *-DQA* and *-B* loci appear to be oligomorphic, whereas only a moderate degree of polymorphism was observed for two of three *Mhc-DRB* loci. The contact residues in the peptide-binding site of the Caja-DRB1*03 lineage members are highly conserved, whereas the -DRB*W16 lineage members show more divergence in that respect. The latter locus encodes five oligomorphic lineages whose members are not observed in any other primate species studied, suggesting rapid evolution, as illustrated by frequent exchange of polymorphic motifs. All common marmosets tested were found to share one monomorphic type of *Caja-DRB*W12* allele probably encoded by a separate locus. Common marmosets apparently lack haplotype polymorphism because the number of *Caja-DRB* loci present per haplotype appears to be constant. Despite this, however, an unexpectedly high number of allelic combinations are observed at the haplotypic level, suggesting that *Caja-DRB* alleles are exchanged frequently between chromosomes by recombination, promoting an optimal distribution of limited *Mhc* polymorphisms among individuals of a given population. This peculiar genetic make up, in combination with the limited variability of the major histocompatability complex class II repertoire, may contribute to the common marmoset's susceptibility to particular bacterial infections.**

The *Mhc* is a cluster of loci coding for polymorphic glycoproteins that provide the context for the recognition of antigens by T lymphocytes and is thought to be present in most, if not all, vertebrate species (1). There are two major types of gene products, named major histocompatability complex (MHC) class I and II molecules. The classical MHC class I molecules are expressed on virtually all nucleated cells and present peptides from intracellular origin to CD8-positive T cells. Such peptides usually originate from intracellular parasites and viruses, and recognition may result in the lysis of the infected target cell. The MHC class II molecules are heterodimeric structures showing restricted tissue distribution. MHC class II molecules present peptides of extracellular origin to T cells of the helper phenotype. Activation of these cells often results in cytokine release leading to a variety of effects, such as antibody production.

In humans, the class I region contains at least 18 highly related genes, which include those encoding the highly polymorphic transplantation antigens (*HLA-A*, *-B*, and *-C*) and the oligomorphic nonclassical *HLA-E*, *-F*, *-G*, *-H*, and *-J* genes (2). Some of the latter show differential tissue distribution, whereas others are pseudogenes. The human class II region is arranged into the *HLA-DP*, *-DQ*, and *-DR* regions, each containing at least one pair of *A* and *B* genes encoding the α and β polypeptide chains (3). Most nucleotide sequence variability is confined to exon 2 of the *Mhc-DPB*, *-DQA*, *-DQB*, and *-DRB* genes. Recent studies have demonstrated that humans, apes, and Old World monkeys (Catarrhini) share numerous *Mhc* class I and II loci (3–9). Such studies also have illustrated that many *Mhc* class I and II lineages predate the speciation of the contemporary living primate species (10–14). In particular, some of the *Mhc* class II lineages are extremely stable and may be >35 million-years-old. *Mhc* class I lineages, on the other hand, seem to evolve much more rapidly (15–16).

Numerous duplications and condensations took place during the evolution of the MHC region, and most species possess *Mhc* genes, which display extensive degrees of polymorphism. There are, however, exceptions. In cotton-top tamarins (*Saguinus oedipus*), low variability was reported for the *Mhc* class I region, as evidenced by sequencing and immunoprecipitation studies (17–19). The *Mhc* class II region of this species exhibits abundant polymorphism, and at least 50 alleles have been described thus far (19–20). The cheetah (*Acinonyx jubatus*) and two species of mole-rat (*Heterocephalus glaber* and *Spalax leucodon*) are thought to have condensed *Mhc* repertoires. Unfortunately, detailed nucleotide sequence analysis of the *Mhc* genes of these species is lacking (21–23). The Syrian hamster was reported to have a monomorphic *Mhc* class I region, but this claim may be the result of a sampling error (24–25).

The Callitrichidae family of New World monkeys (Platyrrhini) comprises marmosets and tamarins, which are used in biomedical research as models for several human diseases, e.g., multiple sclerosis (26). Nonidentical twins are born as natural bone-marrow chimeras because of a sharing of the placental circulation. Their apparently increased susceptibility to several viral, bacterial, protozoan, and helminth agents has been documented (27). In some cases, such as incidence of ulcerative colitis, Epstein–Barr virus, Herpes virus saimiri, and tamarinus infections, there is compelling evidence for a more

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Abbreviation: MHC, major histocompatability complex.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF004741–AF004765, AF044720, and AF044721).

§Deceased July 1, 1997.

resistant status of the common marmoset as compared with the cotton-top tamarin. The increased susceptibility of the cotton-top tamarin to viral infections, in particular, may be due to a severely condensed *Mhc* class I repertoire (17–19). On the other hand, common marmosets seem to be more susceptible than tamarins to fatal infections caused by bacteria such as *Klebsiella*, *Bordetella*, *Clostridium*, and *Shigella* (27). For that reason, we wished to investigate the variability of the *Mhc* class II region of the common marmoset. In this context, genetic variability is defined as the number of allelic lineages and alleles per locus, the number of *Mhc* class II genes present per haplotype, and allelic combinations seen at the haplotypic level also were included in this analysis.

MATERIALS AND METHODS

Animals. The Biomedical Primate Research Centre houses a common marmoset colony of a≈100 pedigreed individuals. New animals were introduced on several occasions to maintain the outbred character of the colony. For this study, 25 monkeys, eight of them consisting of four pairs of twins, were selected. They are recorded descendants of individuals from two former Dutch colonies, one Italian colony, and at least one German colony. Hence, the present selection of animals is considered to reflect a representative sample. To investigate the possibility of inbreeding or potential founder effects, four common marmosets from a Brazilian colony (Primatology Nucleus, Universidade Federal de Rio Grande do Norte, Brazil) were examined. Two of these animals were born in the wild.

DNA Isolation and PCR. B lymphoblastoid cell lines were established from 2-ml blood samples by transformation with a cotton-top tamarin Epstein–Barr virus (B95–8) -producing cell line. These cells were used for genomic DNA isolation as described previously (28). In the case of individuals for which the B cell lines were lacking, DNA isolation was conducted on tissue samples such as frozen spleen and lymph nodes, whereas for the Brazilian animals, 2 ml of peripheral blood was used. Exon 2 of the *Caja* class II genes was amplified by the PCR methodology (29). Negative controls lacking DNA were part of all experiments, and whenever necessary a positive control was included by using samples of rhesus macaque DNA. The primers and protocols used for amplification of the *Mhc* class II *A* and *B* genes were reported previously (19–20, 30–35).

Typing for *Caja-DPB* and *-DPA*-like PCR Products. *DPB* and *DPA* probes, spanning highly conserved regions in the *DPB* (*Caja-DPB-5'*-YAACAGCCAGAAGGAC-3') and *DPA* (*Caja-DPA-5'*-CTGGAGGAGTTTGGCCGAGCC-3') sequences of all primate species studied so far, were used to detect amplified PCR products. One to three microliters of each sample was bound to a nylon membrane (Hybond-N, version 2.0, Amersham) and subsequently crosslinked (Bio-Rad GS Gene Linker UV Chamber) at 250 mJ. Prehybridization took place for 30 min at 58°C (Hybridization Oven, New Brunswick Scientific) in 5 ml of 3 M tetramethylammonium chloride, 5 mM EDTA, 50 mM Tris (pH 7.5), and 1% SDS (TMACl) with denatured herring sperm (1 mg/5 ml). For hybridization, 1 pmol of each oligo was added per milliliter of TMACl, for at least 1 hr, at 60°C (*-DPA*) and 50°C (*-DPB*). After hybridization, each filter was washed 20 min at 61°C (*-DPA*) and 51°C (*-DPB*) in TMACl. Filters were washed afterward twice for 5 min in 1*standard saline phosphate (SSPE) and 0.1% SDS and incubated for 10 min with 1 μg Streptavidin Horseradish Peroxidase Conjugate (GIBCO/BRL) per milliliter of washing buffer. Washing was done as before, and then the membranes were incubated for 5 min in blockbuffer (50 ml of Triton X-100/60 g urea/5.8 g NaCl/10 g dextransulfate, together 1,000 ml, pH 7.3). After further washing, nucleic acids were detected by incubation with ECL detection reagents (Amersham) for 1 min at room temperature; the blots were placed in a cassette with a sheet of autoradiography film for 10 min.

Nucleotide Sequencing. PCR-amplified DNA was prepared for sequencing, as described (28). In brief, a digestion with appropriate endonuclease restriction enzymes was carried out at 37°C, for 2 hr: *Pst*I and *Bam*HI for *GH26/GH27*, *GH98/GH99*, and *DB01/DB03*; *Sal*I and *Xba*I for *5'-DQB SalI/3'-DQB XbaI*, and *5'-DPB SalI/3'-DPB XbaI*; and *Sal*I and *Bam*HI for *Tu215/Tu216* (all enzymes from GIBCO/BRL). The PCR fragments were then cloned into bacteriophage M13 derivatives mp18 and mp19 (vectors from Boehringer Mannheim GmbH) and sequenced by using the Sequenase Version 2.0 DNA Sequencing Kit (Amersham). The reported alleles represent at least three identical clones that were obtained after independent amplifications or in different animals.

Sequence Specific Oligonucleotide Typing for *Caja-DQA1* and *-DQB1* Alleles. After analyzing the *Caja-DQ* locus sequences, we developed an oligotyping method to assay the presence or absence of the known *Caja-DQ* alleles. The biotinylated oligos used were: for *Caja-DQA1*0101*, 5'-YCTCGCTGTGTCAAAACA-CAAC-3'; *Caja-DQA1*2501*, 5'-YATCGCTACGATGAAAC-CCGGC-3'; *Caja-DQB1*2201*, 5'-YCCGCTTGTGACCCGAT-TCATCTAT-3'; *Caja-DQB1*2301*, 5'-YTTTAAGGGTTTCT-GCTAC-3'; *Caja-DQB1*2302*, 5'-YTTTAAGTTTCTCTGCT-AC-3'; *Caja-DQB2*0101*, 5'-YCGGGCGGTGACCGAG-3'; and *Caja-DQB2*0102*, 5'-CGGGCGATGACCGAG-3'. The technical procedure was the same as described in the section "Typing for *DPB* and *DPA*-like PCR products." Hybridization and washing temperatures were, respectively, 58–59°C (*-DQA1, -DQB1*2201*), 50–51°C (*-DQB1*2301*), 48–49°C (*-DQB1*2302*), 49–50°C (*-DQB2*0101*), and 45–46°C (*-DQB2*0102*).

Nomenclature. Marmoset alleles that are similar to human equivalents are depicted by identical lineage numbers. For instance, the Caja-DRB1*03 and HLA-DRB1*03 alleles group into the same lineage. The last two digits are arbitrary and reflect the order in which the alleles were found. The marmoset lineages for which no apparent human equivalent has been identified are designated by a workshop number, such as Caja-DRB*W12. This also indicates that the physical location has not been mapped.

RESULTS AND DISCUSSION

Loci and Lineages

***Caja-DQA* and *-DQB*.** After PCR amplification, only two orthologues of *HLA-DQA1* were identified in our population of marmosets. Phylogenetic analyses demonstrated that both *Caja-DQA1* alleles cluster into separate lineages and are very similar to sequences obtained from an undefined species of marmoset (36). The *Caja-DQA1*0101* allele clusters together with equivalents from Old and New World monkeys, apes, and humans; hence, the *Mhc-DQA1*01* lineage appears to be at least 58 millions of years old. The *Caja-DQA1*2501* allele groups into a younger lineage that seems to be restricted to New World monkeys. The inferred amino acid sequences of *Caja-DQA1*0101* and *-DQA1*2501* alleles are depicted in Fig. 1*a*. Only three *Caja-DQB1* alleles were detected in our colony. The *Caja-DQB1*2201*, *-DQB1*2301*, and *-DQB1*2302* (Fig. 1*b*) alleles group into two lineages earlier observed in cotton-top tamarins (19, 32). However, two alleles were discovered that encode the same Caja-DQB1*2201 amino acid sequence, and differ only for one synonymous substitution at position 168 (CCG→CCC).

No homologue of the *HLA-DQA2* exon 2 gene segment was recovered, as is in agreement with data obtained from studies on other New World monkeys (19, 33, 35). The two alleles retrieved from the common marmoset *DQB2* locus were named *Caja-DQB2*0101* and *-DQB2*0102* (Fig. 1*b*) and share

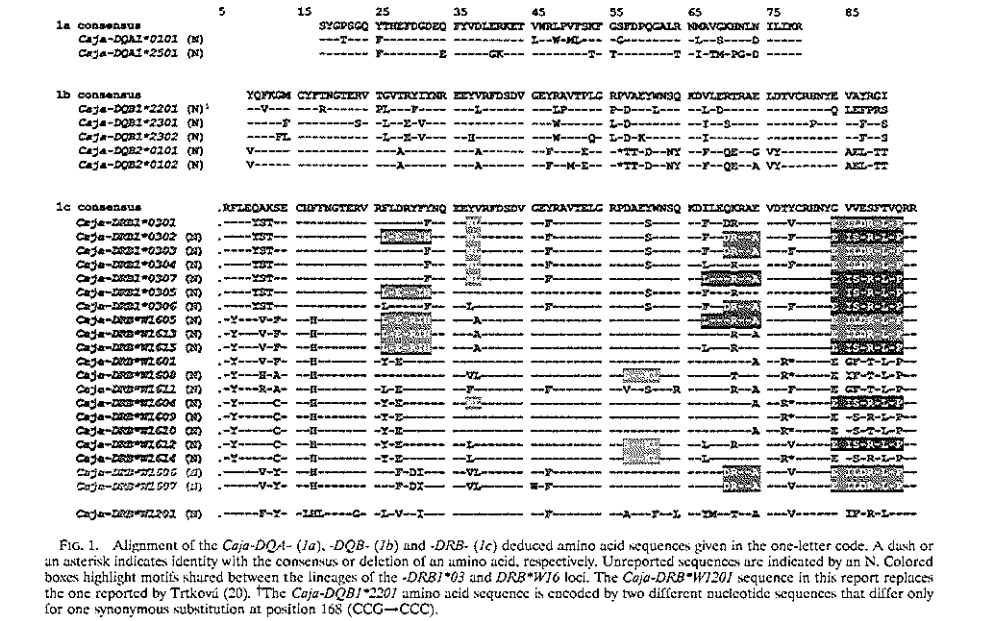


FIG. 1. Alignment of the *Caja-DQA-* (*1a*), *-DQB-* (*1b*) and *-DRB-* (*1c*) deduced amino acid sequences given in the one-letter code. A dash or an asterisk indicates identity with the consensus or deletion of an amino acid, respectively. Unreported sequences are indicated by an N. Colored boxes highlight motifs shared between the lineages of the *-DRB1*03* and *DRB*W16* loci. The *Caja-DRB*W1201* sequence in this report replaces the one reported by Trtková (20). †The *Caja-DQB1*2201* amino acid sequence is encoded by two different nucleotide sequences that differ only for one synonymous substitution at position 168 (CCG→CCC).

a high degree of similarity with their human equivalents. Although *Mhc-DQB2* is considered to be a pseudogene locus, the present results underline its high degree of conservation and antiquity.

On the basis of the nucleotide sequence information, a sequence-specific oligotyping method was developed that allowed us to type all animals for their *DQ* loci (Fig. 2). As can be seen, some animals, like V3 and 742, appear to be homozygous for particular *Caja-DQ* entities. All animals were found to be *Caja-DQA1*2501* positive whereas only animal 9442 lacked the *Caja-DQB1*2201* allele. Segregation data suggest that the *Caja-DQA1*2501* allele is preferentially linked to the *-DQB1*2201* and *-DQB1*2302* alleles, whereas the *Caja-DQA1*0101* allele is linked to *-DQB1*2301* (Fig. 2). Exceptions to the rule exist as is evidenced by animals 9323, 9338, and 9442. It should be stressed, however, that the detection of only two *Caja-DQA1*, three *-DQB1*, and two (nonfunctional) *-DQB2* alleles in common marmosets is in contrast to the observations concerning abundant polymorphism in populations of other primate species, such as humans, chimpanzees, and rhesus macaques (2–6). An overview of these data has been provided in Table 1.

Caja-DP. Despite the fact that several sets of primers were tested in combination with different PCR programs, we failed to clone exon 2 of the *Caja-DPB1* gene. Although PCR products were obtained occasionally, screening with several conserved primate-specific *Mhc-DPB1* probes excluded the presence of relevant exon 2 nucleotide sequences. In contrast, successful amplification was obtained in cotton-top tamarins, even with the primers designed for *HLA-DPB* genes (33). Subsequent Southern blot experiments with conserved *Mhc-DPB1* exon 2 sequences as probes failed to prove the existence of bona fide *Caja-DPB1* genes. We therefore conclude that the *Caja-DPB1* gene, if present, has at least a defunct exon 2 gene segment and is probably inactivated. Studies are underway to determine which genetic defect(s) affect the *Caja-DPB1* gene. Regarding *Caja-DPA1*, only extremely low amounts of exon 2 sequences were amplified, as was evidenced by screening with the relevant probe (data not shown). The same primers, however, worked very efficiently in amplifying *Mhc-DPA1* exon 2 sequences derived from other New World monkey species (34). The detection of low amounts of the *Caja-DPA1* PCR product again suggests that the *Caja-DP* region may have an altered appearance in common marmosets. In all higher primate species studied thus far, three distinct functional *Mhc* class II regions have been detected: namely *-DR*, *-DQ*, and *-DP*. The common marmoset is the first example of a higher primate species in which the equivalents of *HLA-DR* and *-DQ* seem to represent the main types of functional MHC class II molecules.

Caja-DRB. In excess of 500 clones from >30 individuals were sampled. This resulted in the description of 21 *DRB* sequences (Fig. 1c), confirming two of six previously reported, designated *Caja-DRB1*0301* and *-DRB*W1601* (20). In contrast, the existence of the *Caja-DRB11*0101*, *-DRB*W1201*, *-DRB*W1602*, and *-DRB*W1603* alleles was not confirmed (20). To clarify this issue, samples from the same animals as used in the study by Trtková and coworkers or marmosets related in blood line were (re)analyzed with different primer sets. Again, no evidence was found for the existence of the four above-mentioned alleles, which may represent *in vitro* PCR artifacts. Consequently, these alleles were not listed in the alignment, except for *DRB*W1201*, which is a new allele, encoded by a different locus, replacing the one previously reported (Fig. 1c).

In the case of a heterozygous marmoset, one would expect to find a maximum of two alleles per locus. Marmosets are born as natural bone-marrow chimeras and therefore may share blood cells with their nonidentical twin(s). Conse-

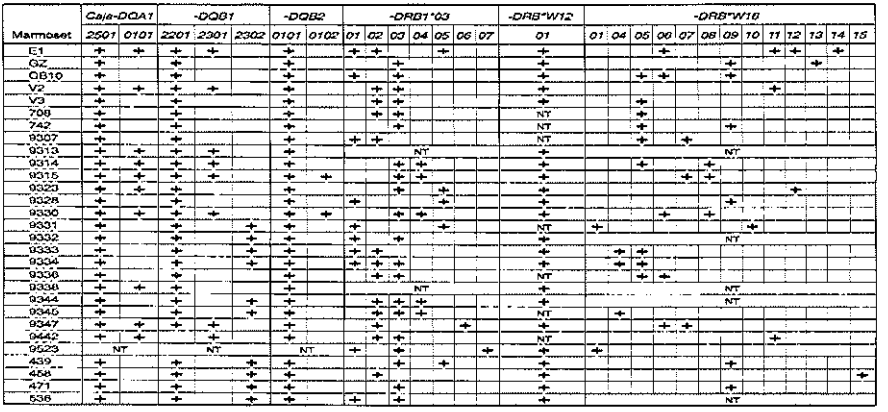


FIG. 2. Distribution of alleles at the *Caja-DQA1*, *-DQB1*, *-DQB2*, *-DRB1*03*, and *DRB*W16* loci in 29 common marmosets. Pairs 9314/9315, 9331/9332, 9333/9334, and 9344/9345 are twins. Animals 439, 458, 471, and 536 are from the Brazilian colony; 439 and 536 were born in the wild. NT, nontested. In the case of *-DRB*, the alleles have been detected by nucleotide sequence analysis after PCR amplification. This means that some *-DRB* alleles may not have been amplified and that their presence was not detected. This phenomenon explains the heterogeneity in the number of *-DRB* alleles as depicted in the last two columns of this table. The presence of *Caja-DRB*W1201* also was confirmed by denaturing gradient gel electrophoresis.

quently, some animals have in their blood circulation cells possessing *DRB* genes that were not inherited in a Mendelian fashion. This situation is evidently applicable to the three *Caja-DRB1*03* and four -DRB*W16 lineage members detected in animal E1 (Fig. 2). All marmosets tested were found to share the *Caja-DRB*W1201* allele. On the basis of segregation studies and nucleotide sequence comparisons, it seems likely that the *Caja-DRB*W1201* allele is controlled by a separate locus. Detailed contig mapping studies are needed to confirm this assumption, which, if true, indicates that the common marmoset is the first known example of a species that possesses a monomorphic *DR* locus that is present in all individuals.

With regard to the level of polymorphism, only 7 *Caja-DRB1*03* and 13 *-DRB*W16* alleles were detected. In one of the four Brazilian animals, *Caja-DRB*W1615* was found, which appears to be absent in the individuals from the Biomedical Primate Research Centre population. This illustrates that the Biomedical Primate Research Centre captive bred population does not differ significantly from other common marmoset populations. To obtain these results, different sets of primers were used. This was done to minimize the chance that not all DRB loci were amplified. Our results contrast the observations done on the MHC Class II repertoire for another New World monkey species, the cotton-top tamarin (Table 1). The question is whether the number of *Caja-DRB* alleles detected indicates a low, moderate, or high degree of polymorphism. Thus far, only a few populations of primate species, such as humans, common chimpanzees, rhesus macaques, and cotton-top tamarins, have been tested thoroughly for *Mhc-DRB* loci diversity at the nucleotide sequence level. Compared with these species the common marmoset displays a low or moderate degree of polymorphism at the *DRB* loci (Table 1).

Convergence, Divergence, and Conservation

The seven *Caja-DRB1*03* alleles share the EYSTS amino acid motif at positions 9–13, characteristic of the lineage. The importance of *Mhc-DRB1*03* is highlighted by the observation that the lineage is evolutionarily stable and its members have been detected in many species and play an important role in mounting immune response against mycobacteria (37). Alter-

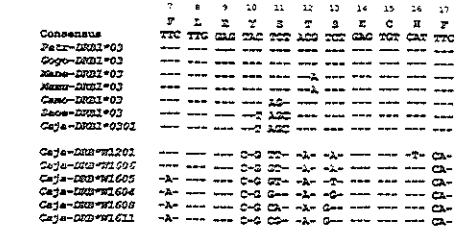
Table 1. Distribution of number of alleles at some *Mhc-DRB*, *-DQA*, *-DQB*, *-DPA*, and *-DPB* loci/lineages among different populations of primate species

Mhc loci/lineages	*HLA*	*Patr*	*Mamu*	*Saoe*	*Caja*
*DRB1*01/10*	5	1	7	—	—
*DRB1*02*	17	11	—	—	—
*DRB1*03*†	133	10	17	5	7
*DRB1*04*	26	—	6	—	—
*DRB1*07*	1	2	1	—	—
*DRB1*09*	1	—	—	—	—
DRB3	11	16	5	16	—
DRB4	9	7	1	—	—
DRB5	12	15	5	1	—
DRB6	3	15	14	—	—
DRB7	2	1	—	—	—
DRB11	—	—	—	10	—
*DRB*W1–7*	—	—	29	—	—
*DRB*W8*	—	1	—	—	—
*DRB*W9*	—	5	—	—	—
*DRB*W12*	—	—	—	4	1
*DRB*W16*	—	—	—	—	13
*DRB*W22*	—	—	—	9	—
DQA1	18	5	15	2	2
DQB1	31	12	28	2	3
DPB1	77	28	15	1‡	—
DPA1	10	3	1	NT	—

The data were taken from ref. 44 and the nonhuman primate MHC database (NHPDB) that is maintained at the Biomedical Primate Research Centre. *HLA*, human; *Patr*, common chimpanzee; *Mamu*, rhesus macaque; *Saoe*, cotton-top tamarin; and *Caja*, common marmoset.

†This lineage includes the *HLA-DRB1* genes encoded by HLA-DR3, -DR5, DR6, and DR8 positive individuals.

‡Only one individual studied (33).



	7	8	9	10	11	12	13	14	15	16	17
	F	L	R	Y	S	T	S	E	C	H	F
Consensus	TTC	TTG	CGA	TAC	TCT	ACG	TCT	GAG	TGT	CAT	TTC
*Patr-DRB1*03*	---	---	---	---	---	---	---	---	---	---	---
*Gogo-DRB1*03*	---	---	---	---	---	---	---	---	---	---	---
*Mane-DRB1*07*	---	---	---	---	---	-A-	---	---	---	---	---
*Mamu-DRB1*03*	---	---	---	---	---	--A	---	---	---	---	---
*Caso-DRB1*03*	---	---	---	---	AG-	---	---	---	---	---	---
*Saoe-DRB1*03*	---	---	---	-T	AGC	---	---	---	---	---	---
*Caja-DRB1*0302*	---	---	---	-T	AGC	---	---	---	---	---	---
*Caja-DRB*W1201*	---	---	---	C-G	TC-	-A-	-A-	---	---	-T-	CA-
*Caja-DRB*W1205*	---	---	---	C-G	TC-	-A-	-A-	---	---	---	CA-
*Caja-DRB*W1605*	-A-	---	---	C-G	TC-	-A-	-T-	---	---	---	CA-
*Caja-DRB*W1606*	-A-	---	---	C-G	G--	-A-	-G-	---	---	---	CA-
*Caja-DRB*W1608*	-A-	---	---	C-G	CA-	-A-	G--	---	---	---	CA-
*Caja-DRB*W1611*	-A-	---	---	C-G	CG-	-A-	G--	---	---	---	CA-

FIG. 3. Nucleotide sequences of the EYSTS amino acid motif as encoded in different primate species, of the infra-orders Catarrhini (*Patr*, common chimpanzee; *Gogo*, gorilla; *Mamu*, rhesus macaque; and *Mane*, pig-tailed macaque) and Platyrrhini (*Camo*, capuchin monkey; *Saoe*, cotton-top tamarin; and *Caja*, common marmoset). In addition, the different DNA motifs that are observed at the *Caja-DRB*W12* and *-DRB*W16* loci are shown. The motifs are depicted in color code and correspond with the alleles listed in Fig. 1c and the phylogenetic tree in Fig. 4.

natively, if a certain motif is functionally of relevance, it may be generated *de novo* by convergent evolution as appears to be the case for the *HLA-DRB1*03*-like lineages in New World monkeys. First of all, in the amino acid motif EYSTS, the serine residue at position 11 can use two different codons. The Catarrhini use the TCT codon, whereas the Platyrrhini make use of codons AGT and AGC (Fig. 3). The EYSTS motif is present also in *DRB* allomorphs of bovids, equids, and murids, where again differential codon usage indicates convergent evolution (38–40). Secondly, all the platyrrhine *Mhc-DRB* sequences described thus far have a characteristic motif at the 3′ end of exon 2, which suggests a common ancestry. Finally, analysis of intron 2, exon 3, and the 3′-untranslated region of *DRB* sequences does not reveal an orthologous relationship of New World monkey *DRB* genes to any of the known catarrhine *DRB* genes (41). Thus the New World monkey *DRB* genes seem to originate from a different ancestor than the progenitor of the catarrhine *DRB* genes.

In the case of the *Caja-DRB1*03* lineage, the contact residues in the β pleated sheets of the peptide-binding site are highly conserved. In this respect, more variation is seen in the *Caja-DRB*W16* lineage, in which five different motifs are present in the first β-pleated sheet section (Figs. 1c and 3). Nevertheless, all the alleles assigned to the *Caja-DRB*W16* lineage share an identical DNA motif at codons 15–25, reflecting their common ancestry. Most of the variation seen at the codons for the contact residues within the first β-pleated sheet of the *Caja-DRB*W16* lineage can be explained by the occurrence of simple point mutations (Fig. 3). This indicates that positive selection for diversity has been acting on the *Caja-DRB*W16* sequences. Many *Caja-DRB*W16* lineage members share patchwork motifs that apparently have been exchanged between alleles, probably as a result of recombination-like events as indicated by colored boxes (Fig. 1c). Some of these motifs are even shared between two different *DRB* loci. This concerted action of point mutations in combination with frequent recombination promotes fast evolution of the *Caja-DRB*W16* locus. Similar findings concerning rapid evolution have been reported for the *Mhc-B* (15–16, 42) and *-DP* (31, 43) loci in primates. Phylogenetic analyses demonstrate that the alleles at the *Caja-DRB*W16* locus cluster into different lineages, each with a remarkably low number of alleles (Fig. 4). The observation that those lineages defined by the various peptide-binding site motifs as depicted in the same color code in Figs. 3 and 4 are arranged in different branches of the phylogenetic tree confirms that recombination-like events must have occurred.

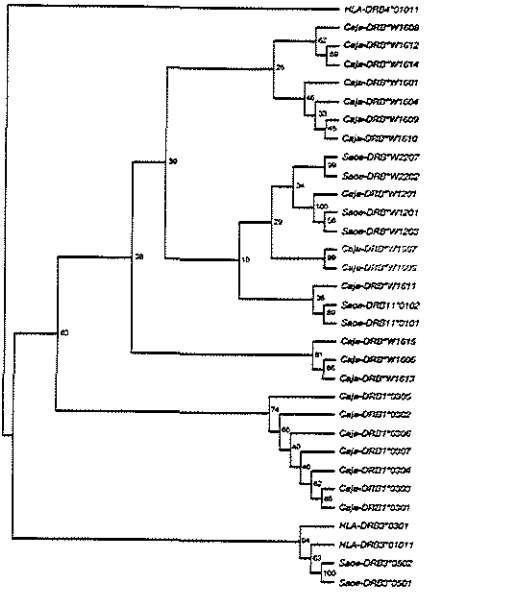


FIG. 4. Phylogenetic tree constructed according to the Neighbor-joining method showing the evolutionary relationships between *DRB* alleles from New World monkeys. Bootstrap values are indicated. The names of the *Caja-DRB* alleles are depicted in colors. These correspond to the color code used in Figs. 1c and 3.

Haplotypes

The primate *Mhc-DR* region contains one monomorphic *A* gene, whereas the number of *B* genes varies between haplotypes and species. As a consequence, the primate *DRB* region displays haplotype polymorphism (45–46). Although rare, *DR* regions or even extended regions can be shared in a similar fashion between species, as has been documented for humans and some ape species (3–4). All common marmosets seem to share a monomorphic *Caja-DRB*W12* locus, a locus controlling the more or less conserved *Caja-DRB1*03* alleles and one locus encoding the highly divergent *DRB*W16* lineage members (Fig. 2). This suggests that all common marmosets tested possess *DR* regions that harbor only three *DRB* loci and lack haplotype polymorphism. Although pedigree data are available, a wide variety of allelic combinations, especially at the *DR* loci, prohibits the definition of haplotypes (Fig. 2). At this stage, the possibility cannot be excluded that different *Caja-DRB* genes are located on distinct chromosomes. On the other hand, alleles encoded in a *cis* configuration may be exchanged frequently between chromosomes by recombination, possibly enhanced due to a high degree of similarity between the loci. This may reflect an optimal strategy to spread the limited number of allelic polymorphisms at the population level in common marmosets.

Chimerism and Disease Susceptibility

In theory, condensation of MHC variability may have been caused by inbreeding, a founder effect, by selective pressures such

as diseases or by a combination of these factors. The sampling procedure excludes the possibility that the observed condensed *Caja* class II repertoire resulted from inbreeding. The chimeric status of the *Callitrichidae* is thought to have an impact on the condensed *Mhc* class I repertoire (17, 18), but this is not the case for the class II region in cotton-top tamarins, which displays abundant polymorphism (Table 1). Despite the limited MHC class II variation seen at the population level, some chimeric marmosets (E1) express up to eight different types of *MHC-DR* molecules (Fig. 2). Such a situation must have an impact on the selection of T cells in the thymus. If, due to the presence of multiple MHC molecules, too many T cells are deleted, an individual may become susceptible to infectious diseases. At some stage, a delicate balance should be reached between the number of MHC molecules present in a individual and the capacity to respond adequately to foreign pathogens. Common marmosets live in relatively small topographic pockets and have adapted to their environment. When taken out of their original habitat, they may, under stressful situations, encounter new pathogens with which they have not learned to cope. That common marmosets are especially prone to die from particular bacterial and helminthic diseases (27) is in line with the present observation that this species possesses limited MHC class II variability. Moreover, all common marmosets tested were found to share the monomorphic *Caja-DRB*W1201* allele. In the marmoset's natural habitat, the wide distribution of this monomorphic DR molecule was probably positively selected because it controlled an important protective function. When common marmoset populations encounter new pathogens, however, such as particular bacterial infections, the presence of a monomorphic immune response gene may turn to their profound disadvantage, especially when such a locus controls a susceptibility trait. The result may be the decimation of a given population of individuals, or in the most severe case, the extermination of a species.

During the course of this study, Susana G. Antunes died trugically on July 1, 1997. To the memory of Sue, whom we cherished for her personal and intellectual qualities, the couthors lovingly dedicate this manuscript. The authors would like to thank Henk van Westbroek for preparation of the figures, Donna Devine for editing, and Dr. Maria de Sousa, Dr. Jon J. van Rood, Dr. Bert 't Hart, and Dr. Jim Kaufman for support and helpful comments. This study was financially supported by the MACROPA Foundation, the European Union human capital and mobility Grant CHGE-CT94-0071 (DG 12 COMA), and Junta Nacional de Investigacio Cientifica e Technológica (Grants PRAXIS XXI BM/2375/94 and PRAXIS XXI BD/9588/96).

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An Extensive Monoclonal Antibody Panel for the Phenotyping of Leukocyte Subsets in the Common Marmoset and the Cotton-Top Tamarin

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New World monkeys are valuable animal models to study human diseases. To determine the phenotype of cells involved in immune responses, we used flow cytometry to screen a large panel of anti-human monoclonal antibodies (mAb) for cross-reactivity with cells of the common marmoset and the cotton-top tamarin. Certain antigens (e.g., CD2, CD8, CD20) are well conserved. However, CD10, CD23, and CD33 showed a clear discrepancy in their reaction patterns in both species, indicating that significant differences on the epitope level occurred during evolution. Epstein-Barr virus-transformed B-cell lines were shown to be a valuable tool for screening B-cell–specific reagents. In some cases, fluorescein isothiocyanate (FITC) and phycoerythrin (PE) modification of mAbs had a negative effect on the binding capacity, which stressed the importance of choosing the right label. Despite the fact that some CD antigens were not detected, adequate numbers of cross-reactive mAbs were identified to perform extensive studies on immunological functions in both the common marmoset and the cotton-top tamarin. Cytometry 45:294–303, 2001. †© British Crown copyright 2001/DSTL—published with the permission of the Controller of Her Majesty's Stationery Office.

Key terms: CD markers; common marmoset; cotton-top tamarin; flow cytometry

The Callitrichidae family of New World monkeys (NWM) comprises marmosets and tamarins. Both NWM species become increasingly important in biomedical research because of both the relatively close immunological relationship with humans and their relative small size (1–7). These features are particularly attractive for preclinical safety and effectivity testing of human-specific, biotechnology-derived pharmaceuticals that are not reactive in less closely related species such as rodents. Furthermore, both species are relatively easy to handle, breeding costs are low compared with Old World monkeys, and their small size keeps test material requirements to a minimum. Valid experimental models for human biology and disease have been developed in common marmosets (*Callithrix jacchus*) for multiple sclerosis (8), various bacterial infections (9), behavioral sciences (10), and the physiological effects of stress (11). They are used frequently in toxicology research (12,13). Cotton-top tamarins (*Saguinus oedipus*) are good animal models for studying viral infections (14,15) and an ulcerative-like inflammatory bowel disease that this species develops in captivity (16). Furthermore, both species are used intensively to study reproductive biology (17–22).

Many disorders in humans are believed to be mediated by a synergy of cellular (T-cell mediated) and humoral (B-cell mediated) immune mechanisms. To gain better insight into immunological pathways in health and disease, it is of prime importance to determine the phenotypes in the involved cell populations. One way to characterize cells is to use flow cytometric analysis using monoclonal antibodies (mAb) directed to cell surface antigens. Few studies have reported on the cross-reactivity of mAbs, raised originally against human CD epitopes, in the cotton-top tamarin (23–25) and common marmoset (26,27). The most extensive study to date (24) reported 62% cross-reactivity of 121 mAbs tested. However, a suitable cross-reactive mAb was not found for 13 markers. Also, triple staining failed because the cross-reactivity of

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mAbs using different fluorochromes was not detected. The number of commercially available anti-human mAbs has increased significantly and new labeling techniques have become available. In this study, the results of an extensive survey with 331 differently labeled mAbs are presented. mAbs conjugated with allophycocyanin (APC), fluorescein isothiocyanate (FITC), phycoerythrin (PE), or peripidin chlorophyll (PerCp) were tested for their cross-reactivity in whole blood samples from both NWM species. Furthermore, common B and T-cell markers were studied in more detail using Epstein-Barr virus (EBV)-transformed B-cell lines and phytohemagglutinin (PHA)-stimulated T cells, respectively.

MATERIAL AND METHODS

Animals

Venous blood was collected from 20 healthy common marmosets purpose bred at the Biomedical Primate Research Centre (BPRC; Rijswijk, The Netherlands) primate facility and from 20 healthy cotton-top tamarins bred at the University of Bristol. In 1995, the cotton-top colony was shipped to the BPRC, quarantined, and housed in group cages. Equal numbers of male and females were used in this study with subjects ranging in age from 2 to 7 years. Their daily diet consisted of food pellets for NWM (Special Diet Services, Witham, Essex, England) supplemented with rice, peanuts, marshmallows, biscuits, fresh fruit, and vegetables. Drinking water was provided ad libitum.

Blood Sampling

Animals were sedated with 6 mg/kg ketamine (AST Farma, Oudewater, The Netherlands) and bled from the medial femoral vein. Up to 2 ml blood was collected using ethylenediaminetetraacetic acid (EDTA-K3) or lithium-heparin (Li-H)-precoated tubes (Greiner BV, Sölingen, Germany).

Sample Preparation

EDTA-K3 whole blood samples were diluted in ice cold phosphate-buffered saline (pH 7.4) containing 1% bovine serum albumin in the presence of 0.1% sodium azide (1% PBS/BSA). Aliquots of diluted samples were placed in Falcon 3052 tubes (Becton Dickinson, Mountain View, CA) and incubated with 5 µl of relevant mAb for at least 30 min on ice. Red blood cells were lysed using an ammonium chloride-based buffer (FACSlyse, Becton Dickinson) for 10 min at room temperature. Samples were washed twice with 1% PBS/BSA after which cells were fixed with PBS containing 0.7% formalin and stored at 4°C until analysis. Samples incubated with unconjugated mAbs were incubated for 15 min with FITC- or PE-conjugated goat anti-mouse IgG (Becton Dickinson), goat anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), or goat anti-mouse IgM (Southern Biotechnology, Birmingham, AL). For stimulation experiments, peripheral blood mononuclear cells (PBMC) were isolated from 2 ml Li-H blood using lymphocyte separation medium (LSM®; ICN Biomedical, Aurora, OH). Cells for T-cell stimulation experiments were transferred into complete culture medium (Hepes-buffered RPMI-1640; GIBCO BRL, Glasgow, England) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, VA), 2 mM L-glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 20 mM 2-mercaptoethanol (2-ME; all from GIBCO BRL). Cells were seeded into 24-well flat-bottom plates (Greiner) at a concentration of 2×10^6/ml. Following this, cells were cultured for 3 days at 37°C and 5% CO_2 with 10 µg/ml PHA (Boehringer Mannheim GmbH, Mannheim, Germany) prior to staining. Stable growing B-cell lines from both species were generated by transformation of peripheral blood mononuclear cells (PBMC) with a cotton-top EBV (B95-8) as described previously (3).

mAbs

All mAbs described in this article are commercially available and were donated kindly by the participating companies: Beckman Coulter, Becton Dickinson, BenderMed Systems, Biotrend, CLB, Connex, Cymbus Biotech, Dako, Diaclone, Hoffman-La Roche, Pharmingen, Serotec, and Tanox Pharma BV. Cymbus Biotech and Pharmingen kindly donated isotype-matched FITC- and PE-labeled mAb as controls. All specificities, clone names, and isotypes are provided in Table 1.

FLOW CYTOMETRIC ANALYSIS

Single-labeled direct immunofluorescence was used to screen mAbs for cross-reactivity against NWM cell surface antigens. Immunofluorescence was measured on a four-color FACSort (Becton Dickinson). Data were obtained without pregating and, generally, 25,000 events were analyzed per sample. After gating T/B cell, monocyte, and granulocyte populations as determined by forward (FSC) and side scatter (SSC) characteristics, data analysis was performed retrospectively using CellQuest software (Becton Dickinson) to determine frequencies and fluorescence intensities using unstained 1% PBS/BSA-incubated cells as a first reference. To compensate for day-to-day differences as well as to set a reference point to determine whether up-regulation of cell surface markers occurred after PHA stimulation (T cells) or after EBV transformation (B cells), the FACSort was calibrated using calibration beads (Becton Dickinson). During the analysis, we discovered that fluorescein-conjugated mAbs from different suppliers could not be compared easily because the amount of fluorescein bound to a single mAb was highly variable. Hence, expression of reactivity by expression of the mean or median fluorescence intensities was considered noninformative. Moreover, the purpose of this study was to identify mAbs of sufficient cross-reactivity rather than to determine the extent of staining. The reactivity of the mAb was therefore scored as the percentage of positively staining cells using the signal on its matched isotype as well as by its staining pattern. In our panel, we found four basic patterns of reactivity. Typical examples are shown in Figure 1 and are represented as colored boxes in Table 1 (0, blank; type 1, yellow; type 2, purple; type 3, green). All

Table 1

Specificities, Clone Names, and Isotypes[a]

					common marmoset					cotton top tamarin				
CD	Number	Clone	Isotype	Label	Tiff cell	Monocyte	Granulo	CBV-B	PHA	Tiff cell	Monocyte	Granulo	CBV-B	PHA
CD2	2	RPA-2	IgG2a	PE	50-75	> 75	< 10	< 10	-	50-75	< 10	< 10	< 10	-
	4, 10	LT2	IgG2b	FITC	50-75	10-25	< 10	< 10	-	50-75	< 10	< 10	< 10	-
	5	CLB-T11/1	IgG1	FITC	10-25	< 10	< 10	< 10	-	50-75	< 10	< 10	< 10	-
	8	9-6F2	IgG2b	FITC	25-50	10-25	< 10	< 10	-	50-75	10-25	< 10	< 10	-
				PE	< 10	< 10	< 10	< 10	-	50-75	10-25	< 10	< 10	-
	9	9-6F2-10	IgG1	PE	50-75	> 75	< 10	10-25	-	50-75	10-25	10-25	< 10	-
CD3	3	SK7	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
				PE	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
	4*	F23	IgG2a	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
	5	CLB-T3/2	IgG2a	FITC	< 10	< 10	< 10	< 10	-	25-50	50-75	25-50	< 10	-
	6	UCHT1	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
	6-7			PE	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
	8	8-B19	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
				PE	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
	9	SP34	IgG2	FITC	50-75	25-50	25-50	< 10	-	50-75	10-25	10-25	< 10	-
				PE	50-75	25-50	25-50	< 10	-	50-75	10-25	10-25	< 10	-
CD4	1	13B8.2	IgG1	FITC	< 10	< 10	< 10		-	< 10	< 10	< 10		-
	2	SK3,SK4	IgG1	FITC	< 10	< 10	< 10		-	10-25	< 10	< 10		-
	3	SK3	IgG1	PE	10-25	< 10	< 10		-	10-25	< 10	< 10		-
				APC	10-25	< 10	< 10		-	10-25	< 10	< 10		-
	4*	LT4	IgG1	FITC	< 10	< 10	< 10		-	< 10	< 10	< 10		-
	5	CLB-T4/2	IgG1	FITC	< 10	< 10	< 10		-	10-25	< 10	< 10		-
	6	RPA-4g	IgG1	FITC	< 10	< 10	< 10			< 10	< 10	< 10		-
				PE	10-25	< 10	< 10			< 10	< 10	< 10		-
	7	MT-310	IgG1	FITC	25-50	< 10	< 10		-	25-50	< 10	< 10		-
				PE	25-50	< 10	< 10		-	25-50	< 10	< 10	< 10	-
				PE-Cy5	25-50	< 10	< 10		-	25-50	< 10	< 10		-
	8	9-F5	IgG1	FITC	< 10	< 10	< 10		-	< 10	< 10	< 10		-
				PE	< 10	< 10	< 10		-	< 10	< 10	< 10		-
	9	M-T477	IgG2a	FITC	< 10	< 10	< 10		-	< 10	< 10	< 10		-
CD5	1	BL1a	IgG2a	PE	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
	2	L17F12	IgG2a	PE	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
				APC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
	4*	LT1	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
	5	CLB-T1/1	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
	6	UCHT2	IgG1	FITC	50-75	< 10	< 10	50-75		25-50	< 10	< 10	< 10	
				PE	50-75	< 10	< 10	> 75		25-50	< 10	< 10	< 10	
	8	5H9	IgG1	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
				PE	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
	10	MT-7/6/5	IgG2a	FITC	< 10	< 10	< 10	< 10		< 10	10-25	< 10	< 10	
CD7	1	8H8.1	IgG2a	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
	2	4H9	IgG2a	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
				PE	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
	4	LT7	IgG2a	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
	5	CLB-3A1/1	IgG2a	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
	8	8H7	IgG1	FITC	50-75	50-75	50-75	10-25		> 75	> 75	> 75	10-25	
				PE	< 10	< 30	< 10	< 10		< 10	25-50	25-50	< 10	
	9	M-T701	IgG1	PE	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
CD8	1	2ST8.5H7	IgG2a	PE	< 10	< 10	< 10		-	< 10	< 10	< 10		-
	2	5H1	IgG2a	FITC	< 10	< 10	< 10		-	< 10	< 10	< 10		-
				PE	< 10	< 10	< 10		-	< 10	< 10	< 10		-
				APC	< 10	< 10	< 10		-	< 10	< 10	< 10		-
	4* 10	LT8	IgG1	FITC	10-25	< 10	< 10	< 10	-	10-25	< 10	< 10		-
	5	CLB-T3/4	IgG2b	FITC	10-25	< 10	< 10		-	10-25	< 10	< 10		-
	6	RPA-8g	IgG1	FITC	< 10	< 10	< 10		-	< 10	< 10	< 10		-
				PE	< 10	< 10	< 10		-	< 10	< 10	< 10		-
	8	UCHT4	IgG2a	FITC	10-25	< 10	< 10		-	10-25	10-25	10-25		-
				PE	25-50	10-25	< 10		-	< 10	< 10	< 10		-
	7	DK25	IgG1	PE	< 10	< 10	< 10		-	< 10	< 10	< 10	< 10	-
	8	5H9	IgG1	FITC	< 10	< 10	< 10		-	< 10	< 10	< 10		-
				PE	< 10	< 10	< 10		-	< 10	< 10	< 10		-
	9	RPA-T8	IgG1	PE	< 10	< 10	< 10		-	< 10	< 10	< 10		-
	11*	MHY054	IgM	FITC	10-25	< 10	< 10		-	10-25	< 10	< 10	< 10	-
				PE	10-25	< 10	< 10		-	10-25	< 10	< 10	< 10	-
CD10	1	ALB1	IgG1	PE	< 10	< 10	< 10	< 10	-	< 10	10-25	10-25	10-25	-
CD11b	1	2B.3	IgG1	FITC	25-50	25-50	< 10		-	50-75	> 75	> 75		-
	2	CLB-2	IgG2a	FITC	10-25	25-50	10-25		-	50-75	> 75	50-75		-
	3	R1	IgG1	FITC	50-75	50-75	50-75		-	25-50	50-75	25-50		-
	5	CLB-LPAM-1/3	IgG2a	FITC	< 10	< 10	< 10		-	< 10	10-25	< 10		-
	6	3B	IgG2a	FITC	25-50	50-75	50-75			< 10	25-50	< 10		-
				PE	25-50	> 75	> 75			< 10	10-25	< 10		-
	8	8-B15	IgG1	FITC	< 10	< 10	< 10			10-25	25-50	25-50		
				PE	< 10	< 10	< 10		-	< 10	< 10	< 10		-
	9	3B11	IgG1	PE	> 75	> 75	> 75			> 75	> 75	> 75		
	10	DP1024	IgG2b	FITC	10-25	> 75	> 75			< 10	25-50	25-50		
CD13	1	3B4.1	IgG1	FITC	< 10	> 75	> 75	< 10		< 10	25-50	> 75	< 10	
	2	D7	IgG2b	PE	< 10	> 75	> 75	< 10		< 10	10-25	10-25	< 10	
	3	LM07	IgG1	FITC	< 10	25-50	10-25	< 10	-	< 10	< 10	< 10	< 10	-
	5	CLB-Mono/gran1	IgM	FITC	< 10	25-50	50-75	< 10		< 10	< 10	< 10	< 10	
	9	ICRF44	IgG1	PE	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-

Specificities, Clone Names, and Isotypes[a] (continued)

CD11c	1	Ra/A	IgG1	PE	< 10	< 10	< 10	< 10	-	< 10	10-25	< 10	< 10	-
	2	S-HCL-3	IgG2b	PE	10-25	> 75	< 10	25-50		< 10	< 10	< 10	< 10	-
				APC	10-25	> 75	< 10	25-50		< 10	< 10	< 10	< 10	-
	3	3.9	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
				PE	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	-
	4	MC101	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
				PE	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	-
	5	Ra/6	IgG1	PE	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
CD13	1	3A1D1	IgG1	FITC	< 10	< 10	< 10			< 10	< 10	< 10		
	2	L138	IgG1	PE	< 10	< 10	< 10			< 10	< 10	< 10		
	3	C. Immunogen	IgG2a	FITC	< 10	< 10	< 10			< 10	10-25	< 10		
	4	WM15	IgG1	FITC	< 10	< 10	< 10			< 10	< 10	< 10		
				PE	10-25	10-25	< 10			< 10	< 10	< 10		
	5	8H10	IgG1	FITC	25-50	25-50	25-50			50-75	> 75	> 75		
				PE	< 10	< 10	< 10			< 10	10-25	< 10		
CD14	1	MEM-87	IgG2a	PE	< 10	< 10	< 10			< 10	< 10	< 10		
	2	LeuM3	IgG2b	FITC	< 10	< 10	< 10			< 10	< 10	< 10		
				PE	< 10	< 10	< 10			< 10	< 10	< 10		
				APC	< 10	< 10	< 10			< 10	< 10	< 10		
	3	CLB-mono/3	IgG2a	FITC	< 10	< 10	< 10			< 10	10-25	< 10		
	4	UCHM-1	IgG2a	FITC	< 10	< 10	50-75			< 10	25-50	< 10		
				PE	< 10	< 10	< 10			< 10	< 10	< 10		
	5	TÜK4	IgG1	FITC	10-25	10-25	75-90			25-50	> 75	> 75		
				PE	10-25	25-50	10-25			< 10	50-75	50-75		
	6	MEM-18	IgG2a	PE	< 10	> 75	50-75			< 10	< 10	10-25		
CD15	1	MMA	IgM	FITC	< 10	< 10	< 10			< 10	< 10	< 10		
	2	CLB-gran/2	IgM	FITC	< 10	< 10	< 10			< 10	< 10	< 10		
	3	28	IgM	FITC	< 10	< 10	< 10			< 10	< 10	< 10		
				PE	< 10	< 10	< 10			< 10	< 10	< 10		
CD16	1	3G8	IgG1	FITC	< 10	< 10	< 10			< 10	10-25	< 10		
	1.9			PE	10-25	10-25	< 10			< 10	10-25	< 10		
	2	MOP15	IgG1	FITC	< 10	< 10	< 10			10-25	25-50	10-25		
	3	1D3.1	IgG1	PE	< 10	< 10	< 10			< 10	< 10	< 10		
	5	CLB-FcgR/mAT	IgG2a	FITC	< 10	< 10	< 10			< 10	10-25	< 10		
	6	DJ130	IgG2a	FITC	10-25	25-50	25-50			25-50	> 75	> 75		
				PE	< 10	< 10	< 10			< 10	10-25	< 10		
CD18	2	L130	IgG1	FITC	10-25	25-50	10-25			25-50	25-50	25-50		
	5	CLB-LFA-1/1	IgG1	FITC	< 10	< 10	< 10			10-25	50-75	25-50		
	6	6.7	IgG1	PE	> 75	> 75	> 75			> 75	> 75	> 75		
	80	YFC118.3	IgG2b	FITC	< 10	< 10	< 10			< 10	10-25	< 10		
CD19	1	J4.119	IgG1	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
				PE	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
	2	HD37	IgG1	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
				PE	25-50	50-75	50-75	10-25		50-75	50-75	50-75	25-50	
				APC	25-50	50-75	50-75	10-25		50-75	50-75	50-75	25-50	
	2.8	SJ25C1	IgG1	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
	2.8			PE	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
	7			APC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
	3	CLB-172	IgG1	PE	< 10	< 10	< 10	< 10						
	7	HD37	IgG1	FITC	< 10	< 10	< 10		-	< 10	< 10	< 10	< 10	-
	8	B43	IgG1	PE	< 10	< 10	< 10	10-25		< 10	< 10	< 10	< 10	
				FITC	< 10	< 10	< 10	< 10		< 10	25-50	10-25	< 10	
	10	FMC63	IgG2a	FITC	< 10	< 10	< 10	< 10		< 10	25-50	< 10	< 10	
CD20	1	2H7	IgG2a	FITC	10-25	< 10	< 10	> 75		25-50	50-75	25-50	> 75	
				RD1	10-25	25-50	50-75	> 75		25-50	50-75	50-75	> 75	
	1.6	B1/B9	IgG2a	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
	6			PE	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
	7	L27	IgG1	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
				PE	10-25					10-25				
				APC	10-25					10-25				
	5	IMG-944	IgG1	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
	6	BC-1	IgM	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
				PE	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
	8	4A20	IgG2a	FITC	< 10	< 10	< 10	< 10		< 10	10-25	< 10	< 10	
				PE	< 10	< 10	< 10	< 10		10-25	10-25	< 10	< 10	
	9	2H7	IgG2b	PE	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
CD21	1	BL13	IgG1	FITC	< 10	< 10	< 10	< 10		10-25	< 10	< 10	< 10	
	4	LB21	IgG1	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
	5	BU33	IgG1	FITC	10-25	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
				PE	10-25	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
	8	BE5	IgG2a	FITC	< 10	< 10	< 10	10-25		10-25	25-50	< 10	< 10	
				PE	< 10	< 10	< 10	< 10		< 10	25-50	< 10	< 10	
	9	2H7	IgG1	PE	10-25	< 10	< 10	< 10		10-25	< 10	< 10	< 10	
CD22	2	S-HCL-1	IgG2b	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
				PE	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
	5	CLB-HuLy8	IgG1	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	
	8	RFB4	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
				PE	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
	10	Mo-bl-12	IgG1	FITC	< 10	< 10	< 10	< 10		< 10	10-25	< 10	< 10	
CD23	2	EBVCS-5	IgG1	PE	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
	6	Tü1	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
				PE	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
	8	BG6	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-
				PE	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-

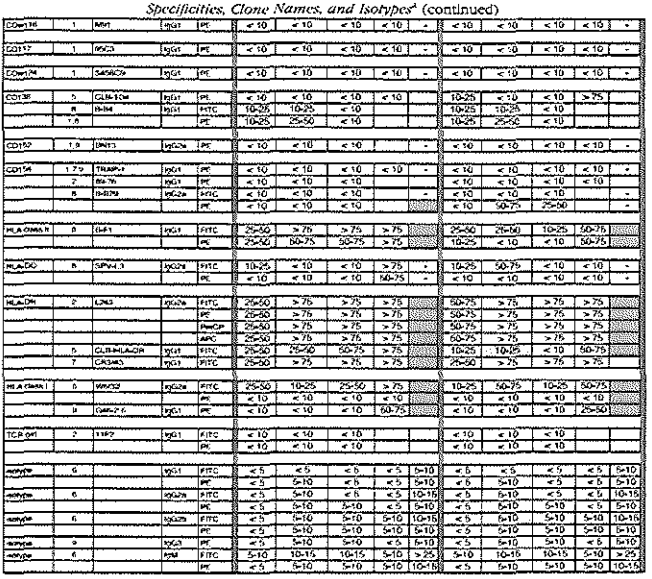
Specificities, Clone Names, and Isotypes[a] (continued)

