BACTERIAL PEPTIDOGLYCAN IN RHEUMATOID ARTHRITIS AND MULTIPLE SCLEROSIS

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BACTERIEEL PEPTIDOGLYCAAN IN REUMATOÏDE ARTRITIS EN MULTIPLE SCLEROSE

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GENERAL INTRODUCTION

CHAPTER 1

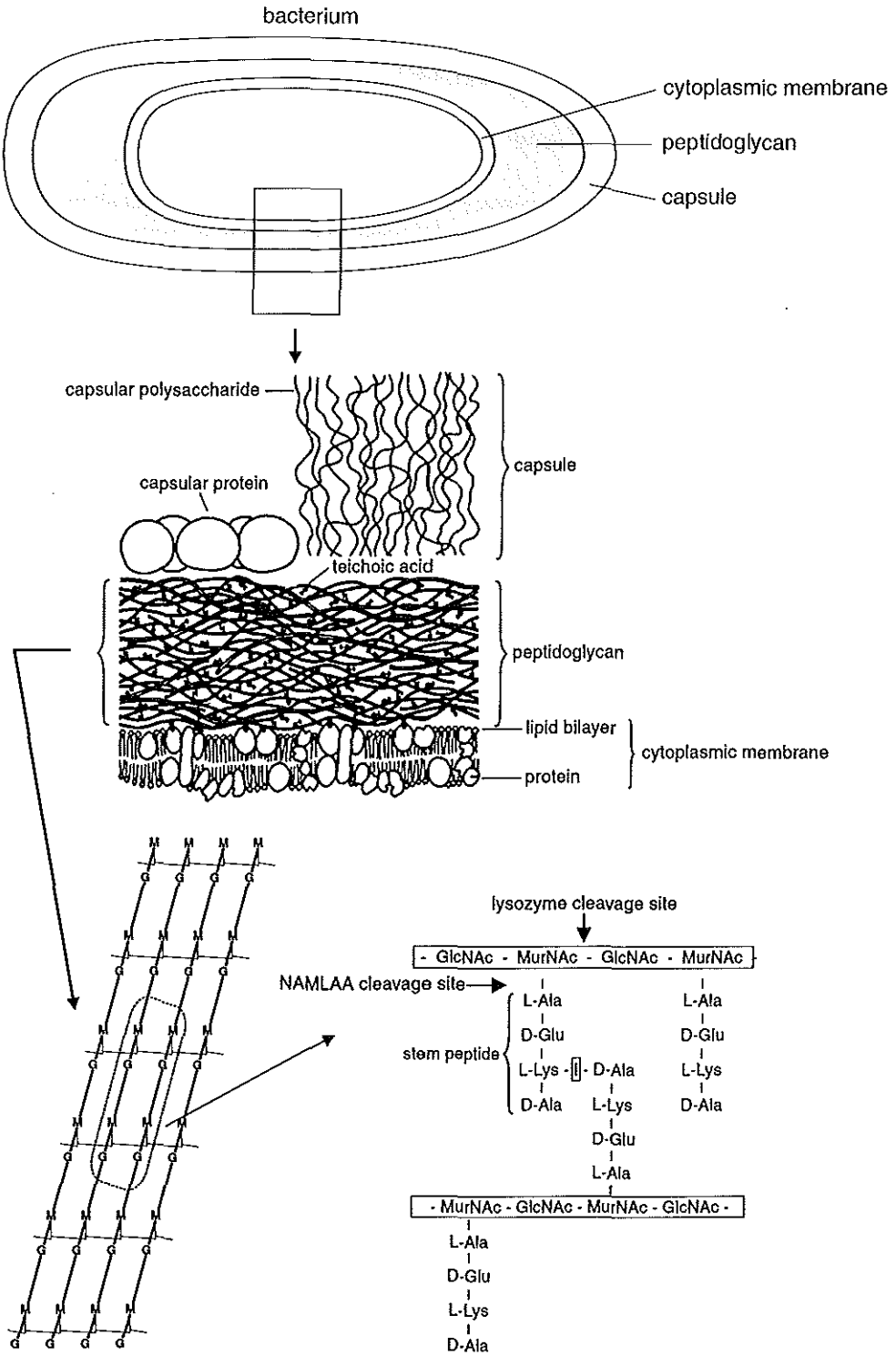
General introduction

The human body is always in close contact with billions of bacteria located at all mucosal sites and prominently in the gut. This bacterial population, commonly referred to as the microflora, is established early following birth, remains constant in a given individual over his or her life span and is of great importance to the host. The bacteria degrade and ferment many of the food components that are not absorbed in the small intestine. Furthermore, the intestinal flora acts as a barrier against food-borne pathogenic bacteria. The mucosal flora also represents a large amount of potent immunostimulatory structures like lipopolysaccharides (LPS) in gram-negative bacteria and peptidoglycan (PG) in both gram-negative and gram-positive bacteria. These structures are important in the resistance to infections but it is commonly believed that these structures are the major cause of bacterially induced sepsis. It has also been suggested that PG is involved in the persistence of chronic inflammation. In this thesis the role of PG in the pathogenesis of the chronic inflammatory diseases rheumatoid arthritis (RA) and multiple sclerosis (MS) is studied. In this chapter an introduction will be given about PG and its biological activities, and about the pathogenesis of RA and MS.

PEPTIDOGLYCAN

Structure

Peptidoglycan is present in the cell walls of most bacteria and is the major constituent (30-70 % v/v) of gram-positive cell walls (figure 1). In gram-negative bacteria PG constitutes only 10% of the cell wall. This bag-shaped molecule surrounds the cell and gives it strength to withstand the turgor pressure exerted by the cytoplasm. The structure of PG was studied extensively by Schleifer (1). Shortly, PG is composed of alternating β1-4-linked residues of N-acetyl glycosamine (GlcNAc) and muramic acid (MurNAc). The latter is usually N-acetylated, but in mycobacteria and some nocardial species the N-acetyl group is oxidized to give the N-glycosylated form. Muramic acid acts as the linking moiety between peptides and sugars through an amide bound at the carboxyl group of the D-lactic ether at C-3. Usually L-alanine is bound to muramic acid, followed by D-glutatic acid, which is linked by its γ-carboxyl group to an L-diamino acid (mostly mesodiaminopimelic acid), and finally D-alanine is attached forming the stem peptide (figure 1). These stem peptides are crosslinked by interpeptide bridges usually between the third amino acid of one stem peptide and the fourth of the other peptide. Although conservative in its general architecture, several hundreds different peptidoglycans have been described. These are distinguished by a large number of variations within the peptide moiety or by minor modifications within the disaccharide unit. The greatest variation of amino acids in the stem peptide occurs in position 3. Variation also occurs in the mode of crosslinking and in the interpeptide bridge. According to the mode of crosslinking PG is divided into group A (cross-linkage between


bacterium
cytoplasmic membrane
peptidoglycan
capsule
capsular polysaccharide
capsular protein
capsule
teichoic acid
peptidoglycan
lipid bilayer
protein
cytoplasmic membrane
lysozyme cleavage site
- GlcNAc - MurNAc - GlcNAc - MurNAc
NAMLAA cleavage site
stem peptide
L-Ala
D-Glu
L-Lys
D-Ala
L-Ala
D-Glu
L-Lys
D-Ala
L-Ala
D-Glu
L-Lys
D-Ala
- MurNAc - GlcNAc - MurNAc - GlcNAc -
L-Ala
D-Glu
L-Lys
D-Ala

positions 3 and 4 of the stem peptide) and the less common group B (cross-linkage between positions 2 and 4 of the stem peptide). The chemical structure of PG monomers theoretically allows the formation of sugar chains of indefinite length. The actual chain length, however, is quite short and differs between bacterial species. In different organisms the average chain length varies between 10 and 65 disaccharide units.

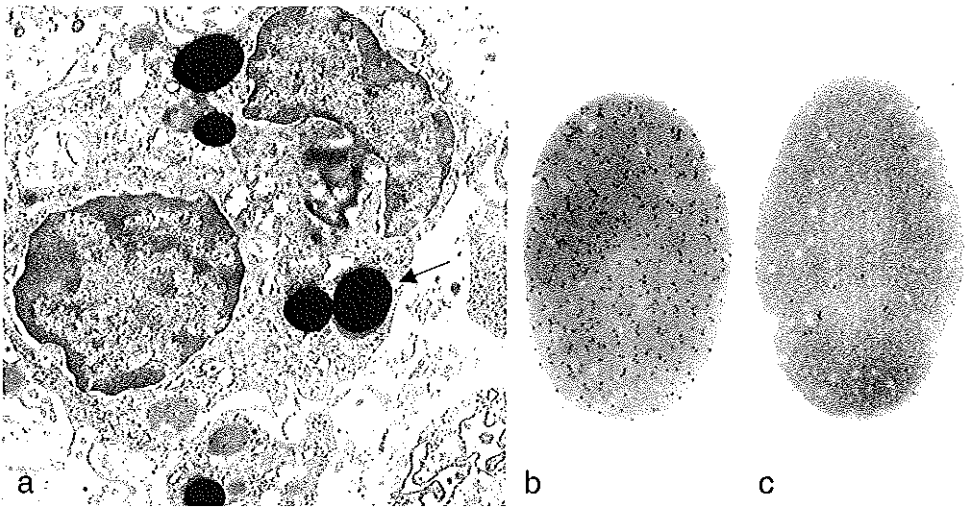


Figure 2. Mab 2E9 detects *S. epidermidis* peptidoglycan (a) Immuno-EM microscopy of *S. epidermidis* (arrow) intracellularly surviving in macrophages (b) Sections labeled with Mab 2E9 directed against PG-PS show high density labeling of coccoid structures. (c) In the sections exposed to the isotype control Mab NS7 labeling was very rare or absent similar to primary antibody omission control labeling (data not shown).

In the gram-negative cell wall, the PG covalently linked to lipoprotein molecules forms a unimolecular rigid layer between the outer membrane and the cytoplasmatic membrane of the cell envelope (figure 1). The N-acetylmuramic acid residues are linked to stem tetrapeptides composed of L-alanyl-D-isoglutamyl-(L)-mesodiaminopimelyl (L)-D-alanine. Approximately two thirds of these stem peptides are crosslinked through D-alanyl (D)-mesodiaminopimelate linkages.
On the outer part of gram-positive bacteria, the PG is covered by a polysaccharide layer covalently coupled to it, protecting the bacterial cell wall against enzymatic breakdown by

Figure 1. Composition and structure of gram-positive peptidoglycan. Peptidoglycan is a major component of the cell wall of gram-positive bacteria. Alternating N-acetyl glucosamine (G + GlcNAc) and N-acetyl muramic acid (M + MurNAc) are connected by peptide bonds resulting in a crosslinked network. These stem peptides and the interpeptide bridges [I] are variable between strains. Peptidoglycan can be degraded by N-acetylmuramyl-L-alanine amidase (NAMLAA) and Lysozym. NAMLAA hydrolyses the lactate bond between N-acetyl muramic acid (MurNAc) and the first amino acid of the peptidoglycan chain L-alanine and lysozyme hydrolyses the bond between MurNAc and GlucNAc.

lysozyme (figure 1). PG contains several structures that make it an unique molecule in biology, e.g. the occurrence of muramic acid, which is a glycosamine connected via an ether bridge with a lactate, the alternating sequence of L- and D-amino acids, and the linkage of a diamino acid to the γ-carboxyl group of glutamic acid. Because the presence of muramic acid can be analyzed with different techniques, it is a valuable clue for the measurement of PG (2,3). Furthermore PG can be detected with a monoclonal antibody (Mab 2E9) directed against PG-PS as shown in figure 2.

Degradation of PG

Degradation of PG in human tissues occurs by three different hydrolytic enzymes: lysozyme, N-acetylglucosaminidase and N-acetylmuramyl-L-alanine amidase (NAMLAA) (4). Lysozyme is able to cleave peptidoglycan sugar chains, producing monomeric muramyl peptides (5). N-acetylglucosaminidase is capable of hydrolyzing free N-acetylglucosamine groups from the non-reducing ends of the PG backbone (6). NAMLAA was shown to hydrolyze the lactamide bound which links MurNAc of the polysaccharide chains to L-alanine of the peptide side chain (7) (figure 1).

Lipopolysaccharide

Another molecule possessing biological properties similar to PG (table 1), which is present in the membrane of gram-negative bacteria, is lipopolysaccharide (LPS), a component of the outer lipid bilayer of gram-negative bacteria. LPS is composed of polysaccharide side chains attached to a basal core oligosaccharide, which in turn is covalently bound to a lipid moiety known as lipid A (8).

Table 1. Similarities and differences between LPS and PG

	LPS	PG
Gram-negative bacteria	+	+
Gram-positive bacteria	-	+++
CD14 receptor	+	+
LPS binding protein	+	-
Toll like receptors	+	+
Cytokine induction	+	+
T cell activation	+	+
B cell activation	+	+

CD14 receptor

Bacterial PG and LPS can interact with cells of the human body. It has been shown that both PG and LPS can bind to the CD14 receptor expressed by monocytes and granulocytes (9,10).

CD14 is a receptor which exists in two forms, the membrane bound form (mCD14), a glycosylphosphatidylinositol (GPI)-anchored glycoprotein present on myeloid cells, and a soluble form (sCD14) present in the circulation (11,12). sCD14 can act as a coligand for the activation of cells devoid of mCD14, such as endothelial and epithelial cells, astrocytes,

or vascular smooth muscle cells (13-16). Recently it has been shown that CD14 is involved in the regulation of apoptosis and apoptotic cell recognition (17) but its major function is the interaction with bacterial membrane molecules. PG is able to bind to CD14 (18,19) and activate cells through CD14 (20). In contrast to LPS, no binding protein is needed (21,22). PG induces CD14 dependent differential activation of the MAP kinases, ERK, p38 and JNK and of CREB/ATF and AP-1 and NK-kB transcription factors (9,20,23). Since CD14 is a GPI-anchored molecule that does not traverse the cell membrane, the mechanisms by which it confers PG responsiveness to cells has remained a long-standing question in the field. This question is not restricted to CD14 since it remains unknown how other GPI-anchored receptors mediate intracellular signaling (24). Most recently some members of the family of human Toll-like receptors (TLR), specifically TLR2 and TLR4, have been linked to CD14 activation (25-30). TLR represent a family of homologous proteins that are characterized by an extracellular leucine-rich domain and a cytoplasmic domain that is responsible for signal transduction. TLR play a crucial role in *Drosophila* and mammalian host defense (26,31,32). Recently it has been shown that the expression of human TLR2, but not TLR4, imparts PG responsiveness to otherwise PG-unresponsive cells. The expression of mCD14 greatly enhanced the responsiveness of the cells to LPS and PG, and CD14 was shown to interact with TLR2 to form the PG receptor (33,34). Therefore, it is hypothesized that binding of PG to a complex of CD14 with TLR2 results in activation of macrophages leading to an immune response (35,36).

Effects of PG on the immune system

PG possesses potent adjuvant properties (37,38) and is able to induce sleep (39). The effects involving the immune system depend strongly on the structure of PG. As described above, the structure of PG differs between bacterial species, explaining why not all effects are shared by all bacterial species. The minimal structure which is required for immunogenic stimulation is thought to be muramyl dipeptide (40). However, it has recently been shown that crosslinked stem peptides obtained after degradation of PG by NAMLAA can also induce TNF-α production in monocytes (41).

PG can activate complement (42), granulocytes (43) and upregulate adhesion molecules on endothelial cells (44). PG is able to induce polyclonal antibody formation in peripheral blood monuclear cells (PBMC) of healthy donors dependent on the presence of T lymphocytes and monocytes (45,46). In addition, it has been shown that PG is able to induce rheumatoid factor (RF) (47,48). IgM, IgA and IgG antibodies specific for PG have been found in sera of all humans (49,50).

A few studies on T cell activation by PG showed that T cells respond to PG only in the presence of monocytes, suggesting that uptake of PG by APC is necessary to elicit T cell responses (45,51). Furthermore, it has been shown that T cell lines specific for PG can induce a delayed type hypersensitivity (DTH) response in Lewis rats (52,53).

The best studied and major effect of PG is on monocytes/macrophages, which are antigen presenting cells of the immune system. PG is a potent inducer of the production of the pro-inflammatory cytokines TNF-α, IL-1 and IL-6 by human monocytes (54-56). The induction of these cytokines can be inhibited by blocking the CD14 receptor (18,56), confirming that binding of PG to CD14 is required for its biological activity. Furthermore it has been shown

that PG can induce production of IL-12p40 by murine macrophages (57).
These functions are all important for the induction of septic shock. It is therefore believed that PG is the inductor of sepsis by gram-positive bacteria, similarly as LPS has been shown to be for gram-negative bacterial sepsis (58,59). It is hypothesized that during a severe bacterial infection massive amounts of PG are released into the bloodstream resulting in pathophysiological reactions due to overstimulation of the immune system. These pathophysiological reactions include fever or hypothermia, hypotension, tachypnoe, multi-organ failure and irreversible shock (60).

Peptidoglycan associated with inflammatory diseases
Besides models in which PG causes septic shock (61) there are also models in which PG causes chronic inflammation. The *in vivo* studies on chronic inflammation have mostly been performed using peptidoglycan-polysaccharides (PG-PS) in which also polysaccharides chains like rhamnose are present. These animal models show that PG-PS can induce multisystemic disease involving injury in liver, spleen, intestines, skin, eyes, heart and bone marrow (62-64), but arthritis is the most extensively studied PG-induced disease.
PG from different sources can induce chronic inflammatory arthritis in susceptible rat and mouse strains (65,66). PG-PS isolated from group A streptococci induces chronic erosive polyarthritis characterized by spontaneous remissions and exacerbations in inbred Lewis rats (67). The chronic features persist for at least 10 months, during which time two to five spontaneous reactivations of inflammation occur. The phenomenon of spontaneous reactivation is important because it is an unique feature of PG-PS-induced inflammation as compared with other agents which cause acute inflammation only. It was also shown that PG is able to reactivate arthritis induced by other agents like superantigen, intraarticular injection of PDGF, IL-1, TNF-α or the creation of small-bowel bacterial overgrowth (68-72). It has been hypothesized that PG-PS accumulates in the liver and that it is released from the liver and redistributed to other tissues including the joint, thus accounting for the reactivation of arthritis (73). Because PG-PS found in the joints was smaller compared to that found in the liver, it is postulated that the liver is able to degrade PG-PS (74). Total degradation of PG by enzymes as lysozyme (75) and NAMLAA (76) inhibits arthritis induction which supports the *in vitro* data that the structure of PG is important .
The induction of arthritis in animal models is probably dependent on the persistence of PG in tissues. After intraperitoneal injection in rats, PG-PS is localized in liver, spleen, lymph nodes and joints (74,77,78). In humans, PG has been detected in liver and spleen (74,79,80), synovial tissues of arthritis patients (81), urine (82), synovial fluid of septic arthritis patients (83,84) and circulating peripheral blood leukocytes (85,86) using different techniques. The latter suggests that circulation of PG also occurs in healthy humans.
Muramic acid has also been detected in brain tissue of conventional rats and it has been shown that PG and different muramyl peptides are able to induce leukocytosis, blood brain barrier permeability and brain edema (87-89).
In this thesis the role of PG in RA and MS is studied. Therefore the pathogenesis of these two chronic inflammatory diseases is discussed below.

RHEUMATOID ARTHRITIS

Rheumatoid arthritis is a chronic and systemic inflammatory joint disease which affects 1-2% of the population world-wide. The primary manifestations are pain, swelling, and limited mobility of joints. Some patients may experience a mild illness, but in the majority of the patients the disease leads to joint destruction, deformity, and disability. The signs and symptoms are not restricted to the joints; a variety of extra-articular manifestations may occur: subcutaneous nodules, vasculitis, pericarditis, pulmonary nodules or intestinal fibrosis, mononeuritis multiplex and scleritis.

The aetiology of RA is still unknown but it is generally accepted that immune-mediated mechanisms are of crucial importance. In RA, the synovial membrane is characterized by a thickened lining layer which consists mainly of type A macrophage-like synoviocytes and type B fibroblast-like synoviocytes, and the infiltration of macrophages, T cells, plasma cells, and to a lesser extent B cells, fibroblasts and dendritic cells into the synovial sublining, forming a pannus. The role of these different cell types present in RA is discussed below.

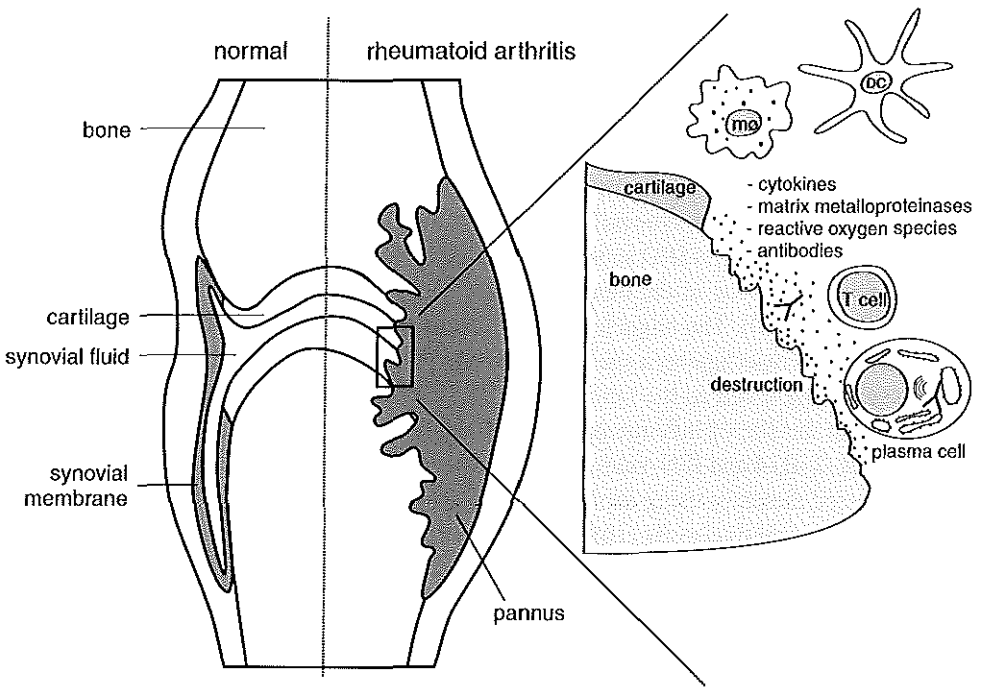


Figure 3. Diagrammatic representation of rheumatoid arthritis in the joint. Pannus forming is mediated by infiltration of T cells, macrophages, dendritic cells and plasma cells. The release of inflammatory mediators like cytokines, matrix metalloproteinases, reactive oxygen species and antibodies lead to destruction of cartilage and bone.

Cells involved in the pathogenesis of rheumatoid arthritis

Resident cells

The macrophage-like synoviocytes, which are particularly found in the more superficial parts of the lining layer (90), are probably bone marrow derived monocytes migrating to the lining layer in response to chemotactic factors (91). The fibroblast-like synoviocytes, which are peculiar to synovium, appear to be of fibroblast origin. Both macrophage-like synoviocytes and fibroblast-like synoviocytes appear highly activated, based on their morphology, expression of HLA-DR and capability to secrete proinflammatory cytokines. These cells may be involved in scavenging of debris released from articular structures and elimination of microorganisms entering the joints via the bloodstream or directly after traumatic events. In arthritis these functions may be involved in the inflammatory process either by a direct contribution through inflammatory mediators or by carrying microorganisms or other antigenic material into the joints (92).

T cells

T cells, in particular CD4+ T cells, are believed to play an important role in the pathogenesis of RA. The association of susceptibility to RA and disease outcome with HLA-DR4/DR1 antigens (93,94) has been regarded as the strongest argument for the T cell-dependent nature of the disease, since the only known function of the major histocompatibility complex (MHC) molecules is to present antigenic peptides to T cells via ligation of their T cell receptor (TCR). Another argument for the role of T cells in RA is the phenotypic evidence of activation displayed by synovial tissue-infiltrating T cells. These T cells predominantly consist of the primed CD45RO subset, expressing early and late activation antigens (95-97) and molecules involved in the interaction between T cells and APC, like CD28, CD40L and CTLA4 (98-100). The oligoclonality of T cells found in the joints of RA patients suggests that T cells react against a specific antigen in RA (101-103). However, several findings argue against the T cell as the initiator of RA. First the hyporesponsive state of the T cell population at the site of inflammation (104,105), second the fact that T cell-derived cytokines like IL-2, IL-4 and IFN-γ can hardly be detected in RA joints (106,107), and third that T cell-directed therapies using depleting anti-CD4 Mabs, and IL-2-receptor immunotoxins have been disappointing so far (108-110).

B cells

Although B cells and plasma cells are not the most prominent cell types present in the synovial infiltrate, evidence has been presented for the involvement of humoral immune mechanisms in the pathophysiology of RA (111). B cells in all stages of differentiation from mature B cells to plasma cells have been detected in the joints of RA patients (111-114) and there is some molecular evidence that B cells are specifically activated and clonally expanded in the synovium (115-117). Within the synovium organized structures can occur resembling germinal centers, which contain follicular dendritic cells and CD4+ T cells and reveal clonal expansion of B cells exhibiting isotype switching and somatic hypermutation (114,116,118,119). Other evidence for the involvement of B cells in RA is the presence of RF in serum (120) and joint fluid in the major part of the RA patients (112). RF repre-

sents an IgM, IgA or IgG antibody reacting with antigenic epitopes in the CH2 and CH3 domains of IgG (121). IgM RF reaction with autologous IgG has been demonstrated to activate the complement system and to stimulate B cell activation (121,122), but may also serve to focus or aggregate antigens or APC and in that way function as an immune response stimulator (123,124). Furthermore, it was shown recently that a T cell receptor transgenic mouse model that spontaneously develops a disease with most of the clinical, histological, and immunological features of RA, critically depends on the presence of B cells and autoantibody production. The specificity of these autoantibodies, however, is still unkown (125).

Antigen presenting cells

Infiltrating APC are also believed to be of crucial importance in the pathogenesis of RA. Peripheral blood monocytes infiltrate into the synovial membrane and mature into macrophages or dendritic cells which are both enriched in the rheumatoid synovium and are also found within the destructive pannus tissue (126,127). In addition, macrophages produce large amounts of cytokines such as IL-1, IL-6 and TNF-α (128) as measured in synovial tissue and fluid, while peripheral blood monocytes show increased phagocytic activity (129) in RA patients. APC are highly activated in RA patients as shown by the expression of costimulatory activation markers, such as B7-1 (CD80), B7-2 (CD86) and CD40 in the synovium (100,130,131). Furthermore, therapies blocking TNF-α, a cytokine produced in large amounts by macrophages in RA have beneficial effects (132,133). The development of spontaneous arthritis in human TNF-α overexpressing mice is a strong indication for the important role of TNF-α in the pathogenesis of RA (134).

Activation of APC contributing to the autoimmune events in RA occur probably by phagocytosis of antigens, although the crucial antigen(s) are not defined yet. Such activation of the phagocytezed antigens results in the production of inflammatory mediators and presentation to T cells leading to T cell proliferation. Antigens can be picked up in the synovium but it is also possible that they are phagocytized by APC elsewhere in the body, for instance in the lymph nodes or at the mucosa and that such macrophages then migrate to the synovium leading to local pathology (135,136). A relation between the gut and synovial tissues has been shown by mucosal macrophages, which preferably migrate to joints by binding to high endothelial venules with P-selectin (137).

Mediators of inflammation and tissue destruction

Although the cell types initiating the inflammatory reaction are not defined yet, our understanding of the mechanisms involved in the pathogenesis of RA has recently substantially increased. The severity and progression of synovitis depend on the local accumulation and activation of cells that release cytokines, which may regulate growth, differentiation and activity of other cells participating in inflammatory and immunological reactions of the rheumatic joint (138,139).

Proinflammatory cytokines

Cytokines can be divided into three groups, the proinflammatory, immunoregulatory and chemotactic cytokines. As reviewed by Feldmann (128) and shown in table 2, cytokines of all three subtypes can be found in the synovium of RA patients. The proinflammatory

cytokines are mainly produced by macrophages and monocytes and can function as growth factors for T, B and NK cells, activate other macrophages, neutrophils and fibroblasts and stimulate the production of other cytokines and proteinases (140-144). These cytokines such as IL-1, IL-6 and TNF-α also have direct effects on cartilage and bone leading to destruction of the joints (145-147). Cartilage destruction can be induced by cytokines by inhibition of new matrix synthesis (148,149) and proteoglycan synthesis (150), activation of chondrocytes (148,151) and activation of plasminogen activator (152) leading to conversion of plasminogen into plasmin (153,154). In addition, bone destruction was induced by induction of prostaglandin production, inhibition of collagen synthesis and induction of alkaline phosphatase activity (143,153,155-159). These *in vitro* studies are supported by studies in animal models where IL-1, IL-6 and TNF-α have been shown to cause destruction of bone and cartilage (134,160-163).

Table 2. Cytokine expression in synovial tissue and synovial fluid of RA patients

Proinflammatory cytokines		Immunoregulatory cytokines		Chemotactic cytokines	
IL-1α/β	++++*	IL-10	+++	IL-8	+++
IL-2	+/-	IL-11	++	GRO-α	+
IL-6	+++	IL-13	+	MIP-1α/1β	+
IL-12	++	TGF-β	+++	MCP-1	+
IL-15	++	IL-16	+	ENA-78	+
IL-17	+			RANTES	+
IL-18	+				
IFN-α/β/γ	+/–				
LIF	+				
LT	+				
GM-CSF	+				
M-CSF	+				
TNF-α	++++				

*relative amounts

Data obtained from: ref 128,166,171,328-332

Immunoregulatory cytokines

Immunoregulatory cytokines such as IL-10 have mainly inhibitory effects on the inflammatory process. It has been shown that IL-10, produced by T/B cells and macrophages, inhibits T cell proliferation and TNF-α and IL-1 production (164). Treatment with IL-10 has shown beneficial effects on the pathogenesis of RA (165). Recently it was shown that IL-11 is also able to inhibit inflammation by downregulating TNF-α and matrix metalloproteinase (MMP) 1 and 3 (166). Besides inhibitory effects these cytokines also have proinflammatory activity such as stimulation of B cells (167), which may account for the production of RF.

Chemokines

Next to their role in the destruction of bone and cartilage, IL-1 and TNF-α can induce the production of IL-8 (168), one of the most important chemokines in RA able to attract T cells and mediate angiogenesis, an important early feature of pannus formation (169,170). Other chemokines present in RA are GRO-a, ENA-78, MIPs and RANTES. The cellular source of these chemokines appeared to be mononuclear and polymorphonuclear cells infiltrating the synovial lining as well as synovial fibroblasts and mononuclear cells found in synovial fluid. These chemokines account for the attraction of monocytes and T cells into the joint (171-173). Neutralization of chemokine activity leads to reduction of joint swelling in animal models (174,175).

T cell derived cytokines

In rheumatoid synovium there are only low levels of the T cell derived cytokines IFN-γ, IL-2 (proinflammatory) and IL-4 and IL-5 (immunoregulatory). Only IL-15 (proinflammatory) is relatively abundant. Cloning studies have showed that the majority of the T cells were Th1 like producing IL-2 and IFN-γ (176,177).

Oxygen species and matrix metalloproteinases

Other mediators involved in the pathogenesis of RA are reactive oxygen species (ROS) and MMP, which can be both induced by TNF-α (178). ROS, among which NO, has been shown to be increased in RA (179). It is involved in RA by degrading the proteoglycans in the cartilage (180-182), by cytotoxic mechanisms of activated macrophages, chemotaxis of polymorphonuclear leukocytes and inhibition of iron-sulfur-centered enzymes (179).
MMP are produced by leukocytes, chondroblasts and fibroblasts (183,184). They play an important role in connective tissue destruction by degrading components of the extracellular matrix (168,185). In RA, large amounts of MMP have been detected in several different types of rheumatoid synovial cells, especially in perivascular infiltrates (168,183,185-188).

Candidate antigens involved in the pathogenesis of rheumatoid arthritis

Collagen

Although a trigger causing the activation of T and B cells or macrophages has not been defined yet, different candidate (auto)antigens have been suggested. Several intra-articular components such as type II collagen and proteoglycan are considered as potential RA specific autoantigens (189,190). Considering the involvement of peripheral joints in RA, immune reactivity to type II collagen (CII) has been extensively investigated since this protein constitutes a major part of joint cartilage and mainly occurs in this tissue. Evidence for the involvement for collagen in RA is found in the detection of antibodies to CII in the serum and synovial fluid of RA patients (191-193). Furthermore, collagen I and collagen II could be recognized by T cell clones isolated from synovial fluid of RA patients (189,194,195). Relevant in this context is also the observation that in rat and mouse strains arthritis can be induced by CII (196). In such models T cells have been shown to play an important role (197,198). Although oral tolerance has been proven to be effective in collagen-induced experimental animal models of autoimmune diseases (199), attempts to

orally tolerize RA patients for collagen have not shown convincing evidence for a beneficial effect so far (200,201).

68kd antigen, P205 and HC-gp39

Recently, three different autoantigens have been described as candidate antigens in RA. First, the ubiquitously expressed 68k autoantigen (thought to be a glycoprotein) (202) has been shown to be a major target for self-reactive antibodies in RA. Autoreactive T cells specific for this antigen could be identified in 19 of 27 RA patients (202). Second, a P205 antigen has been suggested to be a important molecule in RA. P205, which is identical to an immunoglobulin sequence located within a domain that is reactive with RF, appears to be a major target of autoreactive T cells in RA. P205-primed T cells were more abundant in the synovial fluid than in the peripheral blood (203). The third antigen is human cartilage glycoprotein 39 (HC-gp39). HC-gp39 is not expressed in normal cartilage tissues but is detected in cartilage of RA patients. Peptides from HC-gp39 binding to the HLA-DR4 (DRB1*0401) molecule were found to be T cell targets in RA patients, as well as to be arthritogenic in certain BALB/c mouse substrains (204,205).

Heat shock protein

Other antigens possibly involved in RA are the heat shock proteins (HSP). HSP are a family of proteins of which the expression is increased by a number of stress stimuli. They show a high level of sequence homology throughout phylogeny (206,207) and are immunodominant in the humoral response to a number of infectious organisms (208). The 65kD HSP of *Mycobacterium tuberculosis* is of particular interest, since it has been implicated in the pathogenesis of an arthritis model in rats (209) and the disease can be transferred by a T cell clone specific for HSP 65 (210). This has raised the possibility of molecular mimicry as a mechanism for tolerance breakdown (210-212), in which immune responses against an HSP of an infective organism could elicit a cross-reactive autoimmune response to self-protein that shares the same antigenic epitope. Although increased expression of endogenous HSP 60 in RA synovia (213) and T cell reactivity to bacterial and human HSP supported a pathogenic role of HSP 60 in RA, the finding that proliferative responses against HSP correlated with better disease prognosis (214,215) supports a protective role of HSP specific T cells in RA (216).

Infectious agents

A role of infectious agents have also been hypothezised in the etiology of RA. One of the suspects for such an agent is a virus, because viral infections such as rubella, human parvovirus B19, cytomegalovirus, human T cell leukemia virus 1, Epstein-Barr virus and HIV-1 have all been shown to be associated with an acute onset of polyarthritis (217-223).

In addition to viruses an important role for bacteria has been implicated in RA. In other forms of RA like septic arthritis and reactive arthritis the role of bacterial antigens has been confirmed. In reactive arthritis, the triggering antigens are thought to be microbes that cause infections of the gut or urogenital tract, such as *Yersinia* and *Salmonella* species or *Chlamydia trachomatis* (224). Antigens derived from these bacteria have been detected in synovial cells (225,226), and T cells specific for these antigens have been found in the

synovium (227-231). Indications for a microbial cause in RA are the presence of bacterial antigens in synovial tissues (232-234) and the observation that diseases related to intestinal bacteria and intestinal diseases (Crohn's disease, ulcerative colitis, jejunal bypass syndrome) are often accompanied by attacks of joint inflammation (235). Furthermore, an immunological link has been shown between the mucosa and the joint by the fact that mucosal leukocytes preferably home to the joints (137,236-238).
Several authors hypothesized therefore that bacterial fragments, derived from the intestinal flora, may pass the bowel wall, are distributed over the body and cause a local and/or systemic reaction leading to arthritis (239-242). Evidence for this hypothesis has come mainly from animal studies. For instance, cell wall fragments from several human anaerobic intestinal gram-positive bacteria induced severe chronic arthritis when injected intraperitoneally in rats (243-246). In addition, PG-PS isolated from ileostomy effluent induced a severe chronic arthritis in rats (247) and a T cell line specific for PG-PS was also able to induce arthritis (248). Systemic absorption of bacterial cell wall polymers has been demonstrated after feeding and after intracecal injection of streptoccocal PG-PS (249,250). In the serum of rats with a jejunal bypass, an increased titer of anti-PG-PS antibodies was found, indicating that a higher intraluminal bacterial load resulted in an increased uptake of PG-PS by an intact intestine (251). In RA patients differences in the anaerobic flora have been found and a vegan diet has proved to be benefical in RA patients indicating a role of the intestinal flora (252,253). The intestinal permeability is higher in RA patients compared to healthy controls and recently it has been shown that RA patients have ultrastructural bowel lesions (254), but this might also be due to the effects of non-steroidal anti-inflammatory drugs (NSAID) intake (255,256). The beneficial effect of antibiotic agents like minocycline in RA patients (257-259) is also supportive for a microbial cause of RA, although it is suggested that these effects are due to the inhibition of MMP production (260,261).

MULTIPLE SCLEROSIS

Multiple sclerosis is a chronic inflammatory disease like rheumatoid arthritis but affects the central nervous system (CNS) instead of the joints. The incidence in North-America and Europe ranges from 0.1%-0.2%. Different forms of MS occur, the relapsing-remitting form being the most prominent one. This form of MS mostly develops between the ages of 18 and 50 and affects about twofold more females than males. Relapsing-remitting MS begins as a relapsing illness with episodes of neurological dysfunction lasting several weeks, followed by substantial or complete improvement. With the exception of uveitis, pathological findings are confined to the CNS, with prominent involvement of the optic nerves, periventricular white matter, brain stem and spinal cord. The lesion of MS is primarily inflammatory and demyelinating although axonal loss and scarring may be important in the development of irreversible disability. Early in the development of MS lesions, lymphocytes and macrophages can be demonstrated in the perivascular inflammatory cuff, i.e. cells located between the endothelial cells and the basal lamina of blood vessels. When the blood-brain-barrier becomes defective, monocytes derived from the blood circulation and T cells invade the brain parenchyma forming an inflammatory plaque (262).