CD24	1	ALB9	IgG1	PE	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10			
	5	[illegible]	IgG1	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10			
	10	SN3	IgG1	FITC	10-25	< 10	< 10	< 10		10-25	< 10	< 10	< 10			
CD25	1	B1.49.9	IgG2a	PE	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-		
	2	2A3	IgG1	FITC	< 10	< 10	< 10	> 75		< 10	< 10	< 10	10-25			
	10			PE	< 10	< 10	< 10	> 75		< 10	< 10	< 10	< 10			
	2			APC												
	5	[illegible]	IgG2b	FITC	25-50	25-50	50-75	> 75		25-50	50-75	50-75	10-25			
	6	Tu66	IgG1	FITC	< 10	< 10	< 10	> 75		< 10	< 10	< 10	10-25			
				PE	< 10	< 10	< 10	> 75	-	< 10	< 10	< 10	10-25	-		
	6	B4H6	IgG1	FITC	< 10	< 10	< 10	10-25		< 10	< 10	< 10	< 10			
				PE	< 10	< 10	< 10	> 75		< 10	< 10	< 10	50-75			
	12	7G7	IgG1	PE	< 10	< 10	< 10	50-75		10-25	10-25	< 10	< 10			
	12	MAT	IgG1	FITC	10-25	< 10	< 10	> 75		25-50	25-50	< 10	25-50			
				PE	10-25	< 10	< 10	> 75		25-50	25-50	< 10	25-50			
CD26	2	L272	IgG2a	PE	25-50	50-75	> 75			10-25	25-50	25-50				
	3	M-A261	IgG1	FITC	< 10	< 10	< 10		-	< 10	< 10	< 10		-		
	6	1G4-2C2	IgM	FITC	< 10	< 10	< 10		-	< 10	< 10	< 10		-		
				PE	< 10	< 10	< 10		-	< 10	< 10	< 10		-		
	7	M-A5	IgG1	FITC	10-25	25-50	> 75		-	10-25	50-75	> 75	< 10	-		
CD27	2	L128	IgG1	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10			
				PE	25-50	< 10	< 10	< 10		25-50	< 10	< 10	< 10			
	4, 10	L127	IgG1	FITC	25-50	< 10	< 10	< 10		< 10	< 10	< 10	< 10			
	9	M-T271	IgG1	PE	50-75	< 10	< 10	< 10		< 10	< 10	< 10	< 10			
CD28	2	L293	IgG1	PE	< 10	< 10	< 10		-	10-25	< 10	< 10		-		
	4	CLB-CD28/1.7.2	IgG1	FITC	< 10	< 10	< 10		-	< 10	< 10	< 10		-		
	8	9.3	IgG2a	FITC	25-50	10-25	10-25			> 75	50-75	25-50				
				PE	25-50	< 10	< 10			50-75	10-25	< 10				
	9	CD28.2	IgG1	FITC	< 10	< 10	< 10		-	< 10	< 10	< 10		-		
CD29	6	TDM29	IgG1	FITC	25-50	50-75	10-25		-	25-50	25-50	25-50		-		
				PE	< 10	< 10	< 10		-	< 10	< 10	< 10		-		
	8	8-D15	IgG2a	FITC	10-25	25-50	25-50			25-50	50-75	> 75				
				PE	10-25	10-25	10-25			50-75	25-50	< 10				
	9	MAR4	IgG1	PE	< 10	< 10	< 10		-	< 10	< 10	< 10		-		
CD31	1	5.6E	IgG1	FITC	10-25	10-25	< 10	< 10	-	10-25	50-75	10-25	< 10	-		
CD32	9	[illegible]	IgG2b	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-		
	10	AT10	IgG1	FITC	< 10	< 10	< 10	< 10		< 10	10-25	< 10				
CD33	1	D3HL60.251	IgG1	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10			
	2	P67.6	IgG1	PE	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10			
				APC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10			
	5	CLB-MO33.8	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-		
	6	WM53	IgG1	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10			
				PE	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10			
CD34	8,9	581	IgG1	FITC	10-25	< 10	< 10	< 10	-	25-50	10-25	25-50	< 10			
	8			PE	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-		
CD35	6	3A9	IgM	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-		
CD37	1	BL14	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-		
	10	WR17	IgG2a	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-		
CD38	1	T16	IgG1	FITC	< 10	< 10	< 10	< 10	-	25-50	< 10	< 10	< 10	-		
	2	HIT2	IgG1	PE	< 10	< 10	< 10	< 10	-	10-25	< 10	< 10	< 10	-		
	10	AT1/5/5	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-		
CD40	6	5-D/5	IgG1	FITC	25-50	< 10	< 10	> 75		10-25	10-25	< 10	> 75	-		
				PE	< 10	< 10	< 10	50-75		< 10	< 10	< 10	> 75	-		
	9	5C3	IgG1	PE	< 10	< 10	< 10	> 75		10-25	< 10	< 10	> 75	-		
	10	LOB7/6	IgG1	FITC	< 10	< 10	< 10	< 10		< 10	25-50	10-25	< 10	-		
	11*	ADI1	IgG4	FITC	10-25	> 75	< 10	> 75	-	10-25	> 75	< 10	> 75	-		
CD41	1	SP2	IgG1	PE	25-50	25-50	25-50	< 10	-	25-50	25-50	25-50	< 10	-		
CD42b	1	N7	IgG2a	FITC	10-25	10-25	25-50	< 10	-	25-50	25-50	25-50	< 10	-		
CD42b	1	N29	IgG1	PE	10-25	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-		
CD43	6	DF1	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-		
				PE	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10			
CD44	6	IM-7	IgG1	FITC	50-75	> 75	> 75	> 75		> 75	> 75	> 75	> 75			
	8	IM-7.8	IgG1	FITC	> 75	50-75	> 75	> 75		> 75	> 75	> 75	> 75			
	10	F10-44-2	IgG2a	FITC	50-75	> 75	50-75	50-75	-	> 75	> 75	> 75	> 75	-		
CD45	1	J.33	IgG1	PE	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10			
	1	IMMU19.2	IgG1	PE	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-		
	2	2D1	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-		
	4*	9.4	IgG2b	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10	-		
	5	GLM-2/2001	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-		
	6	MV5/1	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-		
				PE	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-		
	7	2B11	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-		
	7	T29/33	IgG1	PE	< 10	< 10	< 10	< 10	-	< 10	< 10	10-25	< 10	-		
	8	9.4A11	IgG1	FITC	25-50	50-75	50-75	< 10		> 75	> 75	> 75	25-50			
				PE	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10			
	9	TU116	IgG1	PE	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-		
	10	F10-89-4	IgG2b	FITC	< 10	< 10	< 10	< 10		10-25	< 10	< 10	< 10			

Specificities, Clone Names, and Isotypes[a] (continued)

CD45RA	1	D46	IgG1	FITC	25–50	< 10	< 10	< 10		< 10	< 10	< 10	< 10		
	1	ALB11	IgG1	FITC	25–50	10–25	< 10	> 75		< 10	10–25	< 10	< 10		
				PE	25–50	< 10	< 10	> 75		< 10	< 10	< 10	< 10		
	4[b]	L748RA	IgG2b	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10		
	6	F8-11-13	IgG1	FITC	25–50	< 10	< 10	> 75		< 10	< 10	< 10	< 10		
				PE	25–50	< 10	< 10	> 75		< 10	< 10	< 10	10–25		
	8	B-C15	IgG2a	FITC	50–75	50–75	50–75	> 75		> 75	> 75	> 75	25–50		
				PE	25–50	10–25	< 10	> 75		10–25	< 10	< 10	< 10		
	9	1A10	IgG1	PE	25–50	< 10	< 10	> 75		< 10	< 10	< 10	< 10		
	10	MEM6	IgG1	Cy5	50–75	50–75	10–75	> 75		50–75	50–75	50–75	> 75		
CD45RO	6	UCHL1	IgG2a	FITC	< 10	< 10	< 10			< 10	10–25	< 10			
	1,2,5			PE	< 10	< 10	< 10			< 10	< 10	< 10	< 10		
CD45R0	1	Cy8	IgG1	FITC	10–25	25–50	10–25	< 10		10–25	10–25	10–25	< 10		
CD45RB	1	MB21	IgG1	FITC	< 10	< 10	< 10	< 10	-	> 75	25–50	< 10	25–50		
CD46	1	3A11	IgG2b	FITC	10–25	25–50	< 10	< 10		10–25	50–75	10–25	10–25		
CD51	6	23C2	IgG1	FITC	< 10	< 10	< 10			< 10	< 10	< 10			
				PE	< 10	< 10	< 10			< 10	< 10	< 10			
CD54	1	84H10	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	
	7	LB-2	IgG2b	PE	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	
	1	RR1/1	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	
	6	11.2	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	
				PE	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10		
	8	3H7	IgG1	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10		
CD56	1	NKH1	IgG1	PE	10–25	< 10	< 10	10–25		10–25	10–25	10–25	25–50		
	7	NCAM16.2	IgG2b	FITC	25–50	< 10	< 10	10–25		< 10	< 10	< 10	25–50		
				PE	25–50	< 10	< 10	10–25		< 10	< 10	10–25	25–50		
	6	MFM18	IgG2a	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	
				PE	< 10	< 10	< 10	25–50		< 10	< 10	< 10	10–25		
	8	B-A31	IgG1	PE	< 10	< 10	< 10	10–25		< 10	< 10	< 10	10–25		
CD57	1	NC1	IgM	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	
CD58	1	AICD58	IgG2a	FITC	< 10	< 10	< 10			10–25	10–25	10–25			
	2	L306.4	IgG2a	PE	25–50	> 75	> 75			> 75	> 75	> 75			
	9	1C3/AICD58.6)	IgG2a	PE	> 75	> 75	> 75		-	> 75	> 75	> 75		-	
CD61	1	DIG.G.56	IgG1	FITC	< 10	< 10	< 10			< 10	< 10	< 10			
	2	1N13	IgG2a	PE	< 10	< 10	< 10			< 10	< 10	< 10			
	3	DIG.G.56	IgG1	FITC	25–50	10–25	> 75		-	25–50	25–50	> 75		-	
	5	FMC46	IgG2b	FITC	< 10	< 10	< 10			< 10	< 10	< 10			
				PE	< 10	< 10	25–50			10–25	10–25	> 75			
CD63	1	NKIM37	IgG1	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10		
CD64	1	22	IgG1	FITC	< 10	< 10	< 10			< 10	< 10	< 10			
CD65s	1	80H3	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	
CD69	1	TP1.55.3	IgG2b	FITC	< 10	< 10	< 10			< 10	< 10	< 10			
				PE	< 10	< 10	< 10		-	< 10	< 10	< 10		-	
	2	L78	IgG1	FITC	< 10	< 10	< 10		-	< 10	< 10	< 10		-	
				PE	< 10	< 10	< 10		-	< 10	< 10	< 10		-	
				APC	< 10	< 10	< 10		-	< 10	< 10	< 10		-	
	9	FN50	IgG1	PE	< 10	< 10	< 10		-	< 10	< 10	< 10		-	
	10	CD69	IgG2a	FITC	< 10	< 10	< 10			< 10	25–50	10–25			
CD71	1	YDJ1.2.2	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	
	10	DF1513	IgG1	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10		
CD80	1	MAB104	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	
	6	B5-1	WM	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	
				PE	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10		
CD83	1	HB15a	IgG2b	PE	< 10	< 10	< 10	10–25	-	< 10	< 10	< 10	10–25	-	
CD86	8	IT2	IgG1	FITC	25–50	25–50	50–75	> 75		50–75	> 75	> 75	> 75		
				PE	< 10	< 10	< 10	50–75	-	< 10	< 10	< 10	25–50	-	
	9	IT2.2	IgG2b	PE	< 10	< 10	< 10	> 75		< 10	< 10	< 10	> 75		
CD90	1	F15-42-1-5	IgG1	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	
CD94	1	HP-3B1	IgG2a	PE	< 10	< 10	< 10		-	< 10	< 10	< 10		-	
CD95	1	DR2	IgG1	FITC	10–25	25–50	25–50	10–25		< 10	25–50	10–25	< 10	-	
	2,9	DX2	IgG1	PE	< 10	< 10	< 10	50–75	-	10–25	< 10	< 10	> 75	-	
	7			APC	< 10	< 10	< 10	50–75		< 10	< 10	< 10	50–75		
	3	APO1-1	IgG1	FITC	< 10	< 10	< 10	< 10		25–50	> 75	50–75	10–25	-	
	1	SM1/23	IgG2b	FITC	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	
	3	APO1-3	IgG3	FITC	25–50	10–25	> 75	25–50	-	< 10	< 10	< 10	< 10	-	
	6	FAS19	IgG1	FITC	10–25	< 10	10–25	> 75		< 10	< 10	< 10	< 10		
	8	B-A24	IgG1	FITC	25–50	25–50	25–50	50–75		25–50	> 75	> 75	< 10		
	8	B-D27	IgG2b	FITC	< 10	< 10	< 10	< 10		< 10	25–50	< 10	< 10		
				PE	< 10	< 10	< 10	< 10		< 10	25–50	< 10	< 10		
CD102	3	CD54-2D2	IgG2a	FITC	< 10	< 10	< 10	< 10	-	< 10	25–50	< 10	< 10	-	
	8	IH-11	IgG1	FITC	< 10	< 10	< 10	< 10		< 10	< 10	< 10	< 10		
CD103	1	2G5	IgG2a	FITC	< 10	< 10	< 10	< 10	-	< 10	10–25	< 10	< 10	-	

Specificities, Clone Names, and Isotypes[a] (continued)



[a]Providers: 1, Beckman Coulter; 2, Becton Dickinson; 3, BenderMed; 4, Biotrend; 5, CLB; 6, Cymbus Biotech; 7, DAKO; 8, Diaclone; 9, Pharmingen; 10, Serotec; 11, Connex; 12, Hoffmann-La Roche; 13, TANOX Pharma BV. Labels: All mAbs were labeled directly with either FITC, PE, or APC unless indicated otherwise. T and B cells (T/B cell), monocytes or large granular lymphocytes (Mono/LGL), and granulocytes (Granulo) populations were gated on the basis of their FSC and SSC characteristics and analyzed in detail. All data presented were corrected for nonspecific background staining because some isotype-matched and/or subclass-matched mAbs showed a type 1 staining pattern (yellow) when compared with unstained, PBS/BSA-incubated cells. For sake of completeness, these percentages are provided in the table. The results are expressed as the percentage of positive cells and graded as <10, 10–25, 25–50, 50–75, >75% positivity. Blank boxes without percentages: not tested. The type of reaction is expressed as blank boxes (type 0), yellow boxes (type 1), purple boxes (type 2), or green boxes (type 3) according to the staining patterns described in Figure 1. EBV-B: Stable-growing B-cell lines from both species were generated by transformation of PBMC with a cotton-top EBV and they were used to test common B-cell markers in more detail. PHA: Cells for T-cell stimulation experiments were cultured for 3 days at 37°C and 5% CO_2 in the presence of 10 μg/ml PHA prior to staining. Blue boxes indicate an upregulation (amount and/or intensity) of the gated cell population after PHA stimulation; –, no increased signal intensity after PHA stimulation. EDTA-K3 blood was used for whole blood analysis. Li-H as anti-coagulant was used for PHA stimulation of T cells.

[b]Indirectly labeled mAbs.

data in Table 1 were corrected for nonspecific staining using isotype and subclass-matched mAbs. However, for the sake of completeness, these results are given in the legend to Table 1.

Ethics

The protocol of this study was reviewed and approved by the institute's animal experimentation committee. All experimental procedures are in accordance with the guidelines of the committee and with Dutch law on animal experimentation.

RESULTS AND DISCUSSION

Similar to human T-cell receptors (TCR), cytokines and the MHC have been reported widely for both the common marmosets and the cotton-top tamarin (1–7). Consequently, these species have been used extensively to model human diseases and to test the therapeutic efficacy of novel compounds, especially those with exclusive primate reactivity. This study was undertaken in an attempt to expand the list of suitable mAbs for their applicability in both species using flow cytometric analysis. These reagents are critical for examining the cells that are instrumental in the immunological pathways of human disease.

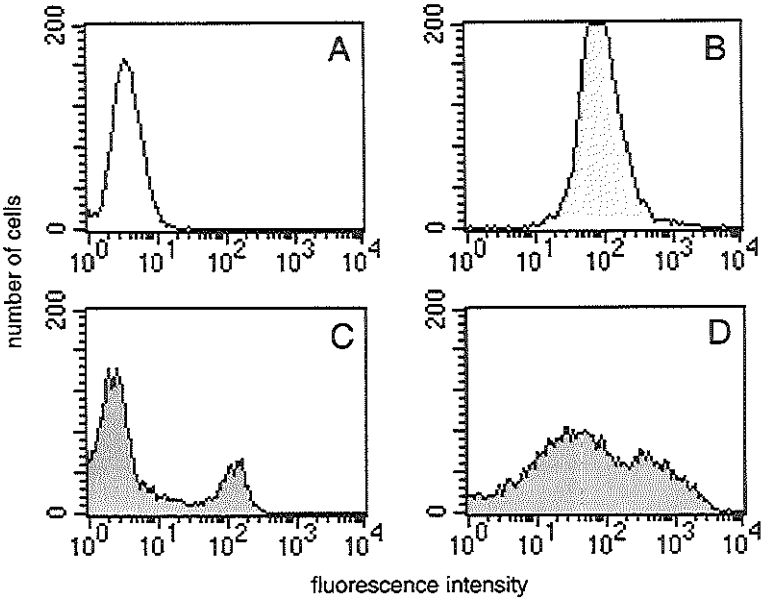


FIG. 1. Histogram plots showing the four fluorescence profiles found in cell samples of the common marmoset and the cotton-top tamarin. Base levels (type 0) were set between fluorescence intensity 10^0 and 10^1 (blank boxes in Table 1). Type 1 reactions were characterized by a complete shift of the analyzed subpopulation (yellow boxes in Table 1). Type 2 was characterized by a peak basal level in combination with a second peak between 10^1 and 10^4 (purple boxes in Table 1). Cells that showed scattered positivity throughout the FL channels, for instance, those observed after MHC class II staining of whole blood, were typed as type 3 reactivity (green boxes in Table 1).

Although several reports have been published for the common marmoset (26,27), data are very limited in the cotton-top tamarin (23–25). The results of this study are summarized in Table 1.

The panel of mAbs tested was raised originally against more than 80 CD-defined molecular structures present on human cells. From the 331 mAbs analyzed, 126 showed positive cross-reactivity with PBMC from the common marmoset (38%) and 152 were positive when tested on cotton-top tamarin cells (46%). After stimulation with the T-cell mitogen PHA, 85 and 76 mAbs showed increased signal intensity with marmoset and cotton-top tamarin PBMC, respectively. Because the T cells in these cultures are enriched preferentially and only one single-labeled mAb per sample was analyzed, little can be said about the relative percentage of cells that show positive staining. However, activation markers like CD25, CD69, and HLA class II were upregulated. Most mAbs that showed good cross-reactivity were also suitable on cryotome-prepared frozen tissue sections (Brok et al., unpublished data). Clone H299 (Beckman Coulter), which recognized CD20, showed good reactivity on B cells. However, we also detected binding to PHA-stimulated T cells, a finding at variance with published data (26). Although this could be the result of nonspecific T-cell binding, no increased background staining was observed with the isotype mAb. Notably, CD20 has been identified as an activation marker on T cells in simian immunodeficiency virus (SIV)-infected rhesus monkeys (28). Positive staining was also found after PHA activation of PBMC from the owl monkey (*Aotus lemurinus griseimembra*; Gonzalez et al., unpublished data).

In this study, directly labeled mAbs conjugated to either FITC, PE, or APC were used in almost all cases. T-cell specific markers like CD2 and CD3 also showed positive staining with the monocyte and/or granulocyte fraction, which is in contrast to the condition in humans. Nevertheless, these mAbs are useful tools for T-cell identification because a positive signal was not found on EBV-transformed B cell lines (Table 1). Moreover, no cross-reactivity of CD2 or CD3 was found on the B-cell population (unpublished observations) after gating of the

T/B-cell fraction of whole blood and double staining using CD20 (clone H299, Beckman Coulter) or CD19 (4G7, Becton Dickinson).

The technical issue that a clear effect of the fluorochrome on the reactivity patterns of some of the mAbs was found needs further discussion. For example, in the common marmoset, CD7 (B-F12; Diaclone) showed positive cross-reactivity only when conjugated with FITC. In contrast, CD19 (4G7; Becton Dickinson) showed positive cross-reactivity when conjugated with PE or APC, but not with FITC. Although different labeling methods are available for coupling the fluorescein to the mAb, to our knowledge, no satisfactory explanation has been published despite the fact that this phenomenon has been reported frequently (23,26,27). One could envisage that under certain experimental conditions, quenching or oxidation/reduction of the fluorochrome can take place. However, this educated guess clearly requires more experimentation, which is outside the scope of this study.

Some PerCp-conjugated mAbs showed positivity. Moreover, of the nine APC-conjugated mAbs tested, all gave a similar staining pattern to those labeled with PE. This demonstration that triple or even quadruple staining of PBMC is now possible may have wide implications because it enables more in-depth analysis of minority cell populations. Although the list of tested APC-labeled mAbs remains small, it may be prudent to test other available APC-labeled mAbs when PE-labeled mAbs gave positive results.

In general, the data presented here show a very good correlation with published data (26). Although some clones in our test panel gave comparable staining patterns, a preferential selection can be made based on signal quality. For example, the CD4 clone MT-310 (Dako) and clone SK3 (Becton Dickinson) both detect $CD4^+$ T cells. MT-310 provides a more distinct positive cell population for both species tested and, therefore, would be the preferred mAb of choice.

Due to the low background staining, the CD8 clone LT8 (IgG_1; Serotec) would be the preferred reagent over CD8 clones 5-T8/4 and 4H8 (IgG_{2b}) from CLB or clone MT-1014 (indirectly labeled anti-IgM; Connex). When available, mAbs of the IgG_1 subclass are generally the antibodies of choice, although the nonspecific reactivity is generally low for most subclasses on resting cells. However, after (mitogen) stimulation in vitro, nonspecific, Fc-tail-mediated binding to PBMC might be increased especially when IgG_{2a} and IgG_{2b} subclasses are used.

This study has identified good cross-reactive mAbs for CD3, CD13, CD27, CD40, CD41, CD42a, CD42b, CD45RO, CD138, CD154, and HLA-DQ, which were not shown previously to be positive in common marmosets. Also, numerous new mAbs have been described for their use in the cotton-top tamarin. mAbs against CD25, CD45RO, CD69, CD71, CD95, HLA class II, and HLA-DR (and CD154 in common marmosets) were suitable for detection of activated T-cell (sub)populations. As anticipated, EBV-transformed and immortalized B-cell lines from both species showed a very distinct staining pattern, indicating suitability to prescreen general B-cell specific markers before their use in whole blood analysis.

Despite the fact that common marmosets and cotton-top tamarins are very closely related NWM species, different cross-reactivities of mAbs between the two species were found. As examples, CD11c (S-HCL-3 clone; Becton Dickinson) showed good cross-reactivity in common marmosets, although it was negative when tested on PBMC from cotton-top tamarins. CD3 (CLB-T3/2 clone; CLB) and CD4 (CLB-T4/2) are positive in cotton-top tamarins, but show no staining on marmoset PBMC. These results indicate that although both species are closely related phylogenetically, significant differences on epitope conservation have evolved on cell surface molecules.

Combined with the data from Neubert et al. (26), the majority of CD markers can now be defined in the PBMC of both NWM species. However, the list is by no means complete. Still, no cross-reactive mAbs have been identified for CD10, 15, 37, 64, 66b, w90, 103, w116, 117, and 152 in the common marmoset and for CD15, 23, 33, 36, 37, 42b, 51, 64, 66b, w90, 94, w116, 117, w124, 152, and 154 in the cotton-top tamarin. However, many new clones were not included in this panel and the list of (differently labeled) mAbs for CD markers continues to grow.

In conclusion, we have identified a large panel of cross-reactive mAbs for use in the cotton-top tamarin and the common marmoset. These mAbs may serve as essential tools in the investigation of specific cell (sub)populations believed to be intrinsic in the immunopathogenesis of human disease modeled in both these NWM species.

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2.
Immunopathogenesis of EAE in the common marmoset

Myelin/Oligodendrocyte Glycoprotein-Induced Autoimmune Encephalomyelitis in Common Marmosets: The Encephalitogenic T Cell Epitope pMOG24–36 Is Presented by a Monomorphic MHC Class II Molecule[1]

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Immunization of common marmosets (*Callithrix jacchus*) with a single dose of human myelin in CFA, without administration of *Bordetella pertussis*, induces a form of autoimmune encephalomyelitis (EAE) resembling in its clinical and pathological expression multiple sclerosis in humans. The EAE incidence in our outbred marmoset colony is 100%. This study was undertaken to assess the genetic and immunological basis of the high EAE susceptibility. To this end, we determined the separate contributions of immune reactions to myelin/oligodendrocyte glycoprotein (MOG) and myelin basic protein to the EAE induction. Essentially all pathological features of myelin-induced EAE were also found in animals immunized with MOG in CFA, whereas in animals immunized with myelin basic protein in CFA clinical and pathological signs of EAE were lacking. The epitope recognition by anti-MOG Abs and T cells were assessed. Evidence is provided that the initiation of EAE is based on T and B cell activation by the encephalitogenic phMOG14–36 peptide in the context of monomorphic Caja-DRB*W1201 molecules. *The Journal of Immunology*, 2000, 165: 1093–1101.

Immunization of common marmosets, a neotropical monkey species, with human myelin in CFA induces a form of autoimmune encephalomyelitis (EAE)[3] that both clinically and pathologically resembles human multiple sclerosis (MS) (1–3). The disease has a relapsing/remitting or progressive course. Radiological and neuropathological analysis of the CNS during clinically active EAE shows demyelinated lesions at different stages, including early active, inactive, and remyelinating lesions. The susceptibility of common marmosets to myelin-induced EAE appears remarkably high in view of the outbred nature of our colony, namely 100% ($n > 75$), although the clinical expression of the disease appeared to differ between individuals (3). In comparison, in a group of 23 randomly selected rhesus monkeys from the outbred colony at the Biomedical Primate Research Centre ($n > 1000$), 15 developed EAE after immunization with human myelin in CFA. Eight monkeys remained completely asymptomatic (4). We undertook the present study to investigate whether the EAE susceptibility of common marmosets has an immunological and/or genetic explanation.

The molecular analysis of the *Mhc* class II genes of the common marmoset revealed functional *Mhc-DR* and *-DQ* regions, and an apparently inactivated *Mhc-DP* region (5). On basis of the number of alleles found, it was concluded that the *Caja-DQA* and *-DQB* loci are oligomorphic. Moreover, three *Caja-DRB* loci were found: two loci with limited polymorphism (*Caja-DRB1*03* and *Caja-DRB*W16*) and one monomorphic locus (*Caja-DRB*W12*). All common marmosets that we have analyzed thus far appeared to share the *Caja-DRB*W1201* allele ($n > 75$). We hypothesized therefore that Caja-DRB*W1201 molecules may function as a major restriction element in the immunopathogenesis of EAE.

The clinical and pathological expression of myelin-induced EAE in marmosets is thought to result from a synergy of cellular and humoral autoimmune reactivity predominantly directed against two Ags, namely myelin basic protein (MBP) (2, 6, 7) and myelin/oligodendrocyte glycoprotein (MOG) (7–9). Hence, for the purpose of the present study, three marmoset twin couples were randomly selected from our colony; one sibling of each twin was immunized with recombinant human MOG (rhMOG), and the other sibling was immunized with purified human MBP (hMBP). The myelin Ags were emulsified in CFA, but usage of *Bordetella pertussis* was avoided. The immune systems of twin siblings can be regarded as highly similar given that they are complete bone marrow chimeras due to the sharing of the placental blood stream *in utero* (10). The cellular and humoral autoimmune responses and the development of clinical and pathological signs of EAE were assessed.

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[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; LNC, lymph node cells; LFB, Luxol Fast Blue; MBP, myelin basic protein; hMBP, human myelin basic protein; MRI, magnetic resonance imaging; MS, multiple sclerosis; rhMOG, recombinant human myelin/oligodendrocyte glycoprotein; PAS, periodic acid-Schiff; phMOG, human MOG peptide; T_2-w, T_2-weighted.

Table I. *Distribution of* Caja-DRB *alleles in the selected panel of marmosets*[a]

Animal Code	Sex	Birth Date (mo/yr)	Weight (g)	*Caja-DRB1*03*	*−DRB*W16*	*−DRB*W12*
9501	M	1/95	291	01/03/07	1604	1201
9502	M	1/95	302	03/05/07	1609	1201
9601	M	1/96	310	03	1605	1201
9602	M	1/96	290	02/03	1605	1201
Escudo	M	5/95	359	01/05	1601/1611	1201
Estrada	F	5/95	347	01/03/05	1601/1611	1201
9347	M	6/93	—	02	1606/1607	1201
9328	F	6/93	—	01/04	1609	1201

[a] Twins 9501/9502 and 9601/9602 were bred and raised at the Biomedical Primate Research Centre, and twins Escudo/Estrada were purchased from the Max von Pettenhofer Institute (Munich, Germany). The sex, birth dates, and body weights at the start of the study of the monkeys are given. Marmosets 9501, 9601, and Escudo were immunized with 100 μg rhMOG/CFA. Animals 9502, 9602, and Estrada were immunized with 1 mg hMBP/CFA. B cell lines from animals 9437 and 9328 were included in the test panel of APC to elucidate the MHC restriction element of phMOG14-36. The presence of *Caja-DRB*W1201* was also confirmed by denaturing gradient gel electrophoresis.

The results show that the rhMOG-immunized monkeys develop severe clinical EAE with specific demyelination of the CNS. The hMBP-immunized monkeys remained asymptomatic and also lacked pathological signs of EAE. The MOG-immunized monkeys proved to share a proliferative T cell response to the same MOG peptide (phMOG14–36), which was found to induce clinical and pathological signs of EAE in four of four monkeys. T cell reactivity to other MOG peptides varied between individual animals.

Activation of phMOG14–36-specific T cell lines appears restricted by the Caja-DRB*W1201 molecule. Anti-MOG IgG molecules appear to bind to peptides contained in two domains of the extracellular domain of MOG, namely between aa 4 and 40 and aa 44 and 76. Because the *Caja-DRB*W1201* allele is present in all monkeys, it is concluded that the 100% incidence of demyelinating EAE in an outbred colony of common marmosets can be explained by a uniform immune response to a single encephalitogenic peptide as EAE initiating event.

Materials and Methods

Animals

Three marmoset twin couples and four single monkeys were randomly selected from the outbred colony at the Biomedical Primate Research Centre. The sex and birth dates (month/year) of the monkeys are depicted in Table I. During experiments, monkeys were housed individually in spacious cages with padded shelters provided in the cage. The daily diet consisted of food pellets for nonhuman primates (Hope Farms, Woerden, The Netherlands), supplemented with rice, raisins, peanuts, marshmallows, biscuits, and fresh fruit and vegetables. Drinking water was provided *ad libitum*.

Typing of Caja MHC class II

Lymphoblastoid B cell lines were generated by transformation of PBMCs with a cotton-top tamarin EBV (B95-8). Genomic DNA was isolated from stable growing B cell lines, and exon 2 of the *Caja-DRB* genes was amplified by PCR (5). Sequence analysis was performed on an ABI prism 310 Genetic analyzer (Perkin-Elmer Applied Biosystems, Foster City, CA) using the ABI Prism dRhodamine terminator cycle sequencing ready reaction kit (Perkin-Elmer). The *Caja-DRB* alleles depicted in Table I represent the consensus sequence of at least four separate clones. All 75 common marmosets tested thus far share the previously described *Caja-DRB*W1201* allele (5).

EAE induction

hMBP was isolated and purified from normal donor brain as described (11). Three animals (9502, 9602, Estrada) were immunized with 1 mg hMBP, emulsified in CFA (Difco Laboratories, Detroit, MI). *Escherichia coli*-derived rhMOG, representing the N-terminal extracellular domain of human MOG (aa 1–123), was purified as previously described (12). Three animals (9501, 9601, Escudo) were immunized with 100 μg rhMOG as an emulsion in CFA.

Under ketamine anesthesia (6 mg/kg, AST Farma, Oudewater, The Netherlands), all monkeys were injected into the dorsal skin with 600 μl emulsion divided over four locations, two in the inguinal and two in the axillary region. *B. pertussis* has been used by others to facilitate development of EAE in marmosets (1, 2, 6–9). In our hands, *B. pertussis* administration is not essential for myelin or MOG-induced EAE in marmosets. Moreover, injection of *B. pertussis* around the time of encephalitogenic challenge results in necrotic lesions in the CNS of marmosets (3).

The four single monkeys were immunized with 100 μg of the synthetic MOG peptide phMOG14–36, also emulsified in CFA, and received booster immunizations with 50 μg phMOG14–36 in IFA after 7, 9, and 12 wk.

Clinical diagnosis

A trained observer recorded daily the clinical course of EAE using a previously described semiquantitative scale (3): 0, no clinical signs; 0.5, apathy, loss of appetite, altered walking pattern without ataxia; 1, lethargy and/or anorexia; 2, ataxia; 2.5, mono- or paraparesis and/or brain stem syndrome; 3, hemi- or paraplegia; 4, quadriplegia; 5, spontaneous death attributable to EAE.

For ethical reasons, monkeys were sacrificed when the clinical EAE score of 3 was reached. The highest per day scores in a week were averaged. Moreover, each monkey was weighed at least three times per week to obtain a surrogate disease marker.

Magnetic resonance imaging (MRI)

For in vivo MRI, animals were anesthetized with 30 mg/kg ketamine in combination with 1 mg/kg valium (Diazepam, Kombivet, Etten-Leur, The Netherlands). Acquisitions were performed as described previously in detail (3). Each slide was recorded with a matrix of 512 × 256 data points and a field of view of 4 × 4 cm. The data sets were analyzed on an Apple MacIntosh Performa 630 (Apple Computer, Cupertino, CA) using the public domain National Institutes of Health program.

Neuropathology

Ketamine-anesthetized monkeys were euthanized by an i.v. injection of 400 mg sodium pentobarbital (Euthesate, Apharmo, Dulven, The Netherlands). The brain and spinal cord were excised in toto and fixed for 3 days in 4% buffered formalin, rinsed with PBS containing 0.05% sodium azide, and embedded in paraffin. Small parts of cervical, thoracic, and lumbar spinal cord were postfixed in PBS, 2.5% glutaraldehyde for 2 days, postfixed in 1% osmium tetroxide in PBS, and embedded in Epon. From some animals, the fresh brain was separated into two hemispheres, one being fixed in formalin and the other snap frozen in liquid nitrogen for immunohistochemical analysis. Paraffin sections of formalin-fixed brain and spinal cord were stained with hematoxylin and eosin, Luxol Fast Blue (LFB) combined with periodic acid-Schiff (PAS) for staining of myelin and Bodian for staining of axons. Immunocytochemistry was performed utilizing the immunoperoxidase method of biotin-avidin with the following Abs: mouse anti-human glial fibrillary acidic protein (Biogenex, San Ramon, CA) for astrocytes; rabbit anti-human CD3 (Dako, Glostrup, Denmark) for T cells and mouse anti-human CD20 (Biogenex) for B cells; mouse anti-

human macrophage (27E10 and MRP14; BMA Biomedicals, Augst, Switzerland) for macrophages; mouse anti-MAG (CD57, Becton Dickinson, San Jose, CA); anti-human MBP (Biogenex); anti-CNPase (Sigma, St. Louis, MO) for myelin and oligodendrocytes. Semithin sections were stained with toluidine blue. Maturation stage and timing of demyelination were classified according to published criteria (3).

MOG and MBP Ab responses

Sera were collected from animals at the time of necropsy and stored in aliquots at −20°C. The Ab responses of individual monkeys directed to MBP, MOG, and MOG epitopes were analyzed using a slot blot assay. Rhesus monkey MBP (0.5, 1.0, and 5.0 μg), rhMOG (0.25, 0.5, and 1.0 μg) and synthetic overlapping peptides spanning the extracellular domain of MOG (phMOG), were spotted onto a polyvinyl difluoride membrane (Hybond, Amersham, Little Chalfont, U.K.) at a concentration of 0.1, 0.5, and 1.0 μg using a Bio-Dot SF blotting apparatus (Bio-Rad, Richmond, CA) (12). To ensure that all peptides remained bound to the membrane, the blots were immersed with 2.5% glutaraldehyde in PBS for 15 min, washed with PBS for 15 min, and the remaining sites were blocked by incubating the membrane for at least 2 h in PBS containing 3% BSA (PBS/BSA). The blots were then incubated for 1 h with the relevant serum diluted 1:1000 with PBS/BSA 1%, washed four times for 10 min with PBS containing 0.05% Tween 20, incubated for 1 h with rabbit anti-human IgA, IgG, IgM (Duko); diluted 1:14,000 in PBS/1% BSA, washed as described above, and processed for ECL detection according to the manufacturer's instructions (Amersham).

MOG and MBP T cell responses

At necropsy, PBMC were isolated from venous blood using lymphocyte separation medium (LSM, ICN Biomedical, Aurora, OH). Lymph node cell (LNC) suspensions were prepared from aseptically removed inguinal and axillary lymph nodes. Cultures were set up in HEPES-buffered RPMI 1640 (Life Technologies, Glasgow, U.K.) supplemented with 10% FCS (Flow Laboratories, McLean, VA), 10 mM MEM with nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 2×10^{-5} M 2-ME (all from Life Technologies). PBMC or LNC (2×10^5/well) were seeded into 96-well flat-bottom plates (Greiner, Solingen, Germany) and cultured with rhMOG (10 μg/ml) or hMBP (50 μg/ml). After 48 h, 0.5 μCi/well of [^{3}H]thymidine was added, and incorporation of radiolabel was determined 18 h later using a matrix 9600 beta-counter (Packard 9600, Packard Instrument, Meriden, CT).

Generation of MOG-reactive T cell lines

T cell lines reactive with rhMOG were generated from LNC of MOG-immunized marmosets. For this purpose, single LNC suspensions isolated at the day of necropsy were used as starting material. LNC (10^6/well) were seeded into 24-well flat-bottom plates (Greiner) and stimulated with 10–15 μg/ml rhMOG. In cycles of 2 or 3 days, one-half of the supernatant was removed, and the cultures were supplemented with fresh medium containing 20 U/ml rIL-2 (Cetus, Amsterdam, The Netherlands). After 14–21 days of culture, the cells were restimulated with rhMOG, using irradiated (50 Gy) autologous EBV-transformed B cell lines as APC.

Peptide specificity of MOG-specific T cell lines

Cells were seeded at 2×10^4 T cells/well into 96-well flat-bottom plates and stimulated with rhMOG or a panel of synthetic overlapping phMOG (1.0 μg/ml) (12, 13). Proliferation was assessed by [^{3}H]thymidine incorporation (0.5 μCi/well) during the final 18 h of a 3-day culture. Incorporated radiolabel was counted as described above. Mean values were calculated from triplicate cultures. T cell lines showing reactivity to a certain phMOG were restimulated with that same peptide at the next round of restimulation until stable growing phMOG-specific T cell lines were obtained.

MHC restriction of phMOG-specific T cell reactivity

The MHC restriction of MOG-induced T cell proliferation was determined by inhibition of responses using mAbs raised to primate MHC isotypes SPVL-3 (anti-DQ), B8.11.2 (anti-DR), B7/21 (anti-DP), PdV5.2 (anti-class II), and W6/32 (anti-class I) at 1:100 ascites dilutions (4). Autologous APC were incubated for 15 min at 37°C with the mAbs and then pulsed with the relevant phMOG for 60 min at 37°C. After extensive washing, the APC were tested for their ability to induce proliferation of specific T cell lines during a 72-h culture. Positive controls consisted of APC pulsed with peptide without mAb and negative controls of APC incubated with mAb without peptide. The restriction elements for presentation of phMOG14–36 were determined by testing T cell proliferation induced by a panel of MHC-typed, EBV-transformed B cell lines from related and unrelated marmosets. Irradiated B cells (50 Gy) were seeded (2×10^4 cells/well) into a 96-well plate, and phMOG14–36 was added. Peptide-induced proliferation of 2×10^4 phMOG14–36-specific T cells was assessed by counting [^{3}H]thymidine incorporation during the final 18 h of a 3-day culture.

Ethics

According to the Dutch law on animal experimentation, the protocol of this study has been reviewed and approved by the Institute's Animal Care and Use Committee. All experimental procedures with the animals are in accordance with the guidelines of the committee.

Results

MOG immunization induces severe demyelinating EAE

All 3 MOG-immunized animals developed clinically manifest neurological disease (EAE score, ≥2), albeit at various times after immunization. The first clinical signs were observed at 38 days in Escudo, at 52 days in animal 9601, and at 64 days in animal 9501. The clinical course of MOG-induced EAE was chronic progressive in all animals, with each monkey developing complete paralysis of the hind part of the body (paraplegia; EAE score, 3.0) within 2 wk after disease onset (Fig. 1*A*). At this stage, the monkeys were sacrificed based on ethical considerations. During the course of the disease, all three MOG-immunized animals lost more than 15% of their body weight (Fig. 1*B*).

The first T_2-weighted (T_2-w) magnetic resonance images were recorded 1 mo after immunization. At this stage, two of three MOG-immunized animals (9501 and 9601) showed MRI-detectable lesions within the brain white matter parenchyma. In addition, high resolution postmortem T_2-w magnetic resonance images were recorded of formalin-fixed brains to determine the total lesion load of the brain (Fig. 2). The lesion load of animals 9501 and 9601 differed remarkably, despite similar EAE severity. In the brain of monkey 9501, several small focal lesions were detected, whereas in monkey 9601 extremely large lesions were found. No MRI-detectable lesions could be observed in the brain of monkey Escudo.

Neuropathological examination confirmed that the abnormalities observed with brain magnetic resonance images represent demyelinated lesions. In monkeys 9501 and 9601, large areas of demyelination were observed in the white matter of the cerebral hemispheres (Fig. 3, *a* and *b*), mainly localized around the wall of the lateral ventricles, in the hemispheric white matter, in the spinal cord, in the corpus callosum, and in the optic nerves and tracts. Light microscopic examination revealed a relevant degree of inflammation within the areas of demyelination, characterized by perivascular cuffs of mononuclear cells, whereas granulocytes were absent. Infiltrates were composed of macrophages, T lymphocytes, and a few B lymphocytes. Inside the cytoplasm of macrophages, LFB- or PAS-positive myelin degradation products were detected. Bodian staining did not reveal apparent axonal damage.

In the brain of Escudo, only a few perivascular inflammatory cuffs around the small venules were observed, without demyelination. Pathological changes were mainly restricted to the spinal cord, where a widespread demyelination was detected. The areas of demyelination involved the ventral, lateral and dorsal columns of the spinal cord, especially in the outer part of spinal tracts (Fig. 3*c*). Diffuse infiltration of demyelinated areas by macrophages was present, but axons were apparently intact (Fig. 3*d*). Both active and inactive lesions were present in the same areas, as indicated by the presence of LFB-positive myelin degradation products and by staining macrophages with MRP14 (Fig. 3*e*) or the presence of only PAS-positive deposits in the macrophages (not shown). Ventral and dorsal roots belonging to the peripheral nervous system, lying nearby the severely affected spinal cord, were completely

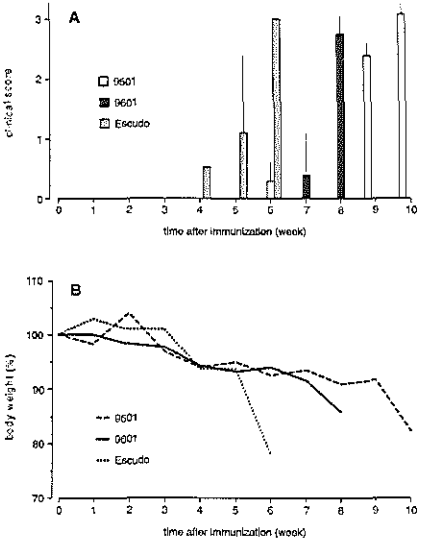

FIGURE 1. Clinical course of EAE in MOG-immunized marmosets. Three twin siblings (9501, 9601, Escudo) were immunized with 100 μg rhMOG in CFA. For each individual monkey, the mean clinical scores per week and SDs are given (*A*). The body weight was measured three times a week. For normalization, the weight at the start of the study was set at 100% (*B*). No clinically definite EAE or weight loss was observed in the other sibling of the twin couple (9502, 9602, Estrada), which was immunized with 1 mg hMBP/CFA (not shown).

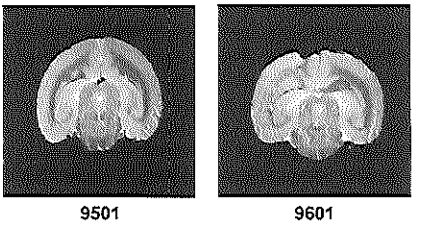

FIGURE 2. Postmortem MRI of MOG-immunized monkeys. Two slices of postmortem T2-weighted brain MR-images in the coronal direction. The lesion load in animal 9501 differed considerably from that of animal 9601, despite the similar clinical expression at the time of sacrifice (EAE score 3). In the brain of monkey 9501 several focal lesions are detectable as hyperintensities, whereas in monkey 9601 there is substantial demyelination covering almost the complete white matter area of one hemisphere. No abnormalities were observed in postmortem brains of MBP-immunized animals.

spared. This indicates that the demyelinating process was restricted to the myelinated fibers of the CNS (Fig. 3*f*).

Absence of clinical and pathological features of EAE after MBP immunization

MBP-immunized animals developed only mild clinical signs of EAE. Animals 9502 and Estrada showed apathy and loss of appetite (EAE score: 0.5) during a period of 2 wk starting at 10 and 11 wk after immunization, respectively. Monkey 9602 remained asymptomatic during the observation period of 178 days. No weight loss was observed. On in vivo T_2-w brain magnetic resonance images recorded 1 mo after immunization, no abnormalities could be detected. However, at 3 mo after immunization, small hyperintense regions were found in the brains of monkeys 9502 and Estrada, but not in the brain of animal 9602. No abnormalities could be detected on postmortem magnetic resonance images, and the neuropathology analysis revealed that no signs of inflammation or demyelination was observed in MBP-immunized animals. The MRI-detectable abnormalities may therefore be an artifact or reflect the edema extravasation associated with perivascular inflammation, which could have been drained by the time the monkeys were sacrificed.

MOG- and MBP-specific Ab responses

In MOG- and MBP-immunized animals, circulating Abs appeared to be primarily directed against the inciting Ag only (Fig. 4). The epitope specificity of anti-MOG Abs present in necropsy sera was analyzed using a set of overlapping 22-mer peptides, spanning the N-terminal extracellular part of MOG (residues 1–116). The main reactivity of anti-MOG Abs in all three MOG-immunized monkeys was directed against two separate regions (Fig. 4). The sera reacted with phMOG4–26, 14–36 and 24–46, but not to phMOG34–56, indicating that one or more B cell epitopes are located within an stretch 4–40. The sera also showed strong reactivity to phMOG44–66 and 54–76, indicating that one or more epitopes are contained within an region 44–76. No Ab reactivity toward rhMOG or phMOG could be detected in necropsy sera of MBP-immunized animals (not shown). The preimmune sera showed no reactivity against whole myelin, rhesus monkey MBP, rhMOG, or phMOG, indicating that Abs were formed after immunization. Notably, with this technique Ab reactivity against discontinuous epitopes of MOG are not detected.

MOG- and MBP-specific T cell responses

Primary LNC cultures of all three MOG-immunized animals displayed strong proliferative responses to rhMOG (Fig. 5) but not to hMBP (not shown). In contrast, LNC from one of the three MBP-immunized animals (Estrada) displayed a significant proliferative response to hMBP (Fig. 5). In conclusion, the different clinical expression and radiological manifestation of EAE as well as the clear immunological and histopathological differences found in MOG- vs. MBP-immunized siblings prompted us to study the MOG-induced EAE in greater detail.

Generation of phMOG-specific T cell lines

After two rounds of restimulation with rhMOG, the epitope specificities of LNC-derived cultures were analyzed using a set of 10 overlapping 22-mer phMOGs (spanning residues 1–116). Positive responses, stimulation index ≥4, are depicted by shaded boxes (Fig. 6*A*). Subsequently, peptide-specific T cell lines were generated. After another two rounds of culture with the specific peptides, the lines were checked for peptide specificity. As shown in Fig. 6*B*, only one T cell line could be established from monkey 9501, reactive with phMOG14–36 [9501 (14–36)] and with phMOG24–46. From animal 9601, 4 different T cell lines could be generated: 9601 (4–26); 9601 (14–36); 9601 (24–46); and 9601 (74–96). Line 9601 (14–36) proved reactive to phMOG24–46. Line 9601 (24–46) recognizes a different epitope in that it is not responding to phMOG14–36. Finally,

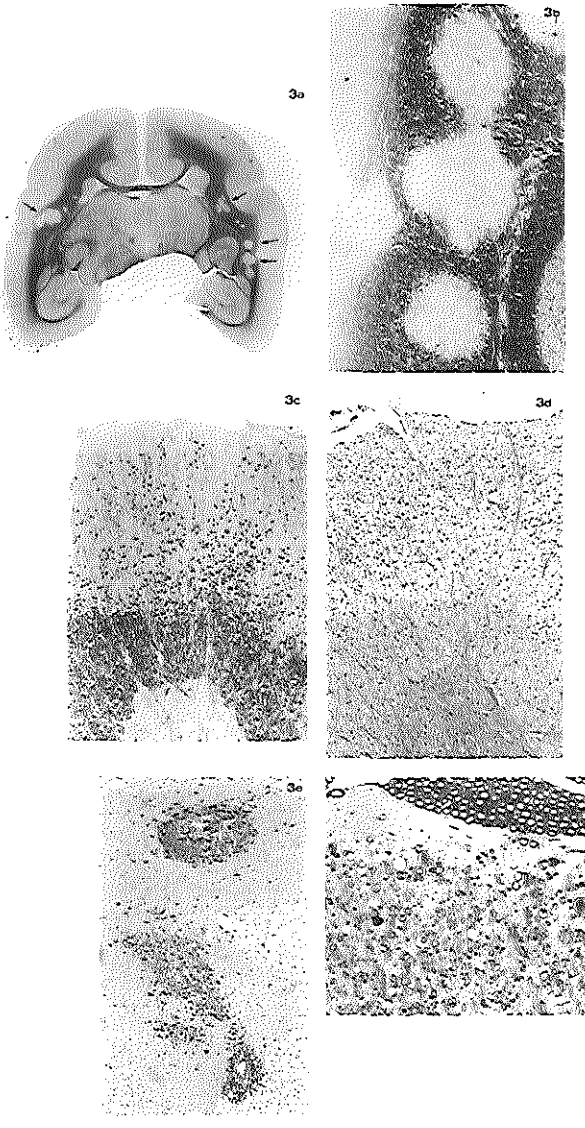

FIGURE 3. Neuropathology of MOG-immunized marmosets. *a*, Coronal section of brain hemispheres passing through the head of caudate nucleus and basal ganglia, showing multiple areas of demyelination (arrows) (9501, LFB-PAS, ×4). *b*, Large demyelinating partially confluent lesions in the hemispheric white matter (section of *a*, 9501, LFB-PAS, ×40). *c*, Areas of demyelination in the spinal cord, involving the outer part of the ventral columns (Escudo, LFB, ×40). *d*, In the same area of *c*, silver staining shows an almost normal density of axons (Escudo, Bodian, ×40). *e*, Diffuse MRP14-positive macrophage infiltration in demyelinating areas surrounding small vessels (9501, anti-MRP14, avidin-biotin method, ×40). *f*, Diffuse infiltration of MRP14-positive macrophages in the demyelinated areas of the spinal cord (being CNS), with complete sparing of myelinated fibers of ventral roots (being the peripheral nervous system) (Escudo, semithin section, toluidine blue, ×250).

T cell line 9601 (74–96) is reactive with a distinct epitope (Fig. 6*B*). From Escudo, three lines were established (eso (14–36), eso (24–46) and eso (34–56)) responding to at least two distinct epitopes (Fig. 6*B*). All presently described in vitro-generated T cell lines were $CD4^+CD8^-$ with high surface expression of Caja-DR molecules reflecting their activated state.

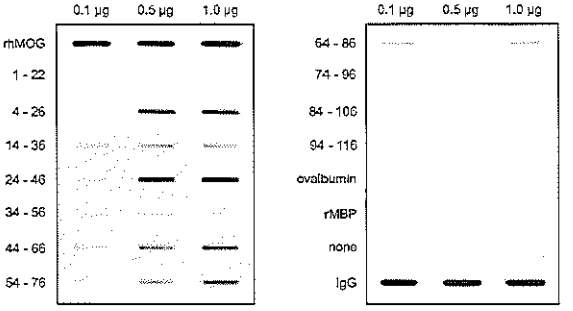

FIGURE 4. Ab responses against rhMOG and MOG peptides (phMOG). Sera were obtained from animals at the time of necropsy. The presence of Abs directed to MBP, MOG, and MOG epitopes was analyzed using a slot blot assay. Values are for a representative example serum from animal 9601, reacting with phMOG4–26, 14–36, and 24–46, but not with phMOG34–56. The sera of all three MOG-immunized monkeys also showed strong reactivity to phMOG44–66 and 54–76 but weak reactivity to phMOG64–86. The reactivity of the anti-MOG Abs in all three MOG-immunized monkeys was directed against these two main regions. rMBP, rhesus monkey MBP.

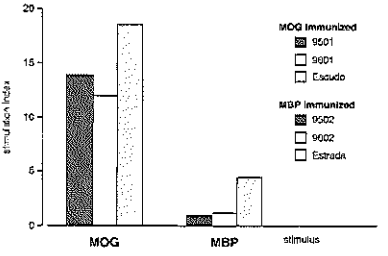

FIGURE 5. Primary responses of LNC in MBP- and MOG-immunized marmoset monkeys. LNC from MOG- and MBP-immunized animals were cultured in the presence of 10 µg/ml rhMOG or 25 µg/ml hMBP/well. All 3 MOG-immunized animals displayed proliferative responses to rhMOG, but not to hMBP (stimulation indexes ≤2; not shown). In contrast, LNC from only one of three MBP-immunized animals showed a proliferative response to hMBP. Results are means of triplicate assays and are expressed as stimulation indexes (stimulation with Ag/stimulation without Ag).

Characteristics of phMOG-specific T cell lines

The fine specificities of T cell lines were delineated using smaller peptides. The fine specificity of the phMOG4–26-reactive T cell line was defined at residue p4–11 (Fig. 7*A*), of phMOG74–96-reactive T cells at p81–96 (Fig. 7*B*) and of phMOG14–36-reactive T cells to p24–36 (Fig. 7*C*). In conclusion, all three MOG-immunized marmosets share T and B cell reactivity to phMOG14–36 (Figs. 5 and 6*B*). To determine the MHC restriction elements that control the T cell reactivity to p24–36, autologous APC were exposed to mAbs directed against primate *Mhc* class II isotypes before they were pulsed with phMOG14–36. Peptide-induced proliferation could be inhibited by both mAbs B8.11.2 and PdV5.2 but not by mAbs SPV-L3, B7/21, and W6/32 (Fig. 8). Hence, it is concluded that p24–36 is presented to the T cell lines in the context of Caja-DR molecules.

For determination of the precise Caja-DR restriction element, an allogeneic panel of transformed B cell lines derived from marmosets with defined *Mhc* class II specificities (Table I), was tested for presentation of phMOG14–36 to Ag-specific T cell lines. phMOG14–36-specific T cell proliferation could be induced by APC from all marmosets (Fig. 9). Because *Caja-DRB*W1201* is shared by all marmosets tested in our population (5), it is the most likely restriction element of p24–36 presentation. This assumption is supported by the fact that transformed B cell lines from the cotton-top tamarin, a new world monkey closely related to the

FIGURE 6. MOG reactivity of lymph node-derived T cells. LNC suspensions isolated at the day of necropsy were seeded (10^6/well) into a 24-well plate and stimulated with 10–15 µg/ml rhMOG. After two rounds of restimulation, cultures were analyzed for specific peptide reactivity. Positive responses (stimulation indexes ≥4) are shown as filled boxes (*A*, gray and black boxes). After two rounds of culture, peptide-specific lines were generated (*A*, black boxes) and were checked for specificity (*B*, gray boxes). The fine specificities of most of the lines were delineated using smaller peptides (for details, see Fig. 7). The summary of the fine specificities in *B* (last column) shows that all monkeys share dominant T cell reactivity to p24–36.

A) LNC reactivity after second round of stimulation

	phMOG									
Monkey	4-26	14-36	24-46	34-56	44-66	54-76	64-86	74-96	84-106	94-116
9501										
9601										
Escudo										

B) Peptide-reactivity of peptide-specific cell-lines

	phMOG										
Cell lines	4-26	14-36	24-46	34-56	44-66	54-76	64-86	74-96	84-106	94-116	Fine-spec.
9501(14-36)											24-36
9601(4-26)											4-11
9601(14-36)											24-36
9601(24-46)											31-40
9601(74-96)											81-96
Esc(14-36)											24-36
Esc(24-46)											24-36
Esc(34-56)											ND

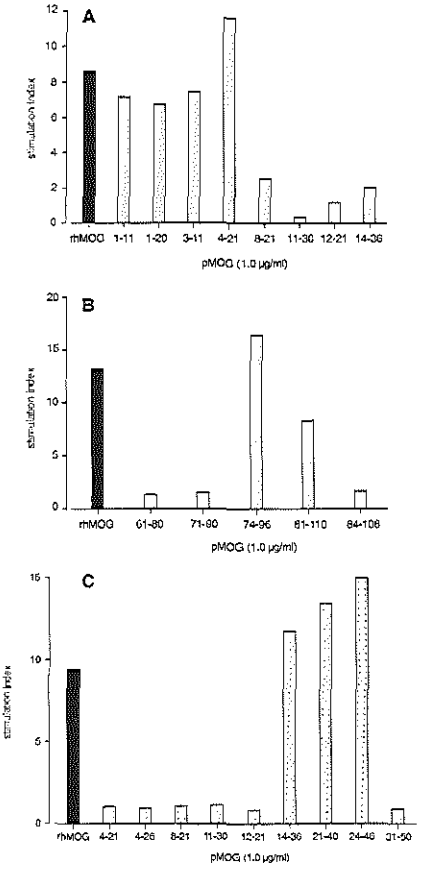

FIGURE 7. Fine specificities of phMOG-specific T cell lines. The fine specificity of most generated lines was assessed using shorter pMOG. The reactivity of the phMOG4–26 T cell line reactive could be delineated to amino acid sequence p4–11 (*A*), whereas the minimal epitope for the phMOG74–96-reactive T cell line was contained within p81–96 (*B*). Fine specificity of phMOG14–36-reactive T cell line could be delinlated to p24–36 (*C*). phMOG14–36-specific T cell lines could be generated from all MOG-immunized monkeys, and all reacted in a similar way as the T cell line from 9601 (*C*).

common marmoset but lacking the equivalent of the *Caja-DRB*W1201* allele (14), were incapable of p24–36 presentation to the T cell lines.

Induction of clinical EAE by immunization with phMOG14–36

To investigate whether the peptide phMOG14–36 is involved in the initiation of EAE, four common marmosets were immunized with the MOG peptide emulsified in CFA, avoiding administration of *B. pertussis*. Fig. 10 shows the clinical course of the EAE (Fig. 10*A*) and the primary LNC responses to rhMOG and phMOG14–36 (Fig. 10*B*). The results show that immunization with phMOG14–36 induces clinical signs of EAE in all four monkeys as well as a cellular immune reaction to the peptide and rhMOG. The time of onset and the course of clinical signs appeared to differ between individual monkeys, as was also found in MOG-immunized monkeys. As could be expected, the earliest responder (QY) displayed the highest proliferative response to MOG protein and peptide. Histopathology analysis of the brain confirmed the diagnosis of EAE, as perivascular cuffs of mononuclear cells could be found (not shown).

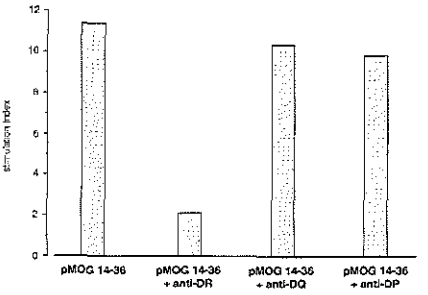

FIGURE 8. Proliferative responses of T cell lines induced by phMOG14–36 primed APC, after blocking with anti-MHC-class II mAbs. Autologous APC were first incubated with blocking mAbs raised against primate *MHC-DR, -DQ,* and *-DP* molecules and subsequently pulsed with phMOG14–36. The pulsed cells were then tested for their capacity to induce proliferation of phMOG14–36-specific T cell lines. Proliferation of the cell lines appeared to be inhibited by anti-MHC-DR (mAb B8.11.2) as well as by anti-MHC class II (PdV5.2; not shown). Results are expressed as stimulation indexes, calculated as T cell proliferation of APC with/without phMOG14–36 added.

Discussion

The close immunological relationship between nonhuman primates and humans (14–16) and the fact that marmoset EAE shares essential clinical, radiological, and pathological features with MS (8, 9, 17) warrant the use of this animal model for the study of genetic and immunological mechanisms leading to CNS inflammation and demyelination in MS. The pathogenesis of EAE in common marmosets involves, as in rodents, at least two separate pathogenic pathways. CNS inflammation can be mediated by T cell reactivity to major myelin Ags such as MBP or proteolipid protein (6, 7). CNS demyelination, however, requires the involvement of Abs, in particular those directed to MOG (7, 9, 18–20). The synergistic action of anti-myelin T cells and Abs in the EAE pathogenesis has been well established in cotransfer experiments, both in rodents (19) and in primates (7).

Where the relevance of the autoimmune reaction to MBP and proteolipid protein for the immunopathogenesis of MS is disputed, evidence is accumulating that T cell and Ab reactivity to MOG play an important, most likely synergistic role. MS patients appear to display a significantly higher level of T cell reactivity to MOG than control individuals (12, 13, 18, 21). Moreover, anti-MOG Abs are localized in CNS areas where myelin disintegration and lesion formation are taking place (8, 17). Finally, in its pathological expression, MOG-induced EAE closely resembles MS (8, 9, 21, 22). However, the Ab dependence of EAE has been challenged by the

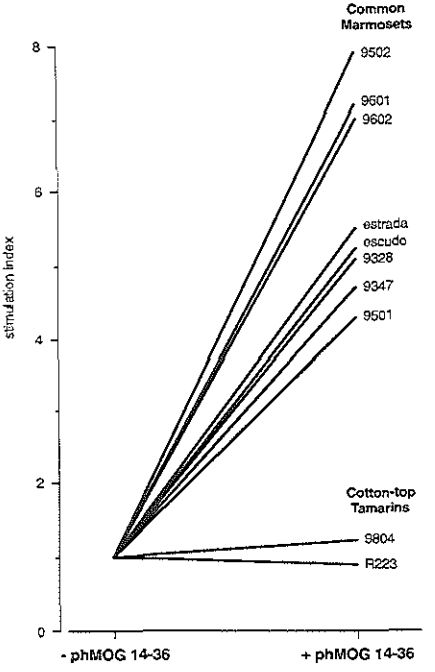

FIGURE 9. Presentation of phMOG14–36 by a panel of APCs from related and nonrelated marmosets. The allelic restriction of phMOG14–36 was determined by a panel of MHC-typed, EBV-transformed B cell lines from related and nonrelated marmosets. 9601, autologous APC; 9602, chimeric APC; 9501, 9502, Escudo, Estrada, 9328, and 9347, allogeneic APC. For distribution of *Caja-DRB1*03, -DRB*W16,* and *-DRB*W1201* alleles, see Table I. R223 and 9804, cotton-top tamarin APC lacking the evolutionary equivalent of the *Caja-DRB*W1201* allele. All marmoset APC in this panel, but not the B cells derived from the cotton-top tamarin, were able to present the phMOG14–36 in a way to induce T cell proliferation. T cell responses in the presence of APC without phMOG were used as negative controls, and the stimulation indexes were set on 1. Proliferative responses using allogeneic APC could be completely blocked by anti-MHC-DR mAb. Reponses of phMOG14–36-reactive T cell lines of all three MOG-immunized animals were comparable with the data as shown for T cell line 9601 (14–36).