In MS four kinds of plaques can be distinguished depending on the presence of only inflammatory cells (inflammatory) or myelin degradation products (demyelinating) or both (inflammatory and demyelinating) or without both signs (inactive) (263). These four types can also be distinguished in acute and chronic plaques. Acute plaques are not clearly demarcated and they contain T cells, B cells and macrophages and myelin fragments which is indicative for demyelination activity. Remyelination and oligodendrocyte proliferation are observed in the majority of acute plaques. Chronic plaques contain hypertrophic astrocytes, microglia and plasma cells, producing antibodies of unknown specificity. Although the cause of MS is still unknown, it seems likely that the disease is the result of an interplay between certain susceptibility genes and environmental factors. Evidence for the genetic influence in MS are the fact that 26% of monozygotic twins were both affected with MS in contrast to only 2.3% of dizygotic twins (264,265). The association of inflammatory cells with new MS lesions implicates an association of the immune system in active tissue destruction. The possible role of mononuclear cell types in these lesions are discussed below.

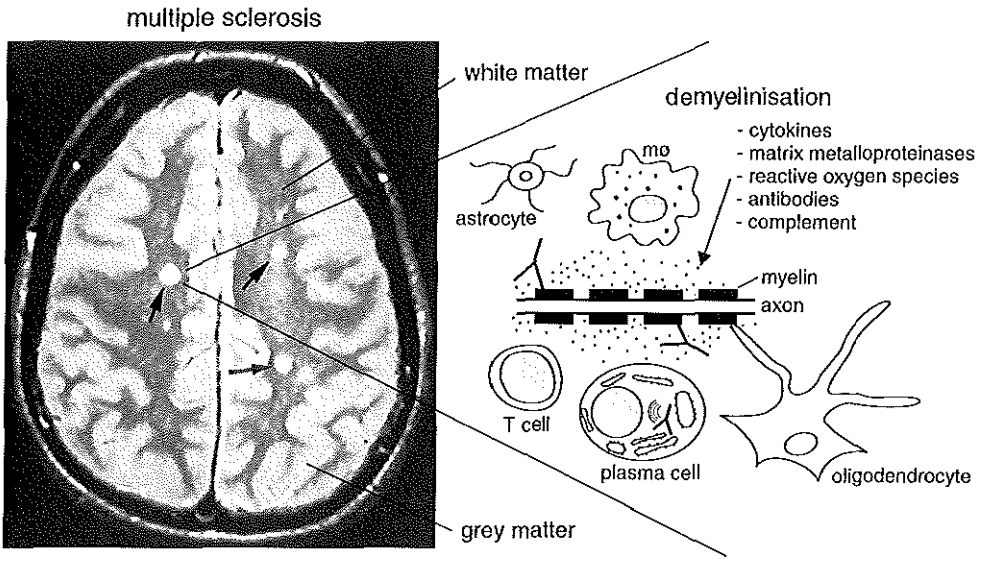


Figure 4. Lesion formation in the central nervous system in multiple sclerosis. Astrocytes, oligodendrocytes and infiltrating T cells, macrophages and plasma cells are involved in the formation of lesions (arrows) in the central nervous system. The release of cytokines, matrix metalloproteinases, oxygen species and antibodies by these cells promote demyelination of nervous tissue.

Cells involved in the pathogenesis of multiple sclerosis

T cells

T cells are thought to play an important role in the pathogenesis of MS. The major hypothesis is that activated autoreactive Th1 cells directed against CNS antigens attack normal brain tissue. Especially CD4+ T cells are present in high numbers at the initial perivas-

cular sites involved in plaque formation (266,267). Most studies provide evidence for an enhanced state of activation of these Th cells (268-270). Like in other inflammatory diseases, costimulatory molecules like CD40L, CD28 and CTLA4 are present on the T cells in MS (268). The concept that MS is a T cell mediated disease is strongly based on studies using animal models. Experimental autoimmune encephalomyelits (EAE) can be induced in susceptible rodents and monkeys by an emulsion that consists of whole spinal cord, purified myelin or specific myelin proteins like myelin basic protein (MBP) or proteolipid protein (PLP) emulsified in an adjuvant like Freund complete adjuvant with killed *Mycobacterium tuberculosis*. The disease can be transferred using CD4+ T cells from animals sensitized against CNS antigens indicating that EAE is a Th cell mediated disease (271). It has to be noted that in many respects acute EAE differs significantly from MS. First, in rodent EAE demyelination hardly occurs in contrast to MS and secondly, most EAE models are in general monophasic diseases while MS follows a relapsing-remitting pattern with cumulative neurologic damage over time.

B cells

There is circumstantial evidence that B cells are also involved in the pathogenesis of MS. Persistent oligoclonal IgG synthesis in the cerebrospinal fluid (CSF) and a high antibody specificity index, which is the CSF/serum quotient of specific antibodies and total IgG level (272), are indicative for sustained B-cell activation within the CNS of MS patients. In both CSF and sera of MS patients antibodies have been detected directed against components of brain tissue (273) and a variety of putative CNS antigens like cerebellar soluble lectin (274), MBP, PLP (275), and myelin associated glycoprotein (276).

Antigen presenting cells

APC are thought to be responsible for most of the damage occurring during relapses of MS (277). Next to cytotoxicity of T cells and antibodies, macrophages are responsible for removal of myelin through phagocytosis. These macrophages can be microglia, the resident macrophages of the CNS or monocytes derived from the blood (278). Demyelination in MS may well be caused by destruction of oligodendrocytes. The involvement of macrophages in EAE has been shown by depleting or inhibiting the recruitment of blood-borne macrophages, which resulted in a strong decrease of clinical signs (279-282).

Mediators of inflammation and tissue destruction

Although the focus in MS research has been on the T cell and its interaction with CNS structures, recently the importance of non-specific inflammatory mechanisms, in particular cytokines and chemokines and induced mediators including proteinases, has come to the forefront (277,283). Multiple mechanisms have been proposed for oligodendrocyte destruction including MOG antibodies (284), activation of death signals (285,286) and cytokines or cytotoxicity (287-289). In vitro studies have implicated the free radical NO in oligodendrocyte death (289,290).

Although cytokines are important in the initiation, propagation and regulation of immune and inflammatory responses, overexpression of these proteins can also result in tissue damage. Different cytokines like IL-1, IL-4, IL-6, IL-8, IL-10, IL-12, IFN-γ, GM-CSF and

TNF-α are abundantly expressed in MS (291,292). TNF-α and TNF-β can damage oligodendrocytes (293) by inducing apoptosis of these cells (294). The importance of proinflammatory cytokine production in MS pathology has been shown by the beneficial effects on disease activity by IFN-α therapy which leads to decreased production of IFN-γ and TNF-α (295). MMP are also expressed in MS tissue and CSF (296,297). MMP are able to degrade the basement membrane and the extracellular matrix and promote extravasation of leukocytes (298), but they also have the capacity within the CNS to cleave MBP into fragments retaining encephalitogenicity (299-301). This implies that MMP can contribute to alterations in the permeability of the blood-brain barrier, enzymatic demyelination, induction of neuronal loss and generation of modified peptides contributing to autoimmunity (299,300).

Candidate antigens involved in the pathogenesis of multiple sclerosis

Autoantigens

A candidate target for the autoreactivity in MS is MBP, the major component of the myelin sheath (302), but other antigens including PLP (303), myelin-associated glycoprotein (MAG) (276), myelin oligodendrocyte glycoprotein (MOG) (304), the HSP α-B-crystallin (305), and the oligodendroglial enzyme transaldolase (TAL) (B10) have also been proposed. MBP is the most studied of all these antigens. MBP-specific T cells have been detected in the sera and CSF of MS patients (306). It was shown that the MBP epitope sequence 154-172 was recognized by T cell clones derived from MS patients. In addition the overlapping epitope sequence 149-171 is able to induce CNS demyelination in rhesus monkeys. α-B-crystallin, generally considered as a HSP has been shown to be an important immunodominant T cell antigen in MS-affected myelin. Immunohistochemical examination of white matter sections containing active MS lesions revealed intense expression of α-B-crystallin in both oligodendrocytes and astrocytes within and adjacent to the lesion (307). The exact role of these T cells specific for autoantigens in MS remains elusive because autoreactive T cells against myelin components are also found in healthy donors (308).

Infectious agents

Autoreactive cells can be activated by cross-reactive epitopes of pathogenic agents like bacteria and viruses. This phenomenon, sharing of antigenic determinants, is called molecular mimicry. Because of the relationship between relapses and viral infections, researchers have considered that viruses may cause MS (309,310). Viruses isolated from MS brain are multiple sclerosis-associated retrovirus (311), and human herpes virus 6 (HHV-6). The latter could be detected in neuroglial cells in active lesions (312-315). Increased HHV-6 IgM titers in and HHV-6 DNA identified in the serum of MS patients indicate recent active viral infection. Finally, it has been postulated that MS might be caused by an initial infection with a common MS-retrovirus followed by an infection with the Epstein-Barr virus in early adulthood or later in life (316). The molecular similarity between virus and myelin antigens may enable immunological cross-reactivity between HHV-6 and myelin antigens. In this model, T cells become activated by exposure to virus, cross the blood-brain barrier and, in genetically predisposed persons, recognize normal myelin antigens as virus resulting in tissue injury (305,317-319). However, the role of viruses in the pathogenesis of MS remains

elusive because recently HHV-6 has also been detected in control brain tissue (318-320).
The role of bacteria in the pathogenesis of MS has not been studied extensively. Recently *Chlamydia trachomatis* and antibodies against *Chlamydia trachomatis* have been detected in CSF of MS patients (321), suggesting a role in the pathogenesis of MS. Although the brain parenchyma is more resistant to acute inflammation compared to other human tissues, injection of LPS has been shown to result in upregulation of adhesion molecules and leukocyte recruitment in the mouse brain (322). Stronger reactions have been observed after inoculation with BCG (*Bacillus Calmette-Guérin*), an attenuated strain of *Mycobacterium bovis*, which after peripheral sensitization resulted in a rapid acute inflammatory response mediated by neutrophils and monocytes followed by an inflammatory lesion consisting of T cells and macrophages at the site of inoculation (323-326). Furthermore, it has been show that LPS is able to enhance the disease severity in virus induced MS models, for example Theiller's murine encephalomyelitis virus induced demyelinating disease (327).

SUMMARY

In summary, RA and MS are both chronic inflammatory diseases with unknown etiology. Bacterial PG has been shown to possess all the biological properties needed to induce inflammation. In this thesis the role of PG in MS but especially in RA has been investigated in detail.

INTRODUCTION INTO THE EXPERIMENTAL WORK

The purpose of the studies described in this thesis was to analyze whether PG is involved in the pathogenesis of the chronic inflammatory diseases RA and MS.
PG has many inflammatory properties such as inducing cytokine production and activation of B cells and T cells. This was studied using PG isolated from bacterial cultures. The presence of PG in different tissues including diseased but also normal tissues has been shown using different methods. One of these methods included the isolation of PG from normal human spleen (80).
In chapter 2 we investigated whether PG present in human tissues, which may be exposed to several PG-degrading enzymes, still has the same biological activities as PG isolated from bacterial cultures. The method used to isolate PG (80) from human spleen tissue was improved to obtain a pure PG fraction suitable for *in vitro* experiments (chapter 2.1).
To analyze whether PG present in human tissues is biologically active, its capability to induce cytokine production was determined (chapter 2.1). Previously, it has been shown that PG induces activation of T cells dependent on monocytes (51), but the relevant mechanisms of T cell activation have not been identified yet. In chapter 2.2 we investigated whether PG isolated from human spleen is able to induce T cell proliferation and in addition whether CD14, the receptor for PG, and antigen processing or presentation are involved.
The presence of PG in synovial tissues and the ability of PG to induce arthritis in rats indicate that PG may be involved in the pathogenesis of RA. Whether immune reactivity to PG

is disturbed in RA patients was investigated in chapter 3. To establish whether antibodies against PG-PS might be involved in protection against RA or conversely in pathological events related to RA, local and systemic levels of antibodies against PG-PS were analyzed in serum and synovial fluid samples (chapter 3.1). Chapter 3.2 presents a study performed to determine disturbances in T cell proliferation against PG and the ability of PG to induce cytokines which are thought to be of crucial importance in RA-related joint pathology.
The biological activity of PG was extensively studied using *in vitro* experiments. However, lymphoid cell functions and interactions analyzed *in vitro* do not always reflect the *in vivo* situation. The immunohistochemical *in situ* approach allows the study of *in vivo* events at a given moment in time. Whether PG is present in synovial tissues and whether these cells are active antigen presenting cells and are able to induce cytokines can therefore be determined using immunohistochemistry as presented in chapter 4. To study costimulatory molecules and cytokines *in situ*, cryosections have to be used. These sections maintain antigenicity but morphology is often poor. In 4.1 an alternative fixation method for cryosections is described which improves the morphology without affecting the antigenicity of the tissues. A Mab, called 2E9, was used to detect PG in such treated tissue sections. 2E9 is a murine Mab (IgG3) raised against a pure fraction of PG-PS isolated from normal human feces. With this Mab 2E9 we were able to study the presence of PG in RA tissues and its involvement in the inflammatory processes (chapter 4.2).
Because PG is also expressed in tissues other than RA synovial tissues, e.g. human spleen, it can be hypothesized that PG can also play a role in other chronic inflammations besides RA. Therfore we examined whether PG is present in healthy brain and MS brain tissues. We also investigated whether PG might be able to contribute to the pathological lesions observed in MS brain (chapter 4.3). The implications of these findings are discussed in chapter 5 together with suggestions for further research.

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Chapter

2

PROPERTIES OF BACTERIAL PEPTIDOGLYCAN PRESENT IN HUMAN SPLEEN

Chapter 2.1

Bacterial peptidoglycan-polysaccharides in sterile human spleen induce proinflammatory cytokine production by human blood cells
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Chapter 2.2

Bacterial peptidoglycan from human spleen elicits proliferation of CD4+ and CD8+ αβTCR+ T cells after proteolytic processing
Submitted for publication

CHAPTER 2.1

Bacterial peptidoglycan-polysaccharides present in sterile human spleen induce proinflammatory cytokine production by human blood cells

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ABSTRACT

Peptidoglycan (PG) is the major component of the cell wall of gram-positive bacteria. *In vitro*, PG isolated from conventional bacterial cultures can induce secretion of proinflammatory cytokines by human monocytes, indicating that PG may be involved in immune responses against infections by gram-positive bacteria. To investigate the biological activity of PG present in human tissues, an improved method was developed to isolate significant amounts of PG from sterile human spleen tissue. Biochemical analysis demonstrated that PG isolated from human spleen is largely intact. Human whole blood cell cultures were able to produce the proinflammatory cytokines TNF-α, IL-1 and IL-6 after stimulation with PG isolated from human spleen. Cytokine induction was not sensitive to inhibition by polymyxin B in contrast to lipopolysaccharide. Collectively, the data show that intact PG present in sterile human tissue is biologically active and may induce local proinflammatory cytokine production.

INTRODUCTION

Peptidoglycan (PG) is the major component of the cell wall of gram-positive bacteria. PG is composed of alternating N-acetyl glycosamine (GlcNAc) and N-acetyl muramic acid (MurNAc) long sugar chains, that are interlinked by peptide bridges resulting in a large complex macromolecular structure (1). During bacterial infection, PG and several other cell wall components are thought to be involved in the inflammatory reaction (2). In gram-negative infections, lipopolysaccharide (LPS) is a well-known activator of the innate immune system (3,4). During gram-positive infections, when no endotoxin is present, PG is able to activate complement (5,6) and granulocytes (7,8), and also to upregulate expression of adhesion molecules on endothelial cells (8). Of importance, PG can also induce produc-

tion of proinflammatory cytokines such as IL-1, IL-6 and TNF-α by monocytes *in vitro* (9,10). Similar to LPS-induced cytokine production, inhibition can be achieved by preventing uptake of PG and/or activation of macrophages and monocytes by blocking the CD14 receptor (11,12).

In addition to the role of PG in gram-positive infections it has been hypothesized that PG is involved in the pathogenesis of chronic inflammation such as rheumatoid arthritis (RA) (13-15). We are interested in the relationship between PG derived from the normal intestinal flora and the pathogenesis of arthritis (16,17). The presence of PG in relevant tissues is a prerequisite for this relationship. Most studies investigating the presence of PG in human tissues focus on the detection of muramic acid, the characteristic aminosugar in the glycan backbone of PG which can be detected after release of the N-acetyl group of MurNac by acid hydrolysis. Furthermore, radiolabeled PG has been used to study the localization of PG *in vivo*. With these different techniques PG was detected in liver and spleen (18,19), synovial tissues (18,19), urine (20), synovial fluid (21) and peripheral blood leukocytes (22). We detected PG in dendritic cells and macrophages of normal human spleen and synovial tissues of RA patients (23) by use of a monoclonal antibody (Mab) directed against bacterial flora-derived PG isolated from human feces (24). We also isolated PG from normal human spleen using biochemical methods (25).

The presence of PG in human tissues other than the gastro-intestinal tract suggests that our body is constantly exposed to bacterial products, even in the absence of clinically apparent infection. PG present in human tissues is probably derived from the normal gut flora. Experiments in rats have shown that PG can be absorbed from the bowel wall (26).
All studies performed to determine the biological activity of PG have focused on PG isolated from bacterial cultures. Thus far the biological properties of PG present in human tissues have never been studied. Therefore, the aim of the study was to determine whether PG present in sterile human tissues has the capacity to induce production of proinflammatory cytokines by human peripheral blood mononuclear cells. To this end, we developed an improved method to isolate high amounts of structurally intact PG with minimal traces of contaminating protein from sterile human spleen tissue. This PG was subsequently demonstrated to induce release of the proinflammatory cytokines TNF-α, IL-1 and IL-6 by human whole blood cell cultures. Biological activity of this PG fraction was determined by analysis of its ability to induce production of proinflammatory cytokines by human blood cells.

MATERIALS AND METHODS

Spleen used for isolation of PG
Seven unfixed human spleens were obtained from the pathology department immediately after surgery (SSDZ, Delft, The Netherlands) and kept frozen (-20°C) until use. Spleens were removed for surgical reasons from three patients with a gastric carcinoma and from four patients because of splenomegaly due to hematological diseases. Spleen samples were aerobically and anaerobically cultured on blood agar base (Oxoid Ltd. London, UK)

during 48 h at 37°C. The anaerobic plates were cultured in a jar with gaspack generator envelopes with palladium catalyst (Becton Dickinson, San José, CA). Bacterial growth was not observed, confirming that no bacteremia *in vivo* nor contamination of the tissue after surgery had occurred.

Immunohistochemistry for detection of PG in spleen

To determine whether the spleens obtained to isolate PG from indeed contained PG in antigen presenting cells, immunohistochemical staining of frozen sections was done according to the method of Kool et al. for the staining of rat spleen (24). We used the same Mab (2E9 ;mouse IgG3) recognizing human intestinal flora-derived PG.

PG isolation from human feces

PG from feces of a healthy subject was prepared as described previously (27). In brief, feces were diluted in distilled water (40 g in 100 ml) and homogenized in a laboratory Blender (model 400, Stomacher, Colworth, UK). After cambric gauze filtration the suspension was centrifuged for 45 min at 5,000 *g*. Four volumes of 96% ethanol were added to 1 volume of supernatant, and after 2 hours at 4°C, the precipitate was centrifuged for 15 minutes at 5,000 *g*. The pellet was dissolved in and dialyzed against Milli-Q water (Millipore, Bedford, MA) for 48 hours. Next, the suspension was centrifuged for 1 h at 100,000 *g* and the clear supernatant was collected. Size exclusion chromatography was done using dilutions of 15-60 mg/30 ml (depending on the viscosity of the solution), with a TSK HW75 column (gel bed 700 x eluent). After passage of 100 ml of void volume, fractions (8 ml/5 minutes) were collected and assayed for their protein and carbohydrate contents. High-molecular-weight fractions containing carbohydrates but no proteins were pooled, dialyzed, and lyophilized. From 100 g of feces about 50 mg PG could be retrieved by this procedure.

Isolation of PG from human spleen

Some 50-100 g of minced spleen tissue in 500-1,000 ml Milli-Q water was homogenized in a homogenizer (Virtis company, New York, NY) at 10,000 rpm for 30 s. The homogenate was sonicated five times for 1 min at maximum amplitude (MSE soniprep 150, UK). Acetic acid (96%) was added to a final concentration of 0.2 M. The extract was incubated at room temperature for 2 hr under rotation and was subsequently heated gradually in a water bath by 100°C over 30 min. The extract was centrifuged at 10,000 *g* for 60 min (4°C). The volume of the supernatant was reduced by lyophilization to 25-50 ml and the extract was then centrifuged at 100,000 *g* for 60 min (4°C). The supernatants were separated by gel filtration on a 275 ml gel bed of Sephadex G-25 (Pharmacia, Uppsala, Sweden). Muramic acid and protein content was determined in all fractions according to the methods of Hadzija (28) with some modifications (29) and Bradford (30), respectively. The high-molecular-weight fraction containing muramic acid was collected and size exclusion was done by loading 1-ml fractions on a Superdex 200 column (Pharmacia) connected to an FPLC (Fast Performance Liquid Chromatography) (Pharmacia). Protein was determined in all fractions according to Bradford, and PG was measured by ELISA. Fractions were pooled (see below), dialyzed against Milli-Q water and lyophilized.

Analysis of PG isolated from human spleen

PG detection by ELISA

To measure PG in the fractions after Superdex 200 (Pharmacia) gelfiltration an ELISA was performed. We coated 50 µl of the fractions overnight at 50°C in 96-well polystyrene microtiter plates. The plates were washed 3 times with PBS-0.02% Tween 20 (Fluka Chemie AG, Buchs, Switzerland). 100 µl of Mab 2E9 (10 µg/ml in PBS-0.2%Tween) was added to the wells. After 1 hour at 37°C, unbound antibody was removed by 3 wash steps. As detecting antibody, we used peroxidase conjugated rabbit anti-mouse immunoglobulin (P260, Dako, Glostrup, Denmark) diluted 1:1000 in PBS-Tween 0.2%. After 3 washes the colorimetric assay was developed at 37°C for 30-45 min after the addition of 100 µl of ortho-phenylenediamine/H_2O_2. The reaction was stopped by the addition of 50 µl 4M H_2SO_4 and the optical density was measured at a wavelength of 492 nm (Titertek Multiskan; Flow Laboratories, Irvine, Scotland). Results were expressed as optical density (OD) units.

Protein analysis according to Lowry

The amount of protein in the pooled fractions was determined according to Lowry (31) with modifications (32). We added 1 ml of 2% Na_2CO_3 in 0.1 M NaOH to 200 µl of the pooled fractions and incubated for 45 min at 70°C. After the samples were cooled, 20 µl of 0.5% $CuSO_4.5H_2O$ was added and the mixture was incubated for 10 min at room temperature. Finally, 200 µl of Folin-Ciocalteen reagent was added during 45 min at 37°C. Absorbance was measured at 620 nm.

Muramic acid analysis with a colorimetric muramic acid assay

In the pooled fractions, the amount of muramic acid was determined in accordance with the Hadzija method (28) with some modifications (29). In short, 100 µl of the samples was hydrolyzed by heating for 2 h at 90°C with an equal volume of 5 M H_2SO_4, then neutralized with 100 µl 10 M NaOH. Hydrolyzed and unhydrolyzed samples (100 µl) were incubated with 50 µl 1 M NaOH at 37°C for 30 min. After the addition of 1 ml 18.8 M H_2SO_4, samples were heated for 3.5 min at 100°C, rapidly cooled on ice and then mixed with 10 µl 0.16 M $CuSO_4.5H_2O$ in H_2O and 20 µl 0.09 M p-hydroxydiphenyl in ethanol. After incubation for 30 min at 30°C, absorbance at 570 nm was determined by Titertek Multiskan. Solutions containing 0-100 µg muramic acid (Sigma, St. Louis, MO) per ml H_2O were used as standards. The data are given as the difference in concentration between the hydrolyzed and non-hydrolyzed samples. In this way, only muramic acid linked to peptides in peptidoglycan was determined and contaminating sugars like rhamnose and lactic acid, which are determined in the non-hydrolyzed sample, were excluded.

Muramic acid assay by high performance liquid chromatography (HPLC)

The presence of muramic acid in the isolated fraction was also determined with an amino-sugar determination using high performance liquid chromatography (HPLC) reversed phase techniques as described by Glauner (33). In short, 300 µl of the isolated PG was hydrolyzed with 9 M HCl for 3 h at 90°C. The sample was frozen, lyophilized and redissolved in 100 µl H_2O. This sample was added to 100 µl 200 mM borate buffer pH 8.8 and 100 µl 20 mM

dansyl chloride (5-dimethylamino-naphthalene-1-sulfonyl chloride, Fluka Chemie AG) in acetone for 2 h at 37°C. The reaction was stopped with 35 µl 200 mM phosphoric acid. Separation of dansylated aminoacids and aminosugars was accomplished by reversed phase HPLC. Samples were analyzed using a single pump solvent delivery system (LAB 2248; Pharmacia) and a UV-VIS monitor (VWM 2141; Pharmacia) both connected to a computer working with HPLC Manager software to control the pump, gradient mixer and UV-VIS detector operating at 330 nm. Integration and analysis of chromatograms was performed using the same software (Pharmacia). Dansylated samples were separated using a Pharmacia SuperPac Sephacil C18, 5 µm, 4x250 nm column. The flow rate used was 1 ml/min and the buffers were as follows: buffer A, 20 mM sodium phosphate pH 5.25 and buffer B: 60% acetonitril and 40% 50 mM sodium phosphate pH 4.0. At 0, 10, 16 and 22 min the concentration of buffer B was 30, 50, 100 and 30 %, respectively. Eluted products were detected at 330 nm, the wavelength that is maximally absorbed by the dansyl groups.

Induction of cytokine release in whole blood after stimulation with PG
Human peripheral blood was obtained from healthy donors and collected in sodium-heparin tubes (Vacutainer, Becton Dickinson). We added 12.5 µl of RPMI 1640 (Gibco, Breda, The Netherlands) containing isolated PG or LPS (Sigma) with or without the LPS antagonist polymyxin B (Sigma) to a polypropylene tube (Falcon) containing 112.5 µl blood. Inhibition assays were performed by incubation of human blood cells with 10 and 5 µg/ml anti-CD14 antibody (My-4, azide-free, Coulter Clone, Hialeah, FL) or isotype control IgG2b antibody (MOPC-195, Coulter clone) for 30 minutes at 37°C or by incubation of the isolated PG fractions and LPS with proteases (1-0.001%) (elastase, trypsin, α-chymotrypsin, (Sigma)) during 1 hour at 37°C. The mixtures were incubated for 5 hours at 37°C in a 5% CO_2 atmosphere. Then 375 µl RPMI was added to each tube and the mixtures were centrifuged for 10 minutes at 400 *g*. The supernatants were tested in bioassays for TNF-α, IL-1, and IL-6 activity.

TNF-α bioactivity was measured using the murine fibroblast cell line WEHI 164.13 by a cytotoxicity assay (34). The cells were plated in flatbottom tissue plates with 96 wells plates (Costar, Cambridge, MA) at a concentration of 10^4 cells/well. The cells were allowed to adhere by incubation overnight at 37°C in a 5% CO_2 atmosphere. At day 2, 50 µl of actinomycine-D (Sigma) (4 µg/ml) and 50 µl diluted sample were added to the cells. Samples were tested in triplicate. After an overnight incubation (37°C, 5% CO_2) the MTT (3-(4,5 – dimethylthiazide-2-yl)-2,5 diphenyltetrazolium bromide, Sigma) cytotoxicity test (35) was used to measure WEHI cell viability.

IL-1 bioactivity was measured by use of an IL-1 dependent subline of the murine T cell line D10.G4.1, designated D10(N4)M, using the method designed by Hopkins and Humphreys (36) with some modifications (37). One hundred microliters of sample was added to 10^4 cells/well and the cells were cultured for 3 days. Proliferation of the cells was measured after 4 h of [^{3}H]-thymidine incorporation (0.2 µCi/well).

IL-6 activity was detected by using the murine hybridoma cell line B9 according to Aarden et al. (38). In the presence of 100 µl of sample the 5 x 10^3 cells/well were cultured for three days. Proliferation of IL-6 dependent B9 cells was measured after 4 hours of [^{3}H]-thymidine incorporation (0.2 µCi/well).

Recombinant human IL-1β (UBI, Lake Placid, NY), IL-6 (a kind gift from Prof. Dr. L.A. Aarden, CLB, Amsterdam, The Netherlands) and TNF-α (Amersham) served as positive controls for the D10, B9 and WEHI-assays. Cytokine activities of the samples were corrected for background activity of cells cultured in the absence of PG, and were expressed as U/ml with 1 U corresponding to a half-maximum response obtained with a titration series of the recombinant cytokine.

TNF-α ELISA

TNF-α production in anti-CD14 and protease treatment experiments was measured by an capture ELISA. ELISA was done according to the manufacturers' guidelines (Biosource, Fleurus, Belgium). In brief, polystyrene microtiter wells (Immuno maxisorp, Nunc, Roskilde, Denmark) were coated overnight at room temperature with monoclonal human anti-TNF-α followed by washing (0.9% NaCl) and 2 h of blocking (PBS/BSA 0.5%). Freshly thawed samples and an human TNF-α standard (calibrated against WHO International Standard, TNF-α 87/650, National Institute for Biological Standards and Controls, Potters Bar, UK) were incubated for 2 h in the presence of a biotinylated second TNF-α antibody, followed by washing steps, poly-streptavidine-HRP (CLB, Amsterdam, the Netherlands) and enzyme substrate (TMB peroxidase (KPL, Gaithersburg, MD)). Optical density was measured at 450 nm.

RESULTS

Immunohistochemical staining of human spleen

To confirm that antigen presenting cells containing PG were present in human spleens to be used for purification of PG an immunohistochemical staining was performed. With Mab 2E9 directed against PG derived from the normal intestinal flora, some macrophages and dendritic cells in all 7 spleens stained positive (figure 1). With a negative control antibody of the same isotype (mouse IgG3) and with irrelevant specificity (anti-sheep red blood cells), no staining was found.

Isolation of PG from human spleen

The purification of PG from 7 individual human spleens was performed as described. After homogenization and sonication the relatively low-molecular-weight fraction was discarded by Sephadex G25 gel filtration. Figure 2 shows the elution pattern after Sephadex G25 gel filtration of 1 human spleen.

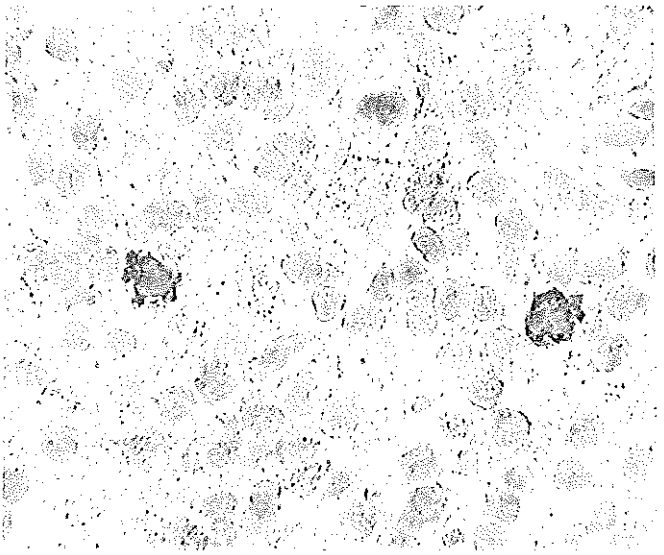

Figure 1. Detection of cells containing peptidoglycan (PG) in human spleen. Immunohistochemical staining was performed using Mab 2E9. Morphology and anatomical localization of the cells containing intracytoplasmatic PG is consistent with that of antigen presenting cells. (magnification x63).

Protein was first found after an elution volume of 120 ml H_2O. Muramic acid was detected after an elution volume of 124 ml until 156 ml H_2O. After an elution volume of 156 ml H_2O the signal for muramic acid increased sharply due to the presence of lactate, which interferes with the muramic acid assay. The fractions between 124 and 156 ml, which represented a relatively high-molecular-weight fraction containing PG were pooled and the

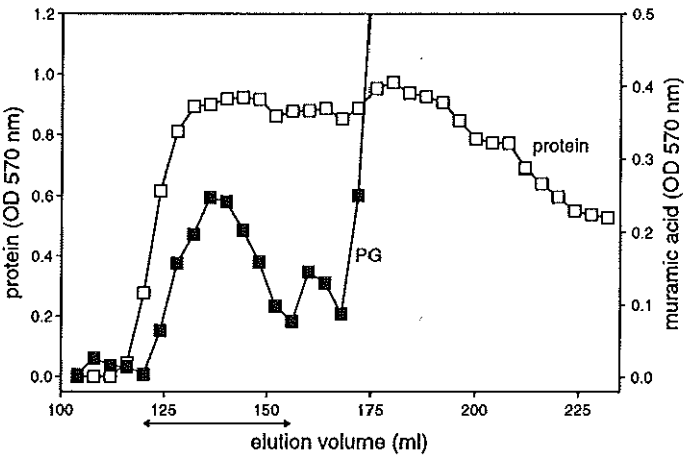

Figure 2. Separation of relatively high and low-molecular-weight fractions from human spleen extract. Twenty-five milliliters of human spleen extract was added to Sephadex G25 column. In all fractions the amount of protein according to Bradford and the amount of PG (muramic acid) was measured. The fractions between 120 and 150 ml of elution volume which represented a relatively high-molecular-weight fraction containing muramic acid, were pooled (↔).

relatively low-molecular-weight fraction was discarded. The pooled fraction was loaded in 1 ml aliquots onto a Superdex 200 column to remove contaminating protein. The presence of PG in the eluted fractions was determined by ELISA. During the column passage the aliquots were diluted 25 times and therefore the colorimetric muramic acid assay was not sensitive enough to detect muramic acid. The protein content of all fractions was measured using the Bradford method. Figure 3 shows the amount of protein and PG in all fractions after Superdex 200 column gel filtration of 1 ml of the extract.

The fractions to be used for *in vitro* studies should contain minimal amounts of contaminating proteins. For this reason fractions with comparable amounts of protein were pooled. This resulted in pooling the fractions 2-5 (P1), fractions 6-8 (P2), fractions 9-16 (P3), fraction 17 (P4), fractions 18-22 (P5) and fractions 23-25 (P6). Fraction 23-25 (P6) was used as a control since this fraction did not contain PG or protein. After all runs of the 7 spleens the corresponding fractions were pooled, dialyzed against Milli-Q water, lyophilized and stored at –20°C.

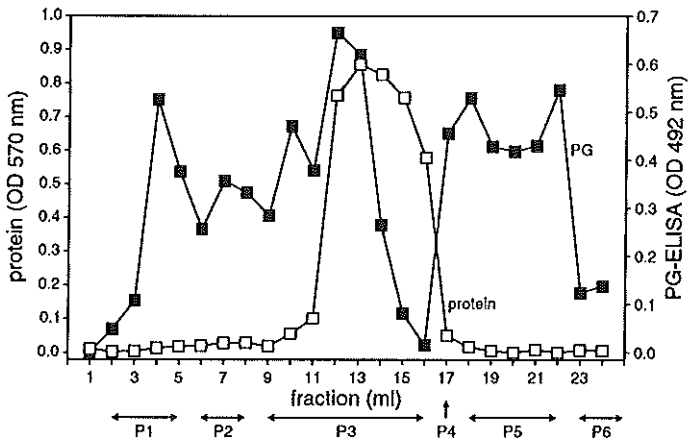

Figure 3. Separation of PG using Superdex 200 gelfiltration. One milliliter of spleen extract after Sephadex gelfiltration was added to the Superdex 200 connected to the fast performance liquid chromotograph. The amount of protein and PG was measured in all fractions with Bradford and ELISA, respectively. Pooled fractions: F2-5 (P1), F6-8 (P2), F9-16 (P3), F17 (P4), F18-22 (P5), F23-24 (P6) (↔).

PG analysis

Protein analysis

After Sephadex and Superdex 200 gel filtration the level of protein in the fractions was measured according to Bradford. With PG preparations of *Eubacterium aerofaciens* and *Brevibacterium divercatium* we showed that the method according to Lowry et al (31) was more sensitive to detect protein (data not shown). Because the dilutions of the pooled fractions used in some of the cytokine induction experiments were based on protein concentrations, we used the method according to Lowry et al.(table 1). The results show that fraction P1 and P4 contain the lowest levels of protein.

Muramic acid analysis by a colorimetric muramic acid assay

To determine the amount of PG in the pooled fractions, a colorimetric muramic acid assay was performed. Results are shown in table 1.

Muramic acid could only be detected after acid hydrolysis to remove peptides from PG, indicating that PG present in human spleen is structurally mostly intact. In contrast, hydrolysis of PG isolated from human feces had no effect on the level of muramic acid measured by the colorimetric muramic acid assay. This indicates that PG in human feces contains no or minimal levels of peptide chains bridging the MurNac backbone. The colorimetric muramic acid assay could not be used to measure the amount of PG in P3 because the level of protein in the fraction was too high.

Table 1. Protein and muramic acid content in pooled fractions isolated from human spleen

	Muramic acid (µg/ml)		Protein (µg/ml)	
Fraction	Before hydrolysis	After hydrolysis	Bradford method	Lowry method
P1	0	70	0	63
P2	0	62	44	170
P3	ND	ND	975	1300
P4	0	42	293	290
P5	0	56	34	75
P6	0	0	0	0

Protein was measured according to Bradford (30) and Lowry et al (31). Muramic acid was measured by colorimetric muramic acid assay. ND, not done

Muramic acid by HPLC analysis

To confirm the presence of PG in the fractions isolated from human spleen, P1 and P4 were analyzed for the presence of muramic acid using HPLC. These two fractions were used because protein levels were low. First, the fractions were applied to the column (figure 4A) and spiked by adding dansylated muramic acid to the fraction. The peak with a retention time of 12.8 minutes was covered by muramic acid added to the fraction (figure 4B). This is also the retention time of muramic acid when it is loaded onto the column on its own.