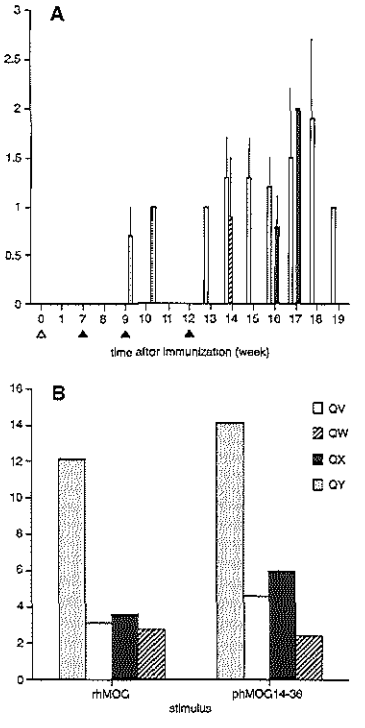

FIGURE 10. Antigenicity and encephalitogenicity of phMOG14–36. Four unrelated common marmosets were immunized with 50 μg phMOG14–36 emulsified in CFA (open arrow). At 7, 9, and 12 wk after the immunization, the monkeys received a booster immunization with 50 μg phMOG14–36 in IFA (closed arrow). For each individual monkey, the mean clinical scores per week (with SDs) are given (*A*). The time of sacrifice was at day 88 for monkey QY, day 95 for QW, day 119 for QX and day 128 for QV. *B*, At necropsy, axillary and inguinal lymph nodes were aseptically removed, and single-cell suspensions were prepared. The proliferative response of the LNC to rhMOG and phMOG14–36 (both at 10 μg/ml) was assessed.

observation that B cell knockout mice also develop clinical signs (23, 24).

Our present results show that in common marmosets MBP is antigenic; MBP-specific T and B cell responses were detected but were only weakly encephalitogenic. The lack of clinical signs and pathological evidence of EAE in our MBP-immunized marmosets seems to contrast with published data (1, 2). It should be emphasized, however, that we avoid usage of *B. pertussis*, which was found an essential component of the EAE induction protocol by these authors (1). The reason is that, besides a direct effect on the blood-brain barrier permeability, *B. pertussis* administration to marmosets immunized with human myelin in CFA appeared to cause lesions by necrosis rather than specific demyelination of CNS white matter (3). This effect might be related to potentiation and/or polarization of the MBP-specific T cell response by *B. pertussis* (25, 26).

The present analysis of the proliferative responses of LNC-derived T cells to rhMOG and the panel of MOG peptides shows that all MOG-immunized monkeys share a T cell reactivity to a single MOG epitope, p24–36. The epitope is contained in the encephalitogenic phMOG14–36 peptide and presented in the context of Caja-DRB*W1201 molecules. The T cell epitope seems not to concur with any of the thus far identified immunodominant T cell epitopes in humans or rodents (18, 27). An individually variable response was found against other MOG epitopes, p4–11, p31–40, and p81–96. Preliminary data from similar cross-presentation studies, as in Fig. 9, indicate that these MOG peptides are likely presented by other Caja-DR molecules (unpublished results). Binding

of anti-MOG Abs proved to be confined to two regions within the rhMOG molecule, namely p4–40 and p44–76. Our results of T cell and Ab epitope mapping are in line with data from Genain et al. (5, 6, 28).

On the basis of the data discussed thus far, we conclude that phMOG14–36 may contain critical T and B cell epitopes for the initiation of EAE in common marmosets. The observation that four of four common marmosets immunized with this peptide emulsified in CFA develop clinical EAE strengthens this assumption. Computer modeling of the three-dimensional conformation of MOG predicts that the 14–36 peptide is exposed on the surface of a homodimer and thus freely accessible to Ab binding (29). The fact that all common marmosets share the Caja-DRB*W1201 molecule, which functions as a major restriction element of the T cell reaction to phMOG14–36, underlies the 100% incidence of severe demyelinating EAE in common marmosets. The EAE-initiating event in myelin-immunized common marmoset monkeys may thus be a remarkably uniform event, namely, the Caja-DRB*W1201-restricted activation of phMOG14–36-specific $CD4^+$ T cells. The subsequent spreading of the T and B cell reactivity to other MOG-epitopes appears to vary between individual monkeys, reflecting the outbred nature of this species. This unique feature, together with the possibility of adoptively transferring T cell lines between fraternal siblings (1) makes the common marmoset a unique model for the detailed analysis of pathophysiological pathways in EAE and MS.

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The relevance of T- and B-cell autoreactivity to myelin oligodendrocyte glycoprotein in common marmoset models of multiple sclerosis

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Abbreviations used in this paper: Ab: antibody (ies); APC: antigen presenting cell(s); CNS: central nervous system; CFA: complete Freund's adjuvant; EAE: experimental autoimmune encephalomyelitis; ^{3}H-Thy: tritium labeled thymidine; LNC: lymph node cells; MBP: myelin basic protein; MHC: major histocompatibility complex; MOG: myelin oligodendrocyte glycoprotein; MRI: magnetic resonance imaging; MS: multiple sclerosis; PLP: proteolipid protein; pMOG: MOG peptide; OVA: ovalbumin; SC: spleen cells; S.D.: standard deviation; S.I.: stimulation index; TCL: T-cell line(s); TCR: T-cell receptor

Abstract

Myelin oligodendrocyte glycoprotein (MOG) is regarded as a key molecule in the induction of autoimmunity to myelin leading to demyelination in the central nervous system (CNS) of patients with multiple sclerosis (MS). This human autoimmune disorder is modeled in the common marmoset. By immunization either with human myelin or recombinant human MOG, clinical and neuropathological signs are incited which mimic chronic progressive cases of MS. Here we report on the relation between the repertoire of T- and B-cell responses in these two non-human primate EAE-models with the initiation and progression of the disease. Our results confirm the earlier finding that $pMOG_{14-36}$ T-cell reactivity is the common trigger for EAE induction in the MOG-induced EAE model. Furthermore, a relation was found between the extent of intermolecular broadening of anti-MOG T-cell response and the time of onset of EAE. Our results provide evidence that antibody (Ab) responses directed against discontinuous epitopes of MOG are a prerequisite for development of demyelinating EAE in both animal models. Ab responses directed against linear epitopes of MOG are completely absent in myelin-immunized animals. Although broadening of the Ab reactivity directed against linear epitopes of MOG was observed in MOG-immunized monkeys, these reactivities did not correlate with disease onset or progression.

Introduction

In multiple sclerosis (MS) autoimmune reactions with central nervous system (CNS) components are thought to contribute significantly to the formation of white-matter lesions. The disease is characterized clinically by neurological impairment of varying severity. Although the initial trigger(s) for MS remains elusive, the most favored hypothesis states that once initiated, perpetuation of the disease is driven by the interplay between myelin reactive T-cells and antibodies (Ab) (1-4).

Humans and marmoset monkeys (*Callithrix jacchus*) share a high degree of similarity at the level of cell surface markers (5), T-cell receptor (TCR) Vβ repertoire (6), co-stimulatory molecules (7), major histocompatibility complex (MHC) class II genes (8,9), immunoglobulin (Ig) V_H repertoire (10) and myelin proteins (11,12). Experimental autoimmune encephalomyelitis (EAE) in the marmoset develops after immunization with CNS myelin or recombinant myelin proteins, myelin oligodendrocyte glycoprotein (MOG) in particular (13-18). Two disease models have been investigated in more detail,

namely MOG-induced EAE (18-20) and the disorder induced using preparations of human white matter (13,16,21,22).
Despite the fact that the common marmoset represents an outbred species the incidence of EAE in both models is 100%. In general, animals display detectable T- and B-cell responses within 2 weeks after immunization (19). Moreover, using *in vivo* magnetic resonance imaging (MRI), alterations in tissue water content around the ventricles can be detected as early as 3 weeks after disease induction (22). The disease finally develops into a chronic progressive paralytic disease, ultimately leading to severe impairment of body functions. After the identification of MOG as a target for Ab-mediated demyelination and the finding that anti-MOG Ab are deposited in MS lesions, the contribution of anti-MOG T-cell and Ab reactivity to the immunopathogenesis of EAE and MS has gained particular interest (23-29). Hence, we investigated the patterns of anti-MOG T- and B-cell responses in three marmoset EAE models and discuss here their relevance in disease pathogenesis.

Material and Methods

Animals

Healthy common marmosets were randomly selected from the outbred colony kept at the Biomedical Primate Research Centre (BPRC, Rijswijk, The Netherlands) or purchased from the German Primate Center (DPZ, Göttingen, Germany). Before the monkeys entered the study a full physical, hematological and biochemical check-up was performed. During the experiments, the monkeys were individually housed in spacious cages with padded shelter provided at the bottom of the cage and were under constant veterinary care. The daily diet consisted of commercial food pellets for New World monkeys (Special Diet Services, Witham, Essex, England), supplemented with rice, raisins, peanuts, marshmallows, biscuits and fresh fruit. Drinking water was provided ad libitum. According to the Dutch law on animal experimentation, the experimental procedures of this study were reviewed and approved by the Institute's Animal Care and User Committee.
With the exception of monkeys immunized with MOG-peptide ($pMOG_{14-36}$), all animals in this study had served as sham-treated controls in preclinical studies where effectivity of new therapies was examined.

Myelin- and MOG-induced EAE

Myelin-induced EAE was evoked by a single immunization with 300 µl human myelin in water (10 mg/ml) emulsified with an equal volume of complete Freund's adjuvant (CFA; Difco Laboratories, Detroit, MI) under ketamin anesthesia (6 mg/kg;

AST Farma, Oudewater, The Netherlands) as described previously (16). RhMOG, representing the extracellular domain of human MOG (amino acid 1-123), was expressed in *Escherichia coli* and purified as previously described (29). Animals were immunized with 100 μg MOG as an emulsion in CFA. All monkeys [n=19 myelin-induced EAE; n=26 MOG-induced EAE] were intradermally injected into the dorsal skin with 600 μl of emulsion divided over four locations; two in the inguinal and two in the axillary region.

The major T-cell reactivity in naïve animals (31) as well as in MOG-immunized animals (18,20) is directed to $pMOG_{24-36}$. To investigate whether autoreactivity towards this peptide is sufficient for initiation of full-blown, demyelinating EAE, four randomly selected monkeys (n=4) were immunized with 100 μg $pMOG_{14-36}$ in CFA as previously described (18). Two animals immunized with 1 mg ovalbumin (OVA) served as adjuvant controls for CFA-mediated immune reactions.

Twice daily, clinical signs of EAE were scored by a trained observer using a previously described semi-quantitative scale (18). Monkeys were sacrificed for ethical reasons once clinically definite EAE (score ≥ 2.0) had been reached, or at the anticipated endpoint of the study. High resolution *in vivo* and *ex vivo* magnetic resonance images (MRI) were recorded on a 4.7 Tesla horizontal bore Varian NMR spectrometer (Varian Palo Alto, CA USA) as described (21). Post-mortem T2-weighted MRI was performed on formalin fixed brains as described previously (18). Parts of the brain and the spinal cord were examined with histopathological and immunohistochemical techniques according to published protocols (18,19,21,22,32).

T-cell responses ex vivo

At necropsy, PBMC were isolated from heparinized venous blood using lymphocyte separation medium (LSM®, ICN Biomedical Inc., Aurora, OH). Mononuclear cell suspensions were prepared from aseptically removed lymph node (LNC) and spleen (SC). PBMC, LNC and SC were cultured in triplicate for detection of proliferative responses towards CNS proteins. Cultured cells were probed for reactivity with rhMOG, $pMOG_{14-36}$, proteolipid protein peptide 139-151 ($PLP_{139-151}$), recombinant human myelin basic protein (MBP) and recombinant human αB-crystallin (both kindly provided by Dr. J.M. van Noort, TNO-PG, Leiden, The Netherlands), while OVA served as the control protein (all at 10 μg/ml). After 48 h, 0.5 μCi/well of [^{3}H]-Thymidine ([^{3}H]-Thy) was added and incorporation of radiolabel was determined 18 h later using a matrix 9600 β-counter (Packard 9600; Packard Instrument Company, Meriden, CT). Results are expressed as the mean stimulation index (S.I.) ± standard deviation (S.D.). Proliferation with an S.I. ≥ 2.0 was considered relevant.

MOG-reactive T-cell lines

MOG-reactive T-cell lines (TCL) were generated from LNC of myelin and MOG-immunized marmosets that were isolated at the day of necropsy. In brief, LNC (10^6/well) were seeded into 24-well plates (cat.nr.: 662102, Greiner, Sölingen, Germany) and stimulated with 10 μg/ml MOG. Every 2 or 3 days, half of the culture supernatant was replaced with fresh medium containing 20 U/ml recombinant human

IL-2 (Proleukin, Chiron Corporation, Emeryville, CA). After 14 to 21 days of culture the TCL were tested for reactivity with a panel of 23-mer pMOG in 96-well flatbottom plates (cat.nr.: 655180, Greiner) as described (18). As APC, immortalized marmoset B-cell lines were used, grown in 75 cm^2 tissue culture flasks (cat.nr.: 658175, Greiner). Peptide-specific TCL were screened for IL-2, TNF-α and IFN-γ production using standard ELISA-assays (U-Cytech, Utrecht, The Netherlands) using 96-wells round bottom plates (cat.nr.: 655092, Greiner) and further characterized by expression of T-cell specific cell surface markers by flow cytometry (5). Isotype controls were kindly provided by J. Miller (Chemicon International, South Hampton, United Kingdom). Some TCL were analyzed for TCR-Vβ gene usage as previously described (6).

B-cell responses

Sera collected from animals at the time of necropsy were stored in aliquots at -20^0C. Ab binding to myelin proteins (MOG, MBP, αB-crystallin, HPLC-purified human PLP [kindly provided by Dr. J. M. van Noort]) or to the panel of 10-mer overlapping 23-mer pMOG sequences was determined using a dot-blot assay (18) and ELISA (19). Ab specific for discontinuous epitopes are considered particularly pathogenic (33). To distinguish Ab directed against linear epitopes from those directed against discontinuous epitopes, serum samples were preincubated with the mixture of overlapping pMOG (10 μg/ml for each peptide) for 1 h at 37^0C before probing them for anti-MOG Ab reactivity with ELISA. As a control, the serum was preincubated with the mixture of pMOG omitting $pMOG_{54\text{-}76}$. The results of the Ab assays are expressed as fold increase of light absorbance at 405 nm using the reactivity to OVA as internal control and using pre-immune sera as a reference for pre-existing Ab reactivity. A more than two-fold increase in signal intensity plus three times the S.D. was considered relevant.

Statistics

Differences between groups regarding time taken to develop clinical EAE were analyzed using Kaplan-Meier survival analysis. Statistical significance of between-group differences was assessed using the Log Rank test.

Results

Clinical and pathological expression of myelin- and MOG-induced EAE
Immunization with human myelin or MOG in CFA resulted in clinical EAE in 100% of the monkeys, although the time of disease onset between individual animals varied considerably (Table I). All myelin- and MOG-immunized animals showed pathological alterations in the brain using *in vivo* and high contrast post-mortem MRI; representative examples are shown in Figure 1. Significant destruction of CNS white matter also occurred in animals that only developed mild disease (EAE score $\leq$ 1.0). The observed pathomorphological alterations in myelin- and MOG-induced EAE were in concordance with previously published results (18,19,21,22,32). $pMOG_{14-36}$ immunized animals all developed clinical signs of EAE (18) associated with MRI-detectable alterations in the brain (Figure 2a). Within the CNS of $pMOG_{14-36}$-immunized animals small cuffs of infiltrated mononuclear cells were found (Figure 2b), but demyelination was absent (not shown). No T-cell infiltrates or myelin-destruction were observed in the OVA-immunized marmosets.

T-cell autoreactivity
As was expected, high proliferative responses of SC, LNC and PBMC against MOG were observed at necropsy in animals immunized with this protein. In animals immunized with human myelin, containing only a minute amount of native MOG, superior T-cell responses towards MOG were found compared to any of the other tested proteins or peptides. In the four cases where clinical EAE was induced by immunization with $pMOG_{14-36}$, proliferative MNC responses remained restricted to $pMOG_{14-36}$ (Figure 3).

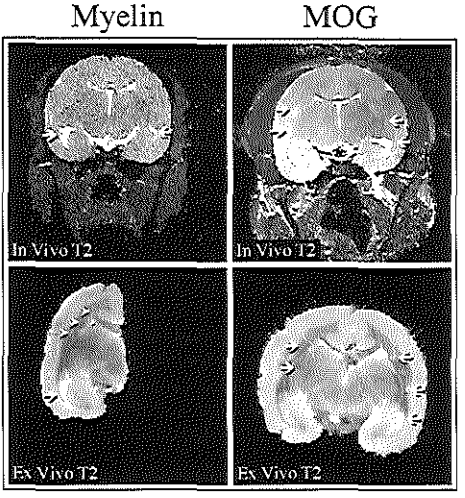

Figure 1. In vivo and post-mortem MRI of myelin- and MOG-immunized marmosets
Representative pictures of *in vivo* (312.5x312.5μm) and corresponding high resolution *ex vivo* (234x234μm) quantitative T2 images of myelin- and MOG-immunized marmosets. Quantitative T2 images sensitively detect edema that appears hyperintense on the images. The *in vivo* images of the MOG immunized animals have a better appearance due to improved hardware adaptations. Both myelin- and MOG-immunized marmosets display strong inflammatory reactions at the lateral horn (white arrows). The white matter of the myelin-immunized marmosets shows multiple small lesions, only visible in the *ex vivo* images. The white matter of the MOG-immunized marmosets shows both in the *in vivo* as the *ex vivo* images clear larger lesions.

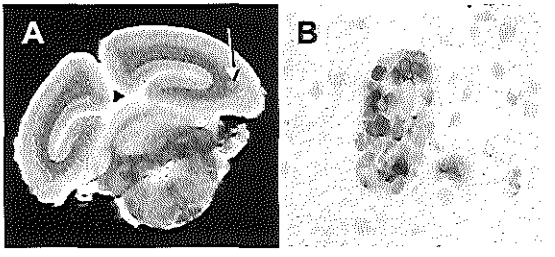

Figure 2. Neuropathological alterations in $pMOG_{14-36}$-immunized marmosets
A: Representative picture of post-mortem T2 image of $pMOG_{14-36}$-immunized marmosets (animal QY). T2 images are sensitive in detecting edema that appears hyperintense on the images (white arrow). No demyelination was found in brain or spinal cord of all $pMOG_{14-36}$-immunized marmosets **B:** Spinal cord section of animal QX showing the presence of infiltrated $CD3^+$ T-cells (grey; x161).

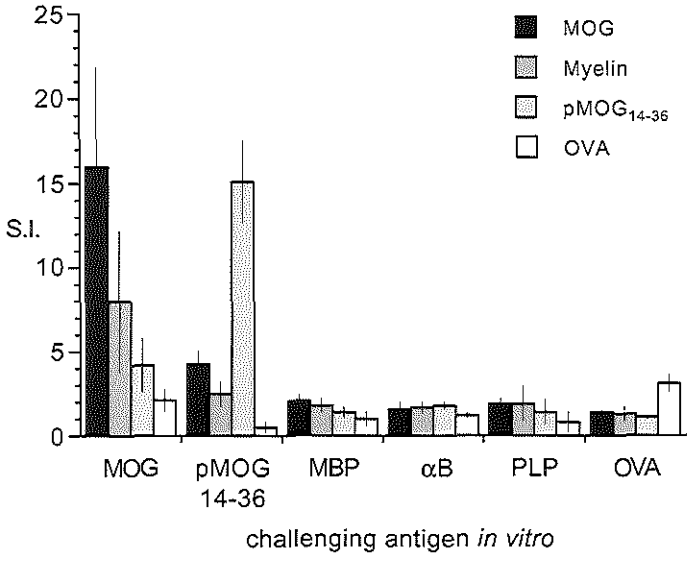

Figure 3. Primary T-cell responses at necropsy
Myelin- and MOG-immunized animals were sacrificed when clinical definite EAE was present (score ≥ 2.0) or at the end of the study period. At necropsy, T-cell proliferation of SC, LNC and PBMC was determined by adding ^{3}H-thymidine during the final 18 h of a 72 h culture. Cells were cultured without additives or in the presence of MOG, $pMOG_{14-36}$ (both 10 μg/ml), MBP, αB-crystallin, $PLP_{139-151}$, or OVA (all 15 μg/ml). Results are expressed as the mean stimulation index (S.I.) with standard deviation. Proliferation with an S.I. ≥ 2.0 was considered relevant. Results are shown for animals immunized with MOG (black bars), human myelin (dark grey bars), $pMOG_{14-36}$ (grey bars) and OVA (white bars). As shown, myelin as well as MOG-immunized animals displayed higher T-cell responses to MOG than to any of the other myelin proteins tested. While clear $pMOG_{14-36}$ T-cell reactivity was present in SC of $pMOG_{14-36}$-immunized marmosets, most animals immunized with MOG and a significant proportion of myelin-immunized marmosets showed reactivity to this pMOG as well.

Characterization of lymph node-derived pMOG-specific TCL

Specificity: Primary TCL were set up by *in vitro* stimulation of LNC with 10 μg MOG. T-cell reactivity was tested at the first round of restimulation. In LNC suspensions of myelin-immunized animals, proliferation in response to MOG or $pMOG_{14-36}$ was found in only 5 out of the 19 cases (Table I). By contrast, in TCL from 25 out of 26 MOG-immunized animals the main reactivity was directed against $pMOG_{14-36}$. By Ab-mediated blocking of

antigen presentation and peptide cross-presentation between non-related monkeys we could confirm our previous observation that the Caja-DRB*W1201 molecule is the restriction element for proliferation of $pMOG_{24-36}$-specific TCL (18). Thus far, this monomorphic MHC-DR allele of the common marmoset has not been described in any other primate species. However, irradiated EBV-transformed B-cells from its evolutionary ancestor, *Callithrix penicillata*, were also able to present $pMOG_{14-36}$ to specific TCL from common marmosets (not shown).

Phenotype: Figure 4 shows that $pMOG_{14-36}$-specific TCL were $CD3^+CD4^+CD8^-$ and expressed T-cell activation markers such as MHC-DR, CD25, and CD154. All TCL had a $Th_{1/0}$-like cytokine profile, production of IL-2, TNF-α, and IFN-γ being clearly detectable.

TCR-Vβ: TCR-Vβ analysis of $pMOG_{14-36}$ specific TCL did show a preferential usage of TCR-Vβ7, and -Vβ9, but TCR-Vβ6, -Vβ8, -Vβ12, and -Vβ15 were also present (Table I).

Epitope pattern: Occasionally, T-cell reactivities directed towards regions of MOG outside the core sequence 24-36 were obtained from bulk LNC cultures of MOG-immunized animals (Table I). We identified TCL reactive for $pMOG_{4-26}$ (core epitopes within $pMOG_{1-11}$ and $pMOG_{12-21}$), $pMOG_{31-46}$, $pMOG_{34-56}$ (core epitope within $pMOG_{34-50}$), $pMOG_{54-76}$, $pMOG_{64-86}$, $pMOG_{74-96}$ and $pMOG_{94-116}$ (core epitope within $pMOG_{94-110}$). In MOG-immunized monkeys a marked correlation was found between the extent of intermolecular broadening of the MOG T-cell response in MOG-immunized animals and the time of EAE onset. As shown in figure 5a, MOG-immunized animals with a relatively broad TCL reactivity at necropsy had experienced an earlier EAE onset than monkeys with a relatively narrow T-cell reactivity ($p<0.001$; Log Rank test).

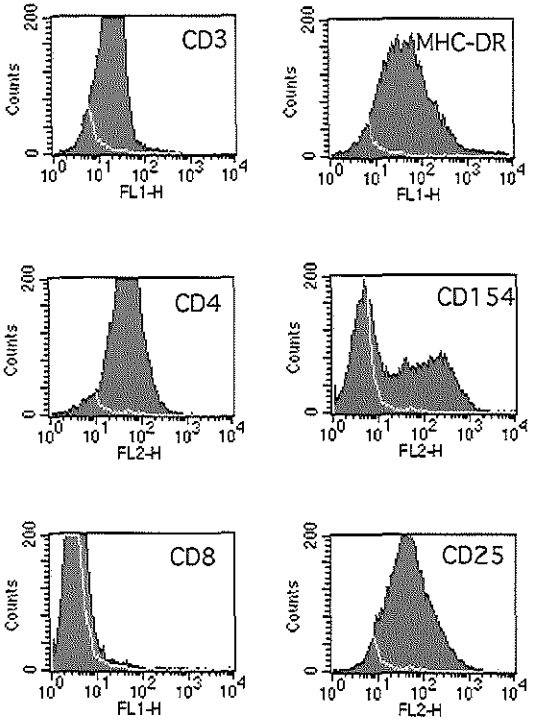

Figure 4: Phenotype of $pMOG_{14-36}$ reactive T-cell lines
T-cell lines reactive with $pMOG_{14-36}$ were characterized using human specific monoclonal antibodies reactive with CD markers at the cell surface. The reaction pattern using isotype specific controls are shown in grey lines, while the pattern after incubation with the antibody is shown in black. All tested $pMOG_{14-36}$-reactive T-cell lines stained positive for CD3 (clone sp34), CD4 (clone MT310), MHC-DR (clone L243), CD154 (clone B-B29) and CD25 (clone CLB-IL2R), but negative for CD8 (clone LT8) (see for details ref. 5).

Myelin-induced EAE

Animal	Gender[a]	sacrificed (days a.i.)[b]	EAE score[c]	T-cell reactivity (pMOG)	Antibody reactivity[d] MOG	MBP	αB	PLP
PE	F	8	5	ND	+ (0)	+	-	-
Mi-036	F	22	3.5	NI	+ (0)	+	ND	ND
Mi-028	M	31	2.5	NI	+ (0)	+	-	-
9607	M	39	4	NI	+ (0)	+	ND	ND
PR	F	39	3	ND	+ (0)	+	-	-
Mi-040	M	40	3	NI	+ (0)	+	-	-
9515	F	41	3.5	4-26; 24-36	+ (0)	+	ND	ND
9461	M	41	2	NI	+ (0)	+	ND	ND
PY	F	45	3	ND	+ (0)	+	-	-
9419	F	47	2	NI	+ (0)	+	ND	ND
Mi-042	M	49	3.5	NI	+ (0)	+	-	+
Mi-032	M	53	3	24-36	+ (0)	+	+	-
Mi-043	M	53	3	NI	+ (0)	+	+	+
9516	F	58	3	NI	+ (0)	+	+	+
Mi-031	M	71	3	24-36; 74-96	+ (0)	+	+	+
9527	M	75	2.5	24-36	+ (0)	+	ND	ND
9507	F	75	3.5	NI	+ (0)	+	ND	ND
Mi-038	M	86	1	NI	+ (0)	+	-	-
9523	F	92	3	NI	+ (0)	+	-	+
9504	M	554	2	24-36	+ (0)	+	-	+

MOG-induced EAE

Animal	Gender	sacrificed (days a.i.)	EAE score	T-cell reactivity (pMOG)	TCR-Vβ pMOG$_{14-36}$ TCL	Antibody reactivity MOG	MBP	αB	PLP
QQ	M	34	3	24-36; 54-76;74-96	9,18	+ (0)	-	ND	ND
Mi-022	M	36	5	24-36; 64-86	ND	+ (0)	-	-	-
KK	M	38	3	24-36; 34-50; 94-110	ND	+ (7)	-	+	-
QR	F	42	3	24-36; 34-50; 64-86	ND	+ (5)	-	+	-
9902	M	44	3.5	24-36; 94-110	ND	+ (6)	+	+	-
QI	M	50	2	24-36	7,9,12	+ (6)	-	+	-
QK	M	50	0.5	24-36	ND	+ (6)	-	+	-
QO	F	50	3	24-36	7,9	ND	ND	ND	ND
Mi-066[e]	M	52	0	24-36;54-76	ND	+ (6)	-	+	-
9601	M	58	3	1-11; 12-21; 24-36; 31-46	ND	+ (8)	-	-	-
Mi-011	M	62	3	1-11; 24-36; 34-50	ND	+ (1)	-	-	-
9854	M	67	2.5	24-36; 54-76; 74-96	ND	+ (3)	-	-	+
9853	F	67	3	24-36	ND	+ (3)	-	-	-
Mi-069	M	67	2.5	24-36; 54-76	12	+ (7)	-	-	+
Mi-065	M	67	2.5	24-36	ND	+ (7)	-	+	-
9501	M	70	3	24-36	6,7,8,9,12,15	+ (8)	-	+	+
9813	M	78	3	NI	-	ND	ND	ND	ND
9814	M	78	2.5	24-36	6,7,8,9	+ (1)	-	-	-

Animal	Gender	sacrificed (days a.i.)	EAE score	T-cell reactivity (pMOG)	TCR-Vβ $pMOG_{14-36}$ TCL	Antibody reactivity MOG	MBP	aB	PLP
9819	M	94	3	24-36	7,9,12	+ (5)	-	-	-
Mi-010	M	106	3	24-36	7,8,9,15	ND	ND	ND	ND
Mi-009	M	112	2.5	24-36	14,16	ND	ND	ND	ND
9841	M	112	2	24-36	ND	+ (1)	-	-	-
9505	M	115	3	24-36; 64-86	ND	+ (1)	-	-	-
Mi-062	M	139	3	24-36	ND	+ (7)	+	+	-
Mi-020	M	155	0	24-36	ND	+ (5)	-	-	-
Mi-021	M	163	0.5	24-36; 74-96	ND	+ (8)	-	+	+
				$pMOG_{14-36}$-induced EAE					
QY	M	88	1	24-36	ND	+ (2)	-	+	-
QW	F	95	1.5	24-36	ND	+ (2)	-	-	-
QX	M	119	2	24-36	ND	+ (3)	-	-	-
QV	F	128	2.5	24-36	ND	+ (1)	-	-	-

Table I: Characteristics of myelin-, MOG- and $pMOG_{14-36}$-induced disease in the common marmoset.
a: Gender is specified as male (M) or female (F). b: a.i. after immunization; c: Clinical signs of EAE were scored as: 0, no clinical signs; 0.5, apathy, loss of appetite and altered walking pattern without ataxia; 1.0, lethargy and/or anorexia; 2.0, ataxia, sensory loss/blindness; 2.5, hemi- or paraparesis; 3.0, hemi- or paraplegia; 4.0, quadriplegia; 5.0, spontaneous death attributable to EAE. days a.i.: days after immunization; The number of Ab reactivities to linear pMOG sequences is given between brackets; +: positive Ab reactivity; - Ab reactivity below detection levels; ND: not done; NI: not identified. e:animal died due to respiratory complications, not to EAE.

Autoantibody reactivity

In monkeys immunized with myelin the main Ab reactivity was with rhMOG. In none of these monkeys was Ab reactivity found towards any of the tested pMOG. The Ab responses to MBP was comparable with the anti-MOG Ab response. In addition, lower levels of Ab responses directed towards PLP and αB-crystallin were detected in a subset of animals (Table I).

In immune sera from 20 out of the 22 MOG-immunized monkeys tested, sera Ab reactivity with MOG peptides could be found. Moreover, Ab reactivity towards both PLP and αB-crystallin was frequently observed, but anti-MBP Ab were never detected (Table I). The main anti-MOG Ab reactivity was directed towards three MOG peptides being $pMOG_{4-26}$, $pMOG_{24-46}$ and $pMOG_{54-76}$. Analysis of longitudinal serum samples from MOG-immunized marmosets showed that the first detectable serum reactivity was directed towards $pMOG_{54-76}$, while the reactivity towards the other peptides emerged during EAE progression (not shown). No correlation was found between the number of different anti-pMOG Ab reactivities at necropsy and the time of onset or clinical severity of EAE (Figure 5b).

The absence of MOG peptide reactivity of immune sera from myelin-immunized monkeys indicates that the main anti-MOG Ab reactivity is directed towards discontinuous epitopes. This was examined by preincubation of immune sera with the mix of all MOG peptides or with a mix lacking $pMOG_{54-76}$ before they were tested with ELISA. Figure 6a shows that a significant proportion of the reactivity with rhMOG is absorbed by the peptide panel. That the absorption of rhMOG reactivity is largely abolished when $pMOG_{54-76}$ is omitted from the peptide mix underscores that this peptide contains an important B-cell epitope. When the same procedure was applied to sera from myelin-immunized monkeys, no evidence for absorption of anti-MOG Ab reactivity was found. This strongly suggests that the anti-MOG Ab specificity in these monkeys is mainly directed towards conformational epitopes.

The Ab reactivity in $pMOG_{14-36}$-immunized marmosets remains confined to peptides overlapping the challenging peptide, i.e. 4-26, 24-36. The detected Ab reactivity towards $pMOG_{4-26}$ as well as to $pMOG_{24-46}$ suggests that different B-cell epitopes are present within $pMOG_{14-36}$ (Figure 6b). The anti-MOG Ab reactivity was completely abolished by preincubation with $pMOG_{14-36}$. This indicates that $pMOG_{14-36}$-immunized marmosets fail to generate Ab responses to discontinuous MOG epitopes (Figure 6b), which might explain the absence of demyelination in these animals.

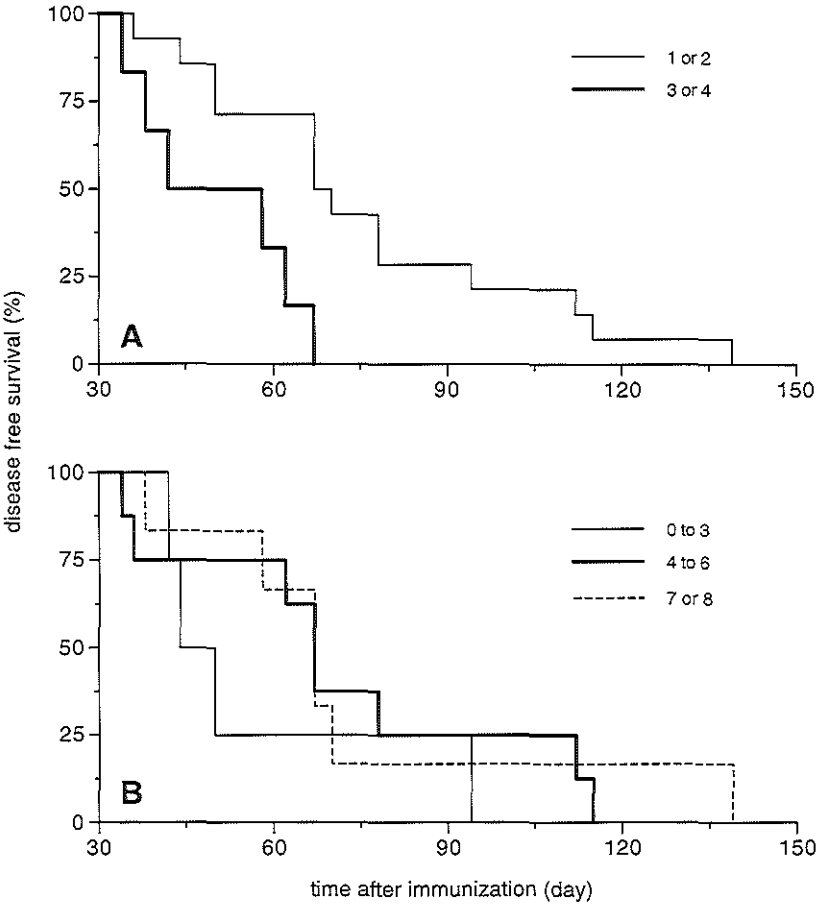

Figure 5: Intramolecular broadening of the MOG T-cell response but not the B-cell response in MOG-immunized animals is correlated to early onset of EAE
A: MOG-immunized animals with three or four TCL with different pMOG specificities (bold line) at necropsy developed severe clinical EAE at an earlier time point than MOG-immunized animals from which only one or two T-cell reactivities (thin line) were found ($p<0.001$; Log Rank test). All animals harboring three to four different T-cell reactivities developed paralyzing EAE within 67 days after immunization. At this time point 50 % of the animals in which only one or two pMOG T-cell reactivities were identified were still clinically healthy. **B:** In contrast to the relation found at the T-cell level, no significant differences in survival times were observed in animals that showed 0 to 3 (thin line), 4 to 6 (bold line), and 7 or 8 (dashed line) different pMOG reactivities.

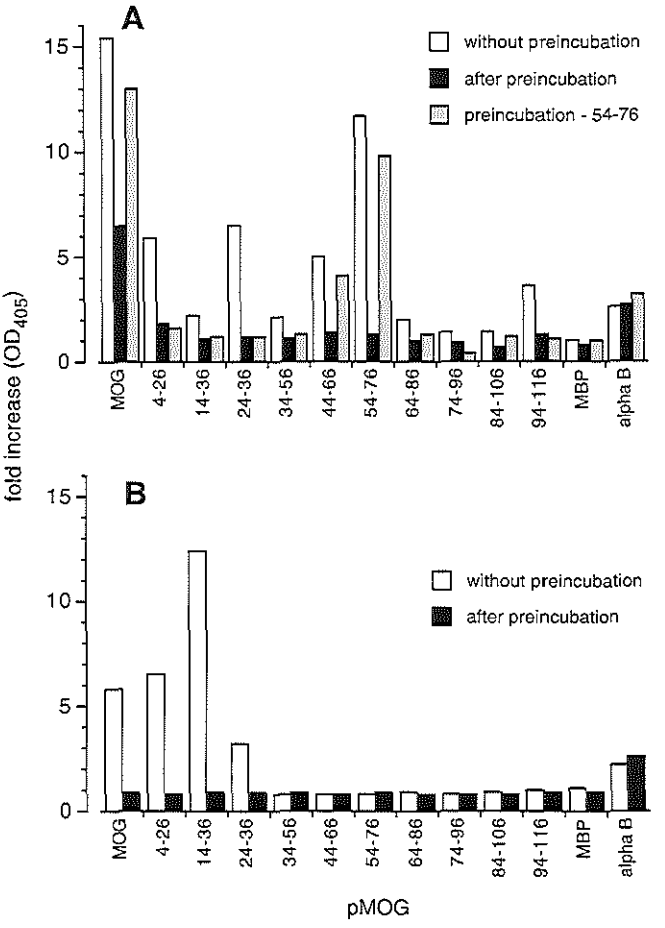

Figure 6: Anti-MOG Ab responses in MOG and $pMOG_{14-36}$-immunized marmosets.
A: Animal QR developed anti-pMOG Ab reactivity to $pMOG_{4-26}$, $pMOG_{24-46}$, $pMOG_{44-66}$, $pMOG_{54-76}$, and $pMOG_{94-116}$ at necropsy (white bars). When the serum was pre-absorbed with a pool of overlapping pMOG (10 µg/ml for each pMOG), all pMOG reactivity dropped below detection levels; however, Ab reactivity to the intact MOG remained detectable (black bars). When $pMOG_{54-76}$ was omitted from the MOG peptide pool, comparable levels of anti-MOG Ab reactivity were present while anti-$pMOG_{54-76}$ Ab reactivity was detectable as well (grey bars). These results indicate that a significant proportion of the Ab reactivity in sera of MOG-immunized animals is directed towards discontinuous epitopes within MOG. **B:** No Ab reactivity to discontinuous epitopes was demonstrated in sera of animals immunized with $pMOG_{14-36}$ (white bars), since all Ab reactivity fell below baseline levels when serum was pre-incubated with the pMOG mix (black bars).

Discussion

Clinical expression and pathology

Immunization of common marmosets with myelin or MOG induces chronic progressive EAE in 100% of the animals. The mean time of onset of disease in both EAE models is around 50 days after immunization, and the majority of the animals develop paralyzing EAE within 80 days. The disease onset in our marmoset EAE models is significantly delayed compared to the models developed by Genain and colleagues (34). Apparently, the usage of *B. pertussis* in the EAE induction protocol causes 70% of the animals to develop clinical EAE within 14-28 days after immunization. The extensive variation in time of onset and progression of the disease between animals likely reflects the outbred nature of this species. MOG-immunized animals showed a variable degree of demyelination in the brain and the spinal cord. In contrast to published findings, we could not establish a good correlation between the extent of demyelination and the clinical expression of the disease (34).

T-cell responses

As MOG is only a minor component of CNS myelin it is surprising that a high proportion of the LNC reaction in myelin-induced EAE is directed towards this protein. Significant proliferative responses to a major myelin component, such as MBP are lacking in this model. In MOG-induced LNC from 14 out of the 19 myelin-immunized animals, anti-MOG responses were no longer detectable after a single round of *in vitro* restimulation. In contrast, LNC stimulated with crude myelin preparations remain responsive to myelin components (unpublished observations). While in all LNC cultures from myelin-immunized animals MOG reactivity has disappeared after several rounds of restimulation, a remarkably high proliferative response remains towards the EBV-transformed B-cells that were used as APC.

A possible explanation for this observation is that the initial LNC suspensions contain APC with ingested myelin (see chapter II.2.3). We assume that T-cells specific for these myelin antigens rapidly overgrow the anti-MOG response in long-term cultures. Since MOG is highly immunogenic, anti-MOG T-cell responses in the myelin-EAE model might be down regulated by the immune system as was hypothesized by Matzinger (35), for example by regulatory T-cells (36). This hypothesis is supported by a recent showing that the blood of naïve common marmosets contains a remarkably high frequency

of MOG-reactive T-cells that are under tight control of regulatory T-cells (31).

Epitope spreading

The progressively broadening epitope reactivity of autoreactive T-cells and B-cells has been put forward as a pathogenic mechanism operating in the progression of MS (37-42). The present study confirms our previous observation that the CD4-cell reactivity to $pMOG_{24-36}$ is an important pathogenic event shared by all monkeys. This reactivity seems to persist throughout the disease, as from all MOG-immunized animals except one we have isolated TCL with $Th_{0/1}$ phenotype reactive with the $pMOG_{24-36}$, regardless of the time point of sacrifice. This is in line with previously published data from naïve as well as from MOG-EAE affected animals (18-20,31). A recent study demonstrated that long term persistence of specific T-cell clones is also a common feature among MS patients (43).

It is clear that adoptively transferred $pMOG_{21-40}$-reactive T-cell clones (34) form perivascular cuffs, indicating that they can transmigrate the blood-brain-barrier. However, neither in $pMOG_{14-36}$ immunized monkeys (this study), nor in monkeys injected with $pMOG_{21-40}$-reactive T-cell clones (34), could abundant demyelination be found. While no preferential TCR-Vβ usage was found in MBP-specific T-cell clones from immunized as well as naïve marmosets (44), we show here that $pMOG_{14-36}$-reactive TCL from MOG-immunized marmosets have a tendency to preferentially use a limited set of TCR-Vβ elements.

Besides the shared $pMOG_{14-36}$ reactivity, MOG-immunized marmosets display a variable T-cell response against epitopes within the MOG peptide panel. These additional peptide responses are likely restricted by members of the two polymorphic Caja-DR loci, since cross presentation by APC from unrelated monkeys occurred only in some combinations (Brok, unpublished observations). The observation that a broadened anti-MOG T-cell response was strongly correlated with an earlier onset of EAE rather than the disease duration marks a clear difference regarding the situation in rodent EAE models. Since comparable anti-MOG T-cell reactivities have been reported in some naïve marmosets (20,31), it would be interesting to investigate whether these animals develop a more rapid onset of EAE upon immunization with MOG.

Antibodies

Several observations support a critical role of anti-MOG Ab in common marmoset models of EAE. First, prominent demyelination in the CNS of monkeys injected with encephalitogenic $CD4^{+}TCR\alpha\beta^{+}$ Th_1-cells only occurs when purified anti-myelin or anti-MOG Ab are injected as well (14). Furthermore, exacerbation of EAE was found associated with increased Ab production (15), while inhibition of the anti-MOG Ab response suppressed EAE (19,22). In addition, McFarland and colleagues observed in monkeys immunized with a chimerical MBP/PLP protein that onset of clinical disease depends on the presence of anti-MOG Ab (17). Finally, a proportion of the auto-Ab in CNS white matter lesions of EAE-affected animals and MS patients is reactive with pMOG (24,25).

In this report we show a different Ab response between myelin- and MOG-induced EAE models. In MOG-induced EAE, Ab directed to both discontinuous and linear MOG epitopes are found, in the absence of significant anti-MBP responses. That Ab responses to CNS-proteins such as PLP and αB-crystallin are found in MOG-induced EAE shows that intermolecular epitope spreading occurs after disease induction. MBP is probably a very weak antigen in marmosets although EAE can be induced with this antigen provided that strong adjuvants have to be used. In myelin-immunized monkeys clear anti-MBP Ab levels were found, while anti-MOG Ab responses were confined to discontinuous structures of the MOG molecule. Importantly, immune sera from MOG- as well as from myelin-immunized animals bind to a cell line expressing a human MOG transgene, as was assessed by flow cytometry (Brok and Linington, unpublished observations). The relevance of Ab reactivity against discontinuous epitopes is illustrated by absence of demyelination in $pMOG_{14-36}$-immunized animals in this study as well as the observation that a combination of overlapping MOG peptides could not reproduce the prominent demyelination as seen after immunization with MOG (34).

One possible explanation for the absence of Ab binding to pMOG in myelin-immunized monkeys is that in its natural glycosylated configuration MOG is protected against extensive processing, allowing only the generation of Ab towards discontinuous epitopes. In animals immunized with recombinant MOG, the bare protein may be degraded and processed by professional APC in peripheral LN. It can be envisaged that under these conditions Ab responses towards processed MOG are generated.

The main reactivity with linear MOG sequences in MOG-induced EAE is directed to three regions of the MOG protein, namely $pMOG_{4-26}$, $pMOG_{24-46}$ and $pMOG_{54-76}$. The peptide inhibition experiments indicate that $pMOG_{54-76}$ is part of an important conformational epitope. No direct relation exists between the diversity of Ab reactivity towards linear sequences and the time of onset or perpetuation of the disease or to the total lesion load in brain and spinal cord. These data seem to contrast with studies in rodents (29) and rhesus monkeys (Brok *et al.*, manuscript in preparation) in which significant demyelination has been observed after immunization with a linear $pMOG_{35-55}$ sequence. Possibly in these cases the generated Ab responses are either cross-reactive with discontinuous epitopes or spreading of the B-cell response occurs.

In conclusion, our results strongly suggest that Ab reactivities towards discontinuous MOG epitopes are needed for induction of demyelinating EAE. This class of Ab is present in myelin- and MOG-induced EAE, while absent in animals that were challenged with pMOG, PLP (34) or MBP (18). Specific Ab reactivity directed against MBP, αB-crystallin and PLP was confirmed in both models but does not seem to provide a major contribution to disease development or progression. While $pMOG_{14-36}$-reactive TCL are shared between all MOG-immunized common marmosets, intra-molecular broadening of the anti-MOG T-cell response contributes significantly to early onset of paralyzing EAE in this model.

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Transfer of central nervous system autoantigens and presentation in secondary lymphoid organs

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Abbreviations: APC, antigen presenting cell(s); CFA, complete Freund's adjuvant; CNS, central nervous system; DC, dendritic cell(s), EAE; experimental autoimmune encephalomyelitis, HRP; horseradish peroxidase, Ig; immunoglobulin(s), LN; lymph node(s), MBP; myelin basic protein; MHC, major histocompatibility complex; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; ORO, oil-red O; OVA, ovalbumin; PLP, proteolipid protein; phMOG, human MOG peptide; rhMOG, recombinant human MOG

ABSTRACT

Dendritic cells (DC) are thought to regulate tolerance induction versus immunisation by transferring antigens and peripheral signals to draining lymph nodes (LN). However, whether myelin antigen transfer and presentation in LN occurs during demyelinating brain disease is unknown. Here we demonstrate redistribution of autoantigens from brain lesions to cervical LN in monkey experimental autoimmune encephalomyelitis (EAE) and multiple sclerosis. Myelin antigens are present within cells, expressing DC/macrophage-specific markers, MHC class II and costimulatory molecules, and which are directly juxtaposed to T cells. Cervical LN also contain myelin antigen-reactive T cells, allowing cognate interactions between myelin containing antigen presenting cells and lymphocytes at this site. Our findings have implications for regulation of autoreactivity in immunoprivileged organs, epitope spreading and targeted therapy.

INTRODUCTION

Current views on the initiation of primary immune responses hold that dendritic cells (DC) transfer antigens from peripheral tissues into secondary lymphoid organs, where they activate or tolerise naive lymphocytes depending on the nature of the antigen and peripheral stimuli (1-7). Although this concept is now fairly well established for model immunogens, microbial antigens and apoptotic cell material, far less is known about transfer and presentation of autoantigens *in vivo* during chronic inflammatory disease. Autoimmune responses directed against myelin components are considered to be pivotal in the development and perpetuation of multiple sclerosis (MS), but the nature of the events leading to the activation of autoreactive lymphocytes is largely unclear. This concerns both the anatomical site of antigen presentation and the type of antigen presenting cell (APC) involved. These issues are particularly relevant in relation to the continuous broadening of the autoimmune response during chronic MS (8, 9), a phenomenon known as epitope spreading. The initial T-cell reactivity is directed towards a narrow set of myelin epitopes, but spreads to other epitopes on the same antigen (intramolecular spreading) or to other myelin antigens (intermolecular spreading). The pathogenic significance of this process was demonstrated in relapsing experimental autoimmune encephalomyelitis (EAE) in mice (10-13). The mechanism underlying epitope spreading is thought to involve APC that present degraded myelin derived from the inflammatory process in the

central nervous system (CNS), but it is unknown where this presentation occurs.

The CNS has long been regarded as an immune privileged site sequestered from the immune system by the blood-brain barrier and the lack of lymphatic vessels, but it is now clear that antigens and cells injected into the brain drain to secondary lymphoid organs (14-16). Outflow of antigens from the brain occurs by interstitial fluid and cerebrospinal fluid drainage to lymphoid organs via various pathways. Antigens in these fluids can drain via the blood to the spleen, can escape along cranial nerves into the nasal lymphatics and drain to the cervical lymph nodes (LN), and can escape along spinal nerves to other LN.

The objective of the present study was to determine whether APC, that have taken up myelin antigens in the brain during an inflammatory process, drain to the cervical LN and other lymphoid organs, and have the potential to present these myelin autoantigens to T and B cells *in situ*. Improved insight into these mechanisms will facilitate elucidation of tolerance induction versus autoimmunity and rational development of immunotherapy of demyelinating disease.

We investigated the possible transfer and presentation of myelin antigens in lymphoid organs during a demyelinating inflammatory process in the CNS in non-human primates and in MS. The EAE model in common marmoset monkeys (*Callithrix jacchus jacchus*) mimics human MS in its clinical presentation and its radiological, neuropathological and immunological aspects of brain white matter lesions (17-20). The close phylogenetic relationship of marmosets and humans makes EAE in this species an unique experimental system for detailed analysis of MS immunopathogenesis. Moreover, this outbred model has several useful features, including (a) a 100% disease incidence, which results from presentation of an encephalitogenic peptide by a monomorphic major histocompatibility complex (MHC) class II molecule (21), (b) primary demyelination occurring after immunisation with whole human myelin or with recombinant human myelin oligodendrocyte glycoprotein (rhMOG), (c) intra- and intermolecular epitope spreading of the cellular and humoral immune response (21, 22) and (d) the fact that the complete disease spectrum is based on the intricate interplay between APC, T cells, macrophages and plasma cells (19, 23). In parallel to lymphoid organs of marmosets with EAE, we investigated cervical LN of rhesus monkeys with fulminant EAE induced by MOG immunisation (24) and MS patient LN from the neck region.

Material and Methods

EAE tissues

Lymphoid organs and brain were isolated from common marmosets, rhesus monkeys (*Macaca mulatta*) and cynomolgus monkeys (*Macaca fascicularis*) raised at the Biomedical Primate Research Centre (Rijswijk, The Netherlands), the German Primate Centre (Göttingen, Germany) and at Inveresk (Tranent, UK) as described previously (21, 24), under conditions approved by the Dutch, German and British law, respectively, on animal experimentation. All experimental procedures were approved by Institutional Animal Care and Use Committees. EAE was induced in marmoset monkeys by immunisation with 20 mg human whole myelin or 0.1 mg rhMOG emulsified in complete Freund's adjuvant (CFA) as described (17, 21). Adjuvant control marmosets were immunised with 1 mg OVA (Sigma Chemical Co, St. Louis, MO) in CFA. Emulsions were injected into the dorsal skin at four different sites; two injections were given in both the inguinal and axillary LN regions. EAE was induced in rhesus monkeys by immunisation with 0.32 mg rhMOG or 0.1 mg MOG_{34-56} (phMOG) in CFA as described (24). The MOG/CFA emulsion was injected into the dorsal skin at 10 different spots on the back. Adjuvant control rhesus monkeys were immunised with 3 to 5 mg bovine type II collagen in CFA as described (55). All myelin antigen immunised animals were examined daily for clinical symptoms of EAE by a trained observer. Brain inflammation was visualised by magnetic resonance imaging and/or (immuno-)histochemistry as described previously (17, 28). According to these examinations, all animals immunised with myelin antigens had clinically active EAE and inflammatory foci in their brain at the time of necropsy. Deep cervical LN and spleen were isolated from animals sacrificed by an intravenous injection of pentobarbital during deep ketamine sedation. In addition, axillary and inguinal LN, draining the immunisation sites were isolated for comparison. All lymphoid tissues were immediately frozen in liquid nitrogen and stored at -80 °C until use.

MS tissues

Human LN taken from a patient with relapsing/chronic/active MS at autopsy, were provided by the Netherlands Brain Bank. LN included two external jugular (lateral superficial cervical) LN from either side of the neck and one supraclavicular LN. After removal, LN were snap frozen in liquid nitrogen and stored at -80 °C until use.

Histochemistry

Myelin degradation products were detected with ORO, which stains neutral lipids, as previously described (56). Briefly, cryosections (6 µm) were stained with 0.3% (w/v) ORO (Gurr Ltd, London, UK) in 60% 2-propanol and counter stained with hematoxylin. Acid phosphatase, a marker of macrophages, was detected in acetone fixed sections as previously described (57) using naphtol-AS-BI-phosphate (Sigma) as substrate.

Immunohistochemistry

Immunohistochemistry was performed as described previously (28, 58) with slight modifications. Sections were fixed in 4% paraformaldehyde in PBS (pH 7.4) and treated with 0.02% (v/v) H_2O_2 in PBS to eliminate endogenous peroxidase activity. Any remaining endogenous peroxidase activity was demonstrated with 4-chloro-1-naphthol (dark blue reaction product; Sigma). Sections were incubated with primary antibodies overnight in humidified atmosphere, followed by biotinylated-secondary antibodies for half an hour and horseradish peroxidase (HRP)-conjugated avidin-biotin-complex (ABC-HRP; Dako, Glostrup, Denmark) for 1 hour. Labelled cells were stained with either 3-amino-9-ethylcarbazole (red reaction product; Sigma Chemical Co) or diaminobenzidine (brown reaction product; Sigma). Finally, sections were counterstained with hematoxylin and embedded in glycerol-gelatin. Double labelling of cells was performed as described previously (58) with alkaline phosphatase-conjugated secondary antibodies and a substrate solution containing Fast Blue BB Base (blue reaction product; Sigma).

The primary antibodies used for immunohistochemistry were polyclonal rabbit anti-bovine MBP (kindly provided by J. Bajramovic, TNO Prevention and Health, Leiden, The Netherlands), rabbit anti-CD3 (Dako) and monoclonal antibodies directed against the encephalitogenic epitope of PLP in SJL/J mice, i.e. $PLP_{139-151}$ (J1/03; ref.(59)), MHC class II (L243; Becton Dickinson, San Diego, CA), and the APC markers CD40 (5D12; Tanox Pharma BV, Amsterdam, Netherlands), CD68 (KP-1; Dako), CD83 (HB15A; Immunotech, ME), CD86 (1G10; kindly provided by Innogenetics NV, Gent, Belgium), DC-SIGN (AZN-D1; kindly provided by Y. van Kooyk, University Medical Center St. Radboud, Nijmegen, The Netherlands) and CCR7 (2H4; BD-Pharmingen, San Diego, CA). Secondary biotinylated antibodies were rabbit-anti-mouse immunoglobulin (Ig; Dako), donkey-anti-rabbit Ig (Amersham, Buckinghamshire, UK), horse-anti-mouse Ig (Vector Laboratories, Burlingame, CA), alkaline-phosphatase conjugated rabbit-anti-mouse Ig, goat-anti-rabbit Ig (both from Dako) and rabbit-anti-goat Ig (Southern Biotechnology Associates, Birmingham, AL).

Quantitation of stained cells

In order to calculate the number of ORO-, anti-MBP- or anti-PLP-stained cells per mm^2 in cervical LN sections, the cells were counted and the area of the sections was determined using a VIDAS-RT image analysis system (Kontron Elektronik GmbH/Carl Zeiss, Weesp, The Netherlands). Area measurements were performed using a 2.5-fold magnification objective.

T-cell proliferation assays

Proliferation assays with cervical LN cells were performed essentially as previously described (21, 60). Briefly, LN cell suspensions were prepared from aseptically removed cervical LN from sacrificed marmoset and rhesus monkeys with EAE. LN cells were cultured with 10 µg/ml rhMOG or phMOG in HEPES-buffered RPMI

1640 (Life Technologies, Glasgow, UK) supplemented with 10% FCS (Flow Laboratories, McLean, VA), 10 mM MEM with nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin and $2x10^{-4}$ M 2-ME (all from Life Technologies). Marmoset cervical LN cells were restimulated with EBV-transformed autologous B cells and 10 µg/ml rhMOG. Proliferation of marmoset cervical LN cells was tested after one or two rounds of restimulation, whereas proliferation of rhesus cervical LN cells was tested directly after the first round of stimulation. Marmoset and rhesus LN cells ($2x10^4$ cells/well) were seeded with or without relevant antigen in the presence of EBV-transformed autologous B cells ($2x10^4$ cells/well) or irradiated spleen cells ($2x10^5$cells/well), respectively, in 96-well flat-bottom plates (Greiner, Solingen, Germany) for 48 h and subsequently pulsed with 0.5 µCi [^{3}H]thymidine for 18 h. [^{3}H]thymidine incorporation was measured in a matrix 9600 beta-counter (Packard, Meriden, CT).

Statistics

Differences in the number of myelin antigen containing cells in cervical LN between treatment groups were determined by a two-tailed Mann-Whitney test. The number of stained cells in the axillary and inguinal LN and spleen was scored per visual field using a 10-fold objective in a semi-quantitative manner as: '-' no positive cells; '+/-' 1 to 5 positive cells; '+' 6 to 20 positive cells; '++' 21-50 positive cells; '+++' >50 positive cells.