There are many peaks in the chromatogram and therefore the peak with retention time 12.8 minutes was collected and reanalyzed to assess whether it had the same retention time as the standard dansyl muramic acid. The only peak in the chromatogram indeed had the same retention time as dansyl muramic acid (figure 4C).

This peak was spiked by adding muramic acid to the sample. The smaller peak with retention time 5.4 minutes belonged to the muramic acid added to the sample (figure 4D). In the PG fraction we could not detect this peak because free dansyl groups were detected after this retention time. These results confirm the presence of muramic acid in both fractions. Because muramic acid is an unique component of PG, the HPLC analysis also confirmed the presence of PG in the fractions.

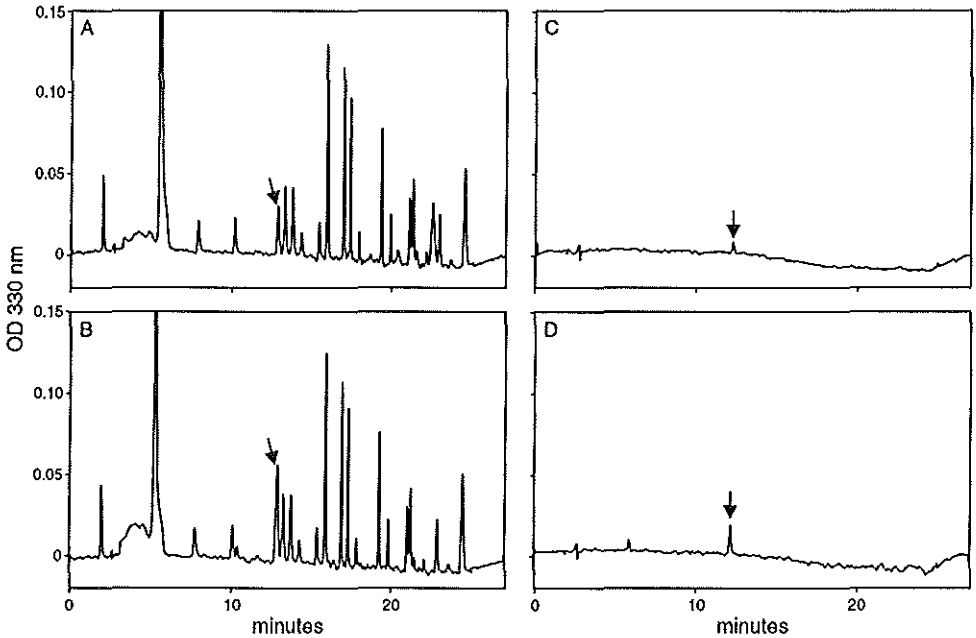

Figure 4. Identification of muramic acid in P5 isolated from human spleen. Reversed-phase HPLC on a C18 column (5 µm, 4 x 250 mm) in a discontinuous gradient 0-15% methanol over 27 minutes at 1.0 ml/min of (A) hydrolysed and dansylated preparation of P5 of spleen from patient 1. B was like A but spiked with dansylated muramic acid..C, collected peak with retention time 12.8 minutes from chromatogram A. D was like C but spiked with dansylated muramic acid. Absorbance was measured at 330 nm. Arrows indicate peak with retention time 12.8 minutes identified as muramic acid.. P1(not shown) shows the same pattern.

Induction of proinflammatory cytokines by PG

To investigate whether PG present in human spleen tissue is biologically active, production of TNF-α, IL-1 and IL-6 was measured *in vitro* after incubation of human blood cells with PG isolated from human spleen. Because P1 contained the lowest amount of contaminating protein, this fraction was used. TNF-α, IL-1 as well as IL-6 were produced after stimulation with PG (figure 5A-C). This production was dose-dependent, correlating with the concentration of P1 added to the blood cell cultures.

Polymyxin B is an efficient antagonist of LPS-activity, thereby inhibiting LPS-induced cytokine production. To exclude the possibility that activity of PG isolated from human spleen is due to contaminating LPS, we analyzed whether cytokine induction by PG was blocked by polymyxin B.

Production of TNF-α, IL-1 and IL-6 induced by P1 was not inhibited by polymyxin B. LPS served as a positive control in all experiments and 1 ng/ml was able to induce the same level of cytokines induced by P1 (75 ng/ml muramic acid). As expected, LPS-driven induction of cytokines was inhibited by polymyxin B. PG isolated from human feces induced TNF-α, but the concentration (based on the amount of muramic acid) needed to induce the same level of cytokine production as P1 was 150 times higher.

To exclude the possibility that contaminating proteins present in the fractions P2-6 induced proinflammatory cytokine production, P1-6 were analyzed on the basis of equal protein concentrations. The amount of protein was based on a 1:800 dilution of fraction P1 (75 ng/ml muramic acid). This fraction contained 78 ng/ml protein (according to the Lowry assay). All fractions were diluted so the concentration of protein added to the blood cells was 78 ng/ml. As expected, P6, which contained no PG or protein, could not induce cytokine production by whole blood cells; however, P1, P2, P4 and P5 all induced TNF-α, IL-1, and

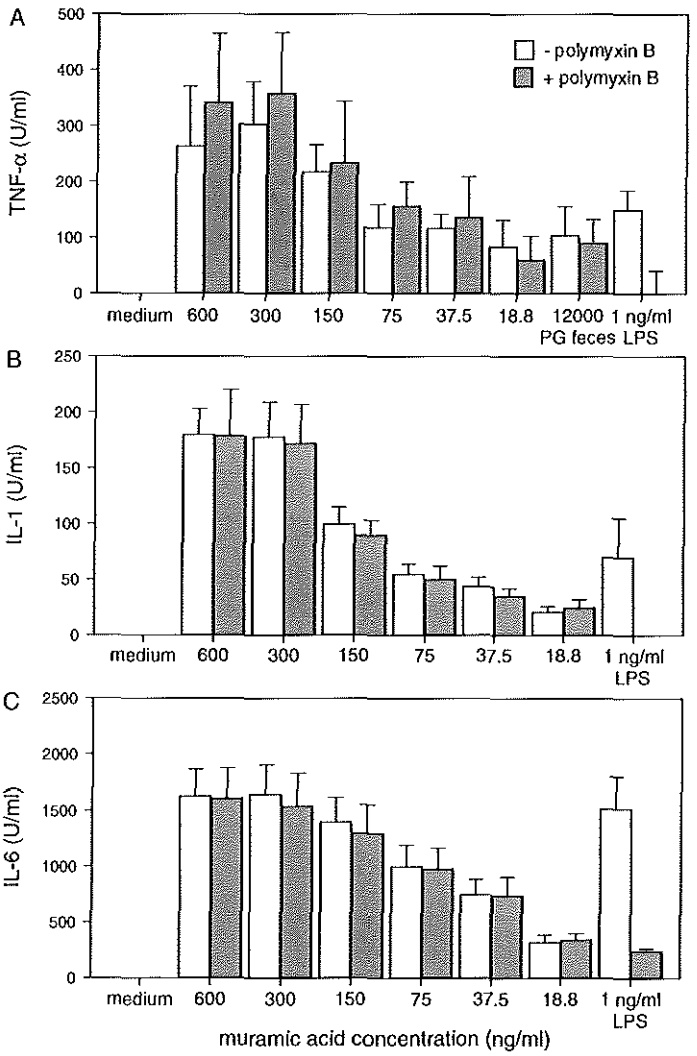

Figure 5. Production of pro-inflammatory cytokines by human blood cells after stimulation with peptidoglycan (PG) isolated from human spleen. Human blood cells were incubated during 5 h in presence of P1 with or without polymyxin B. In supernatants TNF-α production measured by WEHI 164.13 in cytotoxicity assay (A). B, IL-1 production measured using the IL-1 dependent subline of the T cell line D10.G4.1. C, IL-6 production measured by the IL-6 dependent murine hybridoma cell line B9

IL-6 (table 2). P3 did not induce cytokine production. Since P3 contains very high amounts of proteins a dilution to 78 ng of protein results in minimal traces of PG in the fraction. This clearly suggests that contaminating protein does not induce cytokine production. The levels of TNF-α, IL-1, and IL-6 induced by the distinct fractions differed strongly between the fractions. This indicates that protein, present at equal concentrations in all fractions does not induce cytokine production. Consistent with this, the cytokine production directly correlated with the amount of PG in the fractions (correlation coefficients were 0.9, 0.8 and 0.9 for respectively TNF-α, IL-1, and IL-6 production compared to the amount of PG in the fractions). This indicates that PG, but not protein present in the fractions induces the production of TNF-α, IL-1, and IL-6 by human blood cells.

Table 2. Production of TNF-α and IL-1 and IL-6 by human blood cells after stimulation by the different fractions pooled after Superdex 200 gel filtration.

Fraction	Muramic acid	Protein	TNF-α	IL-1	IL-6
P1	75	78	290 ± 25	35 ± 4	853 ± 80
P5	64	78	140 ± 20	55 ± 10	476 ± 55
P2	28	78	64 ± 7	15 ± 2	123 ± 12
P4	11	78	38 ± 4	14 ± 1	89 ± 9
P3	ND	78	0	0	0
P6	0	0	0	0	0
Medium	0	0	0	0	0

Note. Human blood cells were incubated for 5 h with all pooled fractions (P1-P6) diluted to protein concentration of 78 ng/ml. TNF-α, IL-1, and IL-6 measured in supernatants by bioassays, are shown in U/ml; muramic acid and protein are in mg/ml. ND, not done

To confirm that PG and not contaminating proteins causes the cytokine production, human blood cells were incubated with anti-CD14 antibody for 30 minutes by 37°C. All fractions were diluted 80 fold resulting in the following muramic acid concentrations:(P1) 88, (P2) 78, (P3) not done (P4) 53, (P5) 70, and (P6) 0 ng/ml.
Anti-CD14 antibody blocked cytokine production by the different fractions in a dose-dependent manner. TNF-α production was completely blocked in all fractions and LPS using a concentration of 10 μg/ml (figure 6). An isotype matched control IgG2b antibody (MOPC-195) did not affect cytokine production.

DISCUSSION

In the current study we show that structurally intact PG isolated from sterile human spleen can induce production of the proinflammatory cytokines TNF-α, IL-1 and IL-6 by human blood cells. Other studies have demonstrated the capacity of cytokine induction by PG derived from bacterial cultures (9,10), but the ability of PG present in human tissues to induce cytokine production has not been reported.
PG is thought to play an important role in the immunological response against gram-positive

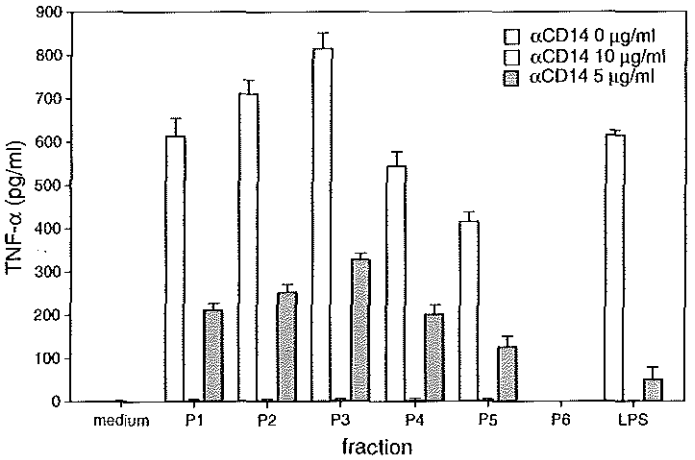

Figure 6. Inhibition of TNF-α production by human blood cells after incubation with anti-CD14 antibody. Human blood cells were incubated with anti-CD14 antibody (5 and 10 µg/ml) during 30 minutes after then stimulated with P1-P6 and LPS during 5 h. TNF-α production was measured using a capture ELISA.

infections but it has also been hypothesized that PG is involved in the pathogenesis of chronic inflammations like RA. Our group investigated the role of PG derived from the normal intestinal flora in the pathogenesis of RA. The presence of PG in several human tissues (18-23) suggests that our body is constantly exposed to PG outside the gut. It is possible that PG in human tissues other than the gut is derived from the normal intestinal flora. The fact that we are able to isolate 50 mg PG per 100 gram feces shows that high amounts of PG are continuously present in the intestine.

We hypothesize that PG is able to traverse the intestinal wall by unknown mechanisms and gain access to other sites of the body through the bloodstream and/or lymph circulation. PG is located in antigen presenting cells in tissues like human spleen and lymph nodes but also in synovial tissues. This was shown by immunohistochemical staining with a Mab against PG isolated from human feces (23). To investigate the relevance of PG in human tissues we studied the biological activity of PG isolated from human spleen. To obtain a high amount of PG it was necessary to modify the method described earlier (25). Because the passage of the spleen extract after Sephadex G25 gel filtration through a DEAE and Dowex column causes a considerable loss of PG, we developed another method to extract the protein. This was performed by size exclusion using Sephadex G25 gel filtration followed by Superdex 200 gel filtration. Because the PG containing fraction is diluted 25-fold by the Superdex 200 column passage the colorimetric muramic acid assay is not sensitive enough to detect PG in each fraction. Therefore we performed an ELISA using a monoclonal antibody against PG which is a more sensitive method to detect PG.

We used two methods to detect PG in pools P1 to P6. The HPLC analysis was used to confirm the presence of muramic acid in the fractions leading to the conclusion that PG was

present. The colorimetric muramic acid assay was used to measure the amount of PG in the fractions. This method was also used to determine whether PG is intact or not in terms of the presence of peptide interlinks. This is important because from earlier studies it is known that the structure of PG is very crucial for its biological activity. For example, muramyl dipeptide (MDP) is the minimal structure of PG which is biologically active, but a much higher amount is needed compared with large molecules of PG which has a repetitive nature (12). Furthermore, PG isolated from the ileum which is mostly intact is able to induce arthritis in rats. PG isolated from feces, which is not intact is not able to induce arthritis in rats (39). This suggests the structural integrity of PG is crucial to its biological activities. PG isolated from human spleen was largely intact because no muramic acid could be detected before hydrolysis. Analysis of PG isolated from human feces revealed that the PG is not intact. With the improved purification of PG we were able to isolate 1.9 μM muramic acid per 100 gram spleen tissue. The amount of muramic acid isolated is similar as the earlier described method (25) (2.3 μM /100 g spleen tissue), but the amount of protein in the fraction is 20-fold lower which means that the fraction is more suitable for *in vitro* studies.

The cytokine induction assays show that PG isolated from human spleen can induce induction of TNF-α, IL-1 and IL-6. Kinetics of cytokine induction by PG was the same as LPS but the amount of PG needed was 100 times higher than LPS. The fact that low amounts of PG isolated from human spleen were required in comparison with PG from human feces indicates that intact PG is more active than a more degraded form.
To exclude contamination of the isolated fraction the sample was analyzed using blood agar plates after every purification step. The whole procedure was also performed with PBS. With this procedure we were not able to induce cytokine production (data not shown). Contamination by LPS was excluded by incubating the fractions with polymyxin B before the various PG preparations to the blood cells. No inhibition of the cytokine production was found in contrast with cytokine induction by LPS. The results show also that the levels of cytokine produced correlated with PG concentrations and not with protein concentrations confirming that PG is responsible for the cytokine induction. This was confirmed with fraction P3 that contained very high concentrations of protein and unknown levels of PG. After the sample was diluted to a concentration of 78 ng/ml protein no cytokine production could be measured.

There are three other indications that the cytokines produced by the different fractions are elicited by peptidoglycan and not by contaminating proteins. First, the incubation of the spleen extract in acetic acid at 100°C for 2 h will cause a loss of the tertiary and secondary structure of the proteins. This will lead to a reduction if not a complete loss of function of the proteins. Second, the fact that TNF-α measured by ELISA could be completely blocked in all fractions by incubating the human blood cells with anti-CD14 indicates that the proteins in the fraction are not responsible for the cytokine production. CD14, which is known as the LPS receptor is also known to bind PG and activate cytokine production (11,12). Binding of proteins to CD14 mediating cytokine production has not been described. The third indication that PG is responsible for the cytokine production is the fact that treatment

of fraction P1-P6 with proteases (elastase, trypsin and α-chymoptrysin) does not affect cytokine production. Using different concentrations of proteases (0.1-0.001% v/v) cytokine induction was equal with and without treatment (data not shown). The use of higher protease concentrations was not possible because the proteases then induced cytokine production directly. The inactivation of protein could not be monitored by measuring the loss of proteins because the protein content of the proteases added to fractions was much higher than of the original low level of contaminating proteins.

Various studies, including the present study, have shown that PG is not restricted to the gut, even in the absence of infection. In all 15 spleens we have obtained thus far, PG-containing cells could be detected by use of immunohistochemistry. All spleens used were removed during surgery, because this is the only way to obtain sterile human spleens. These spleens were removed for various reasons (including when the spleen itself was not in any way diseased) indicating that healthy people contain PG in their spleens. It has been hypothesized that these bacterial products are derived from the intestinal flora. Because the present study showed that PG in human spleen is mostly intact it is likely that PG will pass the bowel wall before it is degraded by enzymes (39).

A prime question is whether the presence of PG in sterile human spleen tissues has any biological significance. To answer this question it is important to know if PG in human tissues has biological activity. This study shows that, *in vitro*, PG has the capability to induce cytokine production by human blood cells. If this activity is the same in the *in vivo* situation, antigen presenting cells containing PG might be activated by PG to produce proinflammatory cytokines. The function of this cytokine production is possibly the triggering of a continuous immune response for controlling normal homeostasis. It was recently argued that continuous exposure to bacterial antigens is important in regulation of T helper 1 (Th1) versus T helper 2 (Th2) CD4 positive T-cells (40). Disturbances of this Th1/Th2 balance may lead to autoimmune diseases. A possible way by which this balance can be disturbed may the presence of too much PG in tissues because of intestinal injury. The presence of PG in tissues where the clearance of antigens is very difficult, for example synovial tissue, could induce local inflammatory processes.

In conclusion, PG isolated from human spleen is largely intact and is able to induce cytokine production by human blood cells at very low concentrations. To further investigate whether PG-containing cells *in vivo* are also able to induce proinflammatory cytokine production in situ, these cells will be analyzed by immunohistochemistry. In addition, immunization of mice with PG will allow kinetic analysis of local cytokine induction by splenic antigen presenting cells containing PG.