RESULTS

EAE and CNS inflammation in common marmosets

To investigate the drainage of myelin degradation products to secondary lymphoid organs under normal physiological conditions and during active inflammation of the CNS, different LN and spleen were isolated from non-immunised marmoset and cynomolgus monkeys, from marmoset and rhesus monkeys immunised to develop EAE, and from adjuvant control marmoset and rhesus monkeys. Tissues were obtained from animals that participated in studies designed for other purposes, thus avoiding sacrifice of animals for the purpose of the present study only. According to clinical, radiological or (immuno-)histochemical examination, all myelin antigen immunised monkeys possessed cellular infiltrates in the CNS at the time of sacrifice (data not shown). (Immuno-)histochemical analysis of inflammatory foci in EAE brains showed the presence of oil-red O (ORO)-positive cells (Fig. 1A), i.e. cells containing neutral lipids as a result of myelin degradation, and proteolipid protein (PLP) containing cells (Fig. 1B), similar to previous findings in marmoset EAE (17). In this respect, the neuropathology of marmoset EAE closely mimics the situation in MS, where abundant ORO-positive cells are

also found in active brain lesions (25, 26). Neutral lipids as visualised by ORO are not just a marker for degraded myelin, but are relevant to pathogenesis since self glycolipids are T-cell autoantigens in MS (27). In the EAE lesions, both ORO and PLP staining were found mainly in the cytoplasm of large cells with a morphology closely resembling macrophages. Furthermore, EAE lesions contained numerous cells expressing MHC class II, acid phosphatase, CD40 (28) and CD83 (data not shown), indicative of the presence of APC.

Myelin antigens in cervical LN and spleen

To determine whether myelin antigens gain access to brain-draining lymphoid organs during demyelinating EAE, sections of cervical LN and spleen were stained with ORO to detect degraded myelin lipids, with a polyclonal antiserum against MBP, or with a monoclonal antibody against $PLP_{139-151}$, a classical encephalitogenic epitope in SJL/J mice.

In the cervical LN of both myelin- and MOG-immunised marmosets, a large number of myelin antigen containing cells were detected (Fig. 1C,D and 2). A striking difference was found in the number of ORO-positive cells, which were detected in 9 out of 10 marmosets with EAE, but in none of the untreated marmosets ($P < 0.05$) or cynomolgus monkeys (Fig. 2A). Moreover, a significantly higher number of myelin basic protein (MBP) and PLP containing cells was observed in the cervical LN of marmosets with EAE as compared to the reference groups (Fig. 2B,C).

To exclude the possibility that myelin antigens were present in the cervical LN as a result of the administration of adjuvant, we analysed the LN from rhesus monkeys immunised with either MOG or type II collagen emulsified in CFA. We chose to examine the LN of these animals, since we were unable to retrieve them from ovalbumin (OVA) immunised marmoset monkeys. Strikingly, the number of MBP- and PLP-containing cells was markedly higher ($P < 0.05$) in the LN of rhesus monkeys with EAE as compared to the adjuvant control animals (Fig. 3A,B). ORO-positive cells were not detected in cervical LN of both rhesus EAE and control monkeys.

Together, these results demonstrate that myelin antigens in the cervical LN result from the demyelination of the CNS and not from the immunisation itself, nor from normal myelin turn-over or from local neurones innervating the LN. The finding that MBP and PLP are present in the cervical LN of marmoset and rhesus monkeys immunised with MOG only, unequivocally shows that myelin antigens in this lymphoid organ are derived from the inflamed CNS rather than from the injected emulsion.

In the cervical LN of marmosets, most myelin antigen containing cells were detected in the medulla. Some myelin antigen containing cells, however, were found in the subcapsular sinus, the site where leukocytes enter the LN, and in extrafollicular areas of the cortex, the site where interaction of APC with T cells occurs. Myelin antigens were found mostly in the cytoplasm of large cells with a morphology resembling macrophages, similar to the findings in the brain. Strikingly, most MBP- and PLP-positive cells expressed macrophage markers, including the lysosomal enzyme acid phosphatase (Fig. 4A) and CD68 (Fig. 4B).

In addition to the cervical LN, the spleen was analysed for the presence of myelin components. ORO-positive cells were detected in none of the spleens of myelin or rhMOG-immunised marmosets, nor of OVA-immunised marmosets or untreated monkeys (Table 1). A significant number of PLP containing cells and a few MBP containing cells, however, were detected in the red pulp and around vessels within the spleen of marmosets with EAE. In contrast, the spleen of adjuvant controls and untreated monkeys was almost completely devoid of cells containing MBP and PLP. These results provide further evidence that myelin antigens gain access to brain-draining lymphoid organs as a direct result of CNS inflammation.

Myelin containing cells in cervical LN express APC markers and co-localise with T cells

Double labelling studies were performed to determine if myelin antigen containing cells in the cervical LN of monkeys with EAE represented cells with the potential to present antigens to $CD4^+$ T cells. Myelin containing cells displayed features of professional APC, including expression of the DC markers CD83 (Fig. 4C) and DC-SIGN (Fig. 4D), which is essential in DC-induced T cell proliferation (29, 30). Staining for both acid phosphatase and CD83 revealed many double positive cells in the cervical LN (data not shown), indicating that macrophage and DC markers can be co-expressed by cells loaded with myelin antigens. The majority of MBP and PLP containing cells in the cervical LN also showed cell-surface expression of MHC class II (Fig. 4E) and of the costimulatory molecules CD40 (Fig. 4F) and CD86 (not shown). In addition, myelin antigen containing cells expressed the chemokine receptor CCR7 (Fig. 4G), the receptor for the LN-derived chemokines CCL19 (MIP-3β or ELC) and CCL21 (6-C-kine or SLC) driving DC migration (31).

To assess whether myelin antigen containing cells co-localised with T cells, indicative of cognate interaction, double labelling for PLP and CD3 was performed on cervical LN of monkeys with EAE. In the medulla, numerous

PLP-positive cells were found in juxtaposition with T cells (Fig. 4H). PLP-positive cells were also found in the subcapsular sinus at the border of the T-cell area and occasionally within the T-cell areas of the cervical LN. These results demonstrate that myelin antigens are present in the cervical LN within cells, which have the characteristics of mature professional APC and which are in direct contact with T cells.

Myelin antigens in axillary and inguinal LN

The axillary and inguinal LN were also examined to determine whether myelin antigens gain access to other lymphoid organs. Analysis of these LN from myelin antigen-immunised and OVA-immunised marmosets revealed a complete absence of ORO-positive cells (Table 1), similar to the spleen. A small to moderate number of cells containing MBP and PLP, respectively, was detected in the LN of both marmosets with EAE and adjuvant control marmosets. These results indicate that myelin antigens in these LN, which drain the immunisation sites, may result from a response to the adjuvant administration rather than from the inflammatory process in the CNS.

Myelin containing cells in cervical LN of an MS patient

To determine whether these findings are relevant for MS, we examined LN isolated from the neck region of a patient with active disease. Similar to rhesus EAE, LN contained cells loaded with MBP and PLP, but not neutral lipids from degraded myelin (Fig. 1E, F). In the external jugular LN, the density of cells containing MBP (mean 6.4 cells/mm^2) or PLP (mean 19.2 cells/mm^2) was similar to the density in cervical LN of marmosets with EAE (mean 4.3 and 13.2 cells/mm^2, respectively). The supraclavicular LN, which are located more remote of the brain as compared to the external jugular LN, contained lower numbers of cells containing MBP or PLP (2.5 and 8.9 cells/mm^2, respectively). These results imply that transfer of myelin antigens during demyelinating inflammation in monkeys and man occurs in identical fashion. Functional phenotyping of these myelin antigen containing cells in external jugular LN revealed that the majority of cells expressed acid phosphatase, MHC class II, CD40, but not CD83 (data not shown), indicating that in human LN these cells also represent immunocompetent APC.

Myelin-specific T cells in cervical LN

In order to determine whether cervical LN contain myelin-specific T cells besides myelin antigen containing APC, cervical LN cells of marmoset and rhesus monkeys with EAE were tested for MOG-specific proliferative

responses. Since primary cervical LN cells showed low proliferative responses as expected, proliferation was determined after a primary stimulation or after one or two rounds of restimulation. Positive responses, stimulation index >2, were found with cervical LN cells from 3 of 4 marmosets and all rhesus monkeys (Fig. 5), indicating that myelin specific T cells are present in these brain draining LN.

DISCUSSION

Autoimmunity towards myelin and other CNS components is considered to play a pivotal role in the development and perpetuation of MS, but the anatomical site of antigen presentation and the type of APC involved in the activation of autoreactive lymphocytes is still largely unclear. In this study we explored the redistribution of myelin components during demyelinating EAE in non-human primates and MS, and characterised the cells loaded with myelin antigens in the draining LN. Using demyelinating EAE models, we demonstrate that (a) myelin antigens (neutral lipids, MBP and PLP) gain access to brain-draining lymphoid organs as a result of a demyelinating process in the CNS, (b) myelin antigen containing cells display characteristics of professional APC, (c) myelin antigen containing cells are in direct contact with T cells, and (d) myelin antigen specific T cells are present at this site. These results indicate that myelin antigens do not remain sequestered behind the blood-brain barrier during EAE, but are transferred to sites that are optimally equipped for priming of myelin specific T and B cells. Moreover, these data strongly suggest that cognate interactions between these myelin containing APC and T cells take place in brain-draining lymphoid organs such as the cervical LN. Clearly, reactivation of myelin-specific antigen-experienced T cells occurs locally in the CNS by virtue of resident APC presenting myelin proteins (32, 33).

Recently, we have provided evidence that MOG-induced EAE in marmosets is associated with intramolecular epitope spreading of the T-cell response (21). MOG-induced EAE is initiated by the presentation of an encephalitogenic peptide of the extracellular domain of MOG (MOG_{14-36}) in the context of a monomorphic Mhc-DR molecule, Caja-DRB*W1201 (21). This disease-initiating step is followed by spreading to other MOG-epitopes. Our present finding of MBP- and PLP-containing cells in the cervical LN of MOG-immunised marmoset and rhesus monkeys strongly suggests that the

priming of lymphocytes specific for (intramolecular and intermolecular) spreading epitopes takes place in this brain-draining LN.
Active demyelinating lesions in both MS patients and marmosets with EAE are characterised by activated macrophages loaded with neutral lipids from degraded myelin, MBP and PLP (17, 25, 26, 34), of which the eventual fate is unclear. Studies tracing the outflow-pathways of the brain in healthy animals revealed that both injected cells (macrophages and lymphocytes), and injected proteins and other substances drain preferentially to the cervical LN (14, 15, 35, 36). These findings imply that the myelin antigens found in the cervical LN within cells with DC and macrophage-like characteristics (Fig. 1,4), have been ingested in the LN or have been transported by phagocytic cells from the brain (Fig. 1) to the LN. The latter notion is supported by studies on inflammatory responses in the kidney (37) and peritoneal cavity (38), which demonstrated that during inflammatory conditions macrophages migrate from these sites to the draining LN. Interestingly, Randolph *et al.* showed in an elegant *in vivo* study that phagocytes at inflammatory sites were not merely macrophages, but that a substantial number of these cells differentiated into DC upon migration to the T-cell area of the draining LN (4). This may explain the mixed phenotype of DC-macrophage-like cells containing myelin antigens in the cervical LN observed in our study. The observation that myelin antigen containing cells express CCR7 further underlines the idea that these cells have migrated from the brain to the LN in a regulated manner (39). However, myelin antigens in LN DC may also result from antigen transfer, i.e. uptake of antigens released by migratory cells, as shown in other systems (40, 41). Although it has been shown that myelin proteins are transiently expressed in various types of leukocytes during immune-stimulatory conditions (42, 43), the presence of neutral lipids from degraded myelin within DC and macrophage-like cells in the cervical LN (Fig. 1C) makes this an unlikely explanation for our findings. The presence of MBP and PLP within cells in the inguinal, axillary and cervical LN of OVA- and collagen-immunised animals may be explained by such *de novo* expression of myelin proteins or may result from normal myelin turn-over (44). Collectively, the findings of the present study imply that during EAE in marmoset and rhesus monkeys, phagocytic cells take up myelin in brain lesions, migrate to the cervical LN and the spleen, and present myelin antigens at these sites to T cells.
Ample evidence is now available that there is functional specialisation of individual LN from distinct anatomical sites. The cervical LN are instrumental in the induction of nasal tolerance (45), i.e. removal of cervical LN abrogated tolerance induction, which could be restored only by

transplantation of cervical LN, but not of other LN. Cervical LN also play an important role in the induction of immune responses to brain-derived antigens (14, 46-48). Interestingly, antigens injected into the CNS evoke a deviant systemic immune response, that is deficient in antigen-specific delayed-type hypersensitivity (49, 50). Recently, it was shown that this deviated immune response could be transferred to naive animals by cervical LN cells from donors that received an injection of antigen in the brain 8 days earlier (50). We hypothesise that during EAE inflammatory signals from the CNS environment prevent the induction of a deviant immune response (2, 3). The significance of cervical LN in the development of EAE is demonstrated by the finding that complete removal of the cervical LN markedly reduced the severity of cryolesion-enhanced EAE in Lewis rats (51). Moreover, there are indications that encephalitogenic T cells primed in the cervical LN may preferentially target the brain (52). The results of our study support the importance of the cervical LN in EAE in the modulation of autoreactivity.

Strategies to treat MS with therapeutic antibodies directed against co-stimulatory molecules and cytokines are currently under development (19, 53). In order to interfere with effector functions of T cells and macrophages, it is generally assumed that such immunotherapeutics should necessarily reach the lesions in the CNS by crossing the blood-brain barrier. However, the results of the present study indicate that the cervical LN, which may be accessible via nasal drainage and the circulation, may also serve as a local target for immunotherapy of demyelinating diseases. In this respect, it is interesting that nasal application of low doses of cytokines such as interleukin 10 ameliorated relapsing EAE in DA rats (54).

In conclusion, cells containing myelin antigens are present in the cervical LN during demyelinating EAE in monkeys and MS. These myelin antigen loaded cells express MHC class II and costimulatory molecules, and are in direct contact with T cells. Moreover, myelin-specific T cells are also present in cervical LN of animals with EAE. These data suggest that professional APC with the potential to modulate the reactivity of encephalitogenic lymphocytes are active in the cervical LN.

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Table I. Myelin antigen containing cells are present in other LN and spleen.

species	immunogen	myelin component	spleen	axillary LN	inguinal LN
marmoset	myelin	neutral lipids	-	-	-
		MBP	+/-	+	+
		$PLP_{139-151}$	++	+	++
marmoset	MOG	neutral lipids	-	-	-
		MBP	+/-	+	+
		$PLP_{139-151}$	+++	++	++
marmoset	OVA	neutral lipids	-	-	-
		MBP	-	+/-	+
		$PLP_{139-151}$	+	+	++
marmoset	none	neutral lipids	-	nd	nd
		MBP	-	nd	nd
		$PLP_{139-151}$	-	nd	nd
Cynomolgus	none	neutral lipids	-	-	-
		MBP	-	+/-	+/-
		$PLP_{139-151}$	-	+/-	+/-

Sections of secondary lymphoid organs were scored for the presence of myelin antigen containing cells (- to +++; nd is not determined; see Methods). The analysis included groups of five marmosets immunised with whole myelin, six marmosets immunised with rhMOG, two marmosets immunised with OVA, one untreated marmoset and five untreated cynomolgus monkeys.

Fig. 1. Myelin antigen containing cells in brain and brain draining LN (page 117)
In situ analysis of myelin antigen containing cells in brain and cervical LN from marmosets with MOG-induced EAE and cervical LN from a patient with active MS. (A,C) Cells containing neutral lipids, stained red by ORO, were found in the brain parenchyma (A) and medulla of the cervical LN (C) of marmosets with EAE. In A, the perivascular infiltrate is indicated (B,D) In marmoset EAE, macrophage-like cell containing the myelin proteins were also present in perivascular brain lesions (B, PLP staining) and the cervical LN (D, MBP staining). (E,F) Similarly, cervical LN of an MS patient with active disease contained numerous cells loaded with MBP (E) and PLP (F). All sections counterstained with hematoxylin. Magnification: A,F, 315x; B-D, 500x; E, 200x.

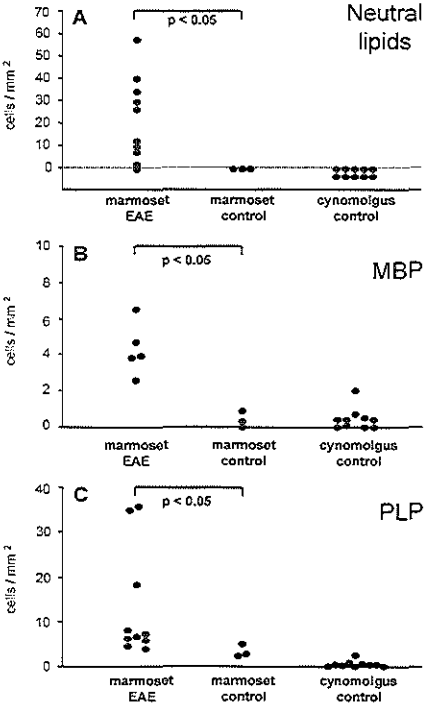


Fig. 2. Myelin antigen load in marmoset cervical LN
The density of cells containing neutral lipids stained by ORO (A), MBP (B) and PLP (C) in cervical LN of marmosets with EAE was compared with healthy marmoset and cynomolgus monkeys. The number of cells per surface area was determined by counting stained cells and measuring the area of the sections at 2.5-fold magnification using the VIDAS-RT image analysis system.

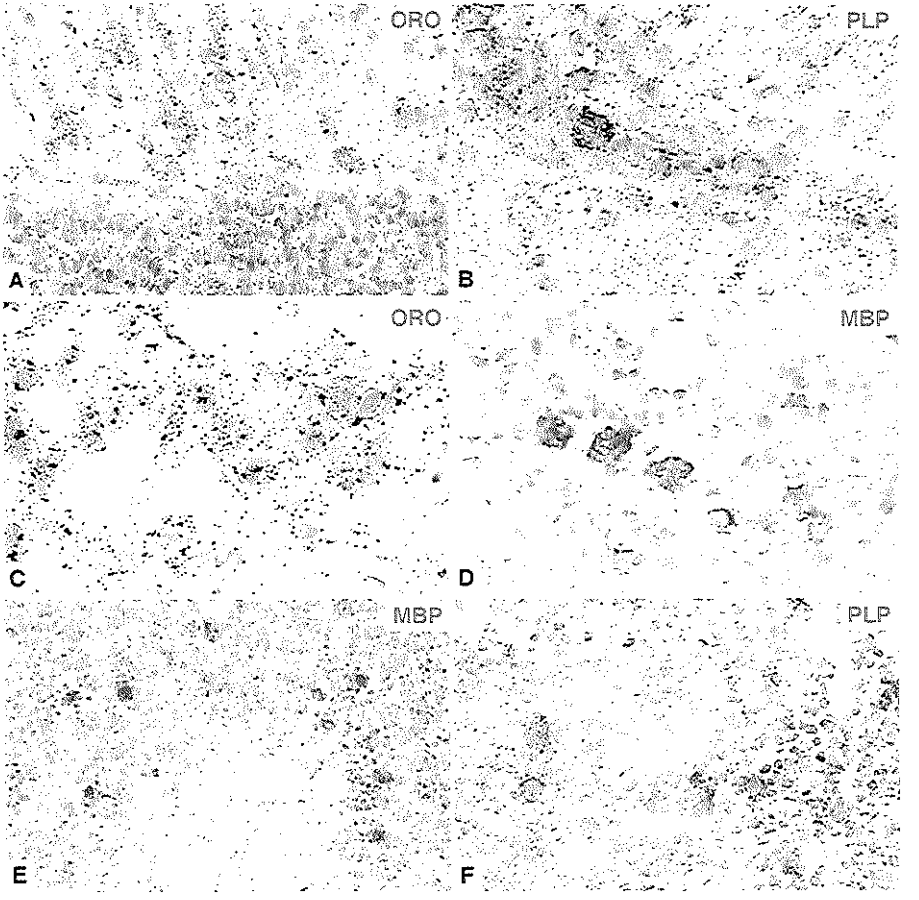
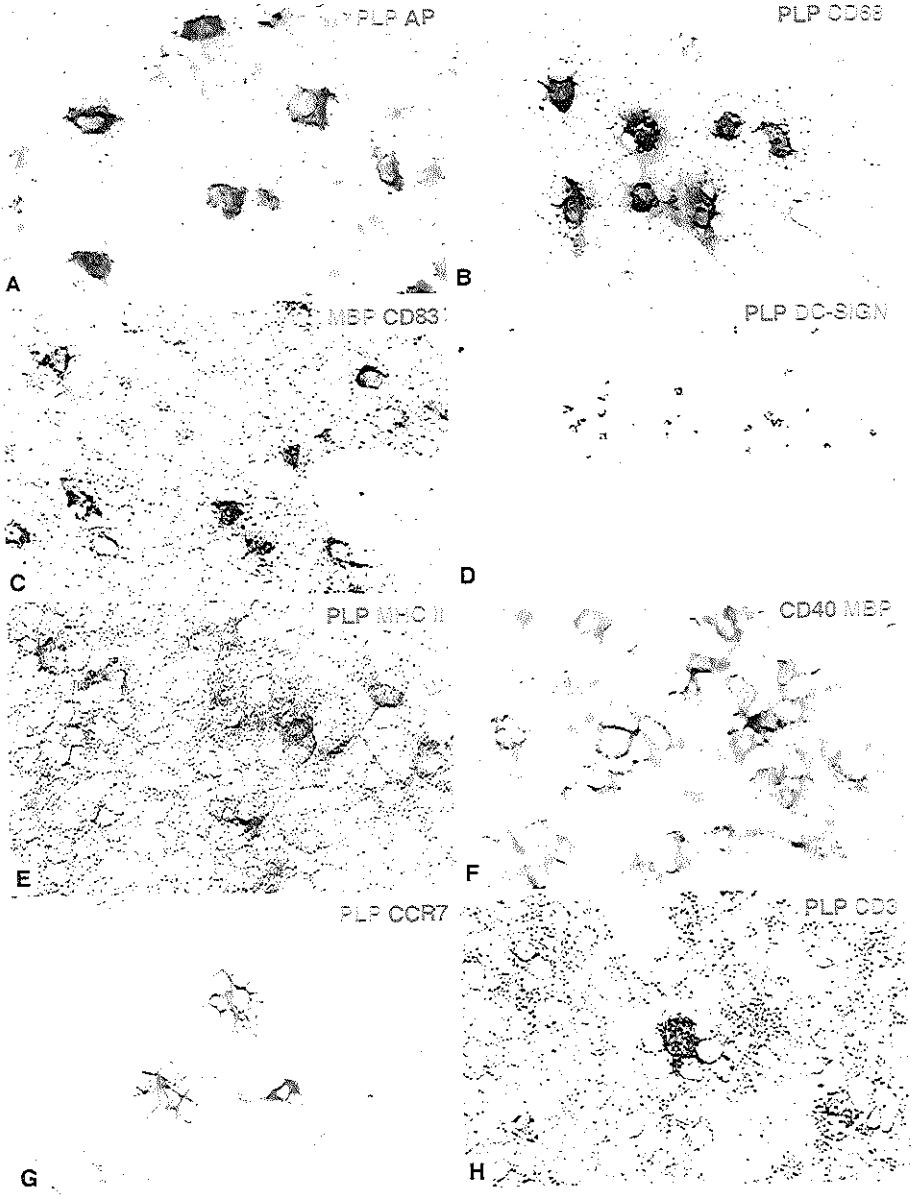

ORO
A
PLP
B
ORO
C
MBP
D
MBP
E
PLP
F

Fig. 4. Myelin antigens are present in immunocompetent APC in cervical LN (page 119)

Myelin antigen containing cells in cervical LN of marmosets with EAE (A,C,E,F,H) or rhesus monkeys with EAE (B,D,G) were characterised by (immuno)histochemical procedures. APC markers were stained in combination with staining for MBP or PLP (no counterstaining). (A) Most PLP containing cells (blue) express the macrophage marker acid phosphatase (red). Indicated are double positive cells and an acid phosphatase single positive cell. (B) In the rhesus cervical LN, many PLP containing cells (blue) expressed the macrophage marker CD68 (red). (C) MBP containing cells (blue) also express the DC maturation marker CD83 (red). Double positive cells stain purple in contrast to CD83 single positive cells. (D) Besides CD83 expression, the majority of PLP containing cells (blue) also express DC-SIGN (red); double positive cells are stained purple. (E) Cell surface expression of MHC class II (stained blue) was found throughout the LN and a subset of class II-positive cells contain myelin antigens. PLP positivity is stained red/MHC class II double positive cells. (F) Expression of CD40 (red) and CD86 (not shown) was found on most myelin antigen containing cells (MBP stained blue). Indicated are CD40 single positive cells and a MBP single positive cell. (G) Example of CCR7 expression (blue) on a PLP containing cells (red). (H) Myelin containing cells (PLP stained red) were found in direct contact with $CD3^+$ T-cells (blue). Magnification: A-C,E-H, 500x; D, 130x.


PLP AP
A
PLP CD68
B
MBP CD83
C
PLP DC-SIGN
D
PLP MHC II
E
CD40 MBP
F
PLP CCR7
G
PLP CD3
H

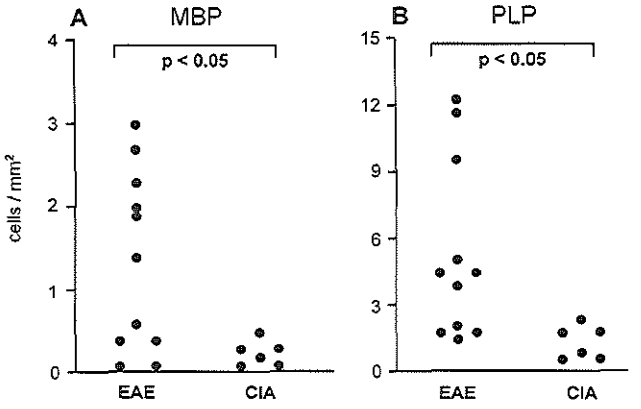


Fig. 3. MBP and PLP containing cells in rhesus cervical LN
Cervical LN of rhesus monkeys with MOG-induced EAE were compared with those of adjuvant controls with collagen-induced arthritis (CIA). The number of MBP-containing cells (A) and PLP-containing cells (B) per surface area was determined as described in the legend for Fig. 2.

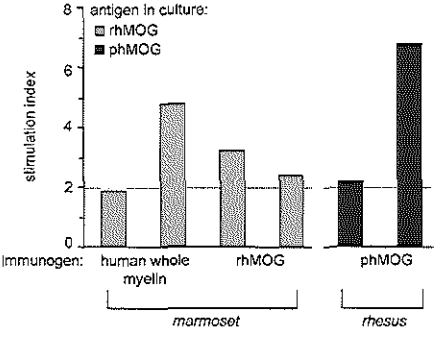


Fig. 5. Myelin antigen-specific T cells in cervical LN of monkeys with EAE
MOG-specific responses of cervical LN T cells of six individual marmoset and rhesus monkeys with EAE induced by immunisation with human whole myelin, rhMOG or phMOG. Cervical LN cells were cultured with 10 µg/ml rhMOG or phMOG. Rhesus cervical LN cells were tested after primary stimulation, whereas marmoset cervical LN cells were assayed after one or two rounds of restimulation. The specific proliferative responses are expressed as the stimulation index, being counts per minute in cultures with 10 µg/ml antigen divided by counts per minute without antigen. Dotted line indicates stimulation index of 2; stimulation indices of >2 were considered relevant.

3.
Immune intervention strategies

Protection of marmoset monkeys against EAE by treatment with a murine antibody blocking CD40 (mu5D12)

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Abbreviations: CNS, central nervous system; iNOS, inducible nitric oxide synthase; MOG, myelin oligodendrocyte glycoprotein; MAMA, marmoset anti-mouse antibody; MBP, myelin basic protein; MMP, matrix metalloproteases; MRI, magnetic resonance imaging; MS, multiple sclerosis; PLP, proteolipid protein

Abstract

CD40-CD40L interactions are crucial to cognate interactions between T cells, B cells and APC, and contribute to non antigen-specific effector functions of APC in inflammatory disorders. Here we demonstrate that functional blockade of CD40 with an antagonist mouse anti-human CD40 monoclonal antibody (mAb mu5D12) effectively prevents clinical expression of chronic demyelinating EAE in outbred marmoset monkeys, a preclinical model of multiple sclerosis (MS). Anti-CD40 mAb interfered with development of clinical symptoms of marmoset EAE during the treatment period, even when treatment was started several weeks after T-cell priming. Magnetic resonance imaging (MRI) demonstrated inflammatory activity in the brain at initiation of antibody treatment, confirming that treatment interfered with the disease process. Access of therapeutic anti-CD40 to potential sites of action, the secondary lymphoid organs and the brain white matter lesions, was visualized *in situ*. The present data are the first to demonstrate the clinical potential of blocking APC and effector cell functions using murine antagonist anti-CD40 mAb in the treatment of chronic inflammatory diseases.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) white matter. In the northern parts of Europe and Northern America, MS is the most important chronic disabling neurological disease in young adults, with a prevalence of 1 in 1.000. The initiating event of MS is not known, but may involve infection of genetically susceptible individuals with viral or bacterial pathogens which induce activation of T cells and production of antibodies cross-reacting with mimicry motifs on myelin antigens. The target of the autoimmune reaction is the myelin sheath, that envelops the axons in the CNS, and which is essential for the saltatory pulse conduction along the axon. The autoantigens involved may be myelin-constituents, such as myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), or neoantigens expressed in the CNS white matter (reviewed by Lassmann and Wekerle[1]).

The histopathological hallmark of MS is the presence of multifocal demyelinated lesions in the CNS white matter. The earliest recognizable stage of lesion development is the presence of perivascular cuffs of mononuclear cell infiltrates, containing predominantly T cells and macrophages. Plasma cells are found only in low numbers (reviewed by Lassmann[2]). The genetic linkage of MS susceptibility with certain major histocompatibility class II

alleles (e.g. HLA-DR2), the beneficial effect of immunosuppressive therapy and the observation that in animal models typical clinical and histopathological features of early MS can be evoked by adoptive transfer of myelin-reactive $CD4^+$ T cells of the helper 1 type (Th1), has made the T cell the main target for therapy in MS patients.

The CNS expression patterns of CD40 and CD40L (CD154) are highly comparable between MS-patients[3] and marmoset monkeys with active disease[4]. CD40 is abundantly expressed and CD40L is present at lower frequencies in inflammatory CNS lesions in both MS and marmoset EAE. The latter finding indicates that this non-human primate species is a suitable model for preclinical testing of immunotherapeutics targeting the CD40-CD40L interaction[3]. We have previously shown that interactions between CD40L, expressed by activated $CD4^+$ T cells, and CD40, expressed by activated APC, are critical to development of EAE in mice. Development of clinical and histopathological features of monophasic acute EAE, induced in SJL/J mice by immunization with PLP139-151 peptide, is abrogated by treatment with a hamster anti-mouse CD40L mAb[3, 5]. This finding was confirmed in mice expressing a transgenic T-cell receptor specific for myelin basic protein[6]. Importantly, this anti-CD40L mAb also interferes with relapsing EAE in a mouse model[7]. The finding that anti-CD40L reduces disease severity even after adoptive transfer of T-cells[7] supports the idea that treatment not only interferes with T-cell priming, but also with effector functions activated by CD40L interaction. Such effector functions may include cytokine production by T cells[8], and a wide range of macrophage activities, including secretion of proinflammatory cytokines, production of inducible nitric oxide synthase (iNOS) and matrix metalloproteases (MMP), and possibly increased phagocytic activity[9, 10]. In general, the protection of mice against EAE provided by anti-CD40L treatment has been attributed to impaired production of the disease-promoting cytokines interleukin-12 (IL-12) and interferon-gamma (IFN-γ)[7, 11].

In the present study we have chosen a fundamentally different approach to interfere with CD40-CD40L interactions in EAE/MS. To effectively block the APC and effector cell side of the autoimmune response instead of the CD4+ T-cell arm, we employed mAb mu5D12, an IgG_{2b} antagonist mouse anti-human CD40 antibody, in the New World monkey common marmoset (*Callithrix jacchus*). This recently developed unique EAE model shares several essential features with human MS, in particular the clinical course and the histopathological as well radiological (MRI) characteristics of the

multifocal lesions within the CNS white matter[12, 13, 16, 18, 20]. The anti-CD40 mAb mu5D12 differs from many other anti-CD40 mAb, since it does not activate macrophages or B-cells, and effectively inhibits a range of APC-activities mediated by CD40-CD40L interactions *in vitro*[14, 15]. We reasoned that treatment with mAb mu5D12 would coat CD40-expressing APC types such as dendritic cells, B-cells and macrophages to prevent productive interactions with CD40L+ T-cells, providing clinical benefit.

2. Results

2.1 Cross-reactivity and half-life of anti-CD40 in marmoset monkeys

The potential *in vivo* effectivity of antagonist mouse anti-human anti-CD40 mAb in marmoset EAE is determined by its (cross)reactivity with marmoset CD40 molecules on the one hand and the biological half-life on the other. Therefore, phenotypical and functional cross-reactivity as well as serum peak levels and clearance rates of mu5D12 were determined.

EBV-transformed B cell lines from humans, cynomologus or rhesus macaques and marmoset monkeys show similar 5D12 staining patterns indicating equal reactivity of the antibody with CD40 molecules between the four species (see Fig. 1 for comparison human versus marmoset). This cross-reactivity confirms previous immunohistochemical detection of marmoset CD40 using 5D12[4].

Functional activity of anti-CD40 mAb mu5D12 with marmoset CD40 was determined in a bioassay measuring reduction of IL-12p40 production as a pathologically relevant readout. IL-12 is a well-recognized central cytokine in development of EAE. PBMC from five experimentally naïve marmoset monkeys were cultured with mouse 3T6 cells expressing human CD40-ligand molecules. Ligation of marmoset CD40 by human CD40-ligand in the presence of IFN-γ induced marmoset monocytes to produce high levels of IL-12p40. In the presence of anti-CD40, IL-12p40 production was markedly reduced (Table 1). These results confirm that anti-CD40 mAb has similar functional antagonist properties for human and marmoset monkey PBMC *in vitro*.

To determine the serum half-life of the mAb *in vivo*, two naive marmoset monkeys were injected i.v. with a single dose of 1 mg anti-CD40 per kg body weight. Blood samples were taken at 1, 2, 4, 8 and 24 h. The peak serum levels of anti-CD40 as detected by ELISA were 23 and 25 μg/ml at 1h,

respectively, and the half-life was calculated at approximately 20 hours (see also Fig. 5). The trough levels at 48 hours after injection were 5 μg/ml, being a sufficiently high concentration for effective blocking of IL-12p40 production *in vitro*. These values are in the expected range for a mouse IgG2b mAb in a non-human primate. Based on these data the dosing schedule for further *in vivo* experiments was an i.v. loading dose of 1 mg/kg body weight, followed by i.p. injection of 1 mg/kg anti-CD40 24 hours later, and then once every 48 hours.

2.2 Access of anti-CD40 mAb to potential sites of action

For therapeutic use of mu5D12, the mAb should be able to not only interfere with CD40-CD40L interactions occurring in the secondary lymphoid organs, but also within the CNS, e.g., between infiltrated mononuclear cells or between mononuclear cells and resident APC. Therefore, the access of anti-CD40 to these sites was determined by immunohistochemistry. Untreated marmoset monkeys with full-blown EAE (9461, 9507, 9527) received a single i.v. bolus-injection of 1 mg of the antibody, corresponding to about 2-3 mg/kg body weight, and were sacrificed one hour later. At necropsy, performed within one hour after sacrifice, secondary lymphoid organs (spleen and lymph nodes draining myelin immunization sites), as well as representative samples of brain and spinal cord were snapfrozen. Abundant anti-CD40 localization *in vivo*, visualized by immunohistochemistry using anti-mouse IgG and IgG_{2b} secondary antibodies, was observed. The same tissues stained with conjugates against mouse isotypes other than IgG_{2b}, as well as tissues from marmosets not treated with anti-CD40 stained with anti-IgG_{2b}, were uniformly negative.

Table 1. Functional cross-reactivity of anti-human CD40 with marmoset CD40

Animal	medium	IFN-γ	anti-CD40	IFN-γ + anti-CD40
9419	61	221	27	102
9453	19	525	17	68
9506	17	271	17	102
9507	28	134	17	41
9511	17	163	17	44

Peripheral blood mononuclear cells from five healthy untreated marmosets were stimulated by co-culture with CD40L-transfected 3T6 cells, in the presence or absence of combinations of IFN-γ (500 U/ml) and mu5D12 anti-CD40 (5 μg/ml). As a readout, secretion of IL-12p40 (in pg/ml), thought to be crucial in both MS and EAE immunopathogenesis, was measured by capture ELISA. A representative experiment out of three is shown.

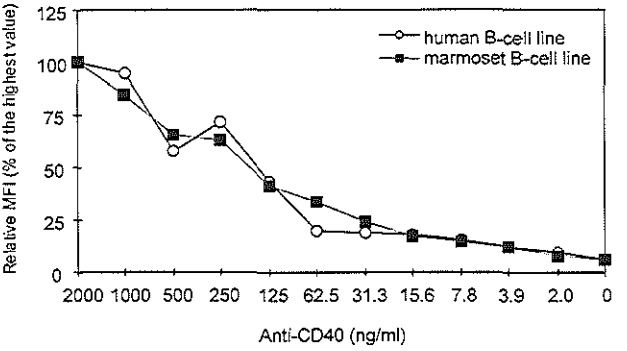
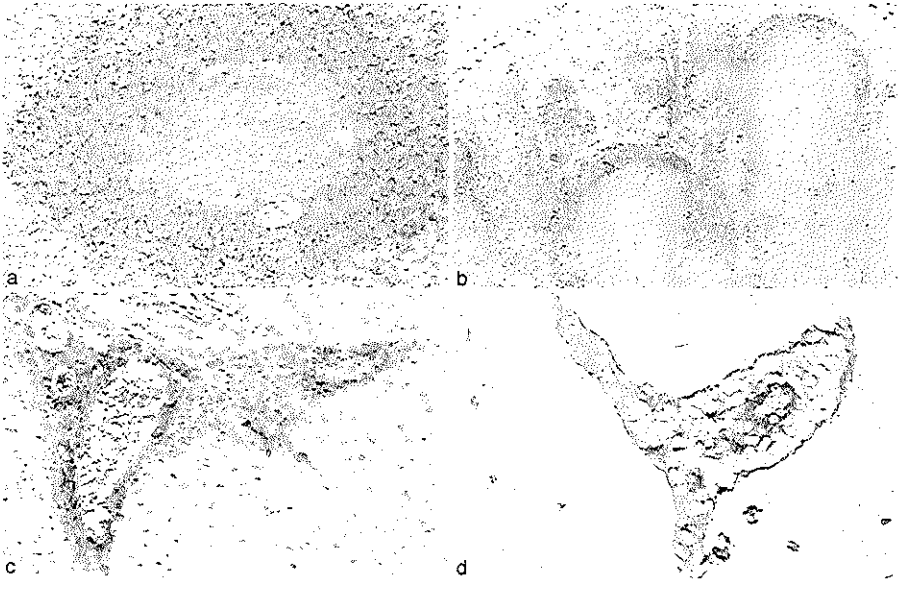


Figure 1. Phenotypical cross-reactivity of mAb mu5D12 with marmoset CD40
Anti-CD40 was titrated on a human and a marmoset Epstein-Barr virus-immortalized B-cell line and binding was evaluated by flow cytometry. Overlapping titration curves indicate similar binding characteristics of mu5D12 for human and marmoset CD40. Relative mean fluorescence intensity (MFI) is shown on the abscissa to normalize data for differential expression of CD40 on the two cell lines, allowing better comparison.



Immunohistochemistry confirmed that mu5D12 had also gained access to secondary lymphoid organs, as evidenced by staining of CD40-expressing follicular B-cells in the spleen (Fig. 2a). Although staining in this figure was amplified by means of tyramide signal amplification (TSA), anti-CD40 could also be detected by staining without amplification (not shown), indicating significant levels of antibody binding *in vivo*. Individual cells distributed in the T-cell areas and the red pulp, also bound anti-CD40, probably representing macrophages and dendritic cells. A similar staining pattern was found when spleen sections of marmosets suffering from EAE that had not been treated with anti-CD40 were stained by applying anti-CD40 on frozen sections, confirming local expression of CD40. Anti-CD40 deposition was also found in the inguinal and axillary lymph nodes, that drain the myelin-injection sites (Fig. 2b). Collectively, these findings indicate that anti-CD40 mu5D12 administered *in vivo* is able to bind to CD40 in the secondary lymphoid organs where primary and secondary lymphoid stimulation occur. Importantly, anti-CD40 could also be detected in marmoset EAE brain tissue after treatment. Here, anti-CD40 was bound to cells present in the perivascular infiltrates (Fig. 2c and d) as well as on occasional cells in the

Figure 2. *In vivo* localization of anti-CD40 mAb 5D12 in secondary lymphoid organs and brain (page 128)

Access of mu5D12 to potential sites of interaction between CD40L-expressing $CD4^+$ T-cells and CD40-expressing APC was assessed by immunohistochemistry in EAE marmosets that were injected with 1 mg of 5D12 one hour prior to sacrifice. Secondary lymphoid organs (spleen, lymph nodes draining myelin-immunization sites, and lymph nodes draining the brain) and brain tissue were evaluated. All sections were counterstained with hematoxylin. 2a. Spleen: Anti-CD40 (red) can be detected bound to B cells, which in marmoset spleen are located around the periarteriolar lymphocyte sheath containing mostly T cells (central area, only stained by hematoxylin)(animal 9461). 2b. Axillary lymph node: Anti-CD40 (red) can also be found in this type of lymph node draining the sites used for myelin immunization (animal 9527). 2c. Brain: Anti-CD40 (red) gains access to perivascular mononuclear cell infiltrates generally containing macrophages, T cells and some B cells. These infiltrates are present in the perivascular space (Virchows-Robin space), located between the endothelium and the remainder of the blood-brain barrier partly formed by astrocytes. In addition, occasional anti-CD40 binding cells are present in the parenchyma (animal 9461). 2d. Brain: The Virchows-Robin space is demarcated by laminin staining (red), which is present both in the endothelium (inner ring), and at the astrocyte end-feet involved in forming the blood brain barrier (outer ring). Anti-CD40 (blue) is bound mostly to cells in the perivascular space, but also to individual cells in the parenchyma (animal 9461).

parenchyma. Similar expression patterns of CD40, mostly by monocytic cells, have been described previously for both marmoset EAE[4] and MS[3]. As functional interactions between $CD40L^+$ T cells and different CD40 expressing APC types (macrophages, perivascular microglia, B cells) leading to cellular activation can take place in the Virchows-Robin space where they likely contribute to the development of EAE pathology[2], binding of anti-CD40 within the CNS may contribute to possible therapeutic effects of the mu5D12 mAb.

2.3 Anti-CD40 protects marmoset monkeys against EAE during treatment

To determine whether immunotherapy with mu5D12 has a beneficial effect in marmoset EAE two independent experiments of similar design were performed, summarized in Fig. 3. In the first experiment, disease was induced in 15 marmoset monkeys, which were then randomly distributed over three groups. A control group of six monkeys was treated with PBS. Five monkeys received intraperitoneal injections of mu5D12 between days 14 and 42, and four monkeys received intraperitoneal injections of mu5D12 between days 25 and 53. In the second experiment, animals (n=3) were either treated with anti-CD40 mAb from day 25-53 after immunization or with PBS (n=3). It is of importance that the EAE induced in marmosets under the chosen conditions has a chronic progressive course leading to complete paralysis in 100% of marmosets in the control group and in previous experiments (n>70). Typically, the first clear sign of neurological abnormality in the marmoset EAE model is inability to maintain balance (ataxia: score 2), progressing to incomplete or complete paralysis. Body weight of monkeys is a useful surrogate marker mainly for acute onset disease, with a reduction up to 15%. For slower onset EAE, body weight is less informative and therefore only clinical scores are depicted. Fig. 3 shows that during anti-CD40 treatment (early and late), 83% of the monkeys were protected against development of EAE, even when administration of the antibody was started as late as 25 days after immunization. Although two animals in the second experiment did develop clinical signs during the late treatment period, onset of disease was considerably delayed compared to the untreated control animals. Following discontinuation of antibody administration, 8 out of 10 animals developed clinical signs of EAE, with progression to hemi- or paraplegia, whereas two of the animals treated late were fully protected against EAE. Disease onset in the early treatment group was significantly delayed compared to the PBS group (p=0.0325), and the delay in disease onset of the late treatment group

compared to the PBS group was also significant (p=0.0327). Comparison of disease onset in the two treatment groups collectively with the control yielded a p-value of 0.0089. It is of note that the fully protected animals are by definition not included in the 'disease onset' parameter, implying that the treatment effect is even stronger than reflected by this statistical analysis.
Pathological investigation of CNS material of all animals for inflammation and demyelination showed no significant difference between control animals and mu5D12-treated animals with EAE (Table 2). This suggests that neuropathologically, EAE developing upon cessation of antibody treatment is not different than in the control group. This was confirmed by detailed immunohistochemistry on frozen brain sections, including cell subset analysis (macrophages, T-cells, B-cells), antigen presenting and costimulatory molecules (MHC class II, CD40, CD80, CD86), as well as pathogenic effector molecules (pro- and anti-inflammatory cytokines, IgG, IgM, MMP-9 and iNOS).

Table 2. Inflammation and demyelination after mu5D12 treatment

treatment	treatment period	spinal cord		brain	
		A	B	C	D
none	--	2.3±1.1	33.7±15.1	+/- (5), + (1)	- (4), +/- (1), + (1)
5D12	d14-52	3.9±0.3	39.7±6.2	+/- (4), + (1)	+/- (4), + (1)
5D12	d25-53	2.0±0.6	33.1±12.5	+/- (3), + (1)	- (1), +/- (2), + (1)

Data refer to the animals of the first experiment shown in Fig. 3. A: Inflammatory index, i.e. average number (± standard error of means) of inflamed blood vessels per spinal cord cross section. B: Demyelination in the spinal cord is given as a percentage (± standard error of means) of spinal cord white matter surface area. C: brain inflammation: +/-;perivascular or paraventricular inflammation, +; parenchymal infiltration. D: brain demyelination: -; no demyelination, +/-; perivascular demyelination, +; confluent plaques. The number of animals is indicated in parentheses.

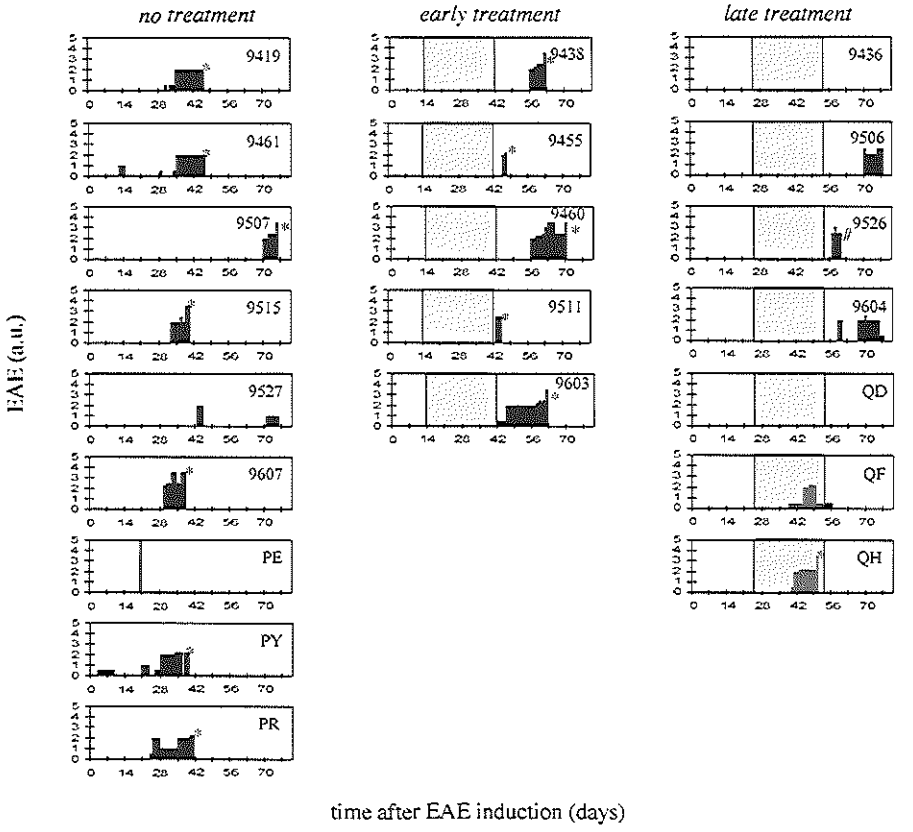


Figure 3. Prevention of marmoset EAE using different mu5D12 treatment windows
First experiment: animals indicated by four-digit codes: No treatment after disease induction (n=6). Treatment with mu5D12 from day 14 to 42 after disease induction (n=5). Treatment with mu5D12 from day 25 to 53 after disease induction (n=4).
Second experiment: animals indicated by two-letter codes: No treatment after disease induction (n=3). Treatment with mu5D12 from day 25 to 53 after disease induction (n=3). EAE scores ranging from 0-5 are shown for individual animals. Treatment period with anti-CD40 is depicted by gray background. Asterisks indicate time point of sacrifice due to severity of EAE. # indicates that animal did not recover from anesthesia for MRI.

2.4 Lesion activity at initiation of treatment

To confirm that during the period of 25 days between disease induction and initiation of anti-CD40 treatment, inflammation of the CNS had already been established, MRI was performed on all monkeys of Fig. 3, on day 26 after myelin sensitisation. Inflammatory activity in periventricular locations in the CNS was indeed observed, as shown in Fig. 4. These findings emphasize that treatment was started well after induction of myelin autoreactive CD4+ T-cells and development of inflammation in the CNS. Ethical considerations disallowed sacrifice of animals for pathology analysis prior to disease, but absence of inflammatory markers in naïve marmoset brain has been demonstrated before [4].

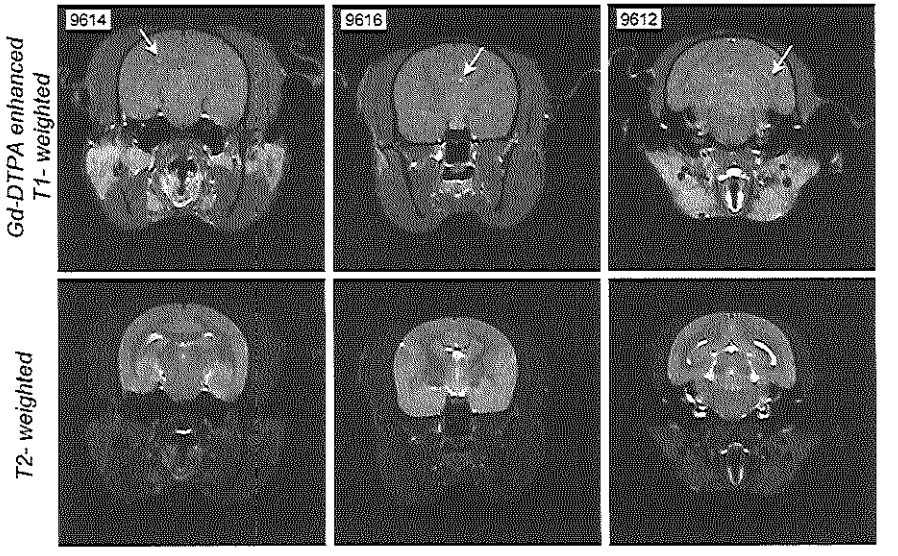


Figure 4. Active CNS inflammation before initiation of anti-CD40 treatment
At onset of therapeutic anti-CD40 treatment, on day 26 after immunisation, brain MR images were recorded of all monkeys from Fig. 3 to assess encephalitis. The figure shows representative cross sections from three monkeys. The T2-weighted images (bottom row) of monkeys 9614, 9616, and 9612 show dilation of the hyperintense region at periventricular location. The top row shows the corresponding slices in T1-weighted images. The arrows point to areas enhanced by Gadolinium administration.

2.5 Serum concentration of anti-CD40

The different effectivity of early (day 14-42) versus late (day 25-53) mu5D12 treatment may be due to different availability of the antibody. Therefore, circulating levels of mu5D12 were measured in the treatment groups described in Fig. 3. Fig. 5 depicts serum peak levels and clearance of anti-CD40 in myelin/CFA-immunized, EAE-affected versus healthy marmosets. Clearly, circulating mAb levels are much lower in EAE marmosets. This is likely due to increased CD40-expression on PBMC and in tissues of myelin/CFA immunised marmosets[4], resulting in increased capture of circulating mAb, and/or to development of marmoset antibody responses against the mouse 5D12 antibody (MAMA). MAMA levels measurable by ELISA were first detected in all 5D12-treated animals 8 days after initiation of immunotherapy and increased to high titers (data not shown). The emergence of MAMA-responses most likely has limited the effective treatment window of mu5D12.

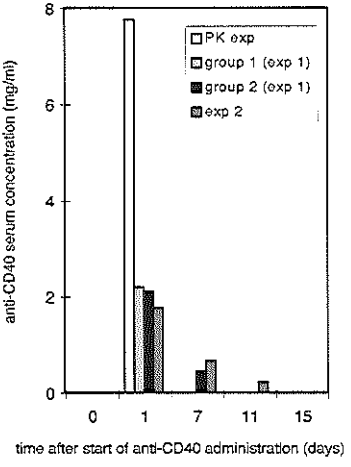


Figure 5. Serum concentration of mu5D12 in marmosets

Levels of circulating mu5D12 were assessed at different time points after administration. Two healthy marmosets were given a single i.v. dose (1 mg/kg body weight) of mu5D12 leading to an average serum level of almost 8 µg/ml in samples taken at 24 h (open bar). In contrast, animals with EAE from the antibody treatment experiments (see Fig. 3 and 4) had much lower levels (around 2 µg/ml) at 24 h after first administration of mu5D12.

3. Discussion

3.1 Anti-CD40 interferes with marmoset EAE

This study demonstrates that immunotherapy with a mAb functionally inhibiting CD40 during the treatment window prevents the development of clinical signs of EAE in marmoset monkeys, a preclinical model of MS. We further show that anti-CD40 mAb mu5D12 penetrated into the secondary lymphoid organs where (cognate) interaction of T cells, B cells and APC take place, and also into perivascular cuffs of infiltrating leukocytes within the brain white matter. These results indicate that mu5D12 may not only interfere with T cell-APC interactions in secondary lymphoid organs, but also within CNS lesions. This study provides the first evidence that an antagonist mAb against CD40 has potential for the treatment of chronic inflammatory diseases. The parallels in pathogenesis between EAE in this nonhuman primate EAE model and MS[4, 16-19] suggest that this finding has important implications for the therapy of MS exacerbations.

3.2 Prevention of EAE in marmosets

The marmoset EAE model as first developed by the group of Hauser[16, 20] and modified by us[18] was used to evaluate the immunotherapeutic potential of antagonist anti-CD40. Distinct advantages of this model for the current study include the cross-reactivity of anti-human CD40 with marmoset CD40[4], allowing testing of human-specific therapeutics in a preclinical model in an animal species closely related to humans. The strong immunological relationship between humans and marmosets is illustrated by the high degree of similarity between the polymorphic MHC[21] and T-cell receptor genes[21]. Despite the outbred nature of the colony, a 100% disease incidence is invariably observed in different experiments (n>50).

The data described show that during mu5D12 treatment, a large majority of animals is effectively protected against clinical expression of the disease. Animals were already suffering from CNS abnormalities as evidenced by MRI when treatment was started. Compiling data from the two experiments, only 2 out of 12 animals showed clinical EAE during treatment, since none of 5 animals treated from day 14-42 developed disease and only 2 out of 7 treated from day 25-53. However, when mu5D12 treatment was stopped, most animals developed late signs of EAE, although one animal remained protected. The limited longterm efficacy of antibody treatment is likely due to

three factors. First, the relatively low frequency administration schedule has lead to suboptimal circulating levels of anti-CD40 in EAE animals as compared to healthy marmosets (Fig. 5), and to insufficient coating of circulating CD40-expressing APC. Second, increased CD40 expression in animals with chronic inflammation may have further reduced anti-CD40 levels. Third, animals developed marked levels of MAMA antibodies to mu5D12, probably leading to complexing and increased clearance. Importantly, the use of CFA for EAE induction in this model may override the inhibiting effect of low serum concentrations of mu5D12 on B-cell responses and stimulate MAMA responses by creating a strong inflammatory environment. In a study with chimerized 5D12, induction of MAMA responses against the complementarity determining regions occurred. These MAMA antibodies blocked IL-8 production by human monocytes stimulated with CD40 L in vitro (Boon et al., submitted)

3.3 Sites and mechanisms of action

Many anti-CD40 antibodies have agonistic properties, and stimulate B-cell responses and T-cell activity through APC activation *in vivo*[22, 23]. In contrast, the current study shows that antagonist mAb 5D12 interferes with EAE. It is not yet known by what molecular mechanism mu5D12 inhibits CD40-mediated activation, and this is under further investigation. Our *in vivo* localization studies clearly show that the mu5D12 mAb penetrates not only into the secondary lymphoid organs, but also into the perivascular infiltrates of the CNS and to a lesser extent in the brain parenchyma. This implies that peripheral and local CD40-CD40L interactions are accessible to the mAb. Hence, anti-CD40 can interfere with stimulation of primary and secondary antigen-specific T-cells and B-cells in the secondary lymphoid organs and CNS lesions. Since peripheral activation of pathogenic T cells continuously occurs during established EAE/MS[2] the relevance of 5D12 activity in secondary lymphoid organs should not be underestimated. Also in rheumatoid arthritis, there is evidence for continuous recruitment of T cells preactivated in the lymphoid tissue into the sites of inflammation[24].

Although the identity of the most relevant APC in the CNS remains controversial (e.g. microglia, infiltrating macrophages or astrocytes), anti-CD40 may be able to interfere with several different interactions taking place during migration of T cells over CD40-expressing endothelium and within the perivascular space. CD40-expression by endothelial cells is thought to be restricted to sites of inflammation, e.g. perivascular and periventricular

lesions. The prevailing view is that T cells receive priming signals in the secondary lymphoid organs, while APC of the CNS provide secondary stimulation. As reviewed extensively elsewhere[9, 10, 25], CD40-CD40L interactions can activate a wide range of functions of B cells, endothelial cells, dendritic cells and macrophages. Many of these effector functions, including improved antigen presentation, induction of proinflammatory cytokines, iNOS, MMP's and phagocytic activity, may be relevant to MS pathogenesis. It is of note that many of these effector functions can be triggered without the necessity of a cognate interaction between MHC-peptide and the T-cell receptor interaction, as soluble CD40L is a sufficient signal *in vitro*. Importantly, a very recent study has elegantly shown that CD40 expressed within the CNS controls the course of EAE in bone marrow chimeric mice[26].

Recent studies show that polyclonal IgG preparations can have beneficial effects in EAE-models[27] and that intravenous immunoglobulins (IVIG) are effective in MS-patients[28]. Nevertheless, it is highly unlikely that the disease-limiting effects of anti-CD40 treatment result from IVIG-like effects since the dose used for IVIG is several orders of magnitude higher than the therapeutic dose of anti-CD40 in our study. In addition, although the protective mechanisms of IVIG-therapy have been poorly defined, it is likely that other serum factors such as Ig-bound cytokines are involved, which are absent in the anti-CD40 preparations.

3.5 Future directions

Rational development of therapeutic regimens requires further insight into the mechanisms of action of anti-CD40 mAb 5D12. Clearly, anti-CD40 therapy in general should be short term or intermittent to prevent prolonged generalized suppression of immune functions. In the case of MS, early identification of exacerbations or even prediction of their onset would be of great value to improve therapy protocols. Anti-CD40 mAb with reduced immunogenicity and lacking complement activating properties (IgG4 backbone) will also improve treatment efficacy. A chimeric 5D12 molecule can completely prevent EAE in marmosets induced by recombinant MOG when treatment is started from immunization onward (33).

4. Material and methods

4.1 Monkeys

21 healthy and naive common marmoset monkeys (*Callithrix jacchus*) were purchased from the breeding colony of the Biomedical Primate Research Centre (BPRC) in Rijswijk, The Netherlands for mu5D12 pharmacokinetics and EAE treatment studies. All studies were approved by the IACUC and performed to regulations in the Dutch law on animal experimentation. The sex (M/F) and birth dates (month and year) of the individual monkeys were: 9218 (M, May 1992), 9309 (F, February 1993), 9419 (F, March 1994), 9436 (M, June 1994), 9438 (M, July 1994), 9455 (M, October 1994), 9460 (F, December 1994), 9461 (F, December 1994), 9506 (M, May 1995), 9507 (F, May 1995), 9511 (M, May 1995), 9515 (F, May 1995), 9526 (November 1995), 9527 (M, December 1995), 9603 (January 1996), 9604 (M, January 1996), 9607 (F, April 1996). The following animals were twins: 9460 and 9461; 9613 and 9614; 9611 and 9612; 9615 and 9616; 9603 and 9604. Before being admitted to the experiments, each monkey received a complete health check and T2-weighted magnetic resonance brain images were made to exclude monkeys with pre-existing neurological abnormalities.

4.2 EAE induction and antibody treatment

Human white matter from MS patients, predominantly from spinal cord, was a kind gift from Dr. Rivka Ravid, coordinator of The Netherlands Brain Bank in Amsterdam, The Netherlands. Myelin was isolated as described[29] and a stock solution containing 0.8 mg dry weight and 1.1 mg protein per ml water was stored frozen at -80°C until further use. For immunization an aliquot of the stock solution was diluted once in buffered saline (PBS, pH 7.4) and emulsified in an equal volume of CFA (DIFCO Lab, Detroit, MI). Under mild ketamin anesthesia each monkey was injected intradermally on the back with 600 µl emulsion, two spots of 150 µl in the axillary and two in the inguinal regions. First antibody treatment at 1 mg/kg was performed i.v. under mild ketamin anesthesia. Subsequent injections were given i.p. to unsedated animals every 2 days. Control animals were treated with PBS.

4.3 Clinical diagnosis

Clinical signs of EAE were scored twice daily by a trained observer using a semiquantitative scoring scale[16]: 0 = no clinical signs; 0.5 = apathy, loss of appetite, altered walking pattern without ataxia; 1 = lethargy and/or anorexia; 2 = ataxia; 2.5 = para- or monoparesis and/or sensory loss and/or brain stem syndrome; 3

= hemiplegia; 3.5 = paraplegia; 4 = quadriplegia; 5 = spontaneous death attributable to EAE. The highest scores per day were taken. Significance of differences in first day of disease onset were determined by Mann-Whitney rank-sum test.

4.4 Blood collection and flow cytometry

Small blood volumes (<100 µl) were collected via a small incision into the *vena saphena*. Larger blood volumes were collected from the groin using heparinized vacutainers (Greiner, Solingen, Germany). Binding of anti-CD40 to human versus marmoset CD40 was assessed by means of flow cytometry. Human and marmoset Epstein-Barr virus (B95-8)-transformed cell lines were cultured under standard conditions in RPMI medium (Biowhitakker, Verviers, Belgium) supplemented with 10% fetal calf serum (Flow Lab, McLean, VA), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (all Gibco BRL, Glasgow, UK). Anti-CD40 was titrated on the cells and detected by means of anti-mouse IgG-FITC (Jackson Immunoresearch Laboratories Inc., West Grove, PA).

4.5 IL-12p40 induction assay

Peripheral blood mononuclear cells (PBMC) from healthy marmosets were isolated by Lymphoprep gradient centrifugation (Nycomed Pharma, Oslo, Norway). Wildtype or CD40L-transfected 3T6 cells (a kind gift from Dr. K. Thielemans, Free University, Brussels, Belgium) were used to stimulate CD40-expressing cells present in marmoset PBMC (50.000 in 1 ml). Anti-CD40 was used at 5 µg/ml, a concentration previously determined to be optimal for inhibition of the CD40-CD40L interaction in human PBMC. IFN-γ (Boehringer-Mannheim, Germany) was added to a final concentration of 500 U/ml. IL-12p40 levels in cultures supernatants were detected by sandwich ELISA Screening Line (Biosource Europe, Nivelles, Belgium).

4.6 In vivo MRI

In preparation for the experiment the monkeys were anesthetized with ketamine/vetranquil (9/1 vol/vol). During scanning the monkey was placed on a 37 °C water filled heating pad (American Pharmaseal) to prevent hypothermia. Just before the monkey was positioned in the magnetic field, Gadolinium-DTPA (Magnevist; Schering, Berlin-Germany) was injected intravenously at a triple dose (0.3 mmoles/kg). MRI was performed on a SISCO 200 MHz spectrometer (Varian, Palo Alto, CA) equipped with an actively shielded gradient (maximum gradient 3,2 G/cm, 33 cm inner diameter). For slice-orientation a sagittal scout-scan was made. The posterior and anterior positions of the corpus callosum were chosen as orientation markers for precise localization of axial slices for the *in vivo* MRI and for post

mortem MR-recordings of isolated brains. First a T2- (TE/TR 60/2500 ms) weighted multislice scan (20 slices of 1 mm thickness) was recorded, followed by a T1-weighted scan (TE/TR 25/1000 ms) with the same coordinates, number of slices and orientation. Each slice was recorded as a matrix of 256x128 datapoints. Data were analysed on an Apple Macintosh performa 630 with the public domain NIH Image 5.1 program.

4.7 Histo- and immunopathology examination

At necropsy one brain hemisphere was snap-frozen in liquid nitrogen for immunohistochemical analysis of infiltrating cellular subsets, expression of costimulatory molecules and cytokines[4] and the other hemisphere and spinal cord were fixed in 4% buffered formalin. Formalin-fixed material was embedded in paraffin and was used for stage-characterization of the lesions by guidance of *in vivo* and postmortem MRI[18]. The inflammatory index was quantified on HE-stained spinal cord cross sections. Demyelination was scored on Klüver-Barrera stained sections by using an ocular grid.

4.8 In vivo localization of anti-CD40 mAb

To assess whether anti-CD40 mAb mu5D12 reaches its potential sites of action in the CNS, selected animals with EAE (n=3) were administered a dose of 1 mg anti-CD40 per monkey i.v. 1 hour before sacrifice. Frozen sections were made of marmoset EAE brain and spinal cord. In addition, secondary lymphoid organs were analysed, including spleen, the lymph nodes draining the sites of immunization (axillary, inguinal), and the cervical lymph nodes draining the brain[30]. Therapeutically administered anti-CD40 (mouse IgG2b) was detected bound to cells in the different tissues by incubating sections with rabbit-anti-mouse IgG-HRP (Dako, Glostrup, Denmark). Staining intensity was improved by tyramide signal amplification according to instructions of the manufacturer (TSA, Life Science Products, Boston MA). Anti-CD40 could also be visualized without amplification using Goat anti-mouse IgG2b-AP (SBA, Birmingham, AL)(not shown). Control experiments included negative control tissues (animals not treated with anti-CD40), and control stainings (conjugate specific for mouse isotypes other than IgG2b: IgM and IgG2a). Laminin was detected with a polyclonal rabbit antibody (ICN Pharmaceuticals). Details of staining methods have been described previously[4, 31, 32].

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Prevention of Experimental Autoimmune Encephalomyelitis in the Common Marmoset (*Callithrix jacchus*) Using a Chimeric Antagonist Monoclonal Antibody Against Human CD40 Is Associated with Altered B Cell Responses[1]

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Inhibition of CD40-CD40 ligand interaction is a potentially effective approach for treatment of autoimmune diseases, such as multiple sclerosis. We have investigated this concept with a chimeric antagonist anti-human CD40 mAb (ch5D12) in the marmoset monkey experimental autoimmune encephalomyelitis (EAE) model. Marmosets were immunized with recombinant human myelin oligodendrocyte glycoprotein (rMOG) and treated from the day before immunization (day −1) until day 50 with either ch5D12 (5 mg/kg every 2–4 days) or placebo. On day 41 after the induction of EAE, four of four placebo-treated monkeys had developed severe clinical EAE, whereas all animals from the ch5D12-treated group were completely free of disease symptoms. High serum levels of ch5D12 associated with complete coating of CD40 on circulating B cells were found. At necropsy placebo- and ch5D12-treated animals showed similar MOG-specific lymphoproliferative responses in vitro, but ch5D12 treatment resulted in strongly reduced anti-MOG IgM Ab responses and delayed anti-MOG IgG responses. Most importantly, treatment with ch5D12 prevented intramolecular spreading of epitope recognition. Postmortem magnetic resonance imaging and immunohistologic analysis of the CNS showed a markedly reduced lesion load after ch5D12 treatment. In conclusion, the strong reduction of clinical, pathological, and radiological aspects of EAE by ch5D12 treatment in this preclinical model points to a therapeutic potential of this engineered antagonist anti-CD40 mAb for multiple sclerosis. *The Journal of Immunology*, 2001, 167: 2942–2949.

CD40-CD40 ligand (CD40L)[4] (CD154) interactions play an important role in B cell activation, APC activation, initiation of Ag-specific T cell responses, and induction of macrophage effector functions (1, 2). The central role of CD40-CD40L interaction in the initiation, amplification, and prolongation of immune responses justifies the choice of this cellular interaction as a target for immunotherapy in disorders based on unwanted immune responses, such as autoimmune diseases and transplant rejection. This has been illustrated in a large number of murine models of autoimmune diseases and transplantation (3). In addition, humanized anti-human CD40L mAbs targeting activated $CD4^+$ T cells prevent kidney rejection in rhesus monkeys (4, 5) and rejection of pancreatic islets in rhesus monkeys and baboons (6, 7). To inhibit the CD40-CD40L interaction we have chosen the CD40 side of this interaction, targeting the APC, B cell, and macrophage activities. Therefore, we previously selected a mouse mAb (mu5D12) that, unlike most other anti-CD40 mAbs, potently inhibits CD40-CD40L-mediated activation in several cell types and is devoid of any CD40 stimulatory activity (8). μ5D12 strongly inhibits Ab production (both IgM and IgG) by human B cells cocultured with activated $CD40L^-$ human T cells (9) as well as production of IL-12 and TNF-α by human monocytes induced by CD40L-CD40 interaction and IFN-γ (10). To reduce the potential immunogenicity and enhance the in vivo half-life of the parent 5D12 mAb for administration in humans, the murine parent Ab was re-engineered as a chimeric mouse-human mAb with a human IgG4 constant region (11).

Multiple sclerosis (MS) is an MHC-associated and T cell-dependent chronic inflammatory disorder of the CNS leading to white matter demyelination. The target of the cellular and humoral autoimmune reactivities in MS is the myelin sheath around axons, which is essential for pulse conduction. A number of myelin proteins, such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and proteolipid protein (PLP) have been described as possible target autoantigens (12). Active MS lesions in humans feature T cells and monocyte/macrophage accumulations around venules and ventricles and at plaque margins (13). The

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[4] Abbreviations used in this paper: CD40L, CD40 ligand; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; h, human; MRI, magnetic resonance imaging; MS, multiple sclerosis; PLP, proteolipid protein.

presence of activated T cells expressing CD40L in close contact with CD40-expressing macrophages in MS lesions, but not in brain sections, of patients with a nondemyelinating neurological disease (Alzheimer's disease) suggests that lesion formation involves APC/T cell interactions (14). Functional evidence for the involvement of CD40-CD40L was provided in the SJL/J mouse model of PLP-induced experimental autoimmune encephalomyelitis (EAE), where injection with an mAb-blocking CD40L at the time of disease induction completely prevented disease. More importantly, treatment initiated well after disease induction strongly suppressed disease severity (14).

Immunization of common marmosets (*Callithrix jacchus*), a neotropical primate species, with human myelin or recombinant human MOG (rhMOG) in CFA induces a demyelinating form of EAE that strongly resembles human MS in its clinical and pathological presentation (15–17). Anti-MOG Abs play an important role in the facilitation of demyelination in marmoset EAE, because adoptive transfer of MBP-specific T cells induced clinical signs of EAE, but no demyelination was observed (18). Interestingly, when T cells were cotransferred with anti-MOG Abs fully demyelinated lesions were formed (19). The accessible location of MOG at the outer surface of the myelin sheet may be an important contribution to this observation. The expression patterns of CD40 and CD40L are highly comparable between MS patients (14) and marmoset monkeys with active disease (20). CD40 is abundantly expressed by macrophages and microglia, and $CD40L^+$ T cells are present at lower frequencies in inflammatory CNS lesions in both MS and marmoset EAE. The high similarity of marmoset EAE and human MS and their close phylogenetic relationship justify the use of EAE in marmoset monkeys as a preclinical model for testing of the engineered antagonist anti-human CD40 mAb.

The major aims of the present study were to prevent clinical signs and symptoms of EAE in outbred marmoset monkeys using mAb ch5D12, and furthermore, we aimed to present functional biological data to confirm the antagonistic characteristics of mAb ch5D12 in vivo.

Materials and Methods

Animals and EAE induction

Eight marmoset monkeys (*C. jacchus*) were randomly selected from the purpose-bred colony at the Biomedical Primate Research Center (Rijswijk, The Netherlands). The body weight of the animals at the start of the study ranged between 213 and 381 g. During the experiments, the monkeys were individually housed in spacious cages with padded shelters provided at the bottom of the cage. The daily diet consisted of food pellets for New World monkeys (Special Diet Services, Witham, U.K.) supplemented with rice, peanuts, marshmallows, biscuits, fresh fruit, and vegetables. Drinking water was provided ad libitum. EAE was induced using *Escherichia coli*-derived rhMOG, representing the extracellular domain of human MOG (21). All animals were immunized with 100 μg rhMOG emulsified in CFA. Under ketamine anesthesia (15 mg/kg; AST Farma, Oudewater, The Netherlands), each monkey was injected with 600 μl emulsion into the dorsal skin divided over four locations: two in the inguinal and two in the axillary region. *Bordetella pertussis* was not included in the immunization protocol. The clinical course of EAE was recorded daily by a trained observer using a semiquantitative scoring system: 0 = no clinical signs; 0.5 = apathy, loss of appetite, altered walking pattern without ataxia; 1 = lethargy and/or anorexia; 2 = ataxia; 2.5 = mono- or paraparesis and/or sensory loss and/or brain stem syndrome; 3 = hemi- or paraplegia; 4 = quadriplegia; 5 = spontaneous death attributable to EAE. The highest per day scores in a week were averaged. For ethical reasons monkeys were sacrificed when the clinical EAE score of 3 was reached. Each monkey was weighed three times a week, and the body weight was used as a surrogate marker of clinical well-being. Animals were treated with 5 mg/kg ch5D12 or placebo on days −1 (before immunization), 0, 2, 4, 6, 8, and 10 and thereafter twice weekly. In the period after day 27 until day 50 the dose frequency was increased from twice weekly to three times per week. PBS was used as placebo. According to the Dutch law on animal experimentation, the experimental procedures of this study were reviewed and approved by the institute's animal care and use committee.

Flow cytometry

Whole blood samples were obtained preimmunization and on days 0, 2, 6, 10, 16, 25, 32, 37, and 44 after immunization. For this purpose, 100 μl blood was drawn via a needle prick into the vena saphena and collected in 3 ml PBS/EDTA. Cells were collected by centrifugation and resuspended, and aliquots were incubated with mAbs for 30 min at 4°C. Subsequently, RBC were removed with lysis buffer (Beckman Coulter, San Jose, CA) for 10 min at room temperature, and the remaining leukocytes were washed twice with PBS. Abs used for staining were anti-CD20, clone B1, directly labeled with RD1 (Beckman Coulter); anti-human IgG (to detect ch5D12 coated cells), directly labeled with FITC (Jackson ImmunoResearch Laboratories, West Grove, PA); anti-CD4, clone MT310 directly labeled with PE (DAKO, Glostrup, Denmark); and anti-CD8 clone MT1014 (Connex, Munich, Germany), indirectly labeled with anti-mouse IgM-PE (Southern Biotechnology Associates, Birmingham, AL). To determine the ch5D12 coating of CD40 expressed by circulating B cells in vivo, incubations were performed with RD1-labeled anti-CD20 mAb in combination with FITC-labeled anti-human IgG mAb. The absence of $CD20^-$ B cell staining with the anti-human IgG mAb might be due to either low ch5D12 serum levels or low CD40 expression. To discriminate between these options extra ch5D12 was added to a parallel FACS incubation.

MOG-specific cellular response at necropsy

Lymph node cell suspensions were prepared from aseptically removed inguinal and axillary lymph nodes, and lymphocytes were isolated using Lymphocyte Separation Medium (ICN Biomedical, Aurora, OH). Cultures were set up in medium (HEPES-buffered RPMI 1640; Life Technologies, Glasgow, U.K.) supplemented with 10% FCS (Flow Laboratories, McLean, VA), 10 mM MEM-nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 2×10^{-4} M 2-ME (all obtained from Life Technologies). PBMC or lymph node cells (2×10^5/well) were seeded into 96-well flat-bottom plates (catalog no. 665180; Greiner, Solingen, Germany) and cultured with rhMOG (10 μg/ml) or hMBP (25 μg/ml). After 48 h, 0.5 μCi/well [^{3}H]thymidine was added, and incorporation of radiolabel was determined 18 h later using a Matrix 9600 beta counter (Packard, Meriden, CT).

ELISA for ch5D12, anti-ch5D12, and anti-MOG

Before each administration of ch5D12 or placebo, serum samples were prepared from venous blood obtained via a needle prick into the vena saphena to determine ch5D12 trough levels, anti-ch5D12 responses, and anti-MOG responses. The ch5D12 concentrations were determined in a competition ELISA. Briefly, plates were coated with Ag (10 ng/ml rCD40-Fc) in PBS. For detection a mu5D12-HRP conjugate was used to compete with free ch5D12 for CD40-Fc. Simultaneous addition of ch5D12 and the mu5D12-HRP conjugate decreased absorbance compared with that observed with mu5D12-HRP alone. To circumvent the influence of serum in the ELISA, the standard matrix contained 1% human serum. For quantification, triplicate standard serial dilutions of mAb ch5D12 (4000–40 ng/ml) were added to each plate.

For the detection of Abs against the variable regions of ch5D12, plates were coated with mu5D12 at a concentration of 2 μg/ml, and for detection an alkaline-phosphatase labeled rabbit anti-monkey IgG Ab was used (Sigma, St. Louis, MO). Marmoset serum samples were serially diluted (1/1) from a starting dilution of 1/100 to a final dilution of 1/12,800. Responses were scored positive for the anti-ch5D12 variable region when the signal exceeded the background signal by a factor of 2. Background was determined in prestudy serum samples of the animal at the same dilution on the same plate.

Anti-MOG IgM and IgG responses were detected with ELISA on plates coated with rMOG. Sera were tested at a 1/50 dilution. Bound Ab was detected using a polyclonal alkaline phosphatase-conjugated goat anti-monkey IgM μ-chain (Rockland, Gilbertsville, PA) or an alkaline phosphatase-labeled swine anti-human IgG γ-chain (BioSource International, Camarillo, CA).

Intramolecular spreading of MOG Ab responses

The Ab responses of individual monkeys to intact MOG and linear MOG peptides were analyzed using a dot-blot assay (22). Recombinant hMOG and synthetic 23-mer peptides overlapping by 10 aa spanning the extracellular domain of MOG were spotted onto a polyvinyl difluoride membrane (Hybond; Amersham, Little Chalfont, U.K.) using a Bio-Dot SF

blotting apparatus (catalog no. 170-6542; Bio-Rad, Hercules, CA). To ensure that all peptides remained bound, the blots were immersed in 2.5% glutaraldehyde in PBS for 15 min and washed with PBS for 15 min, and the remaining binding sites were blocked by incubation for at least 2 h in PBS containing 3% BSA (PBS/BSA). The blots were then incubated for 1 h with the relevant serum at a 1/1000 dilution in PBS/BSA (1%) and washed four times for 10 min each time with PBS containing 0.05% Tween 20. Blots were developed by incubation for 1 h with rabbit anti-human IgA, IgG, and IgM (DAKO); diluted 1/14,000 in PBS/BSA (1%); washed as described above; and processed for ECL detection according to the manufacturer's instructions (Amersham).

Magnetic resonance imaging (MRI) of brain lesions

MRI was performed at the Image Sciences Institute of Utrecht University (Utrecht, The Netherlands). High resolution postmortem T2-weighted MR images were recorded from one formalin-fixed hemisphere of each animal using a 11-cm inner diameter gradient insert (22 gauge/cm). First, a sagittal scout scan was made, and the posterior and anterior positions of the corpus collosum were chosen as orientation markers for precise localization of the axial plane. Slices in the coronal direction were set perpendicular to the axial plane. A T2-weighted (TE/TR 25/7000 ms) multislice scan (50–70 slices of 0.5 mm thickness) was obtained. Each slide was recorded with a matrix of 512 × 256 data points and a field of view of 4 × 4 cm. The datasets were analyzed on an I-MAC G3 using the public domain National Institutes of Health program.

Neuropathology

Ketamine-anesthetized monkeys were euthanized by an i.v. injection of 400 mg sodium-pentobarbital (Euthesate; Apharmo, Duiven, The Netherlands). The brain and spinal cord were excised in toto, fixed in 4% buffered formalin, and processed for neuropathologic examination. Fixed tissues were rinsed with PBS containing 0.05% sodium azide and embedded in paraffin. The extent of inflammation, demyelination, and axonal pathology was evaluated on tissue sections stained for H&E to visualize infiltrated cells with Klüver Barrera (Luxol fast blue combined with periodic acid Schiff) staining myelin and myelin degradation products and with Bielschowsky silver impregnation staining axons. The degree of demyelination (percent loss of total white matter) was quantified on Klüver Barrera-stained spinal cord cross sections (16–21) using an ocular morphometric grid. The degree of inflammation was expressed as an inflammatory index, i.e., the number of inflamed blood vessels per spinal cord cross section (10–15 sections). Immunocytochemistry was performed with a biotin-avidin system. T cells were stained with anti-human CD3 Abs (Dakopatts, Copenhagen, Denmark). Macrophages were detected by staining with the Abs 27E10 and MRP14, which are both commercially available from BMA Biomedicals (Switzerland). Myelin and oligodendrocytes were stained with Abs recognizing 2',3'-cyclic nucleotide 3'-phosphodiesterase (Affiniti Research, Devon, U.K.), PLP-VI (Serotec, Oxford, U.K.), and MOG (provided by Dr. S. Piddlesden, University of Cardiff, Cardiff, U.K.). Ig depositions in the lesions were detected using biotinylated anti-(human) Ig (Amersham) as a primary Ab.

Results

Clinical course

The eight monkeys were randomly distributed over two groups of four monkeys and received injections of ch5D12 at 5 mg/kg body weight or the same volume of PBS two or three times per week for a period of 50 days. Immunization with rhMOG/CFA was performed 1 day after the first dose administration. Fig. 1 shows that all animals in the placebo group had developed clinical signs of EAE by day 41 (disease onset on days 30, 34, 38, and 41). In contrast, all ch5D12-treated animals remained free of clinical signs for the complete 50-day treatment period. In the placebo-treated group, two animals were sacrificed before day 50 according to Institutional Animal Care and Use Committee regulations, because of the severity of their disease. At the end of the 50-day treatment period, the remaining two animals of the control group and two blindly chosen ch5D12-treated animals were sacrificed for neuropathologic examination and postmortem MRI analysis. The two other ch5D12-treated animals (QN and QO) were further followed for possible occurrence of rebound disease. Animal QO developed EAE 9 days after cessation of treatment and was terminated with stage 3 EAE on day 65 after immunization (15 days after cessation of treatment with ch5D12). Animal QN remained asymptomatic for 100 days after cessation of treatment. Then a short episode of visual problems occurred (maximum score, 2.0), which fully remitted. Only after 200 days did this monkey finally develop signs of incomplete paralysis (score of 2.5), at which stage the monkey was sacrificed for neuropathologic examination.

Coating of CD40 on circulating B cells and serum levels of ch5D12 and anti-ch5D12 Abs

Throughout the study, the saturation of CD40 on PBMC by ch5D12 was monitored by flow cytometric analysis. Before day 27 all CD40 on CD20$^+$ B cells was completely saturated. B cell

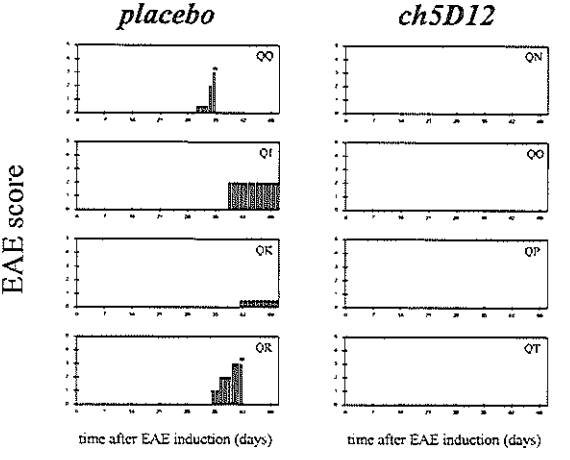

FIGURE 1. ch5D12 prevents EAE in marmoset monkeys. EAE score (ranging from 0 to 5) is shown for placebo-treated (*left*) and ch5D12-treated (*right*) marmoset monkeys. Treatment of ch5D12 was performed every 2–4 days for a period of 50 days. Individual animals are identified by the code in the *upper right corner*. Asterisks indicate the time of sacrifice due to the severity of EAE.

CD40 molecules in animal QN remained fully coated for the duration of treatment. However, it was observed that the ch5D12 coating in vivo decreased in the three other animals on day 27 (data not shown). To determine whether this was due to low serum levels of ch5D12 or to a decrease in B cell CD40 expression, a parallel incubation of PBMC pulsed with extra ch5D12 was included. All animals showed positive staining for CD40 on $CD20^+$ B cells (data not shown). Thus, on day 27 ch5D12 serum levels in animals QO, QP, and QT were insufficient to saturate all CD40 molecules. Therefore, the dosing frequency was increased to three times per week for all animals from day 28 onward. The increased dosing frequency did not completely restore the CD40 coating of B cells. The serum levels of free ch5D12 paralleled the incomplete saturation of CD40 (Fig. 2). Serum concentrations of ch5D12 ranged from 10 to 40 μg/ml during the first 2 wk and subsequently dropped below 10 μg/ml in QO, QP, and QT. Sustained circulating ch5D12 concentrations >10 μg/ml could be measured only in QN, probably explaining the continuous CD40 saturation on $CD20^+$ B cells with ch5D12 in this animal. Apparently, a serum concentration of 10 μg/ml is a threshold for CD40 saturation. Increasing the dosing frequency resulted in only a slight restoration of circulating ch5D12 concentrations in QO, QP, and QT, probably because at that time neutralizing Abs were already being formed (see below). Treatment with ch5D12 had no effect on the percentage of either $CD4^+$ or $CD8^+$ T cells (data not shown). A transient decrease in the percentage of $CD20^+$ B cells from peripheral blood was observed. In three of four animals anti-ch5D12 Abs developed (see below), indicating the functional integrity of thymus-dependent B cell responses in ch5D12-treated animals. Anti-ch5D12 IgM Abs were not detectable throughout the study. In monkeys QO, QP, and QT anti-ch5D12 IgG Abs were first detectable between days 17 and 27. A correlation was found between the circulating ch5D12 concentration, saturation of CD40 with ch5D12, and the development of an anti-ch5D12 IgG response (Fig. 2). The correlation between the magnitude of the anti-ch5D12 response and the area under the curve during the first 3 wk of the study suggests a dose-response relationship. Whereas monkeys QO, QP, and QT developed anti-ch5D12 IgG Abs in the same order at the time when their ch5D12 levels dropped below the 10 μg/ml threshold, monkey QN failed to develop an anti-ch5D12 response after cessation of ch5D12 treatment on day 50 until day 200.

Postmortem MRI and immunohistologic analysis, and neuropathology of marmoset brain

To assess whether ch5D12 treatment affects CNS lesion load, animals were analyzed by postmortem MRI and immunohistochemistry (Table I). The two animals in the placebo group that were sacrificed during the 50-day period due to the severity of their disease, had a very high lesion load in the CNS and overt demyelination in the spinal cord. Also, the two animals from the placebo group that were sacrificed at the end of treatment period day 50 had a very high lesion load in both immunohistochemical and MRI analysis, indicating high inflammatory activity in the CNS. Demyelination was present mainly in the spinal cord, but it was less severe than in the two placebo-treated animals that were sacrificed before day 50 (Table I). These observations were in sharp contrast to those in the two animals from the ch5D12 group (QT and QP) that were sacrificed at the end of the treatment period according to protocol. These two animals that had the lowest ch5D12 serum levels had only minor inflammatory lesions in MRI analysis. In the immunohistochemical analysis no infiltrates were observed in animal QT; besides the inflammation, some limited demyelination in spinal cord and brain was found (Table I). Because ch5D12 levels were higher in the other two animals of the ch5D12 group that were kept alive (QO and QN), this may suggest that the lesion load in these two animals on day 50 was comparable to or less than that in animals QT and QP. Animal QO that developed EAE shortly after cessation of treatment and was sacrificed due to severity of disease had a lesion load comparable to that of the placebo-treated animals. Immunopathology revealed that in this animal large demyelinating infiltrates were present. These infiltrates consisted of T cells and actively demyelinating macrophages and were identical with the early infiltrates in placebo animals QQ and QR, indicating that a rebound EAE developed after cessation of ch5D12 treatment on day 50. There was a complete absence of lesions and demyelination in animal QN.

MOG-specific cellular response, anti-MOG Ab response, isotype distribution, and peptide specificity

To control for the presence of a cellular autoimmune reaction in both groups, MOG- and MBP-specific cellular responses were measured in cultures isolated from peripheral lymph nodes collected at necropsy. Although no response was detectable against MBP, clear T cell proliferative responses against MOG were detected in all placebo- and ch5D12-treated animals (Fig. 3).

Fig. 4 shows that with the exception of monkey QP, treatment with ch5D12 resulted in lower levels of anti-MOG IgM Abs. In the placebo-treated animals anti-MOG IgM Abs first became detectable on day 16 after immunization and remained above background level throughout the treatment period in all animals. Although in the ch5D12-treated animals a small increase in anti-MOG IgM Abs was observed on day 16, the magnitude

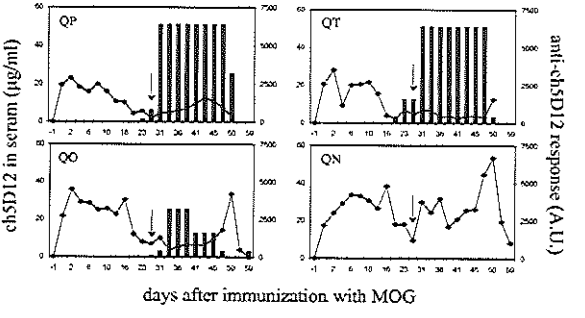

FIGURE 2. Serum levels of ch5D12 and anti-ch5D12. Serum levels of ch5D12 were determined at different time points after immunization with MOG and are depicted on the *left* y-axis. Samples were taken just before the next mAb administration; therefore, serum levels in the figures indicate trough levels of ch5D12. Anti-ch5D12 responses were determined by serial dilution and are depicted on the *right* y-axis and are expressed as the dilution factor necessary to reduce the anti-ch5D12 signal below 2 times absorbance readings of preimmunization serum. Arrows indicate the increase in mAb dosing frequency from two to three times per week.

Table 1. *ch5D12 prevents lesion formation in MOG-induced EAE*[a]

Treatment	Animal	Day of Sacrifice	Spinal Cord Demyelination (%)	Brain Pathology[b]	MRI[c]
Placebo	QQ	35	43.6	+++	+++
Placebo	QR	38	41.2	+++	+++
Placebo	QI	50	8.7	+++	+++
Placebo	QK	50	0.6	++	+++
ch5D12	QP	50	3.3	+	+
ch5D12	QT	50	0.0	+	+
ch5D12	QO	65	30.3	+++	+++
ch5D12	QN	200	0.0	−	−

[a] Animals are listed by treatment and time of follow-up. Lesion load in the CNS was assessed using MRI analysis and immunohistochemical analysis.

[b] Pathology: −, No inflammation, no demyelination; +, inflammation, no demyelination; ++, inflammation and some perivascular demyelination; +++, inflammation and demyelinated plaques.

[c] MRI: −, No lesions detectable; +, limited number of lesions present; ++, lesions present; +++, extensive lesions present.

of this response overall remained lower than that in the placebo group. Treatment with ch5D12 delayed the appearance of the anti-MOG IgG response in all animals (Fig. 5). However, from day 38 onward, most treated animals developed MOG-specific IgG levels comparable to placebo-treated animals. The reduction of anti-MOG IgM and IgG responses demonstrated in vivo functional biological activity of ch5D12 on humoral immunity as predicted by the crucial role of CD40-CD40L interaction in thymus-dependent B cell responses.

To assess the effect of ch5D12 treatment on intramolecular epitope spreading, the reactivity of anti-MOG Abs with MOG protein and MOG peptides was determined in a dot-blot assay. Fig. 6 shows the reactivity of sera with intact rhMOG and a set of 23-mer peptides overlapping by 10, spanning residues 1–116 of the N-terminal extracellular part of human MOG. All monkeys from both groups showed reactivity against rhMOG, confirming the ELISA data. In necropsy sera of three of four monkeys from the placebo group, broad reactivity with the peptide panel was found (Fig. 6) as described previously (22). Only in monkey QQ, which was the first to develop severe EAE and was sacrificed on day 35, was no

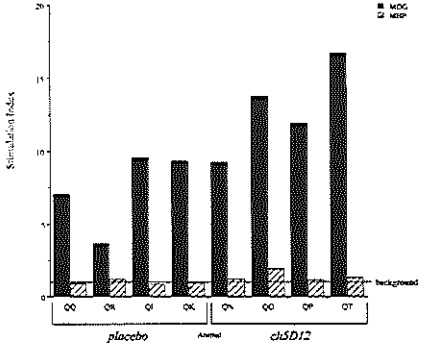

FIGURE 3. MOG-specific cellular response at necropsy. Pooled mononuclear cells isolated from the axillary and inguinal lymph nodes excised at necropsy were probed for proliferative response in culture with rhMOG (10 μg/ml; ■) or hMBP (25 μg/ml; ▨). Placebo-treated monkeys were QQ, QR, QI, and QK. Monkeys treated with ch5D12 were QN, QO, QP, and QT. The specific proliferative reactivities are expressed as the stimulation index, being the counts per minute in cultures with Ag divided by counts per minute in cultures without Ag.

reactivity with the overlapping peptides found, suggesting that epitope spreading takes place thereafter. In three of four ch5D12-treated animals no reactivity with the peptide panel was found (Fig. 6). Interestingly, the only ch5D12-treated animal (QP) that showed reactivity in its necropsy serum against some of the peptides was also the only ch5D12-treated animal that developed high anti-MOG IgM responses during treatment. Possibly this monkey has escaped from the immunosuppressive effects of ch5D12, which is confirmed by the early development of an anti-ch5D12 response. These results demonstrate that ch5D12 treatment not only suppresses anti-MOG Ab production, but also prevents intramolecular spreading of the peptide reactivity of anti-MOG Abs.

Discussion

We have previously demonstrated that interruption of CD40-CD40L interaction by antagonist mAbs is an effective target of therapy in mice (13). This study was undertaken to test the efficacy of a clinically relevant mAb in an animal model closely reflecting MS. The chosen animal model was rhMOG-induced EAE in the common marmoset, a well-established nonhuman primate model in terms of clinical, neuropathologic, and immunologic features (22). This model is a valid preclinical model of inflammatory demyelination of the CNS; therefore, it enabled us not only to obtain proof of our concept that interruption of CD40-CD40L interaction is a feasible immunotherapy of MS, but also to collect data on the possible mechanism of action of ch5D12 treatment.

Within the 50-day treatment period, all animals that received placebo had developed clinical EAE, whereas all ch5D12-treated animals were devoid of any clinical signs. However, induction of clinical signs was observed after withdrawal of the treatment, but only after 2 weeks in one monkey (QO) and after >20 wk in the other (QN). The sustained absence of serious clinical signs in monkey QN until 100 days after the arrest of treatment is certainly impressive. Our results demonstrate that despite the suboptimal dosing for complete saturation of B cell CD40, inhibition of the CD40-CD40L interaction with ch5D12 effectively prevents disease symptoms of EAE in marmoset monkeys. The postmortem MR images and neuropathologic examination confirm the beneficial effect of the Ab with suppression of inflammation and demyelinization.

The results of this study clearly show the therapeutic potential of ch5D12 in EAE. Importantly, although treatment with ch5D12 was started before the immunization, the similar MOG-specific cellular responses at necropsy in both groups of animals clearly demonstrate that the therapeutic effect is not due a general immune suppression. The absence of clinical symptoms of EAE appears to be associated with reduced anti-MOG IgM and IgG responses. In an

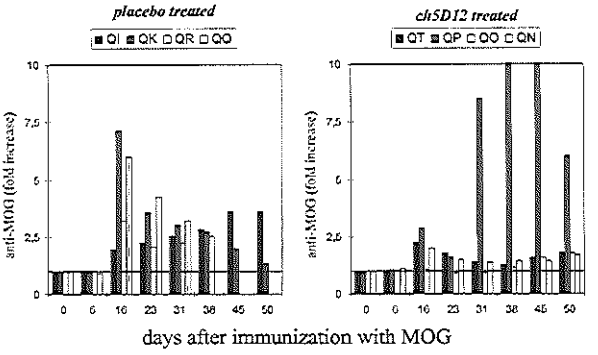

FIGURE 4. Effectofch5D12effectivelyonanti-MOG IgM response. Anti-MOG IgM responses were determined at different time points after immunization with MOG. Values shown are absorbance of postimmunization serum/absorbance of preimmunization serum at 1/50 dilution. Placebo animals are depicted in *A* and ch512-treated animals are depicted in *B*. Animals are indicated by different bars and respective codes.

animal that had sustained elevated anti-MOG IgM Ab levels, intramolecular epitope spreading in Ab responses against MOG and lesions in the CNS were observed. In the ch5D12-treated animals in which anti-MOG IgM Abs were reduced, clinical signs, CNS white matter lesions, and epitope spreading were lacking. It is tempting to hypothesize a causal relation between these phenomena.

The altered profile of anti-MOG Ab reactivity in ch5D12-treated monkeys is of particular interest in view of the important role of this Ab specificity in the facilitation of demyelination (18, 19). The pathogenic mechanism by which anti-MOG Abs mediate demyelination is thought to be mediated by complement activation and subsequent immune activation (23, 24). Anti-MOG mAbs can mediate opsonization of myelin dependent on the isotype, the recognized epitope, and the ability to fix complement (25). Notably, IgM Abs are particularly capable of classical route complement fixation. Therefore, the abolishment of anti-MOG IgM Abs associated with the absence of clinical signs in ch5D12-treated monkeys may explain the absence of clinical signs. We have found a similar mechanism in another primate autoimmune disease model, namely the rhesus monkey model of collagen-induced arthritis. We have demonstrated that disease susceptibility is directly correlated to the capacity to produce anti-type II collagen IgM autoantibodies (26, 27). In addition to the altered isotype distribution of anti-MOG Abs, we found a different reactivity of the necropsy sera with the peptide panel between placebo- and ch5D12-treated monkeys.

What do the modulatory effects of ch5D12 on anti-MOG autoantibodies and the suppression of clinical and pathological aspects in the marmoset model of EAE imply for the possible future treatment of MS patients? The higher incidence of MOG-specific T cell and Ab reactivity in MS compared with non-MS patients or healthy individuals (21, 28, 29) and the localization of anti-MOG Abs in MS brain areas with myelin disintegration underline the relevance of anti-MOG autoimmunity for the MS pathogenesis (29, 30). An issue that is receiving increasing interest in MS is the progressive broadening of the anti-myelin T and B cell reactivity, a phenomenon called epitope spreading. A causal relation between the chronicity of MS and epitope spreading has been suggested (31). Our present results show that the broad serum reactivity with the panel of MOG peptides found in placebo-treated monkeys is absent in the majority of ch5D12-treated animals. Although this assay does not provide formal proof of epitope spreading, the results at least suggest that induction of B cell neoreactivity is abolished in ch5D12-treated monkeys.

The new therapeutic ch5D12 was generated to increase the serum half-life of the mAb and to reduce its potential immunogenicity in humans (11). However, it was observed in this study that the serum half-life of ch5D12 was unexpectedly low and was only

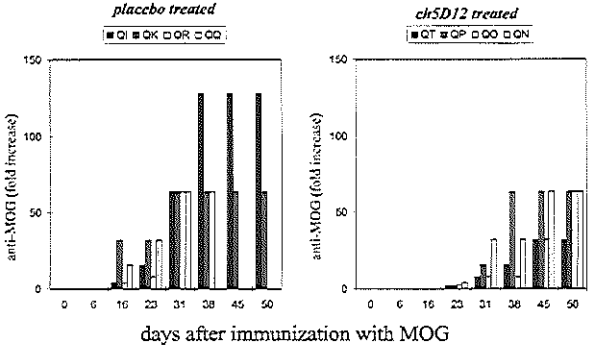

FIGURE 5. Effect of ch5D12 on anti-MOG IgG response. Anti-MOG IgG responses were determined at different time points after immunization with MOG by serial dilution of the samples. Values indicate the dilution factor that is necessary to decrease the signal below 2 times background absorbance. Placebo animals are depicted in *A*, and ch512-treated animals are depicted in *B*. Animals are indicated by different bars and respective codes.

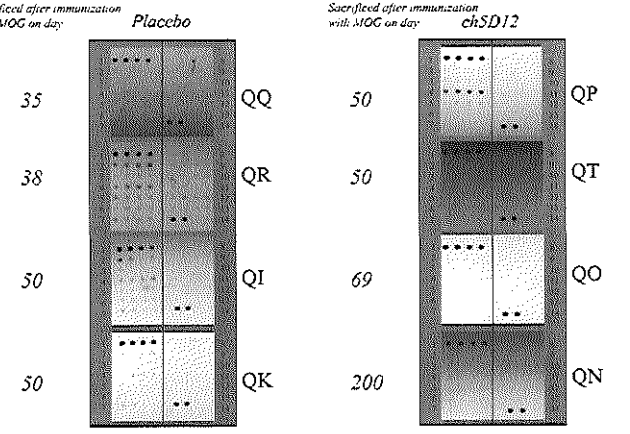

FIGURE 6. Effect of ch5D12 on intramolecular epitope spreading of Ab reactivity. Sera were obtained from animals at the time of necropsy. The presence of Abs against full-length rhMOG and linear MOG peptides was analyzed using a slot-blot assay (20). Recombinant MOG, MOG peptides, or controls were loaded onto the blot, indicated by numbers 1–14: 1) rhMOG; 2) p4–26; 3) p14–36; 4) p24–46; 5) p34–56; 6) p44–66; 7) p54–76; 8) p64–86; 9) p74–96; 10) p84–106; 11) p94–116; 12) OVA (negative control Ag); 13) rhesus MBP; 14) rhesus IgG (*left* two spots) or none (*right* two spots). At numbers 1–13 at the *left* two spots 5 μg was loaded, while at the *right* two spots 2.5 μg was loaded.

slightly improved compared with that of muSD12 in marmoset monkeys. This may be due to insufficient recognition of the human IgG4 backbone of ch5D12 by the Ig-recycling system of the marmoset that uses the FcRn receptor for binding of endogenous Abs and prevents fast elimination of these Abs (32). This is a relevant observation, as marmoset monkeys are increasingly used in safety, tolerability, and pharmacokinetic analyses of therapeutic mAbs before the clinical test phase. On the basis of our recent pharmacokinetic analysis of ch5D12 in cynomolgus monkeys, an Old World primate species more closely related to humans, we expect that the peak serum levels and the half-life of ch5D12 are significantly higher in humans than in marmosets. To compensate for the fast elimination rate of ch5D12 in the marmoset EAE model, frequent administration of high doses of ch5D12 was needed to achieve complete coating of circulating $CD20^+$ B cell. However, from day 27 onward the coating of circulating B cells became incomplete in three of four monkeys as serum levels dropped below a presumed threshold concentration of 10 μg/ml, and neutralizing Abs were formed. At persistent serum levels of ch5D12 above this threshold in animal QN, complete coating of circulating $CD20^+$ B cell could be maintained, and induction of an anti-ch5D12 response was prevented.

In conclusion, in the presence of adequate serum levels of administered ch5D12 (10 μg/ml), the expression of clinical and pathological signs of EAE in outbred marmoset monkeys is prevented. The protective effect of the Ab is probably not due to a generalized immune suppression, as similar MOG-specific cellular responses could be detected at necropsy in placebo- and Ab-treated monkeys. After the arrest of treatment, induction of disease may occur, but only after a variable period of time. CD40-expressing macrophages are abundant in lesions (17, 20), an observation that is indicative for the involvement of macrophages as effector cells in lesions and the role of ch5D12 for treatment of established lesions.[5] In addition, it was observed that the mAb gains access not only to secondary lymphoid organs, but also to the CNS. These observations clearly demonstrate the therapeutic value of ch5D12 in this nonhuman primate model. Extrapolation of these results to MS, indicates that ch5D12 may be effective to reduce the duration and severity of exacerbations and, furthermore, may inhibit progression of the disease by preventing the induction of neoautoreactivities. Thus, disease exacerbations and the concomitant progressive decline of neurological functions may be arrested.

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Prevention of experimental autoimmune encephalomyelitis in common marmosets using a human anti-human IL-12 mAb[1]

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Abstract

The experimental autoimmune encephalomyelitis (EAE) model in the common marmoset approximates the human disease multiple sclerosis (MS) with regard to its clinical presentation, as well as neuropathological and radiological aspects of the lesions in the brain and spinal cord. IL-12 is a pro-inflammatory cytokine that is produced by APC and promotes differentiation of Th1 effector cells. IL-12 is produced in the developing lesions of patients with MS as well as in EAE affected animals. Previously it was shown that interference in IL-12 pathways effectively prevents EAE in rodents. In this study we report on the beneficial effect of CNTO1275, a fully human IgG1κ mAb against human IL-12p40, in the myelin-induced EAE model in common marmosets. Treatment was initiated well after immunization (day 14) and the mAb remained active throughout the treatment period of 72 days. During this period no neutralizing Ab responses against CNTO1275 could be detected. We demonstrate that CNTO1275 treatment has a protective effect on the neurological dysfunction as well as on neuropathological changes normally observed in the brain and spinal cord of EAE affected individuals, stressing its potential for the treatment of MS.

Introduction

Multiple sclerosis (MS)[3] is a chronic inflammatory demyelinating disease of the CNS. The pathological hallmark of MS is the CNS white-matter lesion, a focal area of infiltrated mononuclear cells with a variable degree of demyelination, axonal loss and gliosis. Although susceptibility to MS is thought to be a multifactorial trait, it is generally accepted that disease progression is driven by autoimmune reactions directed against antigens of the CNS white matter (1, 2). A broad pathological analysis revealed that in MS at least four fundamentally different neuropathological patterns can be discerned (3). Pattern I and pattern II lesion pathology are modeled in the current virus- and autoimmune-based animal models of encephalomyelitis that have been established in susceptible rodent strains (4-6) or non-human primates (7).

IL-12 is the predominant cytokine for triggering Th1-mediated (autoimmune) responses and is induced when $CD4^+$ T-cells and APC interact (8-10) and abrogated when CD40-CD154 ligation is disturbed (11-13). Evidence is accumulating that IL-12 plays a pivotal role in the induction of the critical autoimmune responses involved in the initiation of experimental autoimmune encephalomyelitis (EAE), lesion formation, and the progression of the disease

(11-22). Therapies directed at the neutralization of IL-12 or prevention of production by abrogation of CD40-CD154 interaction have proven to be effective in rodent (20, 21, 23-28) as well as in marmoset models of EAE (29), while excess of IL-12 reverses these effects and enhances EAE severity (15-17, 22, 28, 30). Furthermore, local expression of IL-12 within the CNS of rodents (14, 31, 32) and common marmosets (33) during active EAE has been demonstrated.

Although the situation is less clear in MS, IL-12 is locally expressed within the CNS of affected individuals (34-36), and levels of IL-12 in CSF and plasma are increased during active disease (37-39). Moreover, it has been argued that the beneficial effect of IFN-β on MS is exerted via suppression of IL-12 production (40-42). Finally, lower base-line levels of IL-12p35 and p40 mRNA seem to predict clinical responsiveness to IFN-β treatment (43, 44).

In its radiological and neuropathological presentation, the chronic progressive EAE model in the common marmoset approximates the most prevalent lesion type, being pattern II, in MS patients (7, 45, 46). These aspects, added to the MS-like clinical expression of the disease (7, 47) and the close immunological similarity with humans (48-50), make the model an excellent test system for preclinical evaluation of new therapies for chronic MS. The model is particularly important for the safety and effectivity testing of biotechnologically engineered reagents, which by their species-specificity can not be evaluated in rodent EAE models. The present study demonstrates the therapeutic effects of mAb CNTO1275, a fully human IgG_1 molecule directed against human IL-12p40, on the clinical features of EAE. Our results show a reduction of the lesion load, as detected with magnetic resonance imaging (MRI) as well as by neuropathological examination.

Materials and Methods

Animals

Ten non-related healthy common marmosets (Callithrix jacchus) were selected from the experimental stock of the Biomedical Primate Research Centre (Rijswijk, The Netherlands). Before the monkeys entered the experiment a full physical, hematological and biochemical check-up was performed. Individual data of the monkeys, which were identified with an implanted transponder, are summarized in table I.

Ethical regulations limit the total blood volume that can be collected and the frequency of MR imaging. Hence, the monkeys were randomly paired and assigned to the groups receiving placebo or CNTO1275 treatment before the immunization (see

table I). Paired individuals were handled identically throughout the experiment. The time points for collection of larger blood volumes for immunological tests and MR imaging were determined on the basis of the clinical stage of EAE in one of both individuals of each pair.
During the experiments, the monkeys were individually housed in spacious cages with padded shelters provided at the bottom of the cage and were under constant veterinary care. The daily diet consisted of commercial food pellets for New World monkeys (Special Diet Services, Witham, Essex, England), supplemented with rice, raisins, peanuts, marshmallows, biscuits and fresh fruit. Drinking water was provided ad libitum. According to the Dutch law on animal experimentation, the protocol of this study was reviewed and approved by the Institute's Animal Care and User Committee.

Disease induction and clinical read-out
EAE was induced by a single immunization with 300 µl human myelin in water (10 mg/ml) emulsified with 300 µl CFA (Difco Laboratories, Detroit, MI) under ketamin anesthesia (6 mg/kg; AST Farma, Oudewater, The Netherlands) as described previously (51). *Bordetella pertussis* was not used for reasons discussed elsewhere (51).
Twice daily clinical signs of EAE were scored blind by a trained observer using a previously described semi-quantitative scale (51): 0, no clinical signs; 0.5, apathy, loss of appetite and altered walking pattern without ataxia; 1, lethargy and/or anorexia; 2, ataxia, sensory loss/blindness; 2.5, hemi- or paraparesis; 3, hemi- or paraplegia; 4, quadriplegia; 5, spontaneous death attributable to EAE. Body weights were determined once weekly as a surrogate disease marker. Monkeys were sacrificed for ethical reasons once a monkey had reached EAE score 3.0, or at day 86 after immunization, being the planned end-point of the study.

Reactivity, dosing regimen, plasma levels, biodistribution and immunogenicity of CNTO1275
The test substance was produced by clone C379B and purified using standard techniques. CNTO1275 is a fully human $IgG_1\kappa$ mAb specific for the p40 subunit of human IL-12. The effectivity of CNTO1275 in neutralizing marmoset IL-12 was tested using lipopolysaccharide (LPS) stimulated cells. Briefly, plastic adherent cells from common marmosets and human PBMC were stimulated for 24 h with LPS (1 µg/ml). Cell free supernatant was collected and titrated onto 4 day PHA-stimulated human T-cell blasts. After 24 h, IFN-γ levels were determined using standard ELISA techniques. An EC_{90} stimulation level was determined for each preparation and used at this concentration for titration of CNTO1275 to determine the IC_{50} of neutralization.
The animals were treated with antibody between day 14 and day 86 after immunization (a.i.). Five animals received once weekly i.v. injections of 10 mg/kg CNTO1275 in saline into the vena saphena under ketamin anesthesia. Control animals

(n=5) received once weekly i.v. injections with sterile PBS (1 ml/kg) as placebo treatment.
At 3 days after each dosing, serum was collected and stored frozen until determination of CNTO1275 levels with ELISA. Briefly, recombinant human IL-12 (10 μg/ml) was coated on 96-well plates (Costar Corning, New York, NY). Thawed serum samples were incubated for 1 h and, after washing, CNTO1275 was detected by mouse-anti-human Fc Ab, grown from cell line HP6017 (ATCC, Manasas, VA). The Ab was purified via protein A, coupled with sulfo-N-hydroxysuccinimide LC biotin (Pierce, Rockford, IL), and detected using horseradish peroxidase-conjugated streptavidin (SA-HRP; Jackson ImmunoResearch Laboratories, West Grove, MA). Concentrations were calculated using a standard curve prepared with CNTO1275. Anti-CNTO1275 Ab levels were determined by a sandwich ELISA using CNTO1275 binding to the plate and detected with biotinylated CNTO1275 (CNTO1275-biotin) and SA-HRP as described above.

Magnetic Resonance Imaging
High-resolution MRI experiments were performed on a 4.7 T horizontal bore Varian NMR spectrometer (Varian, Palo Alto, CA), equipped with a high-performance gradient insert (11 cm inner diameter, maximum gradient strength 220 mT/m). For in vivo MRI, animals were anesthetized with 30 mg/kg ketamin in combination with 1 mg/kg valium (Diazepam; Kombivet BV, Etten-Leur, The Netherlands). MRI data sets were collected for T1-weighted (T1-w) and T2-w images. A bird cage volume coil (diameter 9 cm) was used for radio frequency transmission and signal reception. For all sequences the field of view was 4x4 cm (matrix 128x128; zero filled to 256x256; in plane resolution 312x312 μm). Post-contrast T1-w images were made after i.v. injection of 0.3 mmol/kg gadolinium-diethylene-triaminepentaacetic acid (Gd-DTPA; Magnevist, Schering AG, Berlin Germany). Post-mortem high contrast T2-w images were made of formalin-fixed brains. A solenoidal-coil (4 windings; diameter 35 mm) was used for radio frequency transmission and signal reception (field of view 3x3 cm; matrix 128x128; zero-filling 256x256; in plane resolution 240x240 μm). The in vivo and post-mortem MRI scores were calculated as proposed by Jordan and coworkers (51). More specific: score 0 = no visible lesions; score 1 = one to five lesions on T2 scans; score 2: six to ten lesions on T2 scans; score 3 = 11 to 20 lesions on T2 scans; score 4: more than 21 lesions or diffuse white matter abnormalities on T2 scans. A score 0.5 is added for one gadolinium-enhancing lesion and a score 1 for two or more enhancing lesions. It should be emphasized here that this scoring is based on the number of lesions, rather than that they provide information on the size or the pathomorphological aspects. The data sets were analyzed on an Apple MacIntosh I-MAC G3 (Apple Computer, Cupertino, CA) using the public domain National Institute of Health Program (NIH Image 1.52).

Neuropathological examination

After formalin fixation, parts of the brain, spinal cord and peripheral nerves were embedded in paraffin and processed as described previously (29). In brief, the cerebrum and cerebellum were divided into seven or eight coronally cut parts and the spinal cord was dissected transversely. The extent of inflammation, demyelination and axonal pathology was evaluated on 3-5 µm tissue sections stained with hematoxylin and eosin (HE) to visualize infiltrated cells, Klüver Barrera luxol fast blue (LFB) combined with periodic acid schiff (PAS) for myelin and myelin degradation products, and with Bielschowsky silver impregnation for axons. The degree of inflammation was expressed as an index, representing the average number of inflamed blood vessels per spinal cord section (n = 10 to 15 sections). Furthermore, the surface area of demyelination was quantified on 10 to 15 spinal cord fields using a monomorphic grid. Macrophages were visualized using mouse anti-human mAb MRP14 (BMA Biomedicals, Augst, Switzerland), while mAb M4 was used to detect amyloid precursor protein (APP; Boehringer Mannheim, Mannheim, Germany).

Expression profiles of pathogenic effector molecules in the CNS

Immunohistochemistry was performed essentially as previously described (33, 53) with minor modifications. Frozen sections of 6 µm thickness were cut, thaw-mounted on glass slides, and kept overnight at room temperature (RT) in humidified atmosphere. After air-drying for 1 h, slides were fixed in fresh acetone containing 0.02% H_2O_2 (v/v), air-dried for 10 min, washed with PBS, and incubated overnight at 4°C with primary Ab. Incubations with secondary and tertiary reagents were performed for 1 h at RT. Between the incubation steps slides were washed twice with PBS. Detection of primary unlabeled mouse Ab was performed with rabbit anti-mouse Ig HRP (Dako, Glöstrup, Denmark), or in the case of a three-step staining with rabbit anti-mouse Ig biotin (Dako) and HRP labeled avidin-biotin-complex (ABC/HRP; Dako). Rabbit polyclonal Ab was detected with biotin labeled donkey-anti-rabbit-Ig (Amersham, Little Chalfond, United Kingdom) as a second step. HRP activity was revealed by incubation for 10 min at RT with 3-amino-9-ethyl-carbazole (AEC; Sigma, Zwijndrecht, The Netherlands), resulting in a bright red translucent precipitate.

For detection of IFN-γ and TNF-α, mAb MD-2 and 61E71 were used respectively (U-Cytech, Utrecht, The Netherlands). MAb against IL-4 and IL-6 were obtained from Genzyme (Cambridge, MA). C8.6, a mouse Ab for the detection of IL-12p40, was from Pharmingen (San Diego, CA). IL-10 was visualized using the B-S10 mAb (Instruchemie, Hilversum, The Netherlands). The Ab against IL-18, M318, was obtained from R&D Systems (Abingdon, United Kingdom). 2D9, a mouse mAb against matrix metalloprotease 9 (MMP-9; gelatinase B), was a kind gift from Dr. G. Opdenakker (REGA Institute, Leuven, Belgium). Rabbit polyclonal Ab against CD3 and iNOS were from Dako and Calbiochem (San Diego, CA), respectively. Finally, CD40 was detected using a mouse anti-human CD40 mAb (mAb 5D12; Tanox Pharma Inc., Houston, TX) known to be crossreactive with marmoset CD40 (29, 33).

In situ detection of CNTO1275 injected i.v.

To determine whether intravenous CNTO1275 traverses the blood-brain-barrier and gains access to the lesions in the CNS, 1 mg of CNTO1275-biotin was i.v. injected into a PBS-treated monkey with an EAE score of 3.0 (Mi-031). At 1 h after injection of the antibody the monkey was sacrificed. For detection of CNTO1275-biotin frozen tissue sections of brain and spleen were incubated with SA-HRP (Jackson) for 1 h at RT, followed by tyramide signal amplification (TSA; NEN Life Science Products, Boston, MA). HRP-activity was revealed as described above. To assess whether CNTO1275 binds to IL-12p40 produced by astrocytes, tissue sections were double stained using the C8.6 mAb against IL-12p40 and donkey-anti-human polyclonal Ab (Sanbio) directed towards glial fibrillary acidic protein (GFAP) characteristic for astrocytes. A combination of HRP and alkaline phosphatase (AP)-labeled conjugates was used, giving a red precipitate for AEC and a bright blue precipitate using Fast Blue BB base and napthol AS-MX phosphate for AP, as described in detail previously (33, 53).