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CHAPTER 2.2

Bacterial peptidoglycan from human spleen elicits proliferation of CD4+ and CD8+ αβ TCR+ T cells after proteolytic processing

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ABSTRACT

Peptidoglycan (PG), the major component of the cell wall of gram-positive bacteria, has potent proinflammatory activity and elicits specific antibody formation including IgG. PG isolated from sterile human spleen is able to induce production of the proinflammatory cytokines TNF-α, IL-1 and IL-6 by human monocytes *in vitro*. In the present study we investigated whether PG isolated from human spleen can also induce T cell proliferation. PG stimulated tritium thymidine incorporation of peripheral blood cells. Flow cytometry showed that Ki-67+ proliferating cells were mainly CD4+ αβ TCR+ T cells but a small fraction of CD8+ αβ TCR+ T cells was also stimulated by PG. γδ TCR+ T cells did not proliferate upon PG exposure. Anti-CD14 inhibited PG-induced T cell proliferation indicating that CD14 is necessary for PG binding by monocytes. Furthermore, the inhibitors of proteolytic processing, chloroquine, leupeptin and brefeldin A efficiently inhibited T cell proliferation in a dose dependent fashion. The presentation of PG was inhibited by anti-HLA class II antibodies and anti-HLA-class I antibodies, suggesting that PG-derived epitopes can be presented by both molecules to CD4+ and CD8+ T cells. These results demonstrate that PG present in human spleen is able to elicit a proliferative response of CD4+ and CD8+ αβ TCR+ T cells after binding to CD14 on monocytes, followed by intracellular processing and presentation mediated by HLA-class II and I. This implies that bacterial PG transported by antigen presenting cells from mucosal surfaces including the gut to secondary lymphoid organs in a continuous fashion can elicit T cell responses, cytokine production and antibody formation in the absence of local bacterial replication.

INTRODUCTION

Peptidoglycan (PG) is the major component of the cell wall of gram positive bacteria. PG is composed of alternating N-acetyl glucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc) residues, forming long sugar chains, which are interlinked by peptide bridges resulting in a large complex macromolecular structure (1). During bacterial infection, PG

and several other cell wall components are thought to be involved in the inflammatory reaction (2). In gram-negative infections, lipopolysaccharide (LPS) is a well-known activator of the innate immune system (3,4). During gram-positive infections, when no endotoxin is present, PG is able to activate complement (5,6), to stimulate granulocytes (7,8), and to upregulate expression of adhesion molecules on endothelial cells (8). Importantly, PG is also able to induce production of proinflammatory cytokines like IL-1, IL-6 and TNF-α by monocytes *in vitro* (9,10). Similar to LPS-induced cytokine production, PG activities can be inhibited by preventing uptake of PG and/or activation of macrophages and monocytes by blocking the CD14 receptor (11,12) in contrary to LPS, PG does not require LBP or serum (13).

It has been hypothesized that PG is involved in the pathogenesis of chronic inflammation like rheumatoid arthritis (RA) (14-16). PG has been detected in liver and spleen (17,18), synovial tissues (17,18), urine (14), synovial fluid (19) and circulating peripheral blood leukocytes (20). The latter finding implies that macrophages may ingest PG at mucosal surfaces or after bacterial translocation, and subsequently transport it to distal non-mucosal sites such as the spleen. PG present in antigen presenting cells could be detected in normal human spleen and in synovial tissues of RA patients (21) using a monoclonal antibody directed against bacterial flora-derived PG isolated from human feces (22). We were also able to isolate bio-active PG from sterile human spleen using biochemical methods (23,24).
The proinflammatory effects of PG on antigen presenting cells have been thoroughly studied (9,10,24) but T cell reactivity against PG has not been fully explored yet.
Healthy donors all have systemic antibody responses against PG, including T cell dependent IgG, strongly suggesting that PG is able to stimulate CD4+ T helper cell proliferation (25).

Previous studies performed on this subject have focused on the mitogenic capacities for PG on B lymphocytes. Mouse studies have shown that PG is not mitogenic for murine T lymphocytes (26), while mitogenic B cell activation by PG is independent of T cells and macrophages (26,27). In humans, B cell activation by PG was dependent on T cells (28-30). Limited studies on T cell activation by PG showed that T cells respond to PG only in the presence of monocytes, suggesting that uptake of PG by antigen presenting cells is necessary to elicit T cell responses (29,31). Furthermore, it was shown that T cell lines specific for PG induced a delayed type hypersensitivity (DTH) response in Lewis rats (32) (33).
These lymphocyte-activating properties, mainly examined on PG isolated from *Staphylococcus aureus*, are not shared by all bacterial PGs. For example, PG from *E. coli* (34) is not able to stimulate immunoglobulin synthesis and *Micrococcus lysodeikticus* (35) has no mitogenic capacities.

In the present study, PG isolated from human spleen was used to further define the immunostimulatory capacities of PG present in human tissues. The origin of spleen PG is unknown but it is most probably derived from the mucosal surfaces, especially the gut, where the body is continuously exposed to gram-positive bacteria. PG isolated from human spleen

might be derived from many various bacterial species and peptide bridges are mainly intact (24). This is crucial in the study of T lymphocyte activation because previous studies have shown that T cell proliferation by stimulation with PG derived from bacterial cultures is abolished after lysozyme degradation (29). Furthermore, PG present in human tissues was approximately 10-100 fold more biologically active than PG isolated from human feces which has been exposed to degrading enzymes in the gut (24).
The aim of this study was to assess whether PG isolated from sterile human spleen is able to elicit T cell responses. Our data show that spleen PG induces αβ CD4+ and CD8+ T cell proliferation after CD14 binding and proteolytic processing.

MATERIAL AND METHODS

Isolation of PG from human spleen
PG used was isolated from human spleen as described before (24). Briefly, minced spleen tissue was homogenized and sonicated. The extract was treated with acetic acid (96%) for 2 h at room temperature and 30 min at 100°C. After centrifugation the volume of the supernatant was reduced by lyophilization. The extract was separated by gel filtration on a 275 ml gel bed of Sephadex G-25 (Pharmacia, Uppsala, Sweden) followed by a Superdex 200 column (Pharmacia) connected to a FPLC (Fast Performance Liquid Chromatography (Pharmacia)). Protein content was determined in all fractions according to Bradford, and PG was measured using an ELISA. Fractions were pooled (see below), dialyzed against milli-Q water and lyophilized. In this study we used a fraction which contained 70 µg/ml muramic acid, a unique component of PG and 0 µg/ml protein according to Bradford and 63 µg/ml protein according to Lowry. Cytokine induction correlated with muramic acid content and not with protein concentration (36).

PG isolation from human feces
PG from feces of a healthy subject was prepared as described previously (37). Briefly, feces was diluted in distilled water and homogenized. After cambric gauze filtration the suspension was treated with 96% ethanol for 2 h at 4°C. The precipitate was centrifuged and the pellet was dissolved in and dialyzed against MilliQ water. Next, the suspension was centrifuged for 1 h at 100,000 *g* and the clear supernatant was collected. Size exclusion chromatography was performed with a TSK HW75 column (gel bed 700 x eluent). Fractions (8 ml/5 min) were collected and assayed for their protein and carbohydrate contents. High molecular weight fractions containing carbohydrates but no proteins were pooled, dialyzed, and lyophilized. From 100 g of feces about 50 mg PG could be retrieved by this procedure.

T cell proliferation
To determine whether T cell proliferation occurred upon PG stimulation, peripheral blood mononuclear cells (PBMC) of healthy donors were isolated from heparin blood using Ficoll (Pharmacia). 100 µl containing $2x10^6$ cells were plated in 96 well round bottom plates. 50 µl of appropiately diluted blocking reagents chloroquine, leupeptin, brefeldin A (all Sigma), anti-CD14 (My-4 Coulter clone, azide-free, Hialeah, FL) anti-HLA-I (3F10 Ancell, azide-

free, Bayport, NM) or anti-HLA-II (TDR31.1, azide-free, Ancell) or isotype-matched control antibodies were added to the wells. Unless indicated otherwise, cells were cultured with 50 µl of PG isolated from human spleen (0.35 µg/ml muramic acid), PG isolated from human feces (4 µg/ml muramic acid) or the recall protein antigen tetanus toxoid (TT) (3Lf/ml)(RIVM, Bilthoven, the Netherlands). Both cells and antigens were diluted in RPMI (Biowittaker, Verviers, Belgium) supplemented with 10% human serum and penicilin/streptavidin. All concentrations mentioned are final concentrations. The cells were cultured during 7 days (unless stated otherwise), the last 8 hours in the presence of tritiated thymidine (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). The cells were then harvested, and tritiated thymidine incorporation was counted in a liquid scintillation counter. The proliferative response was expressed as counts per minute (cpm) or stimulation index (SI), the ratio of mean cpm in the presence and absence of antigen. Each experiment was performed at least four times with PBMC of five to thirteen high responding donors. In the figures representative experiments of one individual donor are shown.

Binding of PG

For determination of binding of PG, PG isolated from human feces was labeled with biotin as described before (38). To assess to which cell type PG binds, double stainings were performed with PG-biotin and the FITC-labeled Mab Leu-4 (anti-CD3), B4 (anti-CD19), Leu-M1 (anti-CD15) (all from Becton Dickinson, San José, CA) or My-4 (anti-CD14) (Coulter Clone) on whole blood cell samples. Kinetics of PG binding were determined by incubating PG-biotin in RPMI + 10% human serum at 37°C in different concentrations for different incubation periods with percoll gradient-enriched monocytes. The number of CD14 positive cells was evaluated in samples incubated without PG-biotin. Binding of PG-biotin was analyzed by flow cytometry.

Flow cytometry

For immunofluorescence staining of proliferating PBMC, anti-Ki-67 (Becton Dickinson) was used. Double staining was performed with antibodies against CD4, CD8, TCRαβ and TCRγδ (all Becton Dickinson). After the PBMC were washed in PBS/0.5%BSA, they were incubated with 50 µl of optimally titrated PE- or Cy labeled Mab Leu-3 (anti-CD4); Leu-2 (anti-CD8), WT31 (anti-TCRαβ), 11F2 (anti-TCRγδ) for 30 min at 4°C. Mouse isotype-matched antibodies with irrelevant specificity were used as a control for non-specific binding. After two washings, cells were permeabilized using Lysing solution® (Becton Dickinson). After washing twice, the cells were incubated with FITC-labeled anti-Ki-67 for 30 min at 4°C. After another two washings, the cells were resuspended for analysis of the fluorescence intensity by means of a FACScan® (Becton Dickinson). For assessment of PG binding to monocytes or whole blood cell samples, the same procedure was followed. Double stainings were performed with PG-biotin and FITC labeled anti-CD3/CD19/CD15 or CD14. PG-biotin was detected with streptavidin–PE.

RESULTS

PG from human spleen induces proliferation of PBMC

To determine whether PG is able to stimulate proliferation of PBMC, cells from four healthy donors were incubated during 9 days with different concentrations of PG isolated from human spleen or human feces. Proliferation was analyzed after 2-9 days by measuring tritium thymidine incorporation. Proliferation could be observed after stimulation with PG from spleen containing a muramic acid concentration higher than 90 ng/ml. 100-fold higher concentrations PG from feces were needed based on muramic acid content, to obtain the same levels of proliferation compared to PG isolated from spleen (data not shown). After 4-5 days of culture with PG, proliferation could be measured and peak proliferation was observed after 7-8 days of culture. Results of a healthy donor are shown in figure 1. These kinetics are consistent with proliferation of antigen specific T cells as measured using TT. In all further experiments a concentration of 360 ng/ml muramic acid and a culture period of 7 days was used.

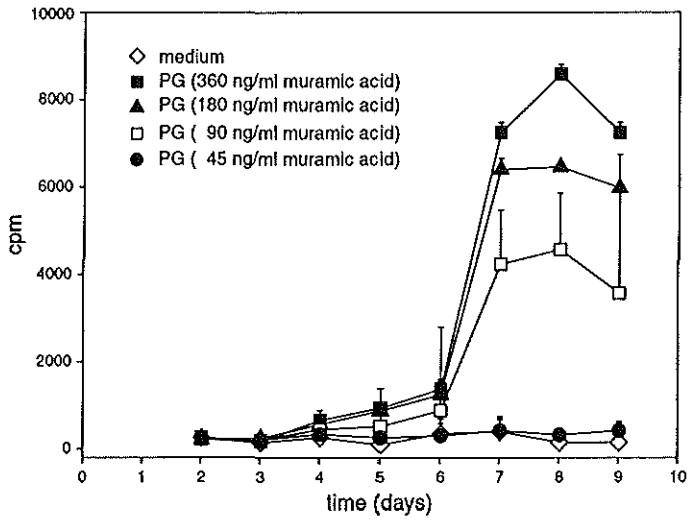

Figure 1. PG isolated from human spleen induces proliferation of PBMC. PBMC isolated from a healthy human donor were incubated during 9 days with different concentrations of PG isolated from human spleen. After a culture period of 4-5 days, proliferation could first be observed with concentrations higher than 90 ng/ml muramic acid (MurNAc) measured by tritium thymidine incorporation. Peak proliferation was observed after 7-8 days of culture. Proliferation is expressed as mean cpm ± SD of triplicate cultures.

CD14 is the receptor for PG

To examine the binding of PG, PG isolated from human feces was labeled with biotin and binding of PG was analyzed by flow cytometry. Binding of PG to cells was analyzed by double staining of whole blood cells of 4 healthy donors with PG-biotin and different cell subset markers (CD3, CD14, CD15, CD19). No double staining could be observed with CD3 or CD19. Double staining with PG was shown with both anti-CD14 and anti-CD15.

CD14 is expressed by both monocytes and granulocytes, while CD15 is only expressed on granulocytes. After gating of monocytes and granulocytes results showed that all monocytes (7% of blood cells) (figure 2A) and 25% of the 40% gated granulocytes bound PG (figure 2B). This was consistent with the percentage of granulocytes expressing CD14. Anti-CD14 treatment prevented PG-binding to monocytes (data not shown). The results suggest that PG from feces binds to CD14 which is present on both monocytes and granulocytes. Kinetic studies on PG binding by monocytes showed that PG binding on CD14 positive cells at 37°C was first detected after 10 min, reaching 100% binding after 20 min of culture. After 30 min PG binding gradually declined, reaching background levels after 4 hrs, probably reflecting internalization (figure 2C).

To examine whether binding of PG by CD14 is a prerequisite for induction of T cell proliferation, anti-CD14 (My-4) known to block PG induced cytokine production (24), was added to PBMC of five healthy donors at different concentrations during cell culture. Proliferation was measured using tritium thymidine incorporation after 7 days of culture. Results showed that anti-CD14 blocked T cell proliferation in a concentration dependent manner. 2 µg/ml of anti-CD14 was able to inhibit proliferation completely. As expected, TT-induced proliferation could not be inhibited by anti-CD14 as this protein antigen does not utilize CD14 for internalization (figure 2D).

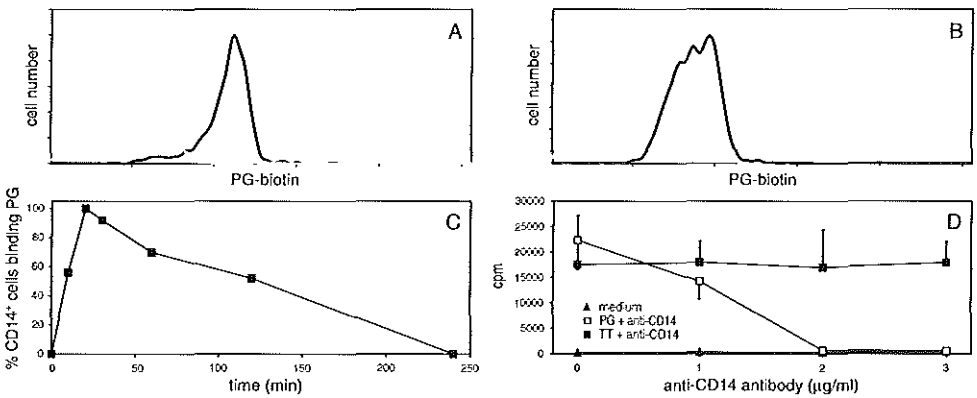

Figure 2. CD14 is involved in the binding of PG and stimulation of T cells by PG. Biotinylated PG isolated from human feces binds to the CD14 receptor on monocytes and granulocytes. Whole blood cells were cultured in the presence of PG-biotin for 30 min. PG-biotin was detected with streptavidin-PE using flow cytometry. On the FSC/SSC scatter monocytes (2A) and granulocytes (2B) were gated. Histograms of the gated cells with (thick line) and without (thin line) streptavidine-PE showed that PG bound to all monocytes and 65% of granulocytes. Experiments on the kinetics of the binding of PG to monocytes showed that binding was detectable after 5-10 minutes and reached binding to 100% of CD14 positive cells within 20 min at 37°C (2C). Data are expressed as percentage of CD14 + cells PG-biotin was bound to. Proliferation of PBMC was dependent on binding of PG to CD14 since 2 µg/ml of anti-CD14 (My-4) was able to completely inhibit proliferation (2D). Proliferation is expressed as mean cpm ± SD of triplicate cultures.

CD4+ and CD8+ αβ TCR+ T cells proliferate after stimulation with PG

To assess which cellular subsets proliferate after PG stimulation, flow cytometry was performed for five PG responders after 7 days of culture.

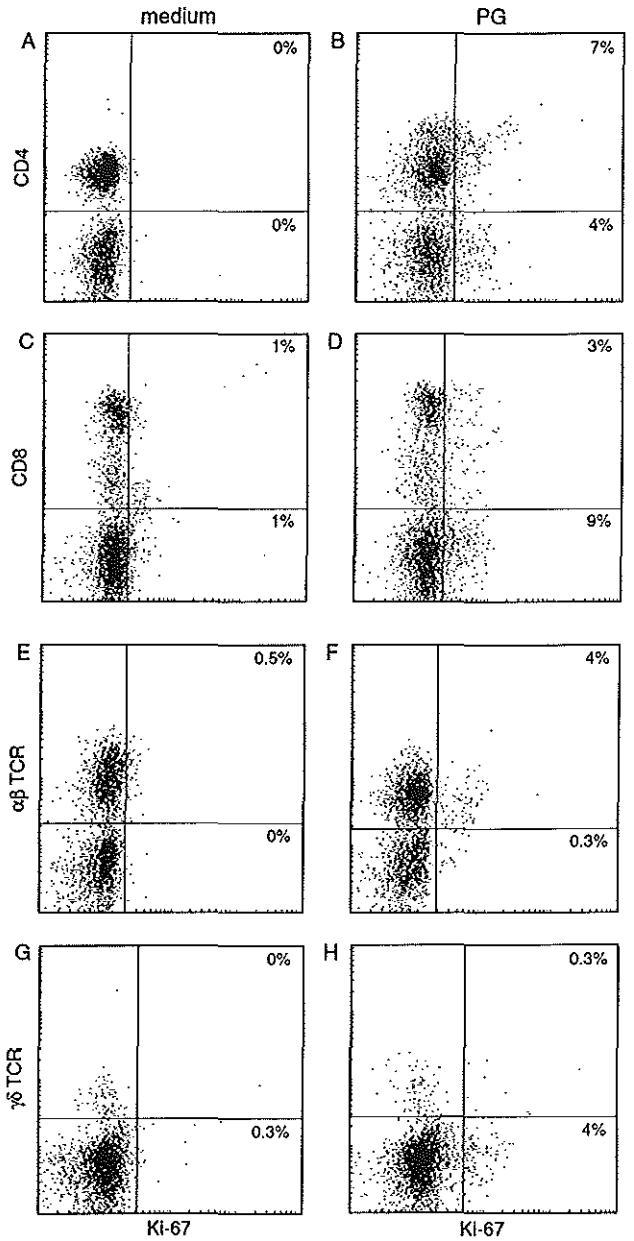

Figure 3. PG stimulates proliferation of CD4+ and CD8+ αβ TCR+ T cells. Flow cytometry with Ki-67 and CD4/CD8/TCRαβ/TCRγδ was performed to identify proliferating cells after stimulation with PG from human spleen. Without stimulation no proliferating cells could be observed (A,C,E,G). Results show that both CD4+ (B) and CD8+ T cells (D) proliferated after PG stimulation. 95% of all proliferating cells were αβ TCR + T cells (F) in contrast to only 5% of γδ TCR+ T cells in a single donor (H).