T- and B-cell functions

Just prior to necropsy, heparinized venous blood was drawn via a needle puncture from the *vena saphena*, after which PBMC were isolated using lymphocyte separation medium (LSM˙, ICN Biomedical Inc., Aurora, OH). Cell suspensions were prepared from aseptically removed lymph node (LNC) and spleen (SC) and cultured in the presence of recombinant human myelin oligodendrocyte glycoprotein (rhMOG; 10 μg/ml) or human myelin basic protein (hMBP; 25 μg/ml) (47).

Serum was isolated from venous blood collected without coagulant. Sera were collected from paired animals at the pre-immunization stage, when one of both monkeys in a pair displayed EAE score 2.0, and at the time of necropsy. Standard ELISA assays were used to determine IgM and IgG Ab levels directed against MBP and MOG as described (29).

Statistics

The χ^2-test was used to determine statistically significant treatment-related effects on the progression to EAE score 3.0; MannWhitney-*U* test for anti-MOG and anti-MBP Ab levels and for MRI-scores. In all cases, $p<0.05$ was considered statistically significant.

Results

Effect of CNTO1275 on clinical EAE

The EAE course in placebo- and antibody-treated monkeys are given as the days of disease onset, when animals showed clear neurological signs (EAE score 2.0), and when the disease score of 3.0 was reached (table I). The percentages of maximal weight loss during the experiment are depicted in the same table. The results show a beneficial effect of CNTO1275 treatment on both aspects of clinical EAE. Four PBS-treated monkeys developed severe progressive EAE and one had a period of mild EAE during the observation period of 86 days. In the CNTO1275-treated group only one monkey, Mi-019, developed clinical signs of EAE ($p<0.001$ vs. PBS treatment; χ^2-test). It should be noted, however, that the time interval between disease onset and EAE score of 3.0 in this animal was considerably longer than in the placebo-treated monkeys (see table I).

Legend to Table 1 (page 161)

Clinical signs of EAE were scored as: 0, no clinical signs; 0.5, apathy, loss of appetite and altered walking pattern without ataxia; 1.0, lethargy and/or anorexia; 2.0, ataxia, sensory loss/blindness; 2.5, hemi- or paraparesis; 3.0, hemi- or paraplegia; 4.0, quadriplegia; 5.0, spontaneous death attributable to EAE. Body weight was determined at the day of dosing as a surrogate disease marker. The maximal weight loss during the experiment is expressed as a percentage of the starting weight. Animals were treated from day 14 after immunization (a.i.) onwards and either sacrificed when a EAE-score 3.0 was reached or at the end of the study period (day 86 a.i.). T1-w (pre- and post-contrast) and T2-w MRI data sets were acquired and scored as described in materials and methods. MRI were made once one of the animals had reached EAE score 2.0 (ataxia), irrespective of the clinical condition of the second monkey. Because of the acute onset of the disease in Mi-032 and Mi-043, both animals were euthanized for ethical reasons before an *in vivo* MRI could be made. Consequently, the *in vivo* MRI of Mi-026 and Mi-023 was recorded at day 55 a.i.. n.d.: not done. The number of infiltrates in the brain were quantified using immunohistochemistry. The number of infiltrates per section were scored as: -, no infiltrates; +, 1-3 infiltrates; ++, 4-10 infiltrates; +++, >10 infiltrates. Results represent the mean of two sections. The size of the largest infiltrate found in two sections was scored as: +, small (< 30 cells); ++, medium (>30 cells); +++, large (>100 cells). The inflammatory index (Infl. Index) in the spinal cord was quantified as being the average number of inflamed blood vessels per spinal cord cross-section (10 to 15 sections). Furthermore, the surface area of demyelination (%) was quantified on 10-15 spinal cord cross sections using a monomorphic grid. In-flammation and demyelination in the brain is expressed as present (+) or absent (-).

Table I: Characteristics of the animals

Animal code	Gender	Pair	Birth (mo-y)	Weight loss (%)	Disease onset (a.i.)	EAE 2.0 (a.i.)	Day of sacrifice	Clinical score
PBS								
Mi-036	F	1	dec-96	20	21	21	22	3
Mi-032	M	2	dec-92	6.5	43	46	53	3
Mi-043	M	3	nov-97	9.1	52	52	53	3
Mi-031	M	4	nov-96	11.1	61	64	71	3
Mi-038	M	5	ayg-97	11.6	63	-	86	1
CNTO 1275								
Mi-019	M	1	aug-98	13.0	56	64	86	2.5
Mi-026	M	2	jul-97	0.6	-	-	86	0
Mi-023	M	3	mar-98	6.3	-	-	86	0
Mi-024	M	4	apr-98	2.8	-	-	86	0
Mi-003	M	5	jun-98	3.4	-	-	86	0

Animal code	*In vivo* MRI	Post mortem MRI	Infiltrates in the brain		SPINAL CORD		BRAIN	
			number	size	infl. index	demyeli-nation (%)	inflam-mation	demyeli-nation
PBS								
Mi-036	4	4	+++	+++	6.6	36	+	+
Mi-032	n.d.	4	++	++	2.3	56	-	-
Mi-043	n.d.	3	++	++	3.5	41	+	+
Mi-031	3	0-1	++	++	1.7	30	+	+
Mi-038	2.5	3	++	++	0.23	1	-	+
CNTO 1275								
Mi-019	1	3	+	++	1.3	23	+	+
Mi-026	2.5	0-1	+	+	0	0	-	-
Mi-023	2	1	-	-	0	0	-	-
Mi-024	4	1	++	+	2.1	22	+	+
Mi-003	2	2	+	+	0	0	-	-

Cross-reactivity, serum levels and anti-Ab responses of CNTO1275

As shown in Fig. 1, the IFN-γ inducing properties of conditioned medium derived from LPS-stimulated marmoset adherent cells were neutralized by CNTO1275. The IC_{50} was comparable with conditioned media from human cells. The antibody levels measured at 3 days after each administration of CNTO1275 were generally maintained at a concentration of 50 to 75 μg/ml throughout the observation period of 86 days (Fig. 2). This is approximately a 50-fold excess of the IC_{50} determined *in vitro*. In one animal (Mi-026) an unexplained disappearance and subsequent reappearance of CNTO1275 serum levels was observed. No Ab responses directed against CNTO1275 were detectable and no alterations in hematological and biochemical parameters were observed during the study period.

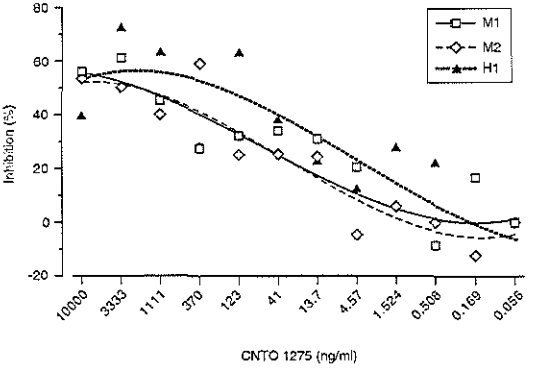

Figure 1. CNTO1275 neutralizes marmoset IL-12

We tested the capacity of CNTO1275 to neutralize IL-12 in culture supernatant obtained from LPS-stimulated marmoset or human PBMC. As shown, the IL-12 neutralization profiles by CNTO1275 in conditioned media of marmoset and human origin were comparable.

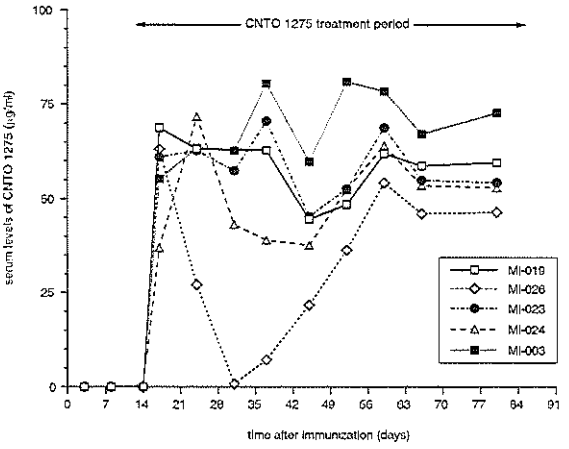

Figure 2. Serum levels of CNTO1275
Animals were intravenously injected with 10 mg/ml/kg CNTO1275 (treatment) or 1 ml/kg PBS (not shown) at 1 week time interval from day 14 until day 84 after immunization. Sera were collected at 3 days after each dosing and CNTO1275 levels were determined using ELISA.

In vivo and post mortem MRI

The a priori condition set before the experiment was to subject paired monkeys to MRI analysis once one of the animals had reached EAE score 2.0 (ataxia), irrespective of the clinical condition of the second monkey. Because of the acute onset of the disease in Mi-032 and Mi-043, both animals had to be euthanized for ethical reasons before an *in vivo* MRI could be made.

The characteristic feature of myelin-induced EAE is a strong periventricular inflammatory reaction that appears as a hyperintense area in T2-w brain MR-images, which is hypointense on T1-w images. Fig. 3 depicts as representative examples for both test groups the MRI-detectable changes in the brains of pair 4. In monkey Mi-031, which had EAE score 2.0 at the time of scanning, large hyperintense areas were found around the ventral horns of both lateral ventricles (A). Such abnormalities, which are typical for this model, were completely absent in the clinically normal CNTO1275-treated monkey Mi-024 which was scanned on the same day (B). Scores of in vivo and post-mortem MRI are given in tabular form. The table shows that post-mortem MRI scores of CNTO1275-treated animals tended to be lower when compared to the scores of control animals ($p=0.08$; Mann-Whitney *U*-test).

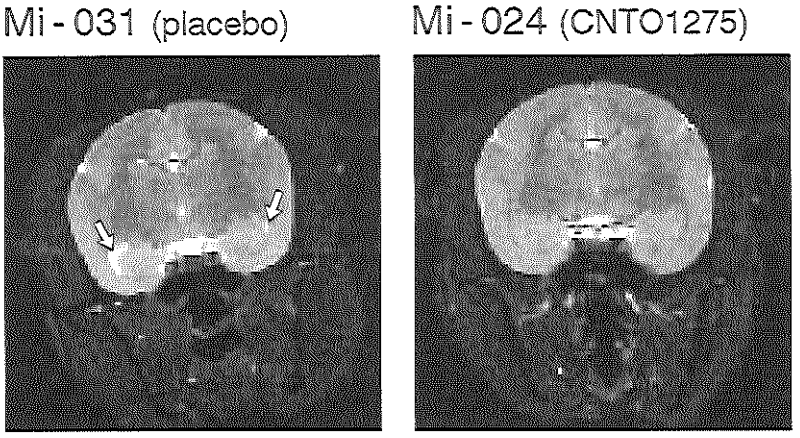

Figure 3. CNTO1275 reduces MRI detectable alterations
T2-w brain images of paired monkeys were recorded at the time that the PBS-treated monkey displayed ataxia (EAE-score 2.0) and the antibody-treated monkey was asymptomatic. The two equivalent slices show in the placebo monkey strong hyperintense areas at the ventral arm of the lateral ventricles (white arrows), indicating periventricular inflammation, which were absent in the paired CNTO1275-treated monkey. This difference is representative for all monkey pairs.

Neuropathology

All PBS-treated control animals displayed cellular infiltrates in the brain and spinal cord. Four PBS-treated animals showed extensive demyelination which was mainly localized in the spinal cord, while in the animal with the mild EAE (Mi-038) the CNS pathology was mainly localized in the brain (see table I). Although inflammation and demyelination of the CNS were detected in two CNTO1275-treated individuals, three animals (Mi-003; Mi-023; Mi-026) were completely devoid of CNS pathology in the spinal cord and the brain ($p=0.03$ for inflammation and demyelination; Mann-Whitney U-test). Fig. 4 shows monkey pair 2 as representative example. The PBS-treated animal Mi-032 displayed large areas with infiltrated mononuclear cells and specific demyelination of the CNS in the spinal cord (Fig. 4A and 4C), while no such pathological changes were observed in the CNTO1275-treated animal of this pair, Mi-026 (Fig. 4B and 4D). Macrophages actively involved in demyelination were present in the lesions as shown in Fig. 4E and 4F, while B- and T-cells also could be detected (Fig 4G and 4H, respectively). The immunoreactivity for APP points at degeneration of the demyelinated axons (Fig 4I).

CNS lesion load and expression of pathogenic molecules

Immunological aspects of the brain lesions were assessed in cryosections on the basis of the number and size of the infiltrates as defined by hematoxylin counter staining, as well as the expression of the pan-T-cell marker CD3, and of acid phosphatase as a marker of infiltrating macrophages. Both the number and the size of infiltrates in the brains of CNTO1275-treated animals were markedly reduced compared to control animals (table I). While $CD3^+$ T-cells were present in the cellular infiltrates of all EAE affected monkeys, these were undetectable in the brains of three out of four CNTO1275-treated animals that remained asymptomatic. Together these data indicate that neutralization of IL-12p40 affects recruitment of mononuclear cells from the circulation into the CNS.

Next we assessed whether treatment with CNTO1275 interferes with CNS expression of Th1 or Th2 cytokines (IFN-γ, IL-4, IL-10, IL-12p40, IL-18, TNF-α) or the inflammatory mediators iNOS, and MMP-9. IL-12p40 was expressed by some mononuclear cells within brain infiltrates, and by many astrocytes (Fig. 5A and 5B). The number of IL-12p40-expressing astrocytes decreased with distance from the infiltrates. IL-12p40 was similarly expressed in both groups of animals (Fig. 5A vs. 5B). This could be expected, since mAb treatment captures released IL-12p40 but likely does not interfere with intracellular synthesis of this factor. Note that in naïve animals or after immunization with ovalbumin emulsified in CFA no IL-12p40 was detectable within the CNS (Fig. 5C).

The staining is specific, as controls omitting the specific antibody step were shown to be negative (Fig. 5D). Mononuclear cell infiltrates in the brain of PBS-treated animals displayed clear expression of IL-4, TNF-α and MMP-9 (Fig. 5E,G,I). In asymptomatic CNTO1275-treated animals the CNS expression of IL-4, TNF-α and MMP-9 was substantially reduced (Fig. 5F,H,J). IFN-γ was also expressed at lower frequency in non-affected mAb-treated animals (data not shown). The two CNTO1275-treated animals that showed CNS inflammation with histology displayed staining patterns similar as the EAE-affected animals in the PBS-treated group. No marked effect of the antibody treatment on the numbers of IL-6, IL-10, IL-18, and iNOS expressing cells was observed (results not shown).

Access of CNTO1275 to lesions in the CNS

We assessed whether intravenously administered CNTO1275 extravasates into CNS lesions, allowing the capture of locally produced IL-12p40. To this end, CNTO1275-biotin was injected i.v. into two PBS-treated animals with clinically manifest EAE (score 3.0) at 1 h before sacrifice. The subsequent immunohistochemical analysis revealed granular staining adjacent to the ventricles, as well as in the cellular infiltrates within the brain of animals injected with the antibody (Fig. 6A and 6B). No such staining was detected in the brains of naïve animals (Fig. 6C), or ovalbumin/CFA-immunized marmosets (not shown). CNTO1275-biotin was found attached to the astrocytes in the immediate environment of the inflammatory infiltrates. This is in conformity with the immunohistochemical demonstration of IL-12p40 by brain astrocytes in EAE-affected animals (Fig. 5A and 5B).

Anti-myelin T-cell reactivity

To investigate the effect of CNTO1275 on anti-myelin T-cell activity we determined the proliferative responses of PBMC, SC and LNC against rhMOG and hMBP in CNTO1275- and placebo-treated animals. The results in Fig. 7 show that in all cases the T-cell response towards MBP was low, while high responses were found against rhMOG (MOG vs. MBP: $p<0.01$; Mann-Whitney *U*-test). In the animals which developed EAE relatively early after disease induction, significant proliferative responses to rhMOG were detectable in SC as well as LNC (Fig. 7A to 7C). In contrast, in all animals sacrificed at the end-point of the study (both PBS- and CNTO1275-treated animals), proliferative responses were mainly restricted to the spleen, while those in LNC were very low or undetectable (Fig. 7E to 7J). As a similar profile was found in the placebo monkey of pair 5, we assume that the number of autoreactive T-cells in the spleen is not affected by the CNTO1275 treatment.

Anti-myelin B-cell reactivity

A critical role of anti-MOG Ab in the marmoset EAE model, mediating demyelination in particular, has been reported (45, 54-57). We therefore determined the serum levels of anti-MBP and anti-MOG Ab in PBS- and CNTO1275-treated animals using ELISA. Anti-MOG and anti-MBP IgM Ab were hardly detectable in the sera of the animals from both groups at any of the evaluated time points. As shown in Fig. 8, increased anti-MOG (A) and anti-MBP (B) IgG serum levels were found in PBS-treated animals at EAE-

score 2.0 when compared with IgG Ab levels in sera from the paired CNTO1275-treated animals (p=0.05; Mann Whitney *U*-test). At necropsy, only serum levels of MBP-specific IgG were significantly reduced in the asymptomatic CNTO1275-treated animals (p=0.03). Taken together, treatment with CNTO1275 seems to have suppressive effect on the production of auto Ab.

Discussion

In this article we report on the beneficial effects of CNTO1275, a human $IgG_1\kappa$ antibody specific for human IL12p40, on EAE in the common marmoset. The non-human primate disease model was chosen because of the insufficient cross-reactivity of CNTO1275 with rodents. To our knowledge this report is the first documented evidence that human Ab produced in transgenic mice are effective in a non-human primate autoimmune disease model.

CNTO1275 shows a comparable bioneutralization profile of marmoset and human IL-12 in vitro. An important result from our study is that the marmosets did not develop a neutralizing Ab response towards the therapeutic Ab. Although the induction of anti-idiotype Ab cannot be excluded, the remarkably stable CNTO1275 serum levels throughout the treatment period of 72 days strongly indicate that the mAb is not or only marginally immunogenic in vivo. This is an important result for the application of therapeutic Ab for chronic diseases. In the past years we have tested a variety of humanized antibodies in non-human primate models of autoimmune arthritis and encephalomyelitis. Although by extensive engineering often up to 99% of the original mouse back bone had been replaced with human Ig sequences the neutralizing immune response towards the remaining 1% mouse part of the molecule formed a limiting factor for a long-standing therapeutic effect. For example, treatment of EAE-affected marmosets with a chimeric anti-CD40 antibody had already resulted in substantial neutralizing Ab activity within two weeks after the first administration (29). Similar findings have been published by other groups (58, 59, 60). All PBS-treated monkeys in this study developed clinical EAE. In contrast, four out of five CNTO1275-treated animals remained asymptomatic. The disease progression in the one CNTO1275-treated animal that developed clinical EAE was substantially delayed compared to the PBS-treated monkeys. Our results also show a diminution of MRI-detectable changes in the CNS white matter in CNTO1275-treated when compared to the paired placebo-treated monkeys. A clear effect of the treatment was that the periventricular inflammatory reactions as observed in all PBS-treated monkeys, were absent in all CNTO1275-treated monkeys, when tested at the same time point. Our observation that a clear beneficial effect of the CNTO1275 treatment on the overall *in vivo* MRI score was not detectable, requires further explanation. T2-w MR images are very sensitive to water.

The fact that all pathological changes in the CNS are associated with altered tissue distribution of water explains that each lesion stage in the common marmoset EAE model has a similar appearance on T2-w images (51). Thus, more sophisticated MRI parameters are needed to reveal the beneficial effects of therapy in vivo. Quantitative MRI parameters are now being developed and implemented for serial imaging, in particular T1-, T2- and MTR maps (manuscript in preparation). That CNTO1275 treatment positively affects structural alterations of CNS white matter was visualized in high contrast T2-w images made of fixed brains. This post-mortem MRI analysis shows clearly reduced MRI scores in the CNTO1275-treated monkeys (with the exception of Mi-019) compared to the placebo group (table I). The beneficial effect of CNTO1275 was confirmed with histology; three out of five CNTO1275-treated monkeys remained completely devoid of CNS infiltration by $CD3^+$ T-cells, while inflammation and demyelination were also prevented.
IL-12 is expressed at sites where T-cells and APC interact (8-10). In the common marmoset EAE model such sites are the secondary lymphoid organs and the developing lesions within the CNS white matter (33, 53). Although microglia are an important source of IL-12 within the CNS (10, 61), our results demonstrate that astrocytes also express intracellular IL-12p40. Intravenously injected CNTO1275-biotin was retrieved at peripheral (spleen/lymph nodes) as well as central (brain) locations, confirming our previous finding that during active disease the blood-brain-barrier is permeable for large molecules like IgG (29). This allows the local action of CNTO1275 within the CNS at locations where infiltrating T-cells interact with resident APC or infiltrating macrophages. As the time needed for 1 mm diffusion of a 20kD protein into unaffected CNS parenchyma has been estimated at about 3 days (62), we assume that the activity range of i.v. injected CNTO1275 (±50 kD) inside the CNS is limited to the perivascular space (Virchows Robin space) and the lesion.
In view of the 100% susceptibility of outbred common marmosets to myelin-induced EAE (7, 45) it is highly unlikely that the observed beneficial effects of CNTO1275 can be explained by the possibility that the antibody-treated monkeys that remained asymptomatic were all non-responders to the disease induction. Hence, the conclusion is warranted that treatment with CNTO1275 protects marmoset monkeys immunized with human myelin in CFA against the clinical and neuropathological expression of EAE. Our present results suggest that (part of) the therapeutic effect of CNTO1275 is exerted via modulation of the autoimmune reaction. Interestingly, recent in vitro studies

revealed that CNTO1275 neutralizes not only human IL-12, but also human IL-23 (D. Peritt, unpublished data). IL-23 seems to play a pivotal role in the activation of memory $CD4^{+}CD45RB$ (low) T-cells (63) and may therefore be an important target for therapy in MS.

Anti-myelin Ab are considered a critical factor in the marmoset EAE model as mediators of CNS demyelination (45, 54-57). It is therefore of great interest that during development of EAE circulating auto Ab levels were consistently lower in CNTO1275-treated animals than in the paired PBS-treated animals. This suggests that, besides preventing T-cell recruitment into the CNS, part of the beneficial effect of CNTO1275 is due to modulation of the auto-Ab reaction as well. Similar observations have been made in marmosets that were protected from EAE by anti-CD40 mAb treatment. In that study the protective effect of the mAb to clinical EAE was associated with abrogation of broadening of the epitope response against MOG (29).

The different reactivity patterns of T-cells present in PBMC, LNC and SC to MBP and MOG between PBS- and CNTO1275-treated monkeys can be explained by the different disease duration. Our data are in line with observations by Targoni and coworkers (64) that T-cell autoreactivity in mice wanes in time from draining lymph nodes and the circulation during EAE development, but persist in the spleen. All CNTO1275-treated monkeys displayed a similar reaction pattern as the one PBS-treated monkey sacrificed at the same time point (day 86). We assume therefore that CNTO1275 treatment modulates the development of autoreactive T-cells yet does not interfere with cell circulation kinetics.

A likely result of CNTO1275 treatment is deviation of the encephalitogenic myelin-reactive T-cells into the Th2 direction as was observed in mice (21). However, we are presently unable to prove this, since reagents for specific detection of Th2 cytokines in common marmosets with ELISA are lacking. Instead, using immunostaining we determined cytokine expression patterns within the CNS of both groups of monkeys.

In three out of five CNTO1275-treated animals neither T-cell infiltrates nor IFN-γ or IL-4 producing cells could be detected. Moreover, inflammatory mediators like TNF-α and MMP-9 were not expressed in the brains of these monkeys. In contrast, in all PBS-treated animals we found brain infiltrates expressing each of the tested inflammatory mediators. The patterns of intracellular IL-12p40 expression within the CNS white matter did not differ between PBS- and CNTO1275-treated animals.

In conclusion, CNTO1275 has direct access to secondary lymphoid organs

and the CNS. Since antibody treatment was initiated at a late stage (day 14 after immunization), it is less likely that CNTO1275 interferes with initial activation of autoreactive T- and B-cells. However, by neutralization of APC-derived IL-12, it affects the influx of autoreactive T-cells into the CNS, inhibits the inflammatory response, and suppresses the auto Ab response against myelin proteins resulting in prevention of EAE development. This, together with the low immunogenicity of CNTO1275, warrants further testing of this mAb as a potential new therapy for MS.

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3: Abbreviations used in this paper: ABC/HRP: horseradish peroxidase labeled avidin-biotin-complex; AEC: 3-amino-9-ethyl-carbazole; a.i.: after immunization; APP: amyloid precursor protein; CNTO1275-biotin: biotinylated CNTO1275; EAE: experimental autoimmune encephalomyelitis; Gd-DTPA: gadolinium-diethylenetriaminepentaacetic acid; GFAP: glial fibrillary acidic protein; HE: hematoxylin and eosin; LNC: lymph node cells; LFB: luxol fast blue; LSM: lymphocyte separation medium; iNOS: inducible nitric oxide synthase; (h)MBP: (human) myelin basic protein; MMP-9: matrix metalloprotease 9; MRI: magnetic resonance imaging; MS: multiple sclerosis; (rh)MOG: (recombinant human) myelin oligodendrocyte glycoprotein; PAS: Periodic Acid-Schiff; ROI: region of interest; RT: room temperature; SA-HRP: HRP conjugated streptavidin; SC: splenocytes, spleen cells; T1-w: T1-weighted; TSA: tyramide signal amplification

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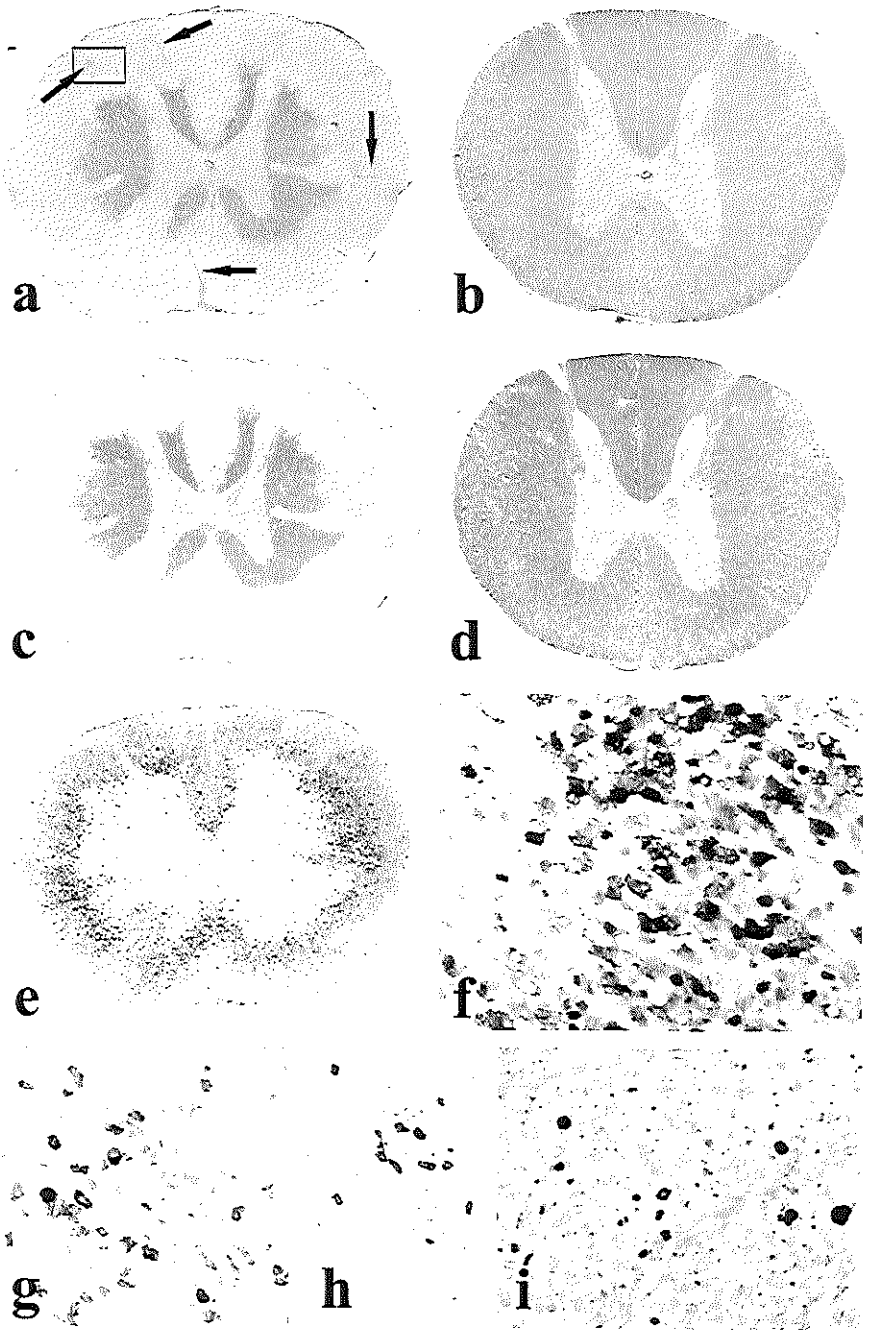
Figure 4. CNTO1275 reduces neuropathological alterations in brain and spinal cord (page 177)

A histological comparison of the spinal cord of Mi-032 (PBS-treated) and Mi-026 (CNTO1275-treated). **A**: HE-staining of spinal cord section of animal Mi-032 showing infiltration of mononuclear cells. Arrows indicate perivascular cuffs (x29). **B**: HE-staining of spinal cord section of animal Mi-026 with no inflammation present (x29). **C**: KLB-staining of Mi-032 showing severe demyelination (myelin stained as blue/green), while **D**: no demyelination was observed in CNTO1275-treated animal Mi-026 (both x29). **E**: MRP14 staining shows the presence of actively demyelinating macrophages (brown) in the spinal cord of Mi-032. **F**: Enlargement of E (x308). **G**: infiltration of $CD3^+$ T-cells (brown) (x308), as well as **H**: $CD20^+$ B-cells (brown) (x161) in the spinal cord of Mi-032 (enlargement of area box in A). **I**: Axonal degeneration as shown by staining for APP (brown) reveals the presence of axonal spheroids in Mi-032 (x308).

Figure 5. CNTO1275 reduces the expression of pathogenic molecules in the CNS (page 178)

Comparison of PBS-treated animals (Mi-032; left column) with CNTO1275-treated animals (Mi-026; right column) for a number of cytokines and enzymes involved in T-cell activation, inflammation and tissue damage. A-B: Similar expression of IL-12p40 (red) in astrocytes around ventricles, by astrocytes around infiltrates and cells within infiltrates. C: Expression of IL-12p40 (red) in a naïve marmoset monkey. D: control staining omitting the first incubation step. E-F: Reduced expression of IL-4 by cells within infiltrates in treated animals (both x250). G-H: Reduced expression of TNF-□ in treated animals (both x160). I-J: Reduced expression of MMP-9 in treated animals (both x160).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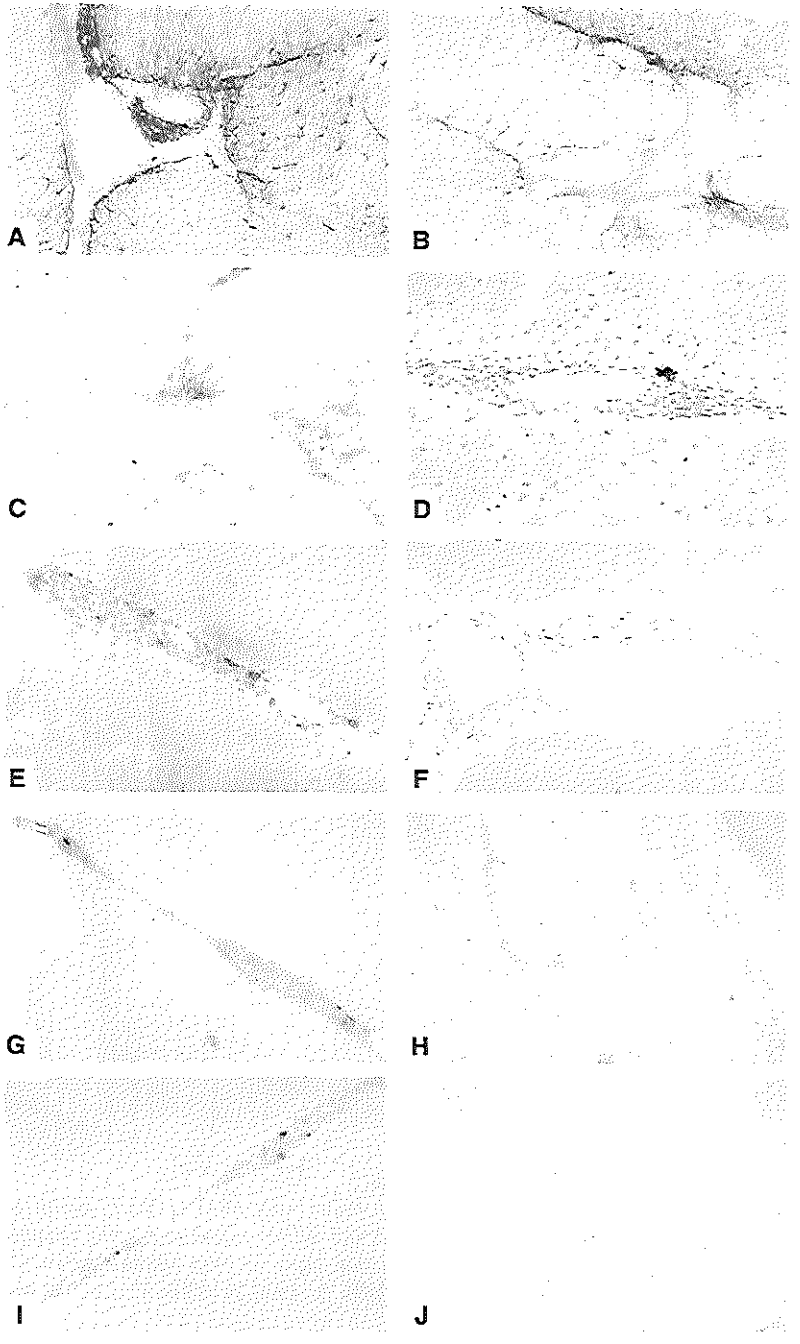
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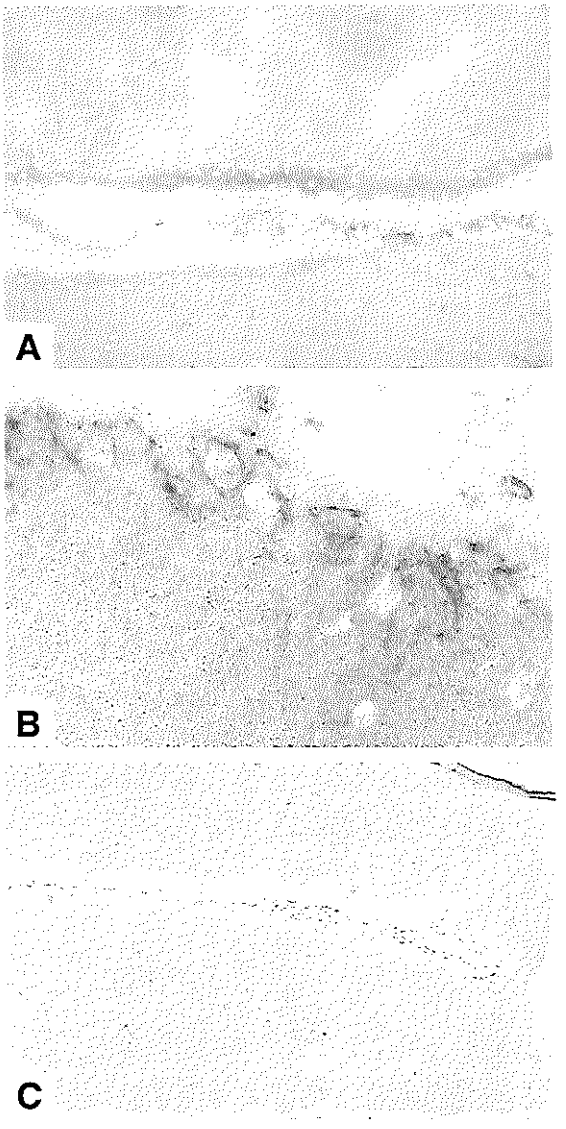

Figure 6. Access of CNTO1275 to CNS lesions
PBS-treated monkey Mi-031 was injected i.v. with 1 mg of CNTO1275-biotin 1 h before sacrifice. Double staining for CNTO1275 (red) and GFAP characteristic for astrocytes (blue) was performed on frozen sections, indicating that upon in vivo administration CNTO1275 binds to astrocytes apparently producing IL-12p40. **A**: 160x, **B**: 630x. **C**: Control staining in a naïve marmoset.

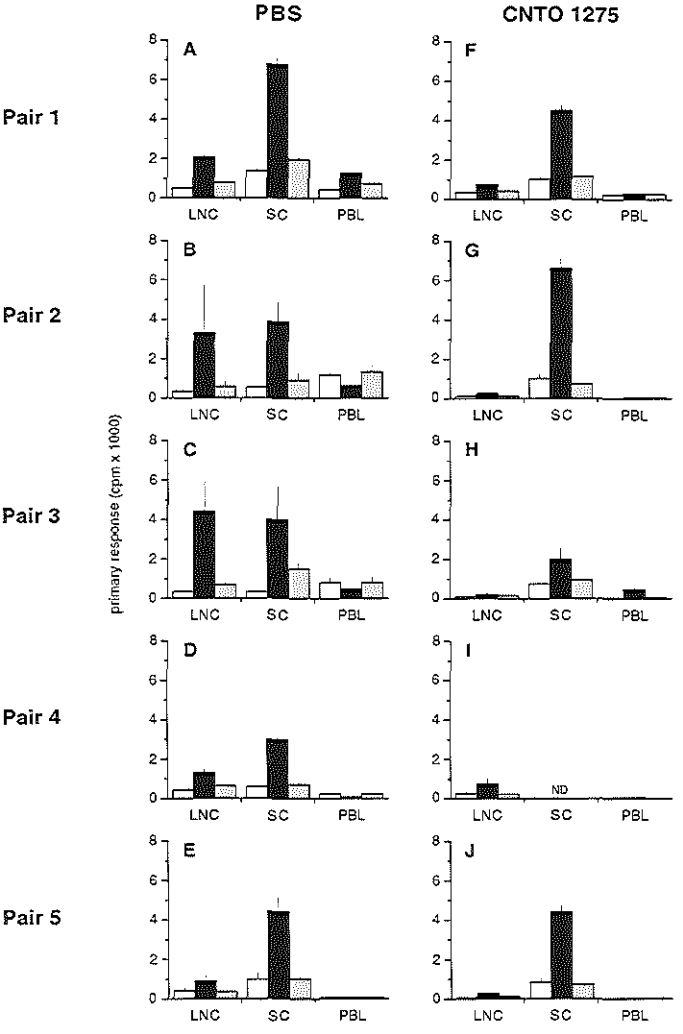

Figure 7. Proliferative responses to MOG and MBP of mononuclear cells (MNC) from lymph nodes (LNC), spleen (SC) or peripheral blood (PBMC)
Proliferation of LNC, SC and PBMC was determined by adding ^{3}H-thymidine during the final 18 h of a 72 h culture. Cells were cultured without additives (white bars) or in the presence of rhMOG (10 µg/ml; black bars) or hMBP (25 µg/ml; gray bars). Animals sacrificed early after disease induction showed proliferative responses to MOG in both SC and LNC (A,B,C). In animals that were sacrificed two weeks before (D) or at the end-point of the study (E,F,G,H,I,J) (both PBS- and CNTO1275-treated animals), MNC proliferation tend to localize primarily in the spleen, while LNC responses wane. n.d.: not done.

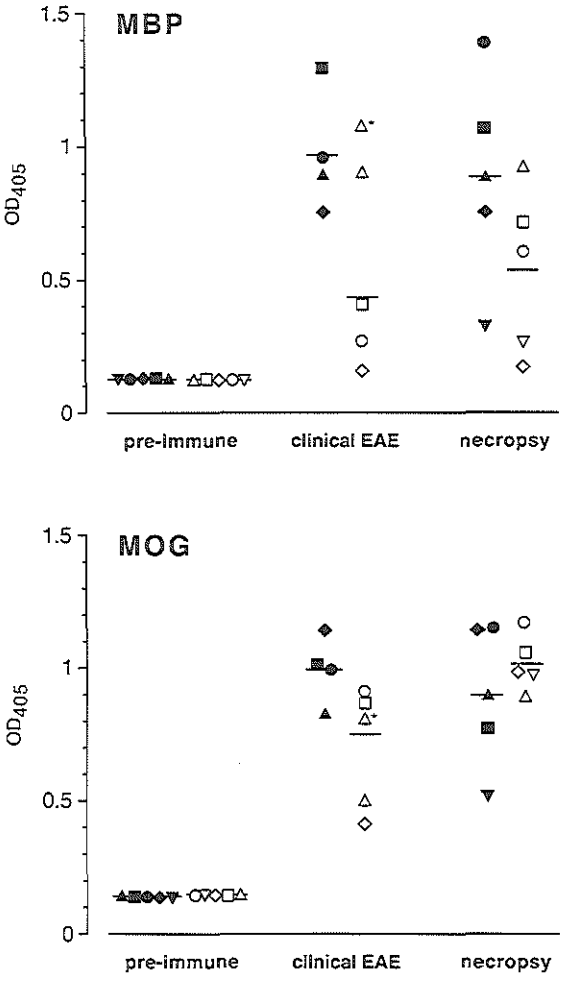

Figure 8. IgG antibody responses against MOG and MBP
Sera from PBS-treated (closed symbols) and CNTO1275-treated animals (open symbols) were screened for the presence of anti-MOG (**A**) and anti-MBP (**B**) IgG antibodies using ELISA. ▲: pair 1; ■: pair 2; ◆: pair 3; ●: pair 4; ▼: pair 5. ✻: Mi-019 at EAE score: 2.0. Sera were collected from paired animals just before immunization (pre-immune), when the PBS-treated animal of a pair presented clear neurological dysfunction EAE score 2.0 (ataxia) and at time of EAE score 3.0 or at day 86 a.i. (necropsy). Sera from PBS-treated monkeys collected at the manifestation of score 2.0 contained significantly higher anti-MBP and anti-MOG IgG Ab-levels than sera from the paired CNTO1275-treated animals (P=0.05; Mann-Whitney *U*-test). At the time of EAE score 3.0 the levels of anti-MBP IgG, but not of anti-MOG IgG remained significantly higher than in the paired CNTO1275-treated animals which were sacrificed much later (P=0.03).

Chapter III

General discussion

A new primate model for multiple sclerosis in the common marmoset

Bert A. 't Hart, Marjan van Meurs, Herbert P.M. Brok, Luca Massacesi, Jan Bauer, Louis Boon, Ronald E. Bontrop and Jon D. Laman

Experimental autoimmune encephalomyelitis (EAE) in outbred marmoset monkeys (Callithrix jacchus) is a recently developed nonhuman primate model of multiple sclerosis. Here, Bert 't Hart and colleagues compare this model to EAE in rhesus monkeys, highlighting autoimmune mechanisms in CNS inflammation and demyelination, including the role of major histocompatibility complex restriction and preclinical evaluation of innovative immunotherapies.

Multiple sclerosis (MS) is regarded as an autoimmune disease caused by $CD4^+$ T cells reactive with myelin proteins and with a T helper 1 (Th1)-pattern of cytokine secretion. This concept is based on data from animal models of experimental autoimmune encephalomyelitis (EAE). Epidemiological and experimental evidence imply that MS results from complex interactions between environmental and host genetic factors, which are difficult to mimic in experimental animals. For instance, because most models require immunization with myelin antigens or adoptive transfer of sensitized $CD4^+$ T cells, it is difficult to assess whether defects in antigen-presenting cells (APCs) precede T-cell reactivity.

Findings supporting an autoimmune anti-myelin origin of MS include the association with certain major histocompatibility complex (MHC) genes, the predominant localization of lesions in CNS white matter, the local presence of T- and B cells and their products, and the clinical and pathological similarities with various EAE models. However, there is also evidence against an autoimmune origin of MS, such as the fact that myelin protein-reactive T cells are found at similar frequencies in healthy subjects and MS patients and that, to date, no single autoantigen has been exclusively identified in patients. Moreover, in MS it has not yet been possible to fully substantiate the concept of sequential Th1 disease-promoting activity and Th2 downregulatory activity as developed in EAE models, and the validity of the Th1/Th2 concept as the basis for immunotherapy in MS has been challenged (reviewed in Refs 1, 2).

The aim of this review is to discuss the new EAE model in the common marmoset monkey (*Callithrix jacchus*), and to compare it with existing rhesus monkey and rodent EAE models, as well as MS in humans. Moreover, we highlight recent findings with respect to MHC restriction of pathogenic autoreactivity and immunotherapy targeting costimulatory molecules, which have immunological implications beyond EAE and MS.

EAE models in nonhuman primates

EAE models in nonhuman primates offer unique features for research into CNS autoimmunity due to their close evolutionary relationship with humans. Nonhuman primate colonies are outbred. The large size and structural complexity of the nonhuman primate brain allows *in vivo* analysis of lesion development using magnetic resonance imaging (MRI). The strong degree of similarity between MHC and T-cell receptor (TCR) genes of higher primate species enables elucidation of T-cell peptide specificity, using human-specific reagents. Finally, nonhuman primates are important for preclinical evaluation of biotechnology-derived pharmaceuticals which, owing to their specificity, are only reactive in primates[3]. As specified by the FDA [Preclinical safety evaluation of biotechnology-derived pharmaceuticals (S6); http://www.fda.gov/cber/publications.htm], relevant species for safety testing of a new drug should be sensitive to the drug's specific pharmacological functions.

EAE in nonhuman primates is induced with protocols very similar to those used in rodents[3] (see Table 1). Commonly used methods include active immunization with brain and spinal cord homogenates, purified myelin preparations or myelin proteins, such as myelin basic protein (MBP), proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG). Dependent on the species, antigen and immunization protocol, a wide array of clinical signs can be found similar to those in MS patients, including monophasic acute, relapsing-remitting, primary progressive and secondary progressive disease courses. Neuropathological presentation covers a wide spectrum including inflammation, demyelination, remyelination, axon loss, gliosis and, in certain cases, necrosis. In contrast to the inbred rodent models, monkeys show much more individual variation within treatment groups. This may be attributable to their outbred nature and microbiological status. Nonhuman primates are susceptible to infection with many human neuroinvasive agents and can carry latent infections with several B-type herpesviruses [e.g. Epstein–Barr virus (EBV), human herpesvirus 5 (HHV-5), HHV-6] that have been associated with demyelinating disease.

EAE in Old World monkeys

Cynomolgus (*Macaca fascicularis*) and rhesus monkeys (*Macaca mulatta*) have been used in EAE research. The relatively close phylogenetic

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Table 1. Non-human primate EAE models versus multiple sclerosis and rodent EAE

	Marmoset EAE	Multiple sclerosis	Rhesus EAE	Rodent EAE[a]
Epidemiology				
Nature of population	Outbred	Outbred	Outbred	Inbred
Prevalence/incidence	100%	1:1000	MBP 60%; MOG 100%	100% in susceptible strains
MHC association	*Caja-DRB*W1201*	*HLA-DR2*	*Mamu-DPB1*01*	Varies between strains
Sex-linkage	No	Yes, female prevalence	No	In some models
Spontaneous onset	No	Yes	No	In transgenic models
Cause	Immunization	Unknown; probably viral or bacterial infection	Immunization	Immunization, viral infection, transgene expression (TNF)
Clinical course	Relapsing-remitting or primary progressive	Rarely acute, mostly relapsing-remitting or chronic progressive	Mostly acute, rarely relapsing-remitting	Monophasic, relapsing-remitting, chronic progressive or acute
Characteristic pathology				
Primary demyelination	Yes	Yes	Probable	In chronic models
Inflammation	Mainly focal	Focal	Mainly focal	Diffuse in some models, focal in others
Myelin antigens in draining lymph nodes	Yes	Not investigated	Not investigated	Yes
Axonal pathology	Present, limited	Extensive in acute MS Marburg type, variable in chronic MS	Strong in all lesions	Strain/model dependent
Remyelination	Present	Present	Present	Strain/model dependent
MRI analysis of lesions	Yes	Yes	Yes	Only in rats
Immunopathology				
Induction	Myelin, MBP/PLP I *B. pertussis*, MOG, MOG14–36	Not testable	Myelin, MBP, MOG, MOG34–56	Myelin-derived proteins, peptides from myelin proteins, viruses
Transferable by $CD4^+$ T cells or T-cell lines	Yes, to chimeric twin	Not testable	Yes, autologous	Yes
B-cell/antibody involvement	Plasma cells, Ig/complement deposits in lesions, anti-MOG enhances demyelination	Plasma cells, Ig/complement deposits in lesions	Not investigated	Controversial; anti-MOG pathogenic in some models
Neuroinvasive viruses	Many similar to humans	Many (e.g. EBV, HHV-5, HHV-6)	Many similar to humans	Very few similar to humans
Preclinical testing of immunotherapies	Possible, low quantity of reagents needed	Limited possibilities	Suboptimal model and high quantity of reagents needed	Primate-specific reagents not effective.

Abbreviations: *B. pertussis*, *Bordetella pertussis*; Caja, HLA-equivalent in marmosets (*Callithrix jacchus*); EAE, experimental allergic encephalomyelitis; EBV, Epstein–Barr virus; HHV, human herpesvirus; Ig, immunoglobulin; Mamu, HLA equivalent of rhesus monkeys (*Macaca mulatta*); MBP, myelin basic protein; MHC, major histocompatibility complex; MOG, myelin oligodendrocyte glycoprotein; MRI, magnetic resonance imaging; PLP, proteolipid protein; TNF, tumor necrosis factor.

[a]The various rodent EAE models have been reviewed in detail elsewhere (see Refs 37–40); here, we highlight only some general aspects for contrast with the non-human primate models and MS.

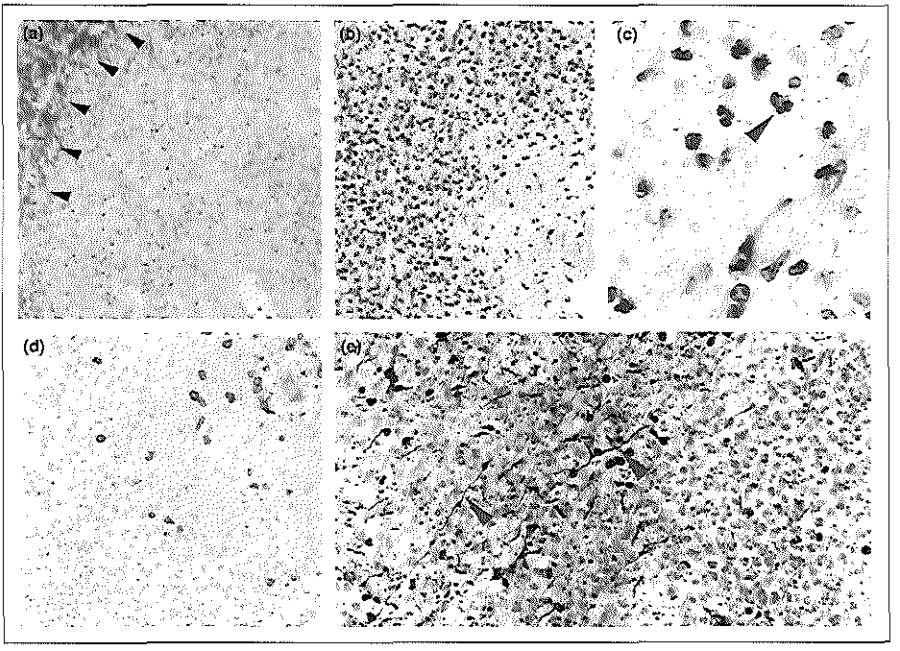

Fig. 1. *Histopathology of a hyperacute lesion in rhesus monkey experimental autoimmune encephalomyelitis (EAE). EAE was induced by immunization with a recombinant version of the N-terminal extracellular Ig-like domain of human MOG (rhMOG[84]; 100 μg in complete Freund's adjuvant). Hyperacute EAE developed after four weeks, at which point the animal was sacrificed. (a) Klüver–Barrera stain (blue) for myelin shows a demyelinated lesion in the brain. Arrowheads indicate the edge of the lesion. Luxol-Fast-Blue (LFB)-positive and Periodic-Acid-Schiff (PAS)-positive (pink) myelin degradation products are present in the lesion (magnification = 161×). (b) Hematoxylin staining (dark pink) shows the presence of numerous neutrophilic granulocytes (246×). (c) In addition, many lesions contain eosinophils (arrowhead) (308×). (d) Staining for CD3 (light brown) reveals the presence of low numbers of T cells (246×). (e) The necrotic character of hyperacute EAE is revealed by Bielschowsky staining (black) showing an almost complete disruption of axonal structures. The arrowheads point at some surviving axons (388×).*

distance between macaques and humans, estimated at 35 million years, is reflected by a high degree of similarity between the immune systems of both primate species[4,5].

The first reports on EAE in macaques date back to 1933 (Ref. 6). In the past decades, EAE models in macaques have been used for the study of genetic, immunological, pathological and radiological features of MS (including MRI) and for therapy evaluation[7–11].

EAE in rhesus monkeys

In its clinical and pathological presentation this EAE more closely resembles post-infectious encephalomyelitis than chronic MS. The disease usually follows an acute course, which may start with convulsions and apathy and leads to death of the animals within two days[11]. By application of immunosuppressive treatment, a relapsing-remitting course can be established[12]. At necropsy, large mainly haemorrhagic or necrotic brain lesions can be found that at histopathological examination show predominance of neutrophil infiltrates and dramatic destruction of axons (Fig. 1). This pathological aspect suggests that lesions are formed by severe inflammatory necrosis rather than by selective demyelination. However, inhibition experiments using anti-CD4 antibodies and autologous adoptive transfer experiments showed that myelin-reactive $CD4^+$ T cells are involved in EAE induction[13,14]. A unique feature of this EAE model is that, in certain MHC class II-compatible combinations, APCs from humans and rhesus monkeys can present the same epitopes from MBP and MOG antigens to their own $CD4^+$ T cells as well as to T cells of the other species[10,15]. This provides the unique possibility of

investigating whether APCs from MS patients can alter the encephalitogenic potential of myelin-specific CD4+ rhesus monkey T cells.

Susceptibility of macaques to EAE

To date, a satisfactory explanation for the high susceptibility of macaques to EAE is lacking, but it appears to depend on genetic factors and on the mode of disease induction. The presence of the MhcMamu (from *Macaca mulatta*) class II allele *Mamu-DPB1*01* is linked with susceptibility to EAE induced with bovine MBP or human myelin in complete Freund's adjuvant (CFA)[15]. However, no linkage was found with EAE induced with a recombinant version of the N-terminal extracellular Ig-like domain of human MOG (rhMOG^{lsd}) in CFA (N. Kerlero de Rosbo, unpublished). Whereas immunization of *Mamu-DPB1*01*-positive monkeys with rhMOG^{lsd} induced acute fatal EAE, repeated immunization with a synthetic peptide encompassing amino acids 34–56 of rhMOG^{lsd} (MOG34–56) induced a relapsing-remitting EAE. By contrast, monkeys lacking *Mamu-DPB1*01* developed acute fatal EAE upon immunization with rhMOG^{lsd} or MOG34–56. Susceptibility to MS is associated with HLA-DR and -DQ alleles, whereas for HLA-DP, a linkage with disease chronicity and epitope spreading has been proposed[16].

EAE in New World monkeys

EAE models in owl monkeys (*Aotus* spp.) and squirrel monkeys (*Saimiri* spp.) have been reported in the early 1970s, but they resemble the macaque models in many respects. In 1995, Hauser and colleagues reported on a new MS model in the common marmoset (*Callithrix jacchus*), which approximates MS in its clinical and pathological expression[17].

The common marmoset is a small-sized monkey, with an adult weight of 300–400 g and an evolutionary distance from humans of 35 million years[4]. Several biological characteristics make the marmoset an attractive model for autoimmune diseases. Marmosets give birth to one or two genetically non-identical sets of twin or triplet siblings per year, which are full bone marrow chimeras as they share the placental blood circulation. Because bone-marrow-derived T cells from both siblings have been educated in the same thymic microenvironment, an alloimmune response between fraternal siblings is absent. This unique feature has allowed transfer of EAE between twin siblings with MBP-specific CD4+ T cells, confirming their central role in the disease[17].

A second attractive feature is the limited polymorphism of MhcCaja (from *Callithrix jacchus*) class II region genes. These encode the evolutionary equivalents of human HLA-DR and -DQ molecules,

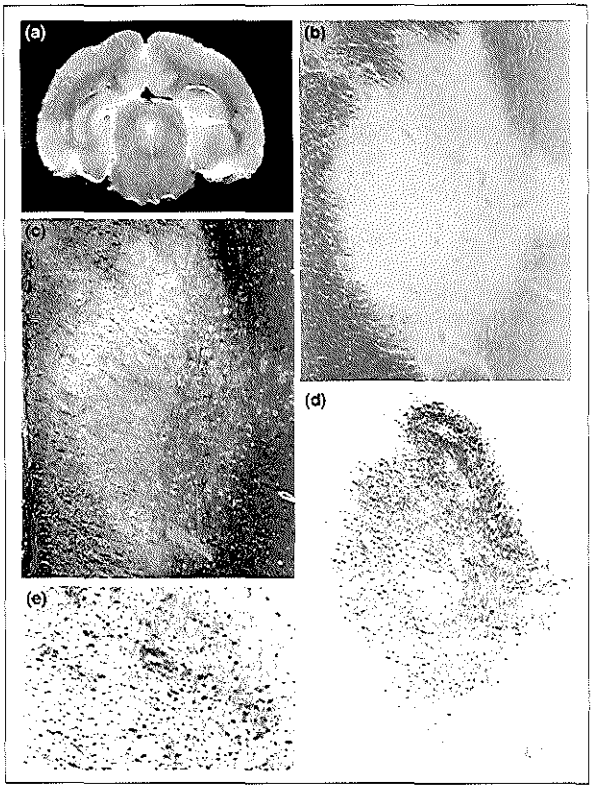

Fig. 2. *Histopathology of a magnetic resonance imaging (MRI)-detectable lesion in common marmosets. Chronic experimental autoimmune encephalomyelitis was induced by immunization with human myelin emulsified in complete Freund's adjuvant. The monkey was sacrificed at 34 weeks after immunization at the stage of ataxia. (a) A coronal slice of a T2-weighted MRI recorded post mortem shows at least three MRI-detectable lesions (top; magnification = 2.5×). The lesion indicated with the arrowhead was processed for histology. (b) Klüver–Barrera staining visualizes myelin in blue, showing strong demyelination (99×). (c) In the same lesion, axonal structures were stained with Bielschowsky silver impregnation, showing axonal conservation (99×). The presence of numerous 27E10-positive macrophages (brown) (d) and CD3+ (T-) cells (brown) (e) classifies the lesion as late-active (246×). Figures reproduced, with permission, from Ref. 22.*

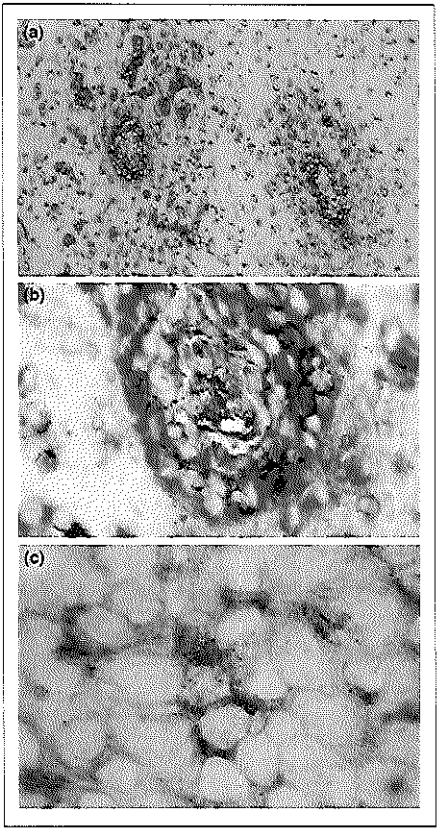

Fig. 3. In situ *analysis of costimulation in a typical early lesion in common marmosets. Cryosections of a marmoset brain affected with experimental autoimmune encephalomyelitis were stained with monoclonal antibody mu5D12, a mouse antibody directed against human CD40 and crossreactive with nonhuman primates. CD40 membrane expression is visualized in blue; activity of acid phosphatase, a lysosomal enzyme abundantly present in the cytoplasm of macrophages, is shown in red. (a) High expression of CD40 by cells is shown within and around two early lesions (130×). (b) At higher magnification (650×), CD40 can be seen expressed by macrophages (blue membrane and red cytoplasm) and cells without acid phosphatase activity (possibly B cells). (c) This shows the co-localization of cells expressing CD40 (red) and gelatinase B (matrix metalloproteinase 9, in blue), an enzyme involved in cell migration and tissue destruction (1300×). Because CD40 ligation in vitro can induce gelatinase B production, these results suggest that local CD40–CD40L interactions induce pathogenic effector functions within lesions. Fig. 3b is reproduced, with permission, from Ref. 24.*

but *Caja-DP* genes were not found. The *Caja-DR* region contains only three loci, two of which (*Caja-DRB*W12* and *-DRB*W16*) have no known equivalent in macaques and humans. By contrast, several members of the *MHC-DRB1*03* allelic lineage are shared between marmosets, macaques and humans[5,18]. The *Caja-DRB*W12* locus in common marmosets contains only one allele *Caja-DRB*W1201*, which is present in all individuals tested from our own and other centres ($n > 100$)[18].

TCR genes in marmosets

A number of rearranged T-cell repertoire β-chain genes were identified (2 *TCRBC*, 13 *BJ*, 2 *BD* and 15 *BV* genes)[19]. The high similarity between human and marmoset V-D-J-CB chain gene sequences (82.6–93.4%) is in line with their close phylogenetic relationship. The length of the *CDR3* region is also similar to that in humans, averaging ten amino acids[19]. Framework regions were conserved to a high extent. Thus, TCR genes are very stable across primate species, and marmosets express a diverse TCRB repertoire, despite their limited polymorphism of MHC class II genes.

Clinical and pathological presentation of EAE in common marmosets

EAE develops in 100% of common marmosets immunized with whole myelin or myelin proteins in CFA. Transfer of anti-myelin $CD4^+$ T cells induces CNS inflammation but, for extensive demyelination, co-transfer of anti-myelin antibodies is needed, confirming the role of plasma cells in EAE (Refs 20, 21). The clinical and pathological expression can be varied by modification of the disease induction procedure. The time of disease onset is variable and clinical signs follow a relapsing-remitting or primary progressive course. It can be shown with serial *in vivo* MRI that lesions first occur around the lateral ventricles. At later stages, large demyelinated areas formed by confluency of smaller perivascular lesions are found in the parenchyma of the white matter. This resembles the situation in MS. Lesions with a similar MRI appearance represent distinct pathological stages with different degrees of inflammation, demyelination, remyelination and axonal pathology[22]. Intravenous administration of *Bordetella pertussis* particles in addition to immunization with myelin/CFA causes simultaneous onset of the first clinical signs and a mainly relapsing-remitting disease course[17,20]. Pathological examination shows a similar topography of smaller-sized lesions in the brain and some larger lesions, that may show destruction of axons[22]. The apparent aggravation of inflammatory reactions in the lesions is consistent with the enhancement of proinflammatory Th1 responses by *B. pertussis*.

Essentially, all clinical and pathological features of myelin-induced EAE can be found in animals immunized with $rhMOG^{1\text{–}125}$ in CFA. The single shared specificity of T-cell lines generated from different MOG-immunized animals is MOG14–36. Activation of these T-cell

lines is restricted by Caja-DRB*W1201 molecules. All marmosets immunized with MOG14–36 develop clinical and pathological EAE with spreading of T- and B-cell reactivity to other MOG epitopes[23]. Because the *Caja-DRB*W1201* allele is present in all our common marmosets, the 100% incidence of demyelinating EAE in our outbred colony can be explained by a uniform EAE-initiating immune response to a single MOG epitope[18].

In situ analysis of immunological activity within lesions

The systematic MRI-guided histopathological and immunological stage-characterization of lesions in the marmoset EAE model has provided the opportunity to relate *in vivo* data to *ex vivo* and *in situ* findings[22] (Fig. 2). Immunohistological characterization of early perivascular CNS lesions in EAE-affected marmosets shows that these are areas of significant immunological activity[24]. Similar to EAE in mice, activated macrophages expressing costimulatory CD40 molecules are dominant in the lesion (Fig. 3), whereas T cells expressing CD40 ligand (CD40L) are present at much lower frequency[25–27]. Myelin-immunized marmosets treated with a mouse anti-human CD40 antagonist antibody (mu5D12) remained free of clinical signs during the treatment period (see below), indicating that the expression of clinical signs is causally related to the peripheral and local humoral and cellular immune reactions.

In vitro interaction of $CD40L^+$ T cells with $CD40^+$ macrophages, dendritic cells or B cells induces several proinflammatory factors with a possible role in EAE pathogenesis. For example, gelatinase B, which is produced by macrophages upon CD40 crosslinking *in vitro* and might be responsible for the increased blood–brain barrier permeability in early lesions, is expressed in marmoset EAE lesions (Fig. 3). The co-localization of Th1- and Th2-type cytokines in the earliest lesion stages might explain why active inflammation and demyelination are present within the same lesion in this model[24]. This is clearly different from many rodent EAE models, which show a polarized and sequential Th1/Th2 reactivity. As a representative example, the expression of the interleukin 12 (IL-12) p35 subunit in an early lesion is shown in Fig. 4. Such early lesions appear surrounded by astrocytes with strong intra-cytoplasmatic staining of the anti-inflammatory cytokine IL-10, pointing at a possible counter-regulatory activity towards the expanding lesion.

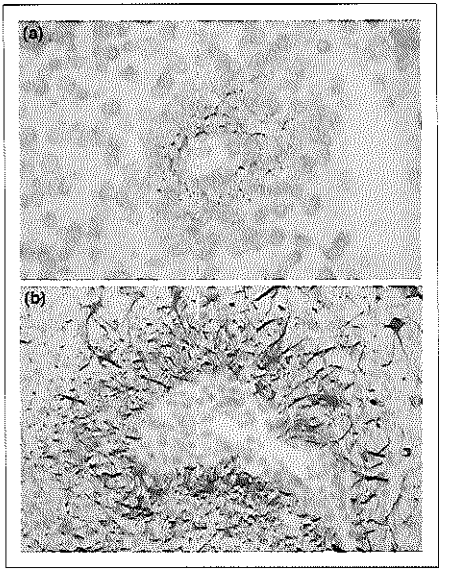

Fig. 4. *Expression of pro- and anti-inflammatory cytokines in lesions of common marmosets. Cryosections of early lesions in experimental autoimmune encephalomyelitis (EAE)-affected marmoset brain were stained with antibodies against human cytokines. (a) Locally restricted expression of interleukin 12 (IL-12) p35 chain within an early lesion (red). IL-12 is regarded as a pivotal T helper 1 (Th1)-derived proinflammatory cytokine in EAE development (magnification = 325×). (b) Expression of IL-10 (red) by cells of astrocyte morphology in the immediate vicinity of a perivascular infiltrate (200×). This demonstrates that expression of pro- and anti-inflammatory mediators can occur simultaneously. Figures reproduced, with permission, from Ref. 24.*

Preclinical evaluation of immunotherapy

An increasing number of new experimental therapies for autoimmune diseases are species specific[3]. This implies that for the preclinical testing of safety and efficacy, disease models in nonhuman primate species are obligatory. Below, some examples are discussed of distinct immunotherapeutical approaches that have been evaluated in marmoset EAE.

Anti-inflammatory therapy

The complete inhibition of myelin-induced EAE in marmosets by Rolipram, a phosphodiesterase inhibitor suppressing tumour necrosis factor α (TNF-α) production, indicates that Th1 cells play an important role in this model[28]. Interferon β (IFN-β) is the most widely used biological therapeutic in MS, but little is known about the actual working mechanism. In the marmoset model, human IFN-β provides some clinical benefit, implying that functional effects of IFN-β in humans can be investigated in this model. In Lewis rats, recombinant rat IFN-β effectively prevented EAE development, but withdrawal induced fatal exacerbation[29]. However, this effect was not observed in common marmosets (B.A. 't Hart *et al.*, unpublished). Hence, compared with Lewis rat EAE, the response to IFN-β treatment in the marmoset EAE model more closely resembles the response in MS.

Tolerance-based therapy

In rodent EAE models, modulation of the anti-myelin T-cell response from a Th1 to a Th2 profile was found to be a safe and effective approach. Because MOG is a likely primary target of the autoimmune

reactivity in MS (Ref. 30), one might assume that deviation of the pathogenic MOG-specific Th1 activity to a protective Th2 activity is beneficial to MS. Although marmosets tolerized to MOG were protected against the acute phase of EAE, they developed an unexpected fatal antibody-dependent demyelinating disease several weeks later[31]. It was concluded that immune deviation can promote Th2-driven anti-MOG antibody responses that exacerbate disease. Detailed examination of the antigen-specificity of the antibody molecules that can be found attached to the myelin sheaths in early active lesions have revealed that these are probably directed towards MOG and MBP (Refs 32, 33).

Costimulation-targeted therapy

The advantage of therapy aimed at blocking costimulatory interactions between APCs and T or B cells over antigen-specific therapies is that the (auto-) antigens involved do not need to be known. Blocking of CD40L on activated CD4+ T cells prevents EAE in mice[25–27]. To demonstrate that blocking APC functions with anti-CD40 antibody provides clinical benefit, marmosets were treated for 28 days with a mouse anti-human CD40 antagonist antibody (mu5D12)[34] starting 14 or 25 days after EAE induction, allowing ample time for T-cell priming to occur. All animals remained free of clinical signs during treatment, but exacerbations occurred in some animals after cessation of therapy. The appearance of marmoset anti-mouse antibodies limited the effective treatment period (J.D. Laman *et al.*, unpublished). Prevention of rhMOG$_{1\text{–}125}$-induced EAE with chimeric antagonist anti-CD40 monoclonal antibody (mAb) showed complete protection against EAE. In addition, anti-MOG antibody responses were dramatically reduced, indicating inhibition of T-B interactions (J. Boon *et al.*, unpublished).

Collectively, these data show that marmoset EAE is an excellent model for the preclinical validation of immunotherapeutic concepts for which proof of principle has been obtained in rodents.

Perspectives

There are obvious limitations to the use of nonhuman primates for research in terms of ethics, availability and cost. Compared with the rhesus monkey and rodents, the marmoset EAE model offers a series of highly useful immunologic and practical features. Its small size and relative ease of breeding and handling in captivity facilitate experimentation and reduce the amount of therapeutic agent needed by a factor of 20, compared with rhesus monkeys. The immune tolerance among chimeric twins allows adoptive transfer experiments assessing relative contributions of T and B cells to pathogenesis. Marmoset MHC typing and characterization of the TCR permit detailed elucidation of epitope specificity. Widespread primary demyelination, the hallmark of MS, occurs in the marmoset CNS. The susceptibility of marmosets to similar neuroinvasive infective agents as humans enables further investigation of environmental factors promoting CNS inflammation, for example by molecular mimicry. Finally, epitope spreading on the B- and T-cell level can be studied in relation to redistribution of CNS myelin antigens to draining secondary lymphoid organs where primary T-cell activation occurs[30].

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Non-human primate models of multiple sclerosis

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Summary: The phylogenetic proximity between non-human primate species and humans is reflected by a high degree of immunological similarity. Non-human primates therefore provide important experimental models for disorders in the human population that are caused by the immune system, such as autoimmune diseases. In this paper we describe non-human primate models of multiple sclerosis, a chronic inflammatory and demyelinating disease of the human central nervous system. While reviewing data from the literature and our own research we will discuss the unique role of such models in the research of basic disease mechanisms and the development of new therapies.

Introduction

There is an increasing need for non-human primates in the safety and effectivity testing of biotechnology-based therapies that are being developed for the treatment of clinical disorders in the human population. The minimum requirement for a relevant animal model for preclinical safety testing of a new therapeutic agent is that the species is sensitive to the drug's specific pharmacological functions. Disease models are important, however, when the target molecules of the therapy are expressed in vital organs only under pathological conditions. As most biotechnology-based therapeutics are exclusively reactive in primates, non-human primates provide the most useful disease models for this purpose.

The chimpanzee is the closest living relative of man in nature. These species share 98.6% identity of their genome and shared an ancestor about 5 million years ago. Chimpanzees would therefore be the animal model of choice, but the usage of this highly endangered species for experimental research is limited by ethical constraints. Useful multiple sclerosis (MS) models have been developed in Old and New World monkeys, such as the experimental autoimmune encephalomyelitis (EAE) models in rhesus monkeys (*Macaca mulatta*) and common marmosets (*Callithrix jacchus*). The implications of the close genetic and immunological similarity of these species

with humans for the research on fundamental pathophysiological processes in autoimmune disease has been well documented (1, 2).

Multiple sclerosis and experimental autoimmune encephalomyelitis

Apart from being a valid experimental model of MS, EAE is one of the most intensively studied experimental models in basic immunology. Concepts developed in the model have taught us a lot about T- and B-cell autoimmunity and the pathogenesis of organ-specific autoimmune diseases. EAE has been successfully induced in each mammalian species tested thus far, including mice, rats, guinea pigs, rabbits and non-human primates, although interindividual (in the case of outbred species) and interstrain (in the case of inbred species) differences with respect to the disease susceptibility do exist. EAE can be induced by inoculation of emulsions containing a strong adjuvant such as complete Freund's adjuvant (CFA) and a preparation from the central nervous system (CNS), such as brain or spinal cord homogenate, semi-purified myelin or pure myelin antigens, either as a purified or as a recombinant protein as well as encephalitogenic peptides within these proteins.

The pathological hallmark of MS and EAE and the most likely cause of the neurological deficit is the lesion, a demarcated area of myelin loss in the CNS white matter with a variable degree of inflammation, axonal pathology and astrocytic scar formation. On the basis of the variable pathomorphological aspects of MS lesions, four patterns of demyelination have been discerned (3). Only two patterns closely resemble the EAE models where lesions are formed by T-cell-mediated (type I) or T-cell- plus antibody-mediated (type II) antimyelin autoreactivity. In the other two patterns (type III and IV), which have no experimental counterparts yet, the primary cause of lesion formation is more likely oligodendrocyte loss caused by direct microbial infection or exposure to cytotoxic agents. As the lesion types found within an individual patient are uniform but can differ between patient groups, it has been suggested that they may represent different types of MS.

In rodent models the concept has been developed that EAE is initiated by the activation of a pre-existing repertoire of myelin-reactive $CD4^+$ T cells in peripheral lymphoid organs. The activated cells are thought to migrate through the blood–brain–barrier (BBB) using adhesion molecules and specific enzymes to split the tight junctions between endothelial cells. At histological examination such infiltrated cells appear organised in perivascular cuffs. The capacity of the infiltrated T cells to enter the CNS parenchyma depends on the interaction with local APC (antigen-presenting cells), presenting the myelin antigens to which the T cells were originally sensitised in the periphery. Cells that do not encounter their specific antigen are drained from the CNS or are locally eliminated (4–6). This mechanism is probably the same in primate models of EAE (7, 8) and MS.

For a long time the dogma has held that demyelination is the main cause of the neurological deficits in MS patients as axonal structures were thought to be (initially) spared. However, on close examination axonal damage and transsection are consistently found in MS lesions, while the extent of axonal pathology correlates with the degree of inflammation (9). The pathological findings have been now confirmed *in vivo* with magnetic resonance (MR) spectroscopy (10).

Primate models of EAE

Of the plethora of transgenic, viral and autoimmune models of MS in laboratory strains of mice and rats, only a few have been explored in non-human primates. Initial attempts to evoke an MS-like syndrome in chimpanzees by inoculation of MS brain material have yielded intriguing results on the viral origin of MS (11–14). However, these experiments have received little follow-up in later years, probably for reasons of high costs and ethical constraints.

Currently, the most investigated non-human primate models of MS are the autoimmune EAE models in rhesus monkeys and common marmosets. An important aspect of both models is that the patterns of neurological deficits are less stereotypical than in rodents and resemble more the clinical and neuropathological heterogeneity seen in MS patients. However, the two models differ fundamentally in their clinical and pathomorphological presentation and possibly represent different forms of MS.

Macaques

The Old World monkey genus *Macaca* comprises medium-sized monkeys having their natural habitat in South-East Asia, with the exception of isolated populations of Barbary macaques in North-West Africa and Gibraltar. The relatively large adult male monkeys can weigh more than 10 kg, and their aggressive nature is a disadvantage of this model as the monkeys have to be sedated for almost every handling.

Macaques have an estimated evolutionary distance to humans of 35 million years. Two species in particular are

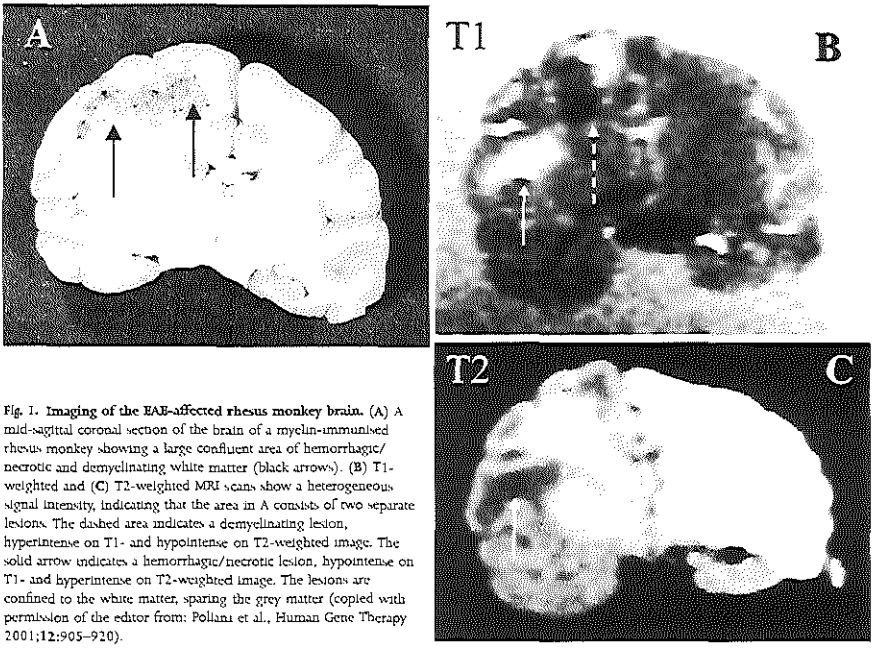

Fig. 1. Imaging of the EAE-affected rhesus monkey brain. (A) A mid-sagittal coronal section of the brain of a myelin-immunised rhesus monkey showing a large confluent area of hemorrhagic/necrotic and demyelinating white matter (black arrows). (B) T1-weighted and (C) T2-weighted MRI scans show a heterogeneous signal intensity, indicating that the area in A consists of two separate lesions. The dashed area indicates a demyelinating lesion, hyperintense on T1- and hypointense on T2-weighted image. The solid arrow indicates a hemorrhagic/necrotic lesion, hypointense on T1- and hyperintense on T2-weighted image. The lesions are confined to the white matter, sparing the grey matter (copied with permission of the editor from: Pollara et al., Human Gene Therapy 2001;12:905–920).

regularly involved in biomedical research, namely the rhesus (*M. mulatta*) and the cynomolgus monkey (*M. fascicularis*). The presence of similar major histocompatibility complex (MHC)-DR and MHC-DQ allelic lineages in humans and macaques has been described and is of great interest because products of both loci have been identified as major regulatory elements of susceptibility to MS in the human population (15, 16).

Some MHC-DR3 lineage members in humans and macaques select the same epitopes from antigens like mycobacterial purified protein derivative (PPD), bovine myelin basic protein (MBP) or human myelin/oligodendrocyte glycoprotein (MOG) and can even present them to T-cell clones of the other species (17–19). This observation not only demonstrates the evolutionary conservation of allelic lineages and their peptide-binding capacities, but also illustrates the high degree of similarity of the human and macaque MHC and T-cell receptor (TCR) gene repertoires. A comparative analysis of TCR-Vβ family members in humans, chimpanzees and macaques has indeed revealed that their genomic and expressed repertoires do not diverge substantially (1). Another important feature of the macaque as a model of MS is that these species are susceptible to infection with several viruses similar to the ones that have been implicated as possible causes of MS in humans, such as type A and B herpesviruses (20).

In conclusion, an MHC-typed and pedigreed macaque colony, such as the one present at BPRC, provides an unique possibility to investigate the activation of immunopathogenic pathways leading to autoimmune encephalomyelitis in a controlled setting and a similar genetic and virological background as in MS patients.

Macaque models of EAE

The first documented EAE case was in monkeys which developed neurological deficit associated with CNS inflammation after injection with healthy CNS tissue (21, 22). Based on this finding, reproducible EAE models have been established in several macaque species for the study of genetic,

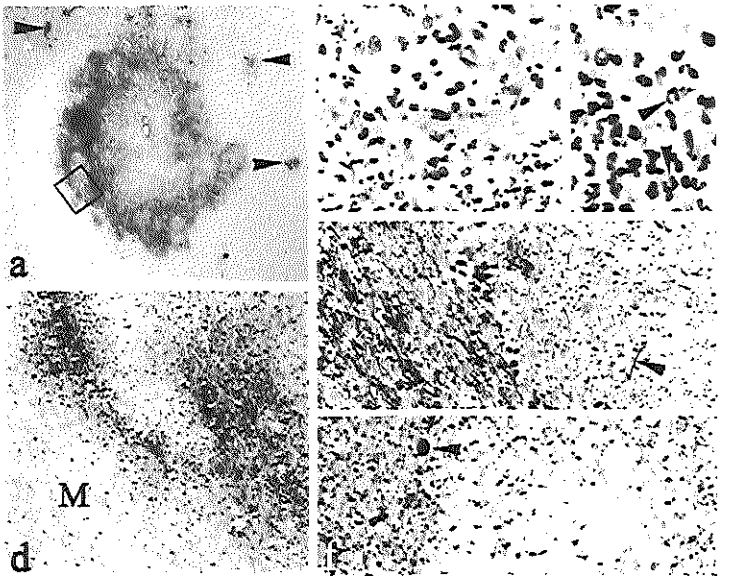

Fig. 2. Characteristic histological presentation of EAE in the rhesus monkey brain. (a) Hematoxylin/eosin staining of the cerebellar white matter of a rhesus monkey affected by rhMOG-induced EAE shows a large hemorrhagic/necrotic lesion. Arrows point to small perivascular infiltrates of leucocytes. The infiltrates consist mainly of granulocytes with few mononuclear cells (**b** and **c**). Picture **d** shows the margin of the lesion (rectangle in picture A) at higher magnification (luxol fast blue and periodic acid-Schiff staining for myelin). The capital M is placed in an area of oedematous myelin. In the centre of the lesion depicted in (**d**) axonal structures (Bielschowski staining in (**e**)) and astrocytes (GFAP staining in (**f**)) have completely disappeared. The arrowhead in (e) indicates an axon with spheroids and that in (**f**), a remaining astrocyte.

immunopathological and radiological features of MS (including magenetic resonance imaging (MRI)) (23, 24).

Of three *Macaca* species tested, *M. mulatta* appeared more susceptible than *M. fascicularis* to EAE induced with whole brain homogenate or myelin basic protein, while *M. nemestrina* was found to be relatively resistant. MBP-induced EAE in *M. mulatta* and *M. fascicularis* responds differently to treatment with combinations of autologous MBP, antibiotics, steroids and copolymer 1, pointing to possibly different immunopathogenic mechanisms (25). Moreover, some evidence was obtained that these outbred species respond to different encephalitogenic fragments of MBP (26).

EAE in myelin-immunised rhesus monkeys usually follows an acute course, although in some unpredictable cases an MS-like chronic disease pattern can be found. The characteristic histological aspect of the model is the presence of large lesions with infiltration of abundant neutrophils, and serious destruction of white matter, affecting both myelin and axons (24, 27). Serial MR images recorded at 24-h time intervals show that in most animals, within 4 days after onset large lesions can develop that at neuropathological examination appear haemorrhagic/necrotic (24). Representative examples of lesions found observed in histology and MRI is depicted in *Figs 1 and 2*. The severe inflammatory aspect suggests that the lesions in this model are formed by an acute pathological event causing severe inflammatory necrosis rather than selective demyelination. EAE in the rhesus monkey therefore most resembles the acute fulminant forms of MS, such as post-infectious leuco-encephalomyelitis, rather than the more common chronic forms of MS.

Table 1. Clinical and pathological heterogeneity of autoimmune encephalomyelitis in rhesus monkeys immunised with pMOG34–56/CFA

Monkey	-B	-A	-DR	-DP	Sex	Birth	EAE-type	Cuff	Demyel.	Death (day a.i.)
Immunisation pMOG34–56/CFA										
9257	19/6	11/26	5/2	4/6	F	'92	hyperacute	y[2]	y[1]	17
RI176	nd	nd	nd	nd	F	'96	hyperacute	nd	nd	14
96054	nd	nd	nd	nd	F	'96	hyperacute	nd	nd	15
95003	23/6	24/13	–/2	6/10	M	'95	mild	y	n	28
Immunisation pMOG34–56/CFA+1 homologous challenge with pMOG34–56/IFA										
V3F	6/10	24/25	1/3	02/08	F	'94	acute	y	y[1]	35
L125	6	11	1/3	8/10	M	'92	acute	y[2]	y	35
94012	23/22	14/14	5/2	6/10	F	'94	optic neuritis	y	y	35
Immunisation pMOG34–56/CFA+1 heterologous challenge with pMOG4–26/IFA										
94002	22/18	24/26	2/1	01/04	M	'94	mild	y	n	138
94052	10/1	2/14	1/	03/11	M	'94	mild	y	y	138
Immunisation pMOG34–56/CFA+2 or more homologous challenges with pMOG34–56/IFA										
2AC	6/8	11/18	3/4	01/10	M	'78	chronic	y	y[1]	112
95063	9/19	13/26	1/3	01/	M	'95	chronic	nd	nd	112
9256	1/1	11/14	8/1	3/11	M	'92	optic neuritis	n	n	73

[1]Demyelination only perivascular; [2]infiltrates contain predominantly neutrophils; a.i., after immunisation.

Acute EAE in rhesus monkeys

On basis of the atypical pathomorphological presentation, the question can be asked whether acute EAE in rhesus monkeys represents an autoimmune disease. The MHC class II association of EAE susceptibility (28), the protective effect of T-cell-depleting therapy (29–32) and the possibility of inducing EAE by (autologous) transfer of an MBP-specific type 1 helper T (Th1) cell line (13) all point to a central role of autoreactive T cells in the ethiopathogenesis of the disease. Several MBP T-cell epitopes have been identified thus far: namely, MBP 29–84 (28), MBP 61–82 and MBP 80–105 (26) and MBP 170–186 (33).

The course of EAE in rhesus monkeys is clearly influenced by genetic factors depending on the mode of disease induction. The presence of the Mamu-DPB1*01 allele was found associated with increased susceptibility to EAE induced with bovine MBP in CFA (28). However, no effect was found in EAE induced with recombinant human MOG (amino acids 1–125; rhMOG) in CFA (19). Compared to HLA-DR and –DQ, little is known about the involvement of HLA-DP molecules in MS, but a role in epitope spreading, a phenomenon that is thought to determine the disease course after clinical onset, has been proposed (34). No data are available on the influence of non-MHC genes on the EAE course in rhesus monkeys.

A remarkable aspect of the rhesus monkey EAE model is that the clinical and pathological presentation is to some extent independent of the antigen preparation used for disease induction. Whereas to our knowledge in all other species immunisation with myelin, MBP or MOG evokes clearly different disease patterns as in rodent and common marmoset models, this seems not to be the case in rhesus monkeys. In all three cases a similar acute EAE with haemorrhagic/necrotic lesions are formed. This intriguing observation prompted us to investigate the immunopathogenesis of EAE in this species in more detail, focussing on the role of MOG, which is quantitatively one of the minor myelin antigens (35) but likely takes a central position in the EAE pathogenesis.

MOG-induced EAE in rhesus monkeys

By the direct exposure on the myelin surface, MOG has now emerged as a potential primary target of the autoimmune reaction in MS patients. This assumption is strengthened by the increased cellular and humoral anti-MOG reactivity of MS patients compared to healthy controls or patients with unrelated neurological diseases (36–38).

The T-cell reactivity of monkeys immunised with rhMOG is at least directed to three main epitopes encompassed within amino acids 4–20, 35–50 and 94–116 (19), of which the first two are also immunodominant epitopes in MS patients (36). The former paper shows also that the main antibody reactivity in the monkey sera occurs with two regions of the MOG molecule, namely amino acids 4–46 and 44–76. As MOG peptide 34–56 is clearly encephalitogenic in several strains of rats and mice, we chose to test the encephalitogenic potential of this particular peptide in rhesus monkeys. To our surprise, all monkeys eventually developed EAE, although a clearly heterogeneous clinical pattern was found (*Table 1*).

Four out of 12 monkeys developed clinical signs after a single immunisation with pMOG34–56/CFA; severe clinical signs were hyperacute in three monkeys and remained mild

in one monkey. Eight monkeys were still asymptomatic at 28 days after immunisation; six were given a challenge immunisation with pMOG34–56 in incomplete Freund's adjuvant (IFA) and two a challenge with an irrelevant peptide (pMOG4–26) in IFA. Three monkeys developed acute clinical signs within 7 days after the homologous challenge, whereas the two monkeys challenged with the heterologous peptide remained asymptomatic. The three remaining monkeys developed clinical EAE only after one or two additional challenges with the homologous peptide.

Table 1 shows that the clinical heterogeneity is reflected by a variable degree of CNS inflammation and demyelination. The immunological basis of the hyperacute and acute cases is still poorly understood. Treatment with the antiviral drug gancyclovir suppresses the expression of clinical signs in these cases, pointing to the possibility that the disease is initiated by an antiviral immune reaction superimposed on an autoimmune reaction to MOG (unpublished results). The pathological aspect of the lesions in the monkeys developing late stage EAE resembles the chronic EAE type in common marmosets.

It is of potential interest that whereas no influence of *Mamu-DPB1*01* on MOG-induced EAE was found, both monkeys developing chronic EAE are positive for this allele. Initial experiments show that in monkeys with chronic EAE, but not in those with acute EAE, a substantial diversification of the T- and B-cell reactivity takes place. Hence, the possibility exists that Mamu-DPB*01 molecules serve as a permissive risk factor determining the outcome of EAE by the presentation of spreading epitopes. A similar mechanism has been postulated for patients with chronic MS (39).

The common marmoset as disease model

Common marmosets (*Callithrix jacchus*) are small neotropical primates weighing between 300 and 500 g when adult. Marmosets breed easily in captivity, giving birth to one or two non-identical sets of twin or triplet siblings per year. As fraternal siblings have shared the placental blood circulation *in utero*, bone-marrow-derived elements developing in a twin or triplet are equally distributed over the siblings. This natural chimerism induces in each monkey permanent tolerance towards its fraternal sibling's allo-antigens. Hence, it is possible to test the pathogenic role of autoreactive T cells by adoptive transfer experiments between such siblings (40).

The similarity of the human and common marmoset immune systems has been well-documented. A high degree of similarity of human and common marmoset *TCRBV-D-J-C* gene sequences has been observed (41). *MHC-Caja* class II region genes were found to encode the evolutionary equivalents of HLA-DR and -DQ molecules (42). Thus far the common marmoset is the only higher primate species in which the existence of *HLA-DPB1*-like sequences could not be demonstrated. All common marmosets from four different centres were found to share the monomorphic *Caja-DRB*W1201* allele. The other *Caja-DRB* sequences cluster into two polymorphic lineages, namely *Caja-DRB1*03*, comprising at least seven alleles, and *-DRB*W16*, comprising at least 13 alleles (2).

The marmoset EAE model

In its clinical presentation and the radiological and pathological aspects of the lesions, EAE in the common marmoset is an excellent model of chronic MS (43, 44). The characteristic lesion type resembles closely the pattern II of active MS lesions, which is the most prominent type in chronic MS (3, 45, 46). The common marmoset is highly susceptible to EAE. Upon a single immunisation with human myelin or recombinant human MOG in complete adjuvant, each monkey tested thus far has developed EAE, although the individual disease course varies. We have collected evidence that this high susceptibility can be attributed to a uniform T-cell response of all marmosets to MOG in the initiation phase of EAE. Although MOG is quantitatively only a minor constituent of CNS myelin (<0.1% of the myelin proteins) a dominant T- and B-cell reactivity towards this antigen was found in myelin-immunised marmosets (unpublished results). Data published by McFarland et al. demonstrate that the presence of an autoimmune reaction to MOG is a key factor in the EAE pathogenesis in common marmosets (47). After having established that all common marmosets share the *Caja-DRB*W1201* allele (42), we were able to demonstrate that the monomorphic Caja-DRB*W1201 molecule is a major restriction element for the activation of $CD4^+$ cells specific for the encephalitogenic peptide pMOG14–36 (48).

As far as the major myelin antigens are concerned, MBP turned out to be a surprisingly poor antigen and weak encephalitogen in common marmosets (48). This observation contrasts with the findings of Massacesi et al., who have reported that clinical EAE can be induced when immunisation with human MBP in complete adjuvant is combined with intravenous administration of *Bordetella pertussis* particles, which is used for permeabilization of the BBB (40). However, we prefer not to use such strong adjuvants as these not only mask the variable disease course between individual monkeys, but also aggravate pathomorphological aspects of the lesions. More specifically, administration of *Bordetella pertussis* to monkeys immunised with human myelin in CFA causes a

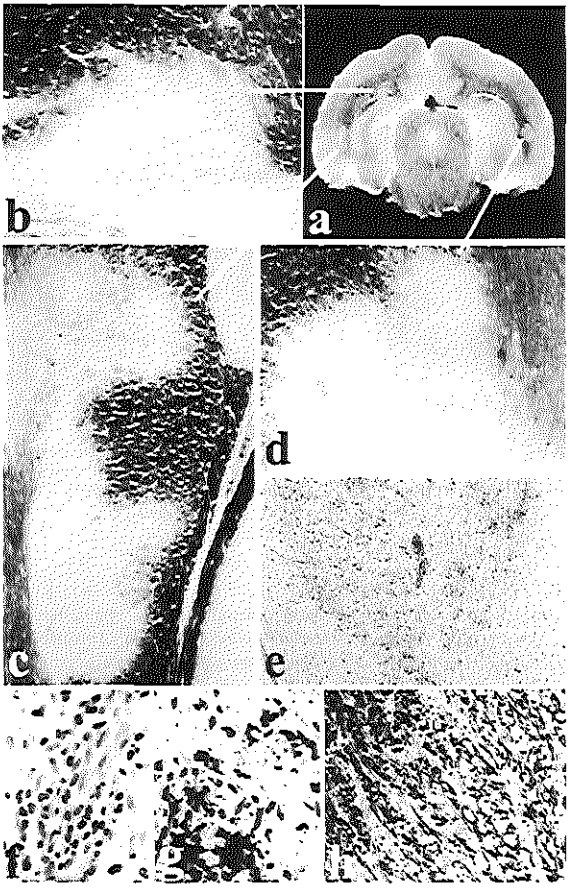

Fig. 3. MRI and histological features of lesions in marmosets immunised with human myelin/CFA. a) A T2-weighted brain MR image showing three confluent lesions. The myelin staining in **b**, **c** and **d** shows that all three lesions are demyelinated. The fine characterisation in ref. 46 show that **b** is an inactive lesion with substantial remyelination, that **c** is a confluence of an active demyelination upper part and an inactive lower part. Lesion **d** is an active demyelinating lesion, with reduced density of axons due to oedema. (e) Spheroids are absent, indicating sparing of axons (h). The perivascular infiltrates in lesion **d** consist mainly of lymphocytes and macrophages, but neutrophils are absent (f, hematoxylin/eosin staining). Many $CD3^+$ T cells are present (g).

synchronised onset of clinical signs with severe inflammatory/necrotic CNS white matter lesions (46).

The heterogeneous lesion pathology in marmosets

Serial MRI is a powerful technique to examine the brain white matter lesion development in common marmosets (49). Our own studies have revealed that at a given time point, lesions of various differentiation stages are present within the CNS (46). As an illustration, one slice of an MR-brain image with three different lesions, which by neuropathological characterisation turned out to represent different stages, is shown in Fig. 3. The continuous formation of new lesions illustrates the chronic nature of myelin-induced EAE in this species.

The obvious advantage of a disease model in non-human primates is that the same reagents can be used for the immunohistological analysis of tissues from MS patients and EAE-affected monkeys. On closer examination, lesions with active inflammation and demyelination also appear immuno-

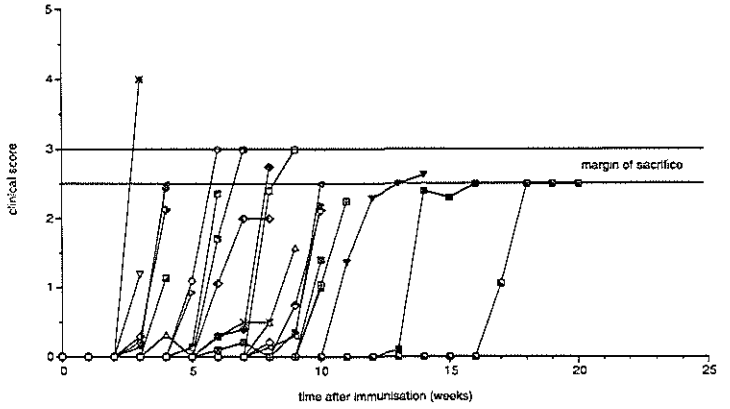

Fig. 4. Clinical heterogeneity in rhMOG-immunised marmosets. A total of 26 marmosets of either sex and between 2 and 5 years of age were immunised with 100 μg rhMOG in CFA. The clinical scores represent: 0.5=apathy, loss of appetite; 1.0=lethargy, anorexia, flaccid tail; 2.0=ataxia, sensory loss, blindness; 2.5=incomplete paralysis one- (hemi-) or two-sided (paraparesis); 3.0=complete paralysis hind part of the body (hemi-/paraplegia); 4.0=complete paralysis whole body= quadriplegia.

logically active. This is illustrated by the presence in active lesions of activated ($CD40^+$) APC in close conjunction with activated ($CD154^+$) T cells (50). At the same locations, high numbers of cells staining positively with antibodies directed to cytokines and matrix metalloproteases were found. Administration of antibodies which block CD40–CD154 interaction (51) or neutralise interleukin (IL)-12 abrogate clinical disease and lesion formation, illustrating that the local immunological interactions mediate the lesion formation (H. P. M. Brok, M. van Meurs, E. Blezer, A. Schantz, D. Peritt, G. Treacy, J. D. Laman, J. Bauer, B. A. 't Hart, submitted for publication).

Neuropathological evidence of axonal damage, intense staining of axons with antibody directed to β-amyloid precursor protein (β-APP), has been found in mice (52), rats (53), common marmosets (54) and MS patients (53). In the marmosets as well as in MS patients the highest density of axons with accumulated β-APP was found in lesions with active inflammatory activity, pointing at a possible causal relation between disturbance of axonal transport and inflammation. A key to the underlying mechanism of the axonal pathology may be the association between inhibition of axonal transport and cytosolic adenosine 5'-triphosphate (ATP) depletion as observed in patients with mitochondrial impairment (55). It is of interest in this context that several pathophysiological conditions considered to be involved in formation of MS lesions can also cause reduced cellular ATP levels, such as metabolic stress (56), oxidative stress (57) and ischaemia (58).

Immunopathogenesis of myelin-induced EAE in marmosets

The data obtained thus far show that the EAE pathogenesis in marmosets bears a much closer resemblance to the situation in the majority of MS patients and rodent EAE models than the rhesus monkey model does. It is becoming clear that the formation of lesions in autoimmune models of inflammatory demyelination in mice (59), rats (60) and marmosets (61) is caused by a similar synergistic action of a T-cell-mediated pathway, leading to inflammation, and an antibody-mediated pathway, leading to demyelination of the CNS white matter. As an example, by active immunization of common marmosets with MBP/CFA or passive transfer of MBP-specific T cells, only CNS inflammation is induced. CNS demyelination is dependent on the co-transfer of anti-MOG antibodies (61). The presence in MS and EAE-affected marmosets of similar depositions of MOG-specific autoantibodies in close interaction with myelin sheaths in white matter areas of myelin dis-

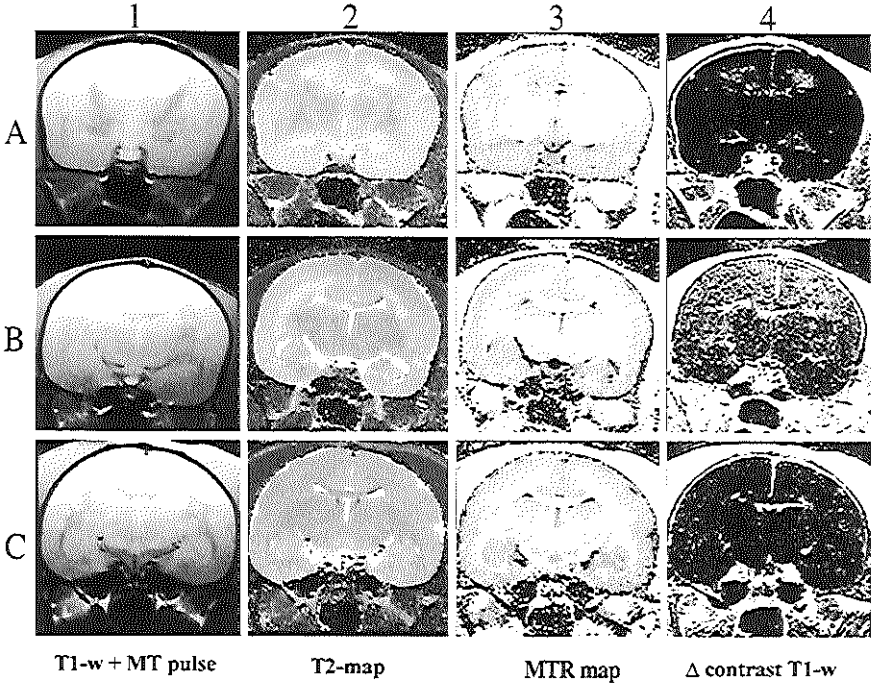

Fig. 5. Neuropathological heterogeneity in rhMOG-immunised marmosets. At an interval of 10–14 days after immunization with 100 μg rhMOG/CFA, MR images were recorded on a 4.7 T horizontal bore Varian NMR spectrometer (Varian, Palo Alto, CA, USA), equipped with a high-performance gradient insert (11 cm inner diameter, maximum gradient strength 220 mT/m). The figure shows representative examples of images collected during clinical EAE (score 2 or 3). Column 1: T1-weighted images with MT pulse; column 2: T2 maps; column 3: magnetisation transfer ratio maps; column 4: subtraction of T1-weighted images recorded after and before contrast enhancement by intravenous gadolinium–DTPA.

integration and lesion formation indicates that MOG may have an equally important role as a target of the autoimmune process in both disorders (62, 63).

Clinical and pathological heterogeneity of MOG-induced EAE in common marmosets

Immunization with rhMOG induces a heterogeneous clinical and pathological disease pattern in monkeys from the outbred colony at BPRC. Although the clinical course is mainly progressive, the time of onset of clinical signs differs considerably (Fig. 4). The neuropathological heterogeneity is illustrated in Fig. 5, displaying three different lesion patterns in brain MR images recorded at the height of the disease. The most commonly observed pattern on T2-weighted images, which are routinely recorded to assess the total number of lesions and their spatial distribution, is depicted in Fig. 5C. This pattern is characterised by the presence of many focal lesions (C1), which are found scattered through the brain white matter, and which display some contrast enhancement due to a leaky BBB (C4). The reduced magnetization transfer ratio (MTR) values of the lesions (C3) point to oedema or loss of tissue mass due to demyelination. The reason that such

lesions are hardly visible on T2 maps (C2) is that the T2-weighted signal intensities between lesions and surrounding normal-appearing white matter differ only marginally (unpublished results). Pattern A, with large inflammatory lesions (note the focal T2 enhancements in A2 and the intense contrast enhancements in A4), is seen much less often. Pattern B was only recently observed for the first time when we applied newly developed quantitative MR strategies, which plot the real nuclear magnetic resonance (NMR) signal intensities per pixel. This unusual pathological pattern possibly represents ubiquitous vesicularization of white matter due to deposition of pathogenic anti-MOG antibodies (62).

Histological examination of MOG-immunised monkeys shows large areas of inflammation and demyelination in the white matter and cortex of the CNS. The myelin sheaths of the peripheral nervous system, however, are not affected (48).

Immunopathogenesis of MOG-induced EAE in marmosets

As was discussed above, the most likely EAE-initiating event in marmosets is the Caja-DRB*W1201-restricted activation of $CD4^+$ T cells specific for the encephalitogenic peptide pMOG14–36. However, cells and sera from monkeys with chronic EAE display a broad reactivity with a panel of 22-mer peptides covering the N-terminal rhMOG domain (48), indicating that intramolecular diversification of the autoimmune reactivity (determinant spreading) has taken place. Determinant spreading is thought to be a major contributor to the chronicity of MS and EAE (64). As already discussed above, the exact mechanism is unknown, but may involve the activation of T and B cells specific for spreading/cryptic epitopes. As cryptic epitopes have escaped tolerance induction due to inadequate processing and presentation during normal development, they are highly immunogenic (65, 66).

The question of where epitope spreading in EAE takes place has been addressed in the marmoset EAE model. Recently submitted data from the lab of co-author J.D.L. indicate that the cervical lymph nodes (CLN) that drain the brain may play a central role in epitope spreading in primates. In the T- and B-cell areas of CLN of MOG-immunised marmosets and rhesus monkeys, activated APC are present containing myelin proteins (MBP and proteolipid protein (PLP)) and expressing co-stimulation molecules on their surface. The fact that such cells were absent in the CLN of control monkeys and monkeys with an unrelated autoimmune disease (collagen-induced arthritis) and that inhibition of demyelination with an antagonist anti-CD40 antibody also abrogates epitope spreading (51), points to a possible causal relation between demyelination and epitope spreading. The simplest explanation of these findings is that after phagocytosing myelin within the lesions, activated macrophages migrate to the CLN where they trigger an expanded repertoire of myelin-specific T and B cells. Experiments in rats show that myelin released from a cryolesion in the CNS leads to T-cell priming within the CLN. The primed T cells were found to home preferentially to the brain of an EAE-affected animal from the same strain, and proved to enhance ongoing EAE (67, 68).

It is unknown whether a similar mechanism exists in MS. However, because of the close immunological and genetic similarity with humans, the outbred character of the species and the possibility of transferring cells between fraternal siblings, common marmosets provide an experimental model 'par excellence' to test whether the concepts hold in primates.

Concluding remarks

Because of their genetic and immunological proximity to humans, non-human primates provide useful models of MS. The two most investigated models are EAE in rhesus monkeys and common marmosets. An as-yet unresolved issue is that rhesus monkeys immunised with myelin or myelin antigens develop a disease resembling acute forms of MS, whereas marmosets immunised following the identical protocols develop a disease resembling chronic MS. We believe that a detailed examination of the pathogenic mechanisms in these models will shed light on the mechanisms that cause the clinical and pathogenic heterogeneity in MS patients. A particularly attractive aspect of non-human primates as disease models is that pathogenic processes can be investigated against a similar genetic and virological background to that of a human patient.

The non-human primate EAE models are of particular importance for the safety and effectivity testing of new biotechnology-derived therapeutics for MS which, because of their species-specificity, do not work sufficiently well in species more distinct from humans. Examples are therapeutical monoclonal antibodies (69, 70) and vaccines (71). Questions concerning the immunogenicity of biological therapeutics have also been addressed in non-human primates (71, 72). Many therapeutic strategies, developed with the aim of treating MS, have been investigated in rodents, such as anti-TNF-α antibodies (73, 74), altered peptide ligands (75) and anti-CD4 antibodies (76). Although these experimental therapies were very effective in controlling EAE in rodents, they are only partially effective in MS patients and in some cases even detrimental (77–80). The question therefore arises whether

the rodent models are 'close enough to MS' to be sufficiently predictive for the success of a new therapeutic strategy. The observation that MOG-based tolerization completely protects mice against EAE, but induces fatal demyelinating disease in marmosets illustrates how useful a preclinical animal model closer to humans can be (81).

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The Major Histocompatibility Complex Influences the Ethiopathogenesis of MS-Like Disease in Primates at Multiple Levels

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ABSTRACT: Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease primarily affecting the central nervous system. Of the many candidate polymorphic major histocompatibility complex (MHC) and non-MHC genes contributing to disease susceptibility, including those encoding effector (cytokines and chemokines) or receptor molecules within the immune system (MHC, TCR, Ig or FcR), human leukocyte antigen (HLA) class II genes have the most significant influence. In this article we put forward the hypothesis that the influence of HLA genes on the risk to develop MS is actually the sum of multiple antigen presenting cell (APC) and T-cell interactions involving HLA class I and class II molecules. This article will also discuss that, because of the genetic and immunologic similarity to humans, autoimmune models of MS in non-human primates are the experimental models "par excellence" to test this hypothesis. *Human Immunology 62, 1371–1381 (2001).* © American Society for Histocompatibility and Immunogenetics, 2001. Published by Elsevier Science Inc.

KEYWORDS: experimental autoimmune encephalomyelitis; multiple sclerosis; primates; immunology

ABBREVIATIONS

APC	antigen presenting cell
BBB	blood–brain barrier
CLN	cervical lymph node
CNS	central nervous system
EAE	experimental autoimmune encephalomyelitis
HLA	human leukocyte antigen
Ig	immunoglobulin
MBP	myelin basic protein
MHC	major histocompatibility complex
MOG	myelin/oligodendrocyte glycoprotein
MS	multiple sclerosis
PLP	proteolipid protein
TCR	T-cell receptor
TMEV	Theiler's murine encephalomyelitis virus
SFV	Semliki Forest Virus

INTRODUCTION

Multiple sclerosis (MS) is generally regarded as an autoimmune disease that develops in genetically susceptible individuals. MS is typically characterized by lesions in the central nervous system (CNS), which are formed by chronic inflammatory demyelination, leading to progressive loss of neurologic functions. MS affects about 1 per 1000 individuals in the moderate climate areas of Europe, the USA, and Southern Australia [1].

The cause of MS and the genetic and immunologic mechanisms that control disease progression are poorly understood. The substantial heterogeneity in clinical course and CNS pathology between MS patients suggests a multifactorial disease cause. More specifically, four main lesion patterns were found in a large number CNS samples from MS patients collected in three different

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centers from Austria, Germany, and the USA [2]. Two patterns reveal close similarity to the T-cell mediated (type I) or T-cell and antibody-induced (type II) encephalomyelitis observed in experimental animal systems. In these types a central role of (auto)immune reactions in lesion formation is likely. In patterns III and IV oligodendrocyte loss by viral infection or exposure to toxic agents seems a more likely primary cause of lesion formation. The fact that all lesions within one patient fall within one category suggests that the four lesion types may represent different forms of MS.

To date, it is not known whether MS patients have inherent neurologic abnormalities or immunologic deficiencies that can explain their susceptibility to the disease. It has been hypothesized that an autoimmune disease, such as MS, may rather be caused by "a high responsiveness to the excess release of antigens from damaged tissue by an antecedent pathological event" [3]. Two obvious core questions in this "primary lesion hypothesis" are as follows: (1) which genes make MS patients high responders; and (2) towards which antigen(s) is the high responsiveness directed?

This article will discuss that the chance to develop MS may be the sum of several risk factors operating at multiple levels of antigen presenting cell (APC)-T interaction involving myelin as well as non-myelin antigens. While reviewing literature data supporting this hypothesis, this article will also discuss the possibilities offered by the presently existing MS-like models in non-human primates in order to investigate whether this is indeed the case.

CLINICAL HETEROGENEITY IN MS

In about 80% of MS patients the disease has a chronic relapsing-remitting course. With time, and after a variable number of relapses, a secondary progressive phase starts in most cases in which full remission no longer occurs and progressive neurologic deficit develops. MS in another 15% of patients is progressive from the onset, with and without episodes of relapses, on a background of chronic progressive neurologic deficit (so-called primary progressive MS). Acute forms of MS, with a rapidly progressing course and severe inflammatory pathology, are relatively rare.

The etiology of relapsing remitting/secondary progressive MS is highly complex and likely involves a combined activity of genetic, endocrine, environmental, and immunobiologic factors. Evidence for a genetic contribution to the disease susceptibility comes from the substantially higher MS concordance between identical (25%) than non-identical (3%) twins [1]. Among the candidate genes, the influence exerted by HLA class II genes seems the most significant; the strongest genetic association in people of Northern European descent being with the HLA-DR2/Dw2, DQ6 (DRB1*1501, DQA1*0102, DQB1*0602) haplotype [1, 4–6]. Polymorphisms in non-MHC genes, such as those encoding cytokines, cytokine receptors, Fc receptors, and chemokines, seem to have a less important contribution to disease susceptibility but have a considerable influence the clinical manifestations of MS [4, 7–10]. The facts that females are approximately 1.5 times more susceptible to MS than men and that pregnancy reduces the relapse rate indicates a hormonal influence on the disease. Migration studies and reports of epidemics of MS indicate a role for environmental influences, viral or bacteriologic infection in particular [1]. The dependence on external factors may explain why the disease concordance is lacking in a significant proportion of identical twins. In addition to the epidemiologic and demographic reports, clinical and experimental studies point to infection with microbial pathogens as a possible trigger for the disease [1, 11]. Although the etiologic agent in MS is unknown, it is intriguing that all the demyelinating diseases in which the etiologic agent is known are caused by a virus.

The general premise that MS is an autoimmune disease is mainly based on the findings that antibodies, T cells, and macrophages are abundant in MS lesions [12, 13] and that experimentally induced antimyelin reactivity in certain experimental animal models gives rise to similar neurologic deficits as in MS (see below). However, the presence of these factors in the MS brain and spinal cord does not necessarily imply that autoreactivity is the primary cause of the disease. Autoreactive T cells and antibodies may also be formed as a reaction to the (massive) myelin release by viral infections of the CNS or oligodendrocyte death.

Experimental Autoimmune Encephalomyelitis

Most current concepts of the immunologic mechanisms that regulate the initiation and progression of MS have been based on rodent and primate models of experimental autoimmune encephalomyelitis (EAE) [14, 15]. Studies in primates report a similar clinical and pathologic heterogeneity in EAE models as in MS, which depends on the immunization protocol, the myelin preparation used, and the animal species in which the disease is induced [2, 16].

In classical EAE models in inbred rodent strains neurologic disease is initiated by the CNS immigration of myelin-reactive CD4+ T cells with a pro-inflammatory helper 1 (Th1) phenotype across the blood–brain barrier (BBB). The interaction of these T cells with resident APC, which locally express the myelin antigen(s) to which the infiltrating T cells were originally sensitized to in the periphery, induces a sequence of events that

leads to inflammation and damage to myelin and axonal structures. In some models the disease is acute, whereas in others a chronic course is observed. Further, some animal models display an MS-like relapsing remitting disease in which, after several disease episodes, chronic unremitting neurologic deficit is observed.

Many therapeutic strategies, with the aim of treating MS have been investigated in animals, such as anti-TNF-α antibodies [17, 18], altered peptide ligands [19], and anti-CD4 antibodies [20]. Although these were found very effective in controlling EAE in rodents, they are only partially effective in MS patients and in some cases even detrimental [21–24]. Therefore, the question is raised whether the aspects of MS that are actually represented by the autoimmune models of MS are "close enough to MS" to be useful for developing therapeutic strategies.

MULTISTEP IMMUNOPATHOGENESIS OF CHRONIC MS

For this article the complex cascade of events, which leads to the lesion formation and neurologic dysfunction in chronic MS, has been separated into different phases. Phase 1 represents the initiation of autoreactivity towards myelin antigens; phase 2 demonstrates the CNS infiltration of autoreactive cells and molecules; phase 3 describes the induction of inflammatory demyelination; and phase 4 characterizes the expansion of the autoreactivity towards myelin as well as non-myelin autoantigens. These phases are not necessarily separated in time, but can coexist at the same time in the CNS of a given MS patient, for example in lesions of different age. This study postulates that in each phase one or more APC–T-cell interactions can occur, which adds a risk factor enhancing the chance to develop clinical MS.

Phase 1: The Initiation of Antimyelin Reactivity

The autoimmune reactions in MS are initiated by the activation of a pre-existing repertoire of T and B cells specific for components of the CNS white matter. It has been well established that myelin specific and potentially encephalitogenic T cells are part of the normal immunologic repertoire of rodents [25] and non-human primate species, such as the common marmoset [26] and the rhesus macaque [27]. Myelin-reactive T cells are also present in the immune system of healthy humans at frequencies that are comparable with those found in MS patients [28]. However, the potential of human T cells to incite encephalomyelitis cannot be tested in transfer experiments, as suitable recipients are lacking for obvious ethical reasons.

The potentially autoreactive T cells are normally kept in a resting state but become activated in MS by environmental triggers, such as viral infection or stress for example. The activation from the resting state requires interaction with "professional" APC, which should not only present the relevant antigen in the context of self MHC-DR molecules, but also provide essential costimulatory signals without which naïve T cells are not activated but anergized instead [29]. Several resident cells in the CNS have the capacity to present antigens, such as microglia cells, astrocytes, and endothelial cells. Resident CNS microglia cells in MS have an activated appearance, expressing the three major MHC class II molecules (HLA-DR, -DQ, and -DP [30]) and several costimulatory molecules [31, 32]. Likewise, in early stages of acute EAE in animals, the resident APC have acquired an activated appearance [32, 33].

A variety of viruses have been associated with MS, including measles virus, rubella virus, influenza virus, respiratory corona virus, and a variety of herpes viruses, such as herpes simplex virus 1, cytomegalovirus, human herpesvirus type 6, Epstein-Barr virus, and Marek's disease virus. However, none of these have been identified as exclusive trigger of MS. Viruses may induce myelin damage through a number of mechanisms either directly or indirectly [34, 35]. An example of a neuroinvasive virus that directly infects and damages oligodendrocytes and myelin is JC virus [36]. Other viruses may incorporate host cell antigens, such as myelin proteins, into the viral envelope thereby allowing the peripheral immune recognition of CNS restricted antigens [37]. A third possible mechanism is that the autoreactive T cells respond to mimicry motifs of myelin antigens in viral proteins [38]. As a general reaction to tissue damage, including cytolytic viral infection, myelin is phagocytosed and processed either locally [38] or carried to the cervical lymph nodes (CLN), which drain the cerebrospinal and interstitial fluids of the brain, where myelin-reactive T cells and B cells are activated *de novo* [39–41].

Many viral infections of animals have been used as models of MS but it is often difficult to dissect the exact mechanisms that lead to myelin damage. The most useful mouse models to investigate the activation of myelin-reactive T cells during a virus infection are Theiler's encephalomyelitis virus (TMEV) and Semliki Forest virus (SFV) [42, 43]. In both models demyelination is immune mediated, although myelin-reactive T cells play a major but not exclusive role in the pathogenesis of disease. The pathogenic mechanisms in these models include the persistence of virus within the CNS and the induction of myelin-reactive T cells and antibodies via molecular mimicry. The specificities and functions (pro- or anti-inflammatory) of the repertoire of activated CD4+ and CD8+ T cells is determined by the interaction of multiple genes. These include genes encoding the MHC class I and II molecules that select and present

viral antigens and genes encoding cytokines and cytokine receptors.