To identify proliferating cells, anti-Ki-67 (39) was used in combination with anti-CD4, anti-CD8, anti-CD19 and anti-CD14. Neither B cells (CD19+) nor monocytes (CD14+) proliferated after stimulation with PG (data not shown). Without stimulation, only 0-1% of total PBMC were positive for Ki-67 (figure 3ADEG), but after stimulation with PG 8%-15% of PBMC were found to express Ki-67 in five different healthy donors dependent on the SI. Double staining with anti-CD4 and anti-CD8 showed that in one healthy donor 75% of all proliferating cells were CD4+ cells and 25% were CD8+ cells (figure 3BD). The mean proliferation in all five healthy donors analyzed was for 70% (50-100%) due to CD4+ cells and for 30% (0-50%) due to CD8+ cells.
Double staining was also performed with anti-TCR αβ and anti-TCR γδ antibodies. In five donors analyzed, 100% of Ki-67 positive cells were αβ positive. In only one person proliferation in the γδ T cell subset (5% of all proliferating T cells), could be observed after PG stimulation (figure 3 G+H).

Proteolytic processing of PG is required to elicit T cell proliferation
To determine whether processing of PG is necessary to stimulate T cells, PBMC of 7 healthy donors were cultured in the presence of chloroquine, brefeldin A or leupeptin, which are classic inhibitors of intracellular proteolytic antigen processing. Results show that all three processing inhibitors were independently able to inhibit T cell proliferation induced by PG in a dose dependent manner (figure 4). To completely block T cell proliferation, 0.8 μM brefeldin A, 30 μM chloroquine or 25 μM leupeptin was needed.

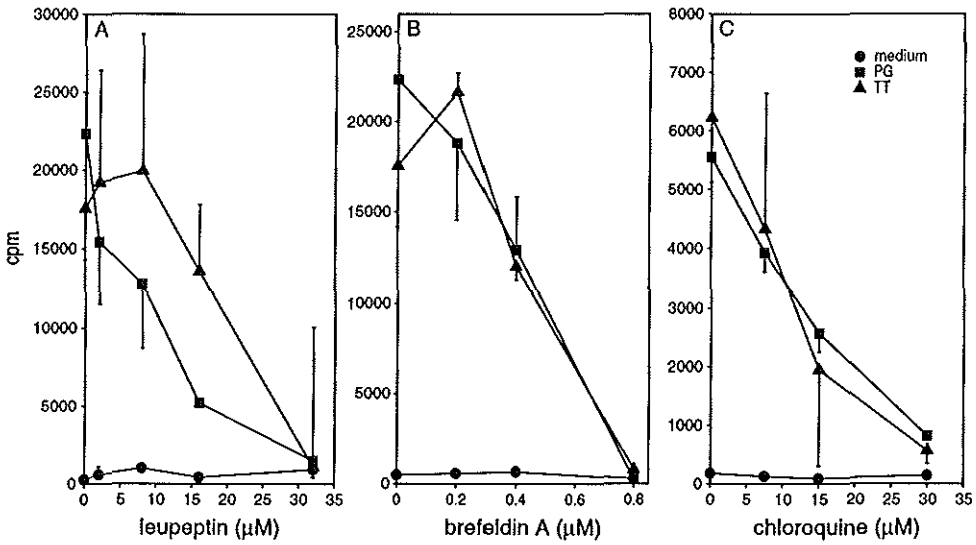

Figure 4. Processing of PG is required to stimulate T cells. To examine whether proteolytic processing of PG from human spleen is required to induce proliferation, the processing inhibitors leupeptin (3A), brefeldin A (3B), or chloroquine (3C) were added to PBMC cultures. Al three inhibitors were able to block T cell proliferation after stimulation with PG or TT stimulation. Proliferation was expressed as mean cpm +/- SD of triplicate cultures. Levels of proliferation differ as several donors were evaluated.

As expected, TT-induced T cell proliferation could also be blocked by all three processing inhibitors independently in the same concentrations.
Cell viability was measured after 7 days of culture using trypan blue staining, confirming that inhibitor treatment did not negatively affect T cell proliferation.

Presentation of PG-epitopes is mediated by both HLA-II and HLA-I
To examine whether HLA is involved in the presentation of PG for T cell proliferation, PBMC cultures of 7 healthy donors were incubated with either anti-HLA class I and II antibodies, known to block antigen presentation for 30 minutes before addition of PG. Both antibodies were able to inhibit proliferation of T cells, although anti-HLA-I antibodies partly depressed the proliferation, and anti-HLA-class II antibodies completely blocked proliferation. This is consistent with the finding that more CD4+ T cells proliferate in response to CD8 + cells (figure 5).

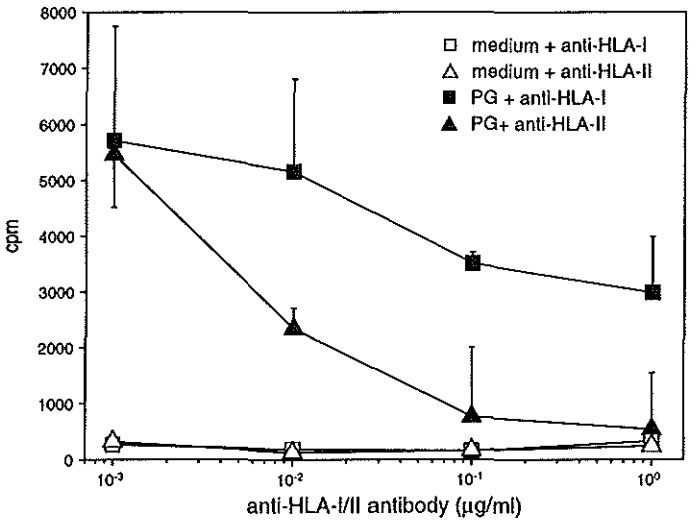

Figure 5. Presentation of PG is mediated by HLA-I and II. To assess whether presentation of PG is required for proliferation of T cells, anti-HLA-I and anti-HLA-II blocking antibodies were added to the PBMC cultures. Anti-HLA-II was able to inhibit T cell proliferation at a concentration of 1 µg/ml. Anti–HLA-I also markedly inhibited PG induced proliferation. Proliferation was expressed as mean cpm +/- SD of triplicate cultures.

DISCUSSION

The present study shows that PG isolated from sterile human spleen is able to induce proliferation of CD4+ and CD8+ αβ TCR+ T cells. CD14 is necessary for uptake of PG by antigen presenting cells and proteolytic processing is required for presentation of PG, involving HLA-class II and I.
It has been reported previously that PG isolated from cultured *Staphylococcus aureus* was able to induce proliferation of human T and B cells (28,29), but the current report, to our

knowledge, is the first to demonstrate that PG present in human tissues is able to induce T cell proliferation. In the present study we analyzed the kinetics of the proliferative response of PBMC after stimulation with PG. It was shown that after 7-8 days of culture the highest proliferation could be measured. Together with the data that the proliferative response can be inhibited with anti-class I and II HLA antibodies, this suggests that PG induces an antigen specific T cell response. Although PG from spleen contained protein, activation of T cells by protein contamination is highly unlikely in view of the following two facts; first, different fractions of PG with different protein and PG concentrations were shown to induce cytokine production dependent on PG concentration and not protein concentration (24). Second, the inhibition assays with anti-CD14 confirmed that the proliferation is CD14-dependent, and it is highly unlikely that contaminating proteins use CD14 as a receptor. Furthermore, LPS contamination of the PG fraction has previously been excluded by coincubation with polymyxin B, an efficient LPS antagonist (24).

Flow cytometry using an antibody recognizing Ki-67, a nuclear antigen of proliferating cells (40), showed that both CD4+ and CD8+ αβ TCR+ T cells proliferate after stimulation with PG. The observation that PG-induced proliferation could be blocked by anti-HLA-class II and I was consistent with proliferation of both CD4+ and CD8+ T cells, respectively. Most proliferating cells were CD4+ , and proliferation could be completely blocked by anti-class II antibody. The observation that CD8+ cells could be activated by (exogenous) PG is consistent with recent studies (41,42), showing that exogenous antigens are able to reach intracellular compartments for loading onto class I molecules for stimulation of CD8+ cells. In macrophages, two fundamentally different pathways for the presentation of exogenous antigens in HLA class I molecules have been described, one involving unconventional post-Golgi loading of HLA class I (43), and another involving unusual access of exogenous antigens into the classical loading pathway (44). Proliferation induced by PG could be inhibited by leupeptin, an inhibitor of lysosomal proteinases, and by chloroquine, a weak base which elevates the pH in endocytic compartments and thereby inhibits the action of acid-optimal proteinases, and finally by brefeldin A, a selective inhibitor of Golgi transport. All three inhibitors blocked T cell proliferation in a concentration-dependent manner at equal as previously used for classic protein antigens (45-47). This suggests that PG induced proliferation is dependent upon proteolytic antigen processing. Our binding studies showed that PG associates with CD14, which is present on both monocytes and granulocytes. After 5-10 min, PG from feces had already bound to monocytes confirming results earlier published for soluble PG (12). The blocking of T cell proliferation by anti-CD14 indicates that monocytes are required for the activation of T cells, probably as antigen presenting cells. This is in accordance with a study in which monocytes were necessary to induce stimulation of T cells by PG derived from cultured *Staphylococcus aureus* (29).

The question remains which PG-epitopes are presented by HLA. HLA molecules mainly present peptides derived from proteins, but recently it has been shown that glycopeptides are also able to elicit CD4+ or CD8+ T cell responses after presentation restricted by HLA class I and II (48-50). Therefore, theoretically, PG provides at least three potential sources of epitopes; peptides, carbohydrates and peptide in combination with one or two carbohy-

drate molecules. T cell proliferation induced by a carbohydrate structure seems unlikely because proliferation of γδ TCR+ T cells which is the major cell population stimulated by carbohydrate molecules, was hardly found after PG stimulation (51,52). To examine whether single PG peptides are able to induce T cell proliferation is difficult because the configuration of the peptides present in PG is complex and cannot be generated synthetically. When carbohydrate together with the peptide structure induces proliferation, degradation of PG is probably necessary because it has been shown that only one or two sugar molecules connected to a peptide can be recognized by αβ TCR+ T cells (51,52). Complete degradation of PG on the other hand prohibits T cell responses as lysozyme digestion of PG resulted in an abolishment of the T cell activation (29). The present study also supports this notion as 10-100-fold higher concentrations of the more degraded PG isolated from the human gut are needed to generate T-cell responses similar to those elicited by the structurally more preserved PG isolated from human spleen.

The present study shows that PG, which is present in various tissues of healthy persons, is able to elicit proliferation of CD4+ and CD8+ αβ TCR+ T cells. The fact that healthy subjects respond to this antigen implies that T cell tolerance is not induced despite the continuous presence of PG in mucosal sites, which are optimally suited for tolerance induction. This lack of tolerance induction may be due to the adjuving properties of PG which elicits cytokine production (24) (9). Recently it has been argued that continuous exposure to bacterial antigens is important in regulation of Thelper 1 (Th1) and Thelper 2 (Th2) CD4 positive T-cells (53). Disturbances of the Th1/Th2 balance may contribute to autoimmune diseases. Reduced gut barrier function may result in increased access of PG to non-mucosal sites (54). Macrophages containing PG migrated to non-mucosal sites, for example synovial tissues, might be able to persist and induce local inflammatory responses (55). To further examine the role of PG in rheumatoid arthritis, T/B cell and cytokine responses induced by PG are under current investigation in a group of rheumatoid arthritis patients.

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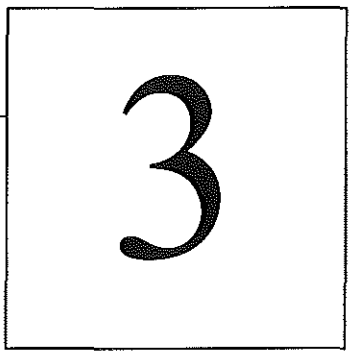
Chapter



ANTIBODY, T CELL AND CYTOKINE RESPONSES INDUCED BY BACTERIAL PEPTIDOGLYCAN IN RHEUMATOID ARTHRITIS PATIENTS

Chapter 3.1

Reduced systemic IgG levels against bacterial peptidoglycan in rheumatoid arthritis patients
Submitted for publication

Chapter 3.2

Bacterial peptidoglycan from human spleen induces T cell proliferation and inflammatory mediators in rheumatoid arthritis patients and healthy subjects.
Submitted for publication

CHAPTER 3.1

Reduced systemic IgG levels against bacterial peptidoglycan in rheumatoid arthritis patients

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ABSTRACT

The gut flora is believed to play a role in the pathogenesis of rheumatoid arthritis (RA). Peptidoglycan, a major cell wall component of gram positive bacteria, is a candidate antigen because of its capability to trigger production of proinflammatory cytokines, to induce arthritis in rodents, and because of its presence in antigen presenting cells in RA joints. We investigated whether the systemic and local antibody levels against an peptidoglycan-polysaccharide (PG-PS) fraction isolated from the human gut is related to presence and disease activity of RA. Systemic antibody levels (IgM, IgG, IgA) against PG-PS were analyzed in sera of 125 healthy donors, 44 early and 63 longstanding RA patients. Local antibody levels against PG-PS were analyzed in synovial fluids of 15 RA patients. All healthy donors had antibodies (IgM, IgG, and IgA) against PG-PS in serum. Significantly lower levels of IgG directed against PG-PS were found in healthy females compared to healthy males. Healthy females showed an age-related decrease in IgA levels specific for PG-PS, while Ig levels of all three isotypes against PG remained stable in males of different ages. Levels of IgG directed against PG were significantly reduced in early RA and longstanding RA patients compared to sex and age-matched healthy controls.
In all synovial fluid samples, antibodies against PG-PS could be detected. Titers of IgA antibodies correlated positively with disease activity. These data showing reduced systemic IgG levels against PG associated with rheumatoid arthritis and the presence of antibodies against PG in synovial fluids, suggest that antibodies against PG are attracted to the synovial sites or that IgG mediates protection against spreading of PG to non-mucosal sites.

INTRODUCTION

The search for the trigger (auto)antigen in the etiology of rheumatoid arthritis (RA) has been the topic of many studies, and in some of those a microbial etiology has been suggested. In reactive arthritis, the triggering antigens are thought to be microbes that cause infections of the gut or urogenital tract, such as *Chlamydia trachomatis*, *Yersinia*, or *Salmonella* species (1). Antigens of these bacteria have been detected in synovial cells (2-4) and T cells specific for these antigens have been found in the synovium (5-7). Because RA is a chronic disease, it can be suggested that the bacterial load in the intestine with which the human body is in lifelong close contact might be important for the induction and perpetuation of RA. This is supported by the observation that some patients with Crohn's disease and ulcerative colitis, both intestinal autoimmune diseases, suffer from joint inflammation (8,9). Furthermore, Crohn's-like inflammatory bowel disease and chronic inflammatory arthritis develop spontaneously in a knockout mice lacking a TNF AU-rich element, responsible for TNF mRNA destabilization and translational repression in hemopoietic and stromal cells (10).

We and others have previously hypothesized that peptidoglycan (PG) plays a role in the pathogenesis of RA (11-14). Peptidoglycan (PG) is the major component of the cell wall of gram-positive bacteria and is composed of long sugar chains of alternating N-acetyl glucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc) residues, which are interlinked by peptide bridges, resulting in a large complex macromolecular structure (15). PG isolated from the anaerobic bacterium *Eubacterium aerofaciens* which is present in numbers > 10^9/g human feces, induces a severe and chronic arthritis in rats (16-18). Furthermore, PG could be detected in APC in synovial tissues of RA patients using a specific antibody (19,20). PG could also be isolated from sterile normal human spleen using biochemical methods (21,22). *In vitro* analysis showed that this PG fraction is able to induce production of the proinflammatory cytokines TNF-α, IL-1 and IL-6 (22) which are crucially involved in RA pathogenesis (23).

Peptidoglycan can also be isolated from feces in soluble form. This macromolecular peptidoglycan polysaccharide complex (PG-PS) was shown to originate from the normal endogenous gut flora (24). Since PG is present at 0.5 mg/g feces (wet weight) it could be calculated that the intestinal anaerobic flora produces up to 200 mg of PG daily in healthy adults.

As high antibody titers of the IgM, IgG and IgA subclasses against PG can be found in healthy persons, it is obvious that the immune apparatus of the body is in continuous close contact with PG possessing strong arthritogenic capacity (25,26). Several studies have shown that increased levels of antibodies against PG are present in patients with bacterial infections (27) and in patients with various rheumatic diseases like ankylosing spondylitis, Reiter's syndrome, juvenile rheumatoid arthritis and rheumatoid arthritis (28-30). However, a study of Rantakokken et al. (31) showed that patients with RA had decreased antibody levels against streptococcal PG compared to healthy controls.
In the present study we analyzed systemic and local antibody levels against PG-PS isolated

from the human gut in a large panel of healthy donors versus early and longstanding RA patients to assess whether antibodies specific may for PG-PS be involved in the pathogenesis of RA.

PATIENTS AND METHODS

Sera

Control sera

Sera of 98 healthy blood bank donors were obtained as a kind gift of the Department of Neurology of the Erasmus University and University Hospital-Dijzigt Rotterdam and 27 additional sera of healthy donors were a kind gift of the Department of Epidemiology from the Erasmus University and University Hospital-Dijzigt Rotterdam (table 1). Additional sera of 6 healthy volunteers (2 females, age 32 and 37 and 4 males age 26, 27, 27, 31) used for determination of optimal serum dilutions were obtained from the Department of Immunology of the Erasmus University Rotterdam.

Patient sera

Sera of 63 RA patients diagnosed with RA fulfilling the American College of Rheumatology criteria (32) were obtained from the Zuiderhospital, Rotterdam and the Leiden University Medical Center. The majority of the patients received no immunosuppressive medication, some patients received low doses of prednisone (up to 10 mg/ day) or methotrexate (up to 30 mg /week). Sera of 44 patients with RA of less than 1 year's duration as measured from the first clinical signs of arthritis were obtained from the Leiden University Medical Center (table 1). The majority of the patients received no immunosuppressive medication.

Standard serum

A pooled standard serum (≈ 500 bloodbank donors) selected for high PG-PS reactivity was used in each ELISA plate as a reference for calculating antibody levels against PG-PS.

Synovial fluid

Paired samples of serum and synovial fluid were obtained from 15 RA patients with longstanding RA from the Zuiderhospital Rotterdam.