Phase 2: The CNS Infiltration of Cells and Molecules

Primed T cells that have encountered their antigen in peripheral lymph nodes are thought to be less dependent on costimulatory molecules than naïve cells and, therefore, can be activated by antigen without the need of costimulation [44]. It is thought that the relative independence of autoreactive T cells of costimulation rescues them from apoptotic cell death [44, 45].

During transmigration across the BBB, T cells encounter brain endothelial cells, astrocytes, and pericytes, all of which have the potential to act as APC [46, 47]. The function of the BBB is mainly affected by the endothelial cells, which are joined by tight junctions of high electrical resistance and lack of fluid-phase endocytosis. In healthy individuals this barrier limits the passage of proteins and cells from the blood. However, during inflammation many changes take place on the surfaces of activated lymphocytes, monocytes, and the endothelial cells themselves [48, 49]. These all act to allow transmigration of leukocytes through the vascular endothelium and into the CNS parenchyma. Activated T cells also express proteases and glycosidases, which augment migration and open the endothelial barrier, allowing the passage of not only additional cell types, such as macrophages, but also various effector molecules (cytokines, complement factors, and antibodies). The transmigrated cells collect in the perivascular Virchow-Rubin space as a characteristic cuff of mononuclear cells. Only T cells recognizing the epitopes to which they were sensitized in the periphery or a mimicry motif thereof, presented by local APC, migrate further into the brain parenchyma [46, 49, 50].

This phase of the disease has been extensively modeled in inbred strains of rodents where EAE is induced by adoptive transfer of myelin-specific T helper 1 cells from immunized animals into naïve syngeneic recipients. In the present model, the selection and presentation by the local APC of mimicry motifs shared by myelin and viral antigens for recognition by infiltrating CD4+ T cells is an important factor in the EAE pathogenesis. The success rate of EAE induction by adoptive transfer of MBP-specific T helper 1 cells in nonhuman primates is high in common marmosets [26], but it is much lower in rhesus monkeys [27]. One explanation may be that, due to the much higher degree of polymorphism of MHC class II genes in the latter species (see below), only in some animals does local presentation of a mimicry epitope take place.

Phase 3: Induction of Inflammatory Demyelination

Thus far, no abnormalities in the CNS of MS patients have been demonstrated prior to initiation of the first lesion. We assume, therefore, that the first infiltrating T cells encounter intact myelin sheaths and inactive local APC, which likely present low levels of myelin antigens released during normal myelin turnover. This is not an optimal environment for T-cell activation as T cells are usually inactivated under such conditions. It is not clear by which mechanisms resident APC become activated. Infiltration of cells that were activated in peripheral lymphoid organs can stimulate APC via the release of cytokines, such as IL-12, IFNγ, and TNFα [49]. Moreover, various products from cell destruction (cell debris, DNA) or viruses and bacteria (double-stranded RNA or cell wall constituents) can directly activate APC via Toll-like receptors [51].

T-cell derived, pro-inflammatory cytokines and chemokines will induce locally enhanced permeability of the BBB, thus facilitating infiltration of B cells, antibodies, and macrophages. Whereas the actual mechanisms involved in the initial myelin damage are unknown, soluble factors such as TNFα are known to induce abnormalities during myelin formation *in vitro* [52] and myelin damage *in vivo* [53]. The initial local destruction of white matter may subsequently lead to increased release of free myelin antigens and further triggering of new T- and B-cell specificities.

The initial damage to the myelin sheaths in chronic MS likely involves a complement-dependent attack of antibodies binding to antigens exposed on the myelin surface [54]. Molecules of the size of antibodies and complement factors can gain access to the CNS via the vasogenic edema at sites where the BBB leaks [55]. Among the various myelin and non-myelin antigens that have been implicated in the MS immuno-pathogenesis, the minor myelin protein myelin/oligodendrocyte glycoprotein (MOG) has now emerged as a likely primary target of the autoimmune reaction. MOG is exclusively located in the CNS where, by its exposure on the outer surface of myelin sheaths and oligodendrocytes, the protein is directly accessible to infiltrating T cells and antibodies. Several groups have reported an increased incidence and more persistent activity of anti-MOG T cells or antibodies in MS than in patients with other inflammatory neurologic diseases or healthy controls. In a variety of animal species (mice, rats, and primates) experimentally-induced autoimmune reactions to MOG give rise to similar clinical and neuropathologic features as found in MS [56, 57]. In both MS and the marmoset model of EAE, anti-MOG antibodies were found localized in areas where pathologic changes of white matter occur [58]. Moreover, a pathogenic role for anti-MOG

antibodies has been demonstrated in rats [59], mice [60], and marmosets [61]. In all three studies it was found that, whereas transfer of antimyelin T cells induces CNS inflammation, the induction of demyelination likely requires the presence of anti-MOG antibodies binding to conformational epitopes on the MOG molecule ([62] and own unpublished observations). It can be envisaged that once an initial lesion has been formed normally sequestered myelin antigens, such as MBP and PLP, become exposed and accessible for antibody binding.

It is unclear by what mechanism(s) anti-MOG antibodies are induced prior to the induction of the first demyelinated lesion. MOG is exclusively localized in the CNS, where it constitutes only a quantitatively minor component of myelin. It is difficult to envisage that sufficient quantities of MOG reach the secondary lymphoid organs. One mechanism that may induce anti-MOG antibodies is a similar molecular mimicry mechanism, as discussed above for T cells, namely that protein conformations in a virus induce antibodies to similar conformations in MOG [63]. However, we regard this unlikely because, in that case, the MS-inducing virus should contain a linear T-cell mimicry epitope as well as a conformational B-cell mimicry epitope.

Recent experiments in rhesus monkeys indicate that a Trojan horse type of mechanism may take place in the initiation of MS, as was described in AIDS-associated dementia [64]. In brief, CD4+ T cells activated in the periphery by infection with a herpesvirus, may transfer that virus across the BBB into the CNS by virtue of cross-reactivity with myelin antigens. We have recently found a candidate mimicry motif shared by dominant epitopes of MOG and cytomegalovirus (manuscript in preparation). Preliminary data indicate that the virus is locally released from the infiltrated cells and infects CNS cells. We hypothesize that the infected CNS cells are destroyed by infiltrating anti-viral cytotoxic T cells and antibodies. Such a mechanism would explain the thus far unexplained dominance of CD8+ over CD4+ T lymphocytes in MS lesions [65, 66].

The described mechanism of lesion initiation implies MHC class I restricted cytotoxic reaction of infiltrated antiviral CD8+ve T cells towards virus-infected CNS white matter cells as an additional risk factor to develop MS. It can be concluded from the TMEV model of MS that chronic inflammatory demyelination within the CNS can be the result of a persistent infection of the brain [42]. Therefore, the question can be asked to what extent the MHC-associated incapacity to effectively clear virus from the CNS may contribute to MS susceptibility [67].

Phase 4: The Expansion of Antimyelin Autoimmune Reactions

Progression of MS seems associated with the appearance in the circulation of T- and B-cell neoreactivities to a variety of myelin and nonmyelin antigens, including stress proteins [68, 69]. The phenomenon that T cells involved in the initiation of disease are specific to a narrow range of myelin epitopes/antigens, but during the later stages of disease T cells respond to a broad variety of myelin epitopes and antigens, is known as epitope spreading. The TMEV model demonstrates that such diversification of the repertoire may also occur after a neurotropic virus infection that leads to subsequent episodes of myelin damage and myelin-specific autoreactivity [42].

In several mouse models of EAE the potentially pathogenic role of T-cell neoreactivity to spreading epitopes has been well established [70–72]. In these models the neoreactivities even overgrow the T-cell autoreactivities that initiated the disease [73]. The activation of a naïve repertoire of T cells responding to spreading epitopes/antigens likely takes place outside the brain, as T cells activated within the CNS do not likely escape to the circulation [74, 75]. It has been well established in rats that myelin antigens released from an experimentally induced cryolesion in the brain white matter are drained to the T- and B-cell areas of the CLN, which drain the cerebrospinal and interstitial fluids from the brain [39, 40]. In the cryolesion model, myelin-reactive T cells activated within CLN were found to preferentially home to the brain and to enhance MBP-induced EAE in syngeneic rats [76]. This suggests that during their priming within CLN (antimyelin) T cells may receive instructions to traffic to the CNS white matter and enhance inflammatory demyelination. In this context the finding that the CLN of EAE-affected marmosets and rhesus monkeys contain significant numbers of APC loaded with immunoreactive myelin antigens (MBP and PLP) is of particular interest [41]. Such activated myelin-loaded APC were lacking in the CLN of monkeys with a nonrelated autoimmune disease, such as collagen-induced arthritis.

The processing of phagocytosed myelin by macrophages within lesions implies that a broad spectrum of previously sequestered antigens and cryptic epitopes becomes available for recognition by the immune system. Also, neoantigens that are induced under pathologic conditions will become available, such as αB-crystallin. This stress protein was found to be expressed in MS lesions and to represent a potential autoantigen in MS [70]. In our model, myelin damage caused by different pathogenic mechanisms, including an anti-viral immune response or an autoimmune attack, may all give rise to the CLN immigration of activated APC. The APC are

not only localized in the CLN areas where T- and B-cell priming can take place but also express high levels of MHC and costimulatory molecules. Therefore, we assume that the APC can potentially induce T- and B-cell neoreactivities directed to the score of myelin and nonmyelin antigens in the white matter material that they have phagocytosed during CNS demyelination.

The *in vivo* diversification of the autoimmune T-cell repertoire has been assumed to contribute mainly to tolerance induction. However, certain MHC class II specificities may instead propagate autoreactivity and disease progression by presentation of spreading/cryptic epitopes to the immune system [77].

MODELING THE MS PATHOGENESIS IN NONHUMAN PRIMATES

To be able to model the complex sequence of events contributing to the MS pathogenesis, lab animals, comprised of a comparable genetic complexity as humans, are needed. Outbred colonies of rhesus monkeys and common marmosets qualify in this respect and valid EAE models have been developed in both species [15, 16]. It has been well established that humans and rhesus monkeys share not only MHC-DP, -DR, and -DQ loci, but also allelic lineages [78, 79]. The sharing of allelic lineages of the MHC-DRB locus has clear functional implications, as was demonstrated by antigen presentation across the species barrier [80, 81].

The susceptibility to myelin-induced EAE in the rhesus monkey colony at the BPRC maps, at least partially, to the Mamu-DP locus; overlapping MHC-DP lineages in rhesus monkeys and humans have not been found [82, 83]. An important additional argument for the use of rhesus monkeys is that this species not only contains natural infections with the equivalent versions of human herpesviruses but also that infections follow a similar course as in humans, for example in the case of herpes simplex virus 1 [84] and cytomegalovirus [85]. Thus, the rhesus monkey provides a potentially interesting model to unravel how viruses (similar to those found in humans) are involved in the induction of inflammatory demyelination of the CNS and how they may influence the course of EAE.

The CNS white matter lesions in the rhesus monkey model of EAE mainly resemble those in the MS type 1, which is characterized by strong inflammation and limited demyelination [2]. This is a remarkable species-related difference with the EAE model in common marmosets. Rhesus monkeys immunized with recombinant human MOG (rhMOG) in complete adjuvant develop acute clinical EAE with predominantly hemorrhagic/necrotic brain lesions [86]. However, the identical immunization procedure in common marmosets induces chronic EAE with lesions formed by selective demyelination of brain and spinal cord [57]. The pathologic pattern resembles the type 2 lesions of chronic MS [2]. We are currently investigating whether this strikingly different disease pattern has a genetic and/or immunologic explanation.

A much more MS-like type of EAE can be induced in rhesus monkeys by immunization with pMOG34-56, a synthetic peptide representing amino acids 34-56 of the extracellular domain of rhMOG (manuscript in preparation). An intriguing finding in that study was that in a randomly collected group of 13 rhesus monkeys sensitized to this MOG peptide a variable disease pattern develops, which ranges from an acute to a chronic relapsing/remitting course.

EAE in the common marmoset is an excellent model of chronic MS because strong similarities between the clinical and pathologic aspects of the model and the human disease exist [14, 87, 88]. However, immunologically, the marmoset stands somewhat more distinct from the humans than rhesus monkeys [79]. The model is unique in a number of respects. Although we deal here with an outbred species, 100% of randomly selected animals from two independent colonies housed at the primate centers in Rijswijk (The Netherlands) and Göttingen (Germany), were found to develop EAE after immunization with human myelin in complete adjuvant, but the disease course varies between individual animals ($n > 50$). The most likely explanation for the high disease susceptibility is that EAE initiation in all animals depends on the same event, namely the Caja-DRB*W1201-restricted activation of CD4+ T cells specific for the encephalitogenic MOG peptide pMOG14-36 [57]. This monomorphic MHC class II molecule is expressed in all marmosets tested thus far [89]. The time of disease onset and the pattern of clinical signs, however, differ between individual animals. We are now investigating whether the clinical heterogeneity is related to a different regulatory role of Caja-DR molecules encoded by the two polymorphic MHC class II loci Caja-DRB1*03 and -DRB1*W16. An indication that this may indeed be the case comes from monkeys immunized with the rhMOG protein. In the few monkeys that did develop acute EAE the T-cell reactivity remained limited to pMOG14-36, whereas T cells from monkeys with chronic EAE responded to a much broader range of MOG peptides. We hypothesize that the activation of T cells specific for different "spreading epitopes," presented in the context of different Caja-DR molecules, gives rise to the variable disease pattern. Marmosets provide a unique system to directly test this hypothesis as T-cell transfer between (nonidentical) fraternal siblings can be performed. The natural bone marrow chimerism between twin animals ascertains that

TABLE 1 A *hypothetical* multistep model of the MS pathogenesis

Step 1:	MS is initiated by a viral infection. The specific viral epitopes presented to CD4 and CD8 cells in peripheral lymph nodes are selected at the level of Mhc-class I and II polymorphisms.
Step 2:	All activated T-cells transmigrate the blood brain barrier, but only those recognising the epitope to which they were primed in peripheral lymph nodes, or a mimicry epitope thereof in myelin antigens, penetrate into the CNS. The specific myelin epitopes presented by resident APC to infiltrated T-cells are selected at the level of Mhc class I and II polymorphisms.
Step 3:	CNS infiltrating CD4+ T-cells or co-infiltrating macrophages, carry (herpes)viruses across the blood brain barrier into the CNS white matter. Shedded virus locally infects white matter cells, including endothelium, oligodendrocytes, astrocytes etc.
Step 4:	The initial attack to the myelin sheaths (Wilkin's "primary lesion") involves the Mhc class I restricted cytotoxic killing of virally infected white matter by infiltrating CD8 cells. Insufficient clearance of the virus may lead to persistent infection of the CNS.
Step 5:	The intra-CNS activation of infiltrated CD4 cells involves Mhc class II-restricted interaction with resident APC inducing release of factors cytokines that enhance BBB permeability, such as cytokines (IL-1, TNF-α) and matrix metalloproteinases. Several of these factors are encoded by polymorphic genes.
Step 6:	The APC-mediated transport or passive drainage of myelin from the "primary lesions" to the cervical lymph nodes triggers a broad repertoire of autoreactive T- and B-cells in susceptible individuals.
Step 7:	Pathogenic T-cells and antibodies specific for cryptic and/or spreading myelin epitopes are released in the circulation and penetrate the CNS to enhance the encephalitis. Disease remission is determined by the patient's capacity to control the activation of autoreactive T- and B-cells.

transferred cells are not rejected despite the genetic disparity [90].

Concluding Remarks

In conclusion, we postulate that during the pathogenesis of MS multiple APC and T-cell interactions take place (summarized in Table 1). MHC polymorphisms operating at each interaction may either enhance or reduce the chance to develop clinical MS. On top of this, polymorphisms in regulatory genes, such as those encoding for cytokines/chemokines and their receptors, may enhance pathogenic and reduce protective activities of the immune system [7–10]. We hypothesize, therefore, that the susceptibility of an individual to MS is determined by the sum of these risk factors.

In our model each renewed exposure to the virus that has initiated MS can induce initial myelin destruction and subsequent autoimmune reactions, giving rise to the exacerbation of clinical signs. We think that remissions are induced by the patient's capacity to control the autoimmune reactions. Several of the viruses that have been implicated in MS cause a latent infection in humans, which can be reactivated without a clear external cause. For example, activation of latent herpes simplex virus 1 or cytomegalovirus infection can occur associated with apparently unrelated events, such as immunosuppression, fever, and stress. Conceptually, activation of a latent infection in individuals with a genetic risk phenotype may exacerbate MS via T cells that respond, for example, to a mimicry motif shared by herpes simplex virus 1 and MBP [91] or one shared by cytomegalovirus and MOG ('t Hart *et al.*, manuscript in preparation).

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Chapter IV

Summary and future perspectives

Multiple sclerosis (MS) is a major cause of disability in young adults affecting approximately 15,000 people in The Netherlands. Critical aspects of the disease have been modeled by experimental autoimmune encephalomyelitis (EAE) in animals. The vast majority of investigators use rats and mice for the unraveling of the MS immunopathogenesis and for the development of new therapies. However, despite decades of intensive research, the primary cause of MS is still unknown and no curative treatment exists.

With the development of EAE in the common marmoset, a new nonhuman primate model for MS has become available that seemed superior to the existing rodent and nonhuman primate models in many aspects (*Massacesi, 1995*). The first experiments performed at the BPRC confirmed this view (*'t Hart, 1998*). The ethical constraints for using nonhuman primates demand that such a model is used only for purposes that can not be investigated otherwise. The prime rationale for nonhuman primate models of human disease is the possibility to evaluate potentially effective therapies that do not sufficiently work in rodents. At the time that our studies were started, only the EAE model in rhesus monkeys could be regarded as being of sufficient scientific maturity. However, both clinically and neuropathologically this model did not sufficiently resemble chronic MS. It was therefore decided to further develop the marmoset EAE model and to scientifically explore whether this model can substantially contribute to our knowledge on the ethiopathogenesis of MS and to the development of new therapeutics for this disease.

The aims of the studies described in this thesis were:

1. To investigate the immunopathogenic mechanisms leading to the specific inflammatory demyelination of the central nervous system (CNS).
2. To investigate the genetic basis of the 100% disease incidence observed in our outbred colony.
3. To validate the model for therapeutic purposes using antibody-mediated intervention in pathogenic pathways.

The genetic regulation of EAE in common marmosets

Cellular responses

The common marmoset colony at the BPRC is fully outbred. As described in chapter II.1.1, all monkeys functionally express the monomorphic MHC class II molecule, *Caja-DRB*W1201,* a phenomenon that has not been described in any other vertebrate species thus far. This monomorphic allele is likely a major susceptibility element for demyelinating EAE as potentially encephalitogenic $pMOG_{24-36}$-specific T-cells are activated via this DR-like molecule. This observation explains most likely the 100% disease incidence in the outbred colony of common marmosets as the same autoimmune reaction induces the disease in all animals (Chapters II.2.1 and II.2.2). This feature provides us with the possibility to study the influence of environmental factors on disease susceptibility and progression in an outbred species. This is particularly true in situations where molecules exerting their effect on cells of the hematopoietic system are evaluated since siblings of chimeric twins can serve as each other's control.

Several studies have demonstrated the existence of autoreactive T-cells directed towards myelin proteins in the naïve repertoire of various monkey species and of human volunteers (*Martin 1990; Jingwu, 1992; Genain, 1994; Meinl, 1997; Lindert, 1999; Diaz-Villoslada, 1999; Goebels, 2000; Uccelli, 2001; Hellings, 2001*). T-cells reactive to epitopes within $pMOG_{21-40}$ are present at a remarkable high frequency in non-immunized marmosets (*Villoslada, 2001*). It is unlikely that the physiological function of these potentially encephalitogenic cells is their autoreactivity towards MOG, a protein exclusively localized in the CNS. As was already demonstrated by Martin and colleagues, highly specific T-cells can recognize completely unrelated peptide sequences in a comparable fashion (*Gran, 1999*).

Having determined a useful panel of mAb for phenotyping of marmoset cells (Chapter II.1.2) we were able to characterize our $pMOG_{14-36}$-specific T-cell lines (TCL) in more detail. Although of polyclonal origin, all TCL were found to share the $Th_{0/1}$-like phenotype, including TCL derived from $pMOG_{14-36}$-immunized animals (Chapter II.2.2). In addition, the TCL also expressed CD56 while being negative for CD16. The subpopulation of $CD3^+CD4^+CD8^-CD16^-CD56^+$ T-cells has been described in humans 15 years ago for the first time (*Lanier, 1986; 1987; 1989*), after which their functional characteristics were published (*Lu, 1994; Barnaba, 1994; Vergelli, 1996; Antel, 1998*). This subpopulation was found to: (1) proliferate in a MHC class II restricted,

antigen dependent fashion; (2) display MHC class II restricted, antigen dependent lysis of target cells; (3) induce MHC non-restricted, antigen independent lysis of target cells that was at least partly mediated through interactions of CD56 with a still elusive ligand (*Vergelli, 1996; Antel, 1998*).

CD56 (neural cell adhesion molecule (NCAM)) plays a role in the adhesion between neural and glial cells and seems to be involved in myelination and remyelination of axons (*Bhat, 1998; Charles, 2000*). Oligodendrocytes do not normally express MHC class II molecules on their surface but are positive for CD56 and are susceptible for lysis by $CD4^+CD56^+$ effector cells (*Vergelli, 1996; Antel, 1998*). This indicates that $CD3^+CD4^+CD8^-CD16^-CD56^+$ T-cells can directly cause CNS demyelination without involvement of antibodies (Ab). T-cells having functional properties of both Th_0 cells and NK cells, to date only reported in man, could shed new light on the role of encephalitogenic $pMOG_{14-36}$-reactive T-cells in the marmoset EAE model.

The data in chapter II.2.2 demonstrate that, although $pMOG_{14-36}$ reactivity is the driving force in disease initiation upon immunization with MOG^{Igd}, new pMOG-specific T-cell reactivities emerge during progression of the disease, a phenomenon known as epitope spreading. The activation of myelin-specific T-cells is thought to occur locally in the CNS by resident antigen presenting cells (APC) (*Cserr, 1992; Katz-Levy, 1999; Harling-Berg, 1999*). However, it is less likely that these new specificities leave the brain to emerge in the circulation (*Schmied, 1993*).

In Chapter II.2.3 we provide evidence that the cervical lymph nodes (cLN) are a more likely anatomical site of naïve T-cell priming. Myelin loaded professional APC are present at substantially higher numbers in T- and B-cell areas of cLN from EAE affected animals compared to animals with a non-neurological autoimmune disease. Therapeutic compounds as big as IgG can cross the blood-brain-barrier and end up in the lesion (Chapters II.3.1 and II.3.3). Cervical lymph nodes can be targeted with small molecules applied via the nasal epithelium to induce tolerance (*Cserr, 1992*). This therapeutic approach can be tested in the marmoset EAE model by using for example blocking peptides for $pMOG_{14-36}$.

The variable broadening of the T-cell reactivity is possibly associated with the heterogeneous disease course in different animals. Further characterization of these T-cell specificities, of which the peptides are likely to be presented by MHC class II molecules of the polymorphic Caja-DRB1*03 and Caja-DRB*W16 lineages, is of interest since these pMOG reactivities also have been found in MS patients (*Kerlero de Rosbo, 1997; 1998*).

Autoantibody responses

Clinical EAE can be induced in common marmosets by immunization with myelin basic protein (MBP) or proteolipid protein (PLP) but strong adjuvantia are needed (*Genain, 1996*). Clinical EAE can also be induced by immunizing marmosets with $pMOG_{14-36}$ (Chapters II.2.1 and II.2.2) or other linear peptide sequences of MOG (*Genain, 2001*). However, the relevance of these models for chronic MS can be disputed since clear demyelination is absent, although anti-MOG Ab reactivity can be present. In chapter II.2.2 we provide evidence that Ab reactivities towards discontinuous MOG epitopes are a prerequisite for CNS demyelination in the marmoset EAE model. These data gain strength by studies of McFarland who demonstrated that demyelinating EAE only occurs in monkeys forming anti-MOG Ab (*McFarland, 1999*). In addition, as in rodents, demyelinating EAE is only induced when in addition to adoptive transfer of myelin reactive T-cells anti-MOG Ig are administered as well (*Iglesias, 2001; Von Büdingen, 2001b; Genain, 2001*). In the MOG^{Igd} model anti-MOG Ab reactivities directed towards linear amino acid sequences are accumulating in time and with disease progression. However, the relevance of this epitope spreading is probably limited since no direct relation of the broadness of the Ab reactivity with disease progression could be demonstrated (Chapter II.2.2). Our results show that immunization with MBP or linear pMOG sequences does not generate Ab responses towards discontinuous MOG epitopes which are critical to disease induction (Chapters II.2.1 and II.2.2). The possibility that only Ab reactivity towards certain pMOG boost the disease remains to be investigated. As specific reagents are lacking, the IgG subclass distribution of the Ab reactivities towards the different linear as well as discontinuous epitopes in the course of the EAE can not be determined. In addition, for technical reasons detailed analysis of the cerebrospinal fluid (CSF) and isolation of the Ab producing cells within lesions could not be performed thus far.

EAE in the common marmoset: pro's and con's

Compared with Old World monkeys, common marmosets are relatively easy to handle and the breeding costs are low. Moreover, due to their small size, only small amounts of test material are needed which can be relevant for preclinical evaluation of potential therapeutics when large amounts of the drug are not (yet) available. The additional value of the use of common marmosets to MS research is that most biotechnology-derived pharmaceuticals show good cross-reactivity in marmosets, while they are often not reactive in more distantly related species such as rodents (Pro). On the other hand, sufficient quantities of CSF can not be easily obtained and the maximum amount of blood that can be frequently sampled is, due to the small size of the monkey, often not sufficient to run the desired number of tests (Con). To compensate for inter-individual variation, commonly observed in outbred animal models, future preclinical trials in the marmoset EAE model should take advantage of the fact that marmoset twins are full bone marrow chimeras. Hence, they share essentially identical immune systems. By treating one animal with the experimental drug and the other sibling with a placebo, one may compensate to some extent for immunological differences that are present between individuals in an outbred population (Pro).

Although the evolutionary distance of man to marmoset is greater than to Old World monkeys, a high similarity at the levels of T-cell receptor (TCR) genes (*Uccelli, 1997*), MHC class II genes (Chapter II.1.1), cell surface structures (Chapter II.1.2), cytokines (*Villoslada, 2001*), IgV_H repertoire (*Von Büdingen, 2001a*), effector molecules (*Villinger, 2001*), and myelin proteins (*Pham-Dinh, personal communication; Della Gaspera, 2001; Mesleh 2002*) has been reported between the two primate species (Pro). However, data on important effector mechanisms such as the complement system, the Ig classes and subclasses, the MHC class I repertoire and MHC class II haplotypes, T-cell subsets, APC subsets as well as their intracellular signaling pathways are still lacking (Con).

As documented in chapter II.3.1, alterations in tissue water content of the brain, indicative for inflammation, can already be visualized by MRI within three weeks after EAE induction, i.e. weeks or even months before clinical signs become apparent. The data described in chapter II.3.3 demonstrate that the effect of therapeutic compounds on the CNS can be directly visualized and evaluated with MRI (Pro). To be able to compare the efficacy of a new therapy with existing ones, quantitative MRI is being developed. The MRI

scoring system as described by Jordan and co-workers and used by us (Chapter II.3.3) is unsatisfactory for this purpose (*Jordan, 1999*). It puts much weight on the number of lesions while the size and pathological characteristics of the lesions are ignored. Moreover, appraising the presence of gadolinium-diethylenetriamine-pentaacetic acid (Gd-DTPA) enhancing lesions by adding only an extra 0.5 to 1.0 point to the total MRI score of 4, probably underestimates the relevance in the disease. As demonstrated earlier, gadolinium-DTPA enhancing lesions are areas of high autoimmune inflammation that most likely is the major cause for the neurological dysfunction (*'t Hart, 1998; Mancardi, 2000*).

Recently developed MRI techniques enable us now to describe MRI detectable changes in the EAE-affected common marmoset brain in quantitative parameters. This provides the possibility to link MRI-characteristics to histopathological characteristics of brain lesions. The use of experimental animals allows analyses at pre-defined time points to study the pathology of EAE in great detail (*Blezer, manuscript in preparation*). The present possibility to monitor brain lesion development by serial imaging at 14 days intervals enables us to test the effect of experimental therapy on already existing lesions. Using the same strategy as above, the effect of therapy on lesion development can be followed with quantitative MRI (Pro).

The morphological aspects of EAE-induced CNS white matter lesions resemble those in chronic MS cases (Chapters II.2.1, II.3.1-3, *Lucchinetti, 2000; Genain, 2001*). However, thus far we were unable to directly correlate the number or size of the brain lesions and the clinical deficit in the marmoset EAE model. It has been proposed that in this EAE model the major neurological deficits such as paralysis are caused by lesions in the spinal cord. Due to the small size, with the current available MRI technology we can not obtain useful information on the spinal cord in the common marmoset. The efficacy of therapeutic compounds is therefore still mainly evaluated on the basis of clinical scoring and brain MRI (Con). To compensate for this, other tests need to be included. Implementation of the expanded scale of disability score at the BPRC as published by others should be a first step in this direction (*Villoslada, 2000*). Furthermore, the integrated usage of validated neurophysiological tests (*Philippens, 2000; Roberts, 2000*) will definitely be of additional value for predicting the efficacy of new therapeutics in MS patients (Pro).

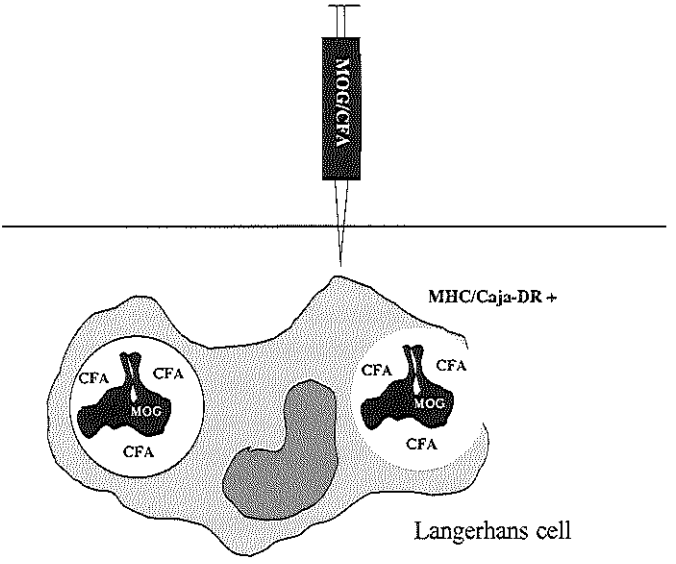
Successful induction of EAE in marmosets precludes the use of strong adjuvantia. Besides the fact that the use of CFA induces significant physical

discomfort, the immunization procedure also induces an unphysiological situation by the systemic activation of myeloid lineage cells (*Billiau, 2001*). By using additional adjuvantia these effects are even further enhanced since severe clinical EAE may already develop within 7 days after immunization when CFA containing *M. tuberculosis* and *B. pertussis* are used for disease induction (*Genain, 2001*). Our studies have shown that in some cases the additional use of *B. pertussis* induces aspecific necrosis with brain lesions which in our view is a likely cause for the acute onset of this model (*'t Hart, 1998*). Although skin ulcerations are diminished by using *M. butyricum* containing CFA, undesired effects of CFA still remain (Con). For example, in CFA-treated monkeys a neutralizing immune response towards test compounds is much more rapidly induced than in naïve animals. This is especially true with Ab produced in prokaryotes or with engineered Ab of mouse origin (Chapter II.3.1 and II.3.2; *Hakimi, 1991; Boon, 2000; Brok, 2001a*). However, by using fully human Ig molecules a significant neutralizing Ab response that prevents the efficacy of the compound is absent, even in the presence of CFA. (Chapter II.3.3).

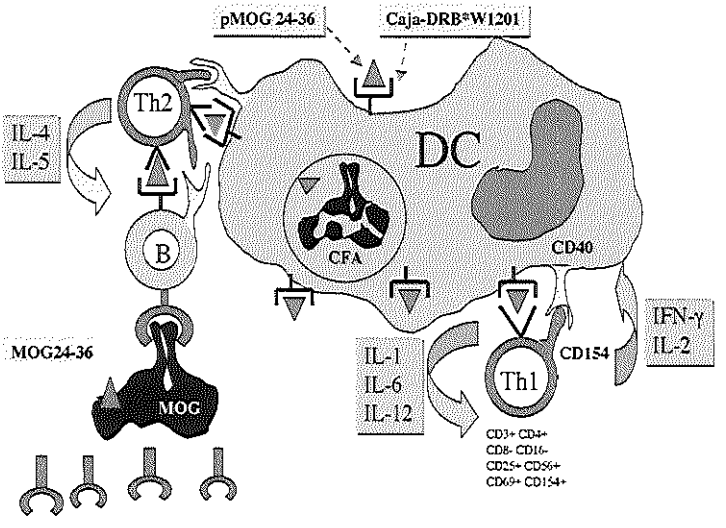
Detailed genetic and immunological characterization of this New World monkey species should have a high priority. Knowledge of the innate and adaptive immune system of this unique species is a prerequisite for enhancing the applicability of the marmoset EAE model. Hopefully these efforts will increase our knowledge of the MS pathogenesis and disease progression. This should result in the development of a definite cure for this neurological deficit in the next decade. Until then, MS will keep affecting the quality of life of millions of people.

SCHEMATIC VIEW OF EAE DEVELOPMENT
IN COMMON MARMOSETS

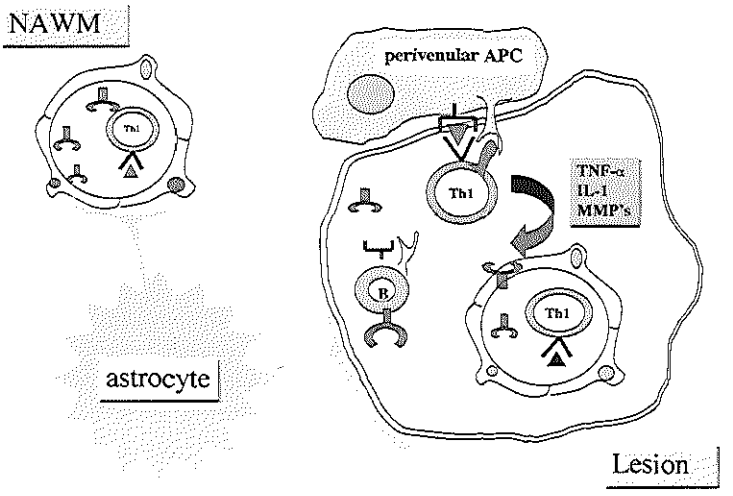
Box 1: The skin: immunization



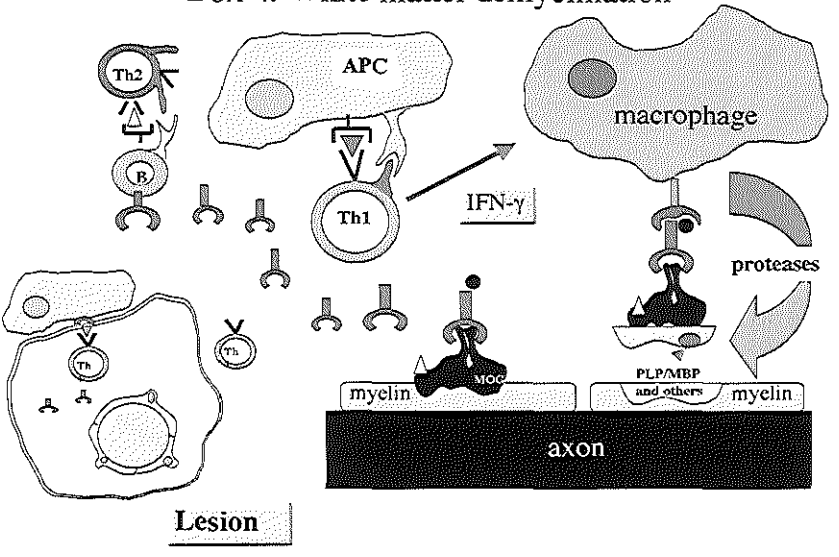
Box 2: the draining lymph node



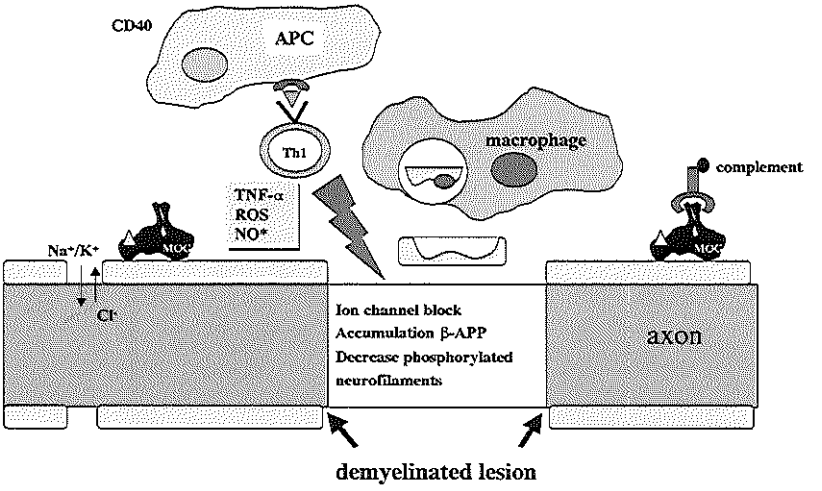
Box 3: blood-brain barrier leakage



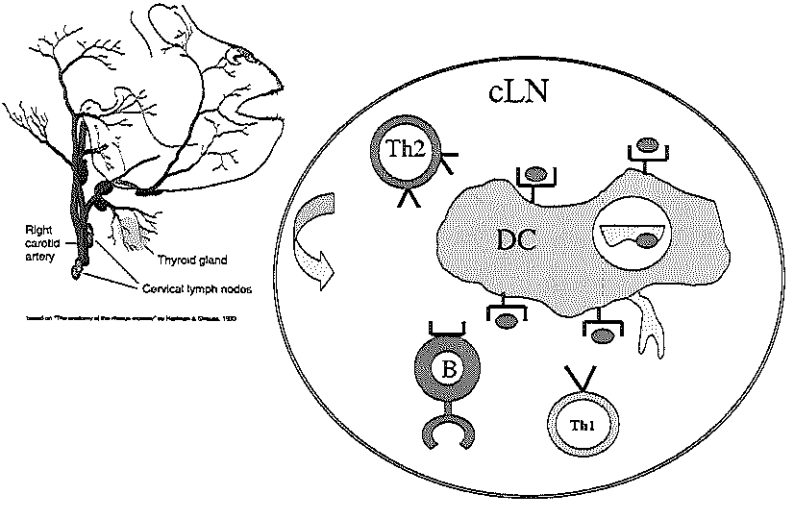
Box 4: White matter demyelination



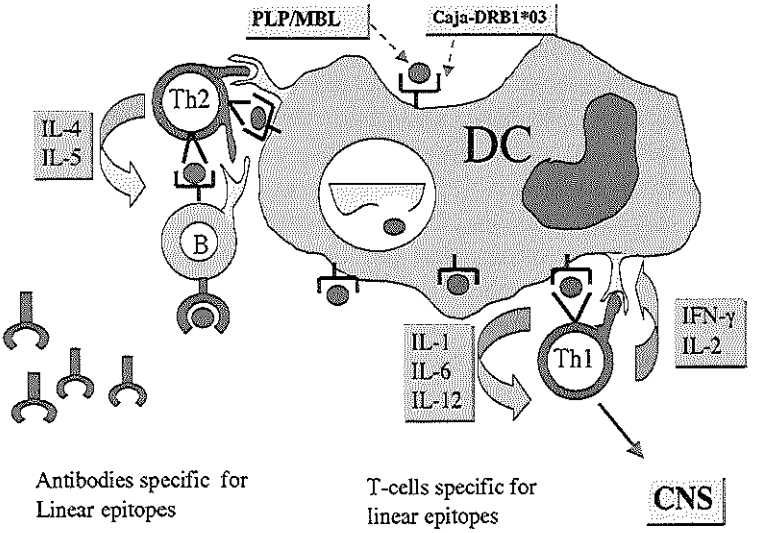
Box 5: Interaction of demyelination and inflammation



Box 6: drainage of myelin-loaded macrophages to cervical lymph nodes



Box 7 = inter-molecular epitope spreading



A case for mimicry of MOG and avian herpesviruses: Marek's disease virus as the cause for MS-like disease in common marmosets ?

Based on the data obtained in cotton-top tamarins it was postulated that chimerism has an impact on the diversity of the MHC class I repertoire (*Watkins, 1988; 1990b*). The MHC class I genes in New World monkeys show similarities with the MHC class G locus in humans and seem to have evolved by an active process of loci turnover (*Watkins 1990a; Cadavid, 1999*). Nucleotide sequence analysis of the polymorphic exon 2 segments of *Mhc* class II genes demonstrates that the common marmoset has a limited variability of the MHC class II region, a phenomenon which may be associated with chimerism as well (Chapter II.2.1). Whether the presence of the monomorphic *Caja-DRB*W1201* allele is related to the fact that common marmosets normally give birth to chimeric twins is not known. Nevertheless, the chimeric state seems to have a function in compensating for the limited class II polymorphism at the population level. Although there are only limited data on the extent of MHC class I polymorphism in common marmosets, the low degree of polymorphism between MHC class II genes at least may explain why this species is highly susceptible to bacterial infections (*Potkay, 1992*).

We hypothesize that the monomorphic *Caja-DRB*W1201* allele has been positively selected during evolution, probably because it controlled an important protective function. The natural habitat of the common marmoset has largely been destroyed but new habitats are populated. The presence of this gene, however, can be of great disadvantage when new pathogens are encountered, especially when this molecule serves as a susceptibility trait. The fact that primate centres in the north and north-east of Brazil are facing regular extinctions of their captive marmoset colonies by a paralyzing disease is of major concern in our endeavor to prevent the extermination of this species. Unfortunately, no screening for neuropathological abnormalities has been performed thus far and hence a possible similarity with EAE is unclear (*Menezes, Natal; Porforio, Joao Pessoa; Aarestrup, Juiz de Fora: personal communication*).

In view of the data above it is interesting that the specific epitope of Caja-DRB*W1201-restricted encephalitogenic $CD3^+CD4^+CD8^-CD16^-CD56^+$ $pMOG_{24-36}$-specific T-cells shares a high degree of amino acid similarity with glycoprotein A of Marek's disease virus (MDV) serotype 1 and 2 and with Turkey herpes virus (HTV), also known as MDV serotype 3 (*Kato, 1985; 1989*). Glycoprotein A is a virion surface protein anchoraged in the viral envelope of the class of Gallid herpes viruses of which homologues have been described in Herpes Simplex virus, Pseudorabies virus and

Varicella Zoster virus (*Ihara, 1989*), all being double stranded DNA viruses known to infect humans (*Stohlman, 2001*). To date, a vaccine consisting of infectious, tissue-culture propagated HTV is still widely used in The Netherlands to protect chicken against MDV. In this context it should be noted that MOG has not yet been demonstrated in birds and reptiles (*Birling, 1993*).

After infection, MDV has a life-long latent persistence in T-cells but can be reactivated (*Delecluse, 1993*). Marek's disease in susceptible lines of chicken was initially characterized by lesions in the peripheral and central nervous system, and by leukocyte malignancies, particularly in the viscera (*Wight, 1962; Payne, 1976; Prineas, 1972; Lawn, 1979*). Susceptible animals showed electroencephalographic abnormalities correlating with clinical signs (*Kornegay, 1983*). Recent electron microscopic studies of the brain have demonstrated that MDV infection induces a similar vacuolation of myelin with dilation of the intramyelinic space as has recently been described in MOG-immunized marmosets (*Genain, 1999; Raine, 1999*) and *in vitro* (*Zeine, 2001*). Moreover, mononuclear cell infiltrates were found within the parenchyma. In addition, active myelin phagocytosis by macrophages was occasionally observed but fully demyelinated axons were rare. Notably, MDV remained undetectable at these sites (*Kornegay, 1988*). Demyelination in MDV-infected fowl is caused by blocking of the hydrolyzation of cholesterol esters leading to accumulation of myelin blocks around the vessels which became a target in the chronic autoimmune reaction. Taken together, these observations in MDV affected chickens resemble the findings of electron microscopic studies of MS during the early stages of lesion formation (*Shah, 1980; Raine, 1999*)

Other birds may be susceptible to MDV induced disease as well (*Abujoub, 1995*). Infectious MDV is spread by feather follicles and the virus is highly resistant (*Calnek, 1969*). It is worth mentioning that the primate colonies in Brasil are in open contact with the environment, so zoönotic transfer of viral particles from wild birds is feasible. Although MDV and HTV are not considered to be a public health hazard (*Sharma, 1973*) and direct evidence for a significant role of MDV in MS is lacking (*McStreet, 1992; McHatters, 1995; Hennig, 1998*), herpesviruses have been frequently associated with MS (*Bilzer, 1996; Cermelli, 2000; Atkins, 2000; Ascherio, 2001; Stohlman, 2001*). Experiments are in progress to test whether infection with MDV can lead to EAE-like disease in the common marmosets in an attempt to obtain a "natural" animal model to elucidate the role of viruses in the pathogenesis of MS.

Chapter V

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Abbreviations

APC	antigen presenting cell(s)
BBB	blood-brain-barrier
CFA	complete Freunds' adjuvant
cLN	cervical lymph node(s)
CMV	cytomegalovirus
CNS	central nervous system
CSF	cerebrospinal fluid
CTL	cytotoxic T cell
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
FDA	American Food and Drug Administration
Gd-DTPA	diethylenetriaminepentaacetic acid
HHV-6	human herpes virus-6
HLA	human leukocyte antigen
HPA	hypothalamus-pituitary-adrenal
HTV	Turkey herpes virus
IFN	interferon
Ig	immunoglobulin(s)
IL	interleukin
(m)Ab	(monoclonal) antibody(ies)
Mϕ	macrophage
MBP	myelin basic protein
MDV	Marek's disease virus
MHC	major histocompatibility complex
MMP	matrix metalloproteinase(s)
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	multiple sclerosis
NAWM	normal appearing white matter
NCAM	neural cell adhesion molecule
PBMC	peripheral blood mononuclear cells
PLP	proteolipid protein
SFV	Semliki Forest virus
TCR	T-cell receptor
Th	T helper cell
TMEV	Theiler's murine encephalomyelitis virus
TNF	tumor necrosis factor
VDJ	variable, determining, joining region of the T-cell receptor
VCAM	vascular cellular adhesion molecule

Samenvatting voor niet-ingewijden

Wanneer ziekteverwekkers, zoals bacterien of virussen, het lichaam binnendringen worden deze herkend door ons afweersysteem (immuunsysteem) als "vreemd". Als gevolg hiervan komen er een aantal reakties op gang die uiteindelijk leiden tot vernietiging van de indringers: zo blijven we gezond (immuun). Soms kan ons immuunsysteem echter geen goed onderscheid maken tussen wat nu lichaamsvreemd is en wat nu lichaamseigen ("zelf") is. Als gevolg van deze fout begint ons afweersysteem lichaamseigen strukturen aan te vallen. We spreken in dit geval van een autoimmuunreaktie.

Multipele sclerose (MS), een voorbeeld van een autoimmuunreaktie, omvat een verzameling van ziektebeelden waarbij de beschermlaag (het myeline) rondom de zenuwuitlopers (axonen) in het centrale zenuwstelsel (hersenen en ruggenmerg) wordt aangetast. Dit resulteert in een verstoorde prikkeloverdracht van de hersenen en het ruggenmerg naar de spieren en organen. De zichtbare beschadiging van het myeline (lesie of plaque) in de hersenen en ruggemerg van patienten met MS kan leiden tot allerlei neurologische problemen zoals verlamming, spasmen, tintelingen, zicht afwijkingen en incontinentie. MS komt voor bij 1 op de 1.000 mensen in de westerse wereld (15.000 in Nederland) en is één van de belangrijkste redenen tot invaliditeit. Ondanks intensief onderzoek is de oorzaak van MS nog onbekend – algemeen wordt er gedacht aan een rol van virussen – en is een behandeling die de ziekte definitief een halt toeroept of geneest niet beschikbaar. Tevens is nog niet volledig duidelijk hoe en waarom de fout door het afweersysteem gemaakt wordt en tegen welke componenten van het myeline de reaktie in beginsel gericht is.

In principe kan onderzoek naar het ontstaan en de ontwikkeling van MS het best bij patiënten worden uitgevoerd. Echter, mensen gaan pas naar de dokter wanneer de ziekte zich uit in lichamelijk ongemak en dus al enige tijd (vaak jaren) aan de gang is. Ook kan er niet zomaar zonder problemen hersen- of zenuwweefsel van deze mensen afgenomen worden voor verder onderzoek en is het inschakelen van gezonde personen volstrekt onacceptabel. De ingewikkelde ziekteprocessen die leiden tot MS, als ook de complexiteit van de aangetaste organen, maken dat er nog geen model is buiten het lichaam (*in vitro*) waarin de ziekte tot in detail nagebootst kan worden. Als gevolg hiervan zijn we voor een beter inzicht in het vroege ziekteproces en voor de

ontwikkeling van geneesmiddelen vooralsnog aangewezen op proefdieren, waarin ziektemodellen in non-humane primaten (apen) een belangrijke schakel vormen.

De gewone marmoset – *Callithrix jacchus jacchus* of penseelaap – is oorspronkelijk afkomstig uit Brazilië. Een volwassen dier weegt 300-350 gram, is ongeveer 20 cm groot met een even lange staart. De witte pluimpjes op de oren zijn het meest markante kenmerk van deze soort. In 1995 is de Amertikaanse neuroloog Prof. dr. Steven Hauser er als eerste in geslaagd om een experimenteel model in de marmoset te ontwikkelen dat sterke overeenkomsten vertoont met de chronische vorm van MS in de mens en wordt betitield als experimentele autoimmuun encephalomyelitis (EAE). De ziekte kan worden opgewekt door de dieren componenten van het centraal zenuwstelsel toe te dienen.

Een belangrijke voorwaarde voor het gebruik van de aap als ziektemodel is dat zo'n model alleen gebruikt wordt als vraagstellingen niet op andere manieren kunnen worden onderzocht. In tegenstelling tot de meest gebruikte muizen- en rattensoorten, verschillen apen, net als mensen, onderling in de opbouw van hun afweersysteem, waardoor elk dier anders reageert. Door wat bloed van dieren af te nemen kunnen de genetische kenmerken die het afweersysteem reguleren (het zogenaamde major histocompatibility complex of MHC) voor elk individu in kaart worden gebracht. De bijzonderheid van dit systeem is dat de genprodukten een grote verscheidenheid (polymorfisme) op populatie nivo vertonen. Het grote voordeel van deze genetische typering is dat men vooraf – maar ook achteraf - inzicht krijgt in de factoren en eigenschappen van het immuunsysteem die mogelijk een rol spelen bij het ontstaan van MS, temeer daar de bouwstenen van het afweersysteem in de aap zeer sterk vergelijkbaar zijn met die van de mens. Verder is de complexiteit van de hersenen van apen goed vergelijkbaar met die van mensen en zijn apen vatbaar voor dezelfde infecties met dezelfde soort virussen en bacteriën als mensen. Het EAE model in de marmoset is enige jaren geleden ook in Nederland op het primatencentrum BPRC geïntroduceerd. Enkele resultaten staan beschreven in dit proefschrift.

Eerst zijn we begonnen om het MHC van de marmoset in kaart te brengen. Uit de resultaten bleek dat de MHC klasse II moleculen een sterke gelijkenis vertoonden met die van de mens. Echter, in tegenstelling tot de mens hebben alle penseelapen één bepaalde MHC klasse II molecuul gemeen (hoofdstuk II.1.1). Uit verder onderzoek is gebleken dat juist deze struktuur verantwoordelijk lijkt te zijn voor de bevinding dat de ziekte in alle dieren succesvol is op te wekken (hoofdstuk II.2.1). Dit gegeven geeft ons de mogelijkheid om tijdens toekomstig onderzoek de rol van erfelijke en omgevingsfaktoren voor de ziekte verder in kaart te brengen.

De cellen van het immuunsysteem waarvan algemeen aangenomen wordt dat ze een belangrijke rol spelen bij het onstaan en de verdere ontwikkeling van de ziekte zijn antigeen presenterende cellen (APC), T-cellen en B-cellen. Om deze cellen beter te kunnen identificeren zijn specifieke antistoffen getest voor strukturen die zich op het oppervlak van deze cellen bevinden (hoofdstuk II.1.2). Met behulp van de hierbij behaalde resultaten was het mogelijk om de T-cellen en B-cellen verder te karakteriseren.

In hoofdstuk II.2.2 worden de resultaten beschreven voor de twee op dit moment meest gangbare ziektemodellen, gebruik makend van humaan myeline of myelin oligodendrocyte glycoprotein (MOG) als trigger. MOG is een eiwit dat slechts in geringe hoeveelheid, maar specifiek aanwezig is in het centraal zenuwstelsel. De autoimmuunreaktie tegen MOG speelt een belangrijke rol bij de afbraak van myeline (zie ook hoofdstuk II.2.1). Uit ons onderzoek is gebleken dat niet de kwantiteit of diversiteit maar de kwaliteit en specificiteit van de geproduceerde antilichamen van belang zijn bij het ontstaan van EAE. Antistoffen gericht tegen – waarschijnlijk – 2- en 3-dimensionale strukturen van het MOG molekuul bleken een essentiële schakel te zijn om het myelin rondom de zenuwuitlopers af te breken. Bij dieren waarin dit soort antistoffen niet aantoonbaar aanwezig waren, trad geen lesie vorming op. Antilichamen gericht tegen – waarschijnlijk – lineaire strukturen lijken niet wezenlijk bij te dragen aan het ziekteproces. T-cellen daarentegen kunnen alleen maar reageren op lineaire strukturen (peptiden). In het MOG model in de marmoset bleek dat het aantal verschillende peptiden waarop T-cellen reageren positief gekorreleerd is met de snelheid waarmee de ziekte zich ontwikkeld. In het myeline model kon dit nog niet overtuigend worden aangetoond. De betrokken T-cellen vertoonden sterke overeenkomsten met elders eerder beschreven T-cellen in knaagdieren en mens (zgn. $CD4^{+}Th_1$ cellen). Uit verder onderzoek

bleek dat ze ook een molekuul, genaamd CD56, tot expressie brengen op hun oppervlak. Van dit type T-cellen – tot nu toe alleen nog beschreven in de mens – is bekend dat het in staat is om cellen uit het centrale zenuwstelsel op een aantal manieren te doden. Wat de rol van deze cellen is in mensen met MS en of dit ook in de marmoset het geval is, wordt op dit moment verder onderzocht.

Lang werd aangenomen dat hersenen en ruggenmerg organen waren die niet of nauwelijks beïnvloedbaar waren door signalen van het afweersysteem van buitenaf. In hoofdstuk II.2.3 beschrijven wij echter dat de diepgelegen lymfeknopen uit de hals (cervicale lymfeknopen) een belangrijke rol spelen bij de chroniciteit van het ziekteproces. Wij hebben aanwijzingen dat hier de aktivatie en programmering plaatsvindt van een nieuwe golf aan T-cellen die het myeline aanvallen. Deze informatie opent nieuwe wegen voor toekomstige therapiën, aangezien de autoimmuunreaktie in deze lymfeknopen met bepaalde technieken direct geneutraliseerd kan worden (tolerantie). Of dit ook zal werken in het marmoset EAE model en MS patiënten vereist verder onderzoek.

Het testen van potentiële effectieve therapiën in proefdiermodellen – zogeheten preklinisch onderzoek – is nodig teneinde de veiligheid en werkzaamheid van een medicijn voor de mens afdoende vast te stellen. De nieuwe generatie geneesmiddelen zijn gebaseerd op biologische moleculen, die met biotechnologische technieken worden geproduceerd. Naar verwachting zijn deze middelen veiliger en blijven deze langer werkzaam in de patiënt dan de huidige middelen, waardoor betere resultaten behaald kunnen worden. De Amerikaanse Food and Drug Administration (FDA) - de organisatie die het gebruik van een nieuw medicijn moet goedkeuren alvorens het kan worden toegelaten op de markt – stelt de eis dat de veiligheid van biotechnologisch geproduceerde stoffen tevoren aangetoond is in een diersoort die gevoelig is voor de werking van de stof. Een belangrijk nadeel van de biologische geneesmiddelen is dat ze door hun specificiteit alleen werkzaam zijn in de mens, of in diersoorten die nauw aan de mens verwant zijn. De enige diersoort die dan nog voor preklinische evaluatie in aanmerking komt is de aap. Een tweetal van deze moleculen is getest in het marmoset EAE model. Hierbij werd gekeken naar de klinische verschijnselen, lesie vorming in het centraal zenuwstelsel en werden de cellen en produkten van de immuunreaktie met elkaar vergeleken *in vitro*. Zowel de werking van anti-CD40 (hoofdstuk II.3.1 en II.3.2) als van anti-IL-12 antilichamen (hoofdstuk II.3.3) gaven

bemoedigende resultaten te zien en worden momenteel verder ontwikkeld voor toepassing in MS.

De in dit proefschrift beschreven resultaten geven aan dat het marmoset EAE model een belangrijk ziektemodel is om de autoimuunreakties in mensen na te bootsen (zie ook hoofdstuk III). Toch zal er voor toekomstig onderzoek verder geïnvesteerd moeten worden om zowel de marmoset als het ziekteproces in dit dier verder in detail te karakteriseren. Juist dan zal dit diermodel significant bij kunnen dragen tot de ontwikkeling van een behandeling die kan leiden tot genezing van de ziekte multipele sclerose.

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Curriculum vitae

De auteur van dit proefschrift werd op 1 augustus 1965 geboren te Udenhout. Na het behalen van het HAVO diploma aan het Cobbenhagen College te Tilburg, werd in 1982 begonnen met het Hoger Laboratorium Onderwijs aan de Hogeschool West-Brabant te Etten-Leur. Gekozen werd voor de afstudeerrichting Medische Microbiologie. In het kader hiervan werd een 9-maands onderzoeksstage uitgevoerd op de afdeling Gnotobiologie van het TNO-instituut, het Radiobiologisch Instituut te Rijswijk, onder leiding van Prof. dr. P.J. Heidt. Na het behalen van het diploma in 1987 werd aangevangen met een baan als microbiologisch analist bij de Stichting Samenwerking Delftse Ziekenhuizen. Na korte tijd werd deze functie ingeruild voor een functie als cytogenetisch analist op de afdeling Tumorgenetica van de Universiteit van Amsterdam onder leiding van Dr. R. Sweeting. Na ruim 2 jaar werd er op het Instiuut voor Radiotherapie en Immunologie begonnen met het bestuderen van de rol van IFN-γ in het ontstaan van Graft-versus-Host ziekte na beenmerg-transplantatie onder leiding van Prof. dr. J.M.J.J. Vossen en Prof. dr. P.J. Heidt. Dit project mocht slecht 3 jaar duren voordat het door interne bezuinigingsmaatregelen abrupt werd beeindigd. In Leiden werd op de afdeling Pathologie van de Universiteit aanvang gemaakt met het bestuderen van het EMS1 gen bij het proces van metastasering onder leiding van Dr. E.M. Schuuring. Op 1 augustus 1995 keerde hij weer terug op het oude nest. Bij de, inmiddels uit TNO geprivatiseerde, stichting Biomedical Primate Research Centre (BPRC) werd begonnen als research analist bij de afdeling Immunobiologie alwaar het collageen-geinduceerde arthritis model in de resusaap nader werd bestudeert. Na een 3-tal jaren werd in augustus 1998 aangevangen met het promotie onderzoek onder leiding van Dr. L.A. 't Hart. De hierbij behaalde resultaten staan beschreven in dit proefschrift. Medio 2002 is de auteur werkzaam als manager van de sectie Animal Care van het Animal Science Department van het BPRC. Verder werkt hij aan een meer gedetailleerde immunologische en genetische karakterisering van Nieuwe Wereldapen, de penseelaap in het bijzonder.

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