Clinical scoring

RA disease activity of 39 patients at time of donation (including the synovial fluid patients) was determined by the DAS (disease activity score)-28 score (33):

$0.56\sqrt{TJC} + 0.28\sqrt{SJC} + 0.7 \log ESR + 0.014\ GH$

were TJC= number of painful joints (out of 28)
SJC= number of swollen joints (out of 28)
GH= general health score on a visual analogue scale (0-100) (table 1).

Erythrocyte Sedimentation Rate (ESR) (mm/hour) was used as a measure of disease activity of all other RA patients (table 1).

Table 1. General features of the donor groups

Group	Source	Number male/ female	Age (years)	Duration (months)	DAS score* (mm/hour)	ESR
Healthy controls	serum	67	52(30-83)	n.d.	n.d	n.d.
		58	50(19-88)	n.d.	n.d.	n.d.
RA patients:						
Early	serum	17	64(28-84)	< 12	n.d.	n.d
		27	50(23-82)	< 12	n.d.	n.d.
Long-term	serum	20	62(33-84)	89(8-240)	5.6(3.3 - 7.4)	71(23-113)
		43	63(31-82)	132(13-420)	5.1(1.9-7.2)	68(15-140)
	Synovial fluid	3/12	63(46-82)	66(8-180)	5.7(3.4-7.4)	n.d.

expressed as mean with the range shown in parentheses
erytrocyte sedimentation rate, n.d.= not done

Isolation of PG from human feces

PG-PS from feces of a healthy subject was prepared as described previously (24). Briefly, feces was diluted in distilled water (40 g in 100 ml) and homogenized in a Model 400 Lab Blender (Stomacher, Colworth, UK). After cambric gauze filtration the suspension was centrifuged for 45 minutes at 5,000 *g*. Four volumes of 96% ethanol were added to 1 volume of supernatant. After 2 hours at 4°C, the precipitate was centrifuged for 15 min at 5,000 *g*. The pellet was dissolved in and dialyzed against milliQ water for 48 h. Next, the suspension was centrifuged for 1 hour at 100,000 *g* and the clear supernatant was collected. Size exclusion chromatography was performed using dilutions of 15-60 mg/30 ml (depending on the viscosity of the solution), with a TSK HW75 column. After passage of 100 ml void volume, fractions (8 ml/5 minutes) were collected and assayed for their protein and carbohydrate contents. High-molecular-weight fractions containing carbohydrates but no protein were pooled, dialyzed, and lyophilized. From 100 g of feces about 50 mg PG could be retrieved by this procedure.

Enzyme-linked immunosorbent-assay (ELISA)

To measure anti-PG-PS-antibodies in serum and synovial fluid an ELISA was performed. 50 µl of PG-PS (10 µg/ml) was coated overnight at 50°C in 96-well polystyrene microtiter plates. The plates were washed three times with PBS-0.02% Tween 20. 100 µl serum or synovial fluid diluted in PBS-0.2% Tween was added to the wells and after 1 hour at 37°C unbound antibody was removed by three wash steps. As detecting antibody, peroxidase conjugated rabbit anti-human IgM, IgG or IgA (Jackson Immunoresearch, Inc., Westgroove, PA) diluted in PBS-Tween 0.2% was used during 1 hour at 37°C. After washing three times the development of the colorimetric assay took place at 37°C for 30-45 minutes after the addition of 100 µl of ortho–phenylenediamine/H_2O_2. The reaction was stopped by adding 50 µl 4M H_2SO_4 and the optical density was measured at a wavelength of 492 nm with a Titertek Multiskan (Flow Laboratories, Irvine, Scotland). Results were expressed as optical density (OD) units. On each plate the standard serum was included and each sample was

tested in triplicate. Background absorption of the conjugate was also tested in triplicate. The average OD reading of the sera and standard serum minus the background staining was used to calculate concentrations of antibodies in a ratio versus the standard serum according to the following formula:

$$\text{ratio} = OD_{sample} - OD_{background} / OD_{standard\ serum} - OD_{background}$$

This ELISA has been validated previously (19), we excluded crossreactivity with rheumatoid factor (RF), by preincubating sera with known amounts of RF with PG. This did not interfere with subsequent detection of RF.

Determination of optimal dilutions for measuring antibody levels against PG-PS
Standard serum, sera of six healthy volunteers, as well as synovial fluids of five RA patients were titrated from 1:2 to 1:6400 and added to the wells coated with either 5, 10 or 20 µg/ml of PG-PS to determine the optimal concentration of serum and antigen. Optimal serum concentration was the concentration in the linear area in the dilution curve. Based on checkerboard titrations, anti-PG-PS antibodies were measured after coating plates with 10 µg/ml PG-PS and serum dilutions of 1:400, 1:1600 and 1:200 for IgM, IgG, and IgA, respectively. To analyze PG-PS antibodies in synovial fluid, dilutions of 1:20, 1:200 and 1:80 were used respectively for IgM, IgG, and IgA.

Total immunoglobulin determinations
Of 11 randomly chosen sera of RA patients total IgM, IgG and IgA concentrations were measured using immunonephelometry (34) to examine whether total immunoglobulin concentrations were in the normal range.

Statistical analysis
The ratio's of IgM, IgG and IgA measured in the PG-PS ELISA of RA patients versus standard serum and controls were compared with Student's T test to analyze significant differences between the two groups. Linear regression was used to detect any age-related influence on the levels of immunoglobulin specific for PG-PS in healthy controls and to evaluate correlation between disease activity and anti-PG-PS antibodies in synovial fluid.

RESULTS

Systemic antibody levels against PG in healthy females compared to healthy males
Antibody levels against PG-PS were analyzed in the healthy control group to examine whether levels against PG-PS differ between male and female donors. Figure 1 shows that females have a significantly lower mean ratio of anti-PG-PS IgG antibodies to standard serum, compared to males ($p<0.01$). Levels of IgA and IgM antibodies against PG-PS were not different in females compared to males (figure 1). These results show that IgG antibody levels against PG-PS differ in healthy male and female donors and have to be analyzed separately.

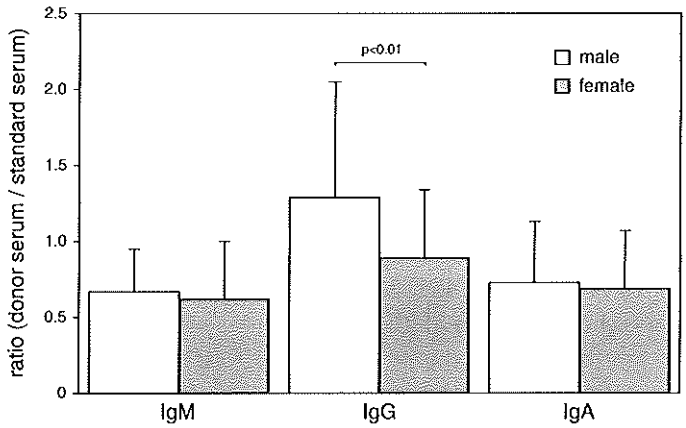

Figure 1. Systemic antibody responses against PG in healthy females compared to males. Levels of IgM, IgG and IgA against PG-PS were measured in 67 healthy men and 58 healthy women (table 1). Levels of IgG were significantly reduced in women compared to men ($p<0.01$). Results are indicated as mean values + standard deviation.

Systemic antibody levels against PG in healthy donors in relation to age

To examine whether antibody levels against PG-PS are related to age, the same sera used for determination of the sex difference in systemic antibodies against PG-PS were analyzed. Figure 2 shows that in females a decline occurs in antibody levels of all three isotypes with advancing age. This correlation was only significant for IgA antibodies. In males, a minor decline could be observed in IgA titers with age but this difference was not significant. These results imply that age-matched controls have to be used for the examination of PG-PS antibodies in female RA patients compared to healthy controls.

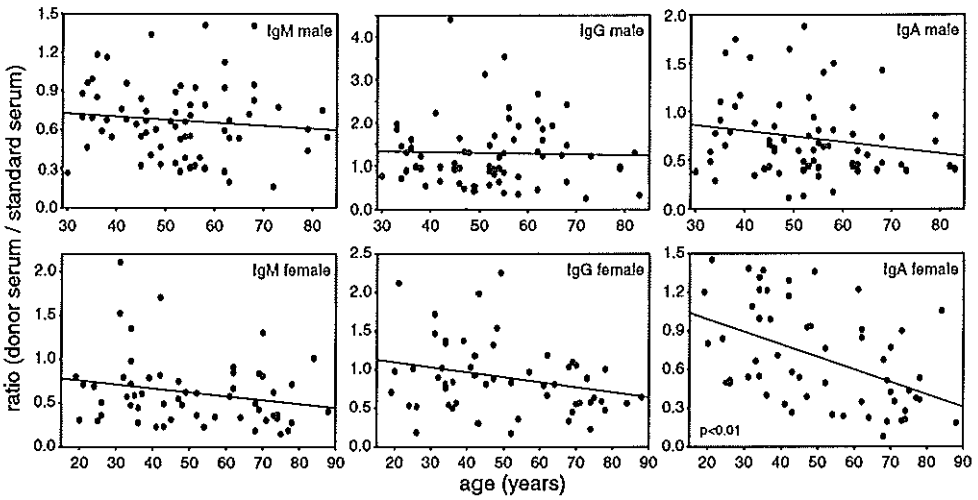

Figure 2. Systemic antibody responses against PG-PS in relation to age of healthy females and males. Levels of IgM, IgG and IgA against PG-PS measured in 67 healthy males and 58 healthy females were related with age. The decline of IgA antibodies in female was significantly related to increasing age ($p<0.01$) ($r^2=0.24$).

Systemic antibody levels against PG in RA patients

To determine whether the systemic antibody levels against PG-PS are different in RA patients compared to healthy controls, sera of 111 healthy controls and of 61 RA patients were compared. Males and females were compared separately and RA females were compared with age matched controls. Analysis of 67 male donors (mean age 52, range 30-83) and 21 RA patients (mean age 62 range 33-82) showed that RA patients had lower IgG antibody levels against PG-PS compared to healthy controls (figure 3).

Figure 3 also shows that IgG antibody levels against PG-PS were lower in 40 sera of RA females (mean age 63 range 30-84) compared to 34 sera of healthy controls (mean age 63, range 31-88). Using Student's T test, this difference was found to be significant for both males ($p<0.01$) and females ($p< 0.01$). No differences could be observed in IgM and IgA antibody levels against PG-PS in male or female RA patients compared with healthy controls.

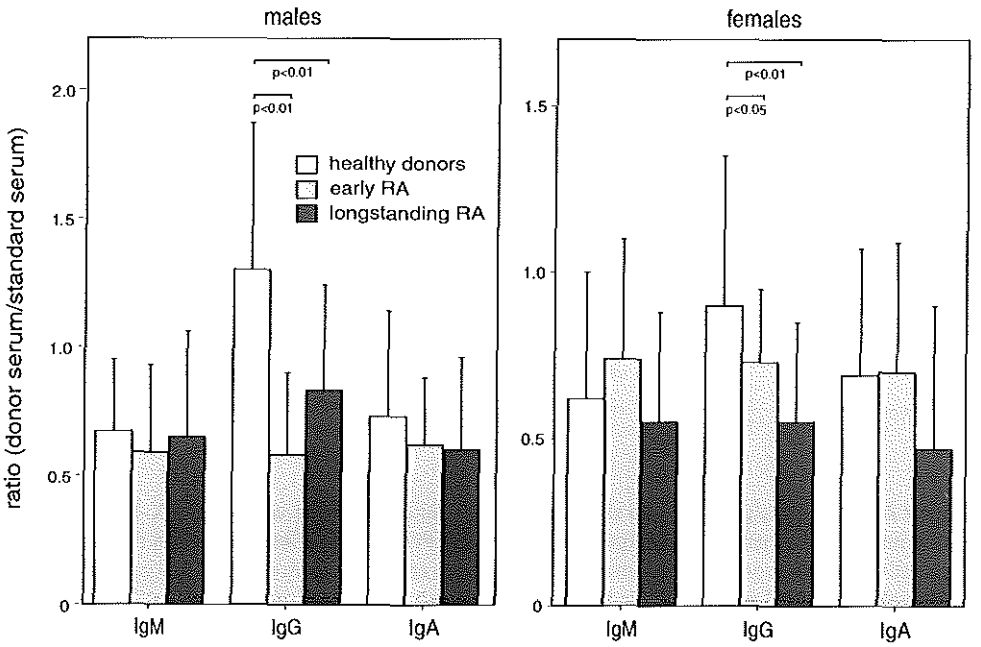

Figure 3. Systemic antibody responses against PG-PS in early and long-term RA patients. Levels of IgM, IgG and IgA against PG-PS were compared in early and long-term RA for male and female patients. Levels of IgG were reduced in RA patients compared to healthy controls. This difference was significant for long-term RA males and females ($p<0.01$) and for early RA male and female donors ($p<0.01/p<0.05$). Results are indicated as mean values + standard deviation.

As antibody levels shortly after the disease onset might be more informative than those in long term RA, we also compared patients with newly developed disease (less than one year) and healthy controls.

Sera of 27 females with early RA (mean age 50, range 23-84) were compared with 58 sera of healthy controls (mean age 50, range 19-88).

The level of IgG specific for PG-PS was reduced compared to healthy controls. No dif-

ferences were observed between levels of IgM and IgA of females with early RA and healthy controls. Sera of 17 male donors with early RA (mean age 64, range 28-84) and of 67 healthy controls (mean age 52, range 30-83) were compared. Again IgG levels against PG-PS were reduced and no differences were observed in IgM and IgA antibodies. The reduction in levels of IgG antibodies against PG-PS was significant for both male and female donors ($p<0.01$, $p<0.05$ respectively).

Antibody levels specific for PG in synovial fluid of RA patients

To examine whether antibodies can also be detected locally in synovial fluids of RA patients where they might affect inflammation, antibody levels against PG-PS were determined in synovial fluid of 15 RA patients. In all synovial fluids IgM, IgG and IgA antibodies against PG-PS could be measured.

To assess whether anti-PG-PS antibody levels occurring in the joints correlated with disease activity, linear regression was performed. No correlation could be observed between disease activity and anti-PG-PS IgG. In contrast, synovial IgM and IgA titers against PG-PS were related positively with disease activity (figure 4), but only the correlation found for IgA against PG-PS and disease activity was significant.

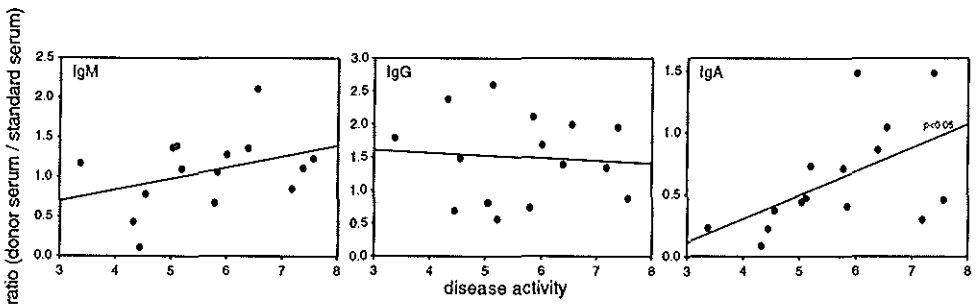

Figure 4. Antibodies against PG-PS synovial fluid in relation to disease activity in RA. In 15 synovial fluid samples of RA patients IgM, IgG and IgA against PG-PS were measured and in relation to disease activity (DAS score). All 15 synovial fluid samples contained IgM, IgG and IgA against PG-PS. IgA levels against PG were significantly positively related with disease activity ($p<0.05$) ($r^2=0.3$).

DISCUSSION

The present study showed that early and long term RA patients had significantly lower systemic IgG antibody levels against PG-PS than healthy controls, and that the amount of IgA antibodies against PG-PS in synovial fluid samples of RA patients is positively correlated with disease activity. Furthermore, this study showed that IgG antibody levels against PG-PS were significantly lower in healthy females than in healthy males, and levels of IgA against PG-PS are negatively correlated with age in healthy female donors.

This study was performed to analyze systemic and local antibody levels against PG-PS in an extended panel of RA patients and healthy controls. As shown before (25,26), all healthy donors have serum antibodies against PG-PS indicating that no tolerance at the B cell level

occurs despite continuous exposure to PG-PS at the mucosal surface of the gut, which is thought to be optimally suited for tolerance induction.

A lower antibody level was found in serum of females compared to males, which was significant only for IgG ($p<0.01$). We also observed a negative relationship between age and PG-PS-specific IgA antibodies in females. Although there is no evidence of causal linkage, it is of interest to note that the lower antibody levels against PG-PS in females compared to males correlates with the 2-3 fold higher general incidence of RA in females. In addition, the decrease of antibody levels against PG with age in females parallels the rise of RA incidence with advancing age (35,36).

RA patients had significantly lower IgG antibodies against PG-PS compared to healthy controls. To exclude the possibility that the decrease in antibody levels to PG-PS in RA patients was due to generalized depression of antibody levels, total IgG, IgM and IgA levels were measured in 15 randomly selected RA sera. These levels were either normal or slightly increased, indicating that no general decrease of immunoglobulin titers had occurred (data not shown). To analyze whether the decrease of immunoglobulins against PG-PS in RA was also apparent during early stages of the disease we analyzed patients with early RA (< 1-year disease duration). Both male and female patients had significantly lower IgG levels specific for PG-PS, suggesting that this phenomenon is apparent already early in the disease. Of five early RA patients, we also obtained a blood sample one-year after the first sample. The antibody levels in the paired samples were comparable indicating that disease progression has no influence on antibody levels specific for PG-PS (data not shown). Decreased antibody levels against PG-PS possibly were not due to disease progression as can be concluded from the fact that in early RA male patients the level of IgG specific for PG-PS was even lower than in longstanding RA patients.

The present study also showed the presence of IgM, IgG and IgA antibodies specific for PG-PS in synovial fluid of RA patients and that the level of IgA anti-PG-PS antibodies correlated positively with disease activity of the patients. At present it is unclear whether these antibodies aggravate local inflammation (e.g. due to immune complex formation or activation of cells through Fc-receptor binding), or that they locally capture PG with beneficial results. Neither is it known whether antibody is produced locally by plasma cells, or leaks into the synovium from the circulation. Inflammation can enhance permeability of the synovial membrane (37). Paired analysis of serum and synovial fluid showed a positive relationship between synovial fluid and serum IgM, IgG and IgA levels specific for PG-PS, suggesting that at least part of the antibodies present in the synovial fluid could be derived from the circulation (data not shown). The local inflammatory response in the synovium may attract lymphocytes. It is known that especially mucosal lymphocytes are able to home to the synovium by expression of relevant adhesion molecules. (38-40). Trapping of PG in the synovium may result in the attraction of PG specific plasma cells, due to local antigen deposition as well as antigen-driven T cell activation which can provide important 'second signals' for expansion and terminal differentiation of mucosal Ig-producing immunocytes at secretory effector sites (41).

Some earlier studies focussing on antibody levels against PG in RA reported elevated and not decreased antibody titers against PG (27-29). An explanation for this discrepancy might be that the immunoglobulin levels were measured using PG isolated from cultured bacteria, which may be structurally distinct from PG physiologically present at the mucosa. We used PG-PS derived from the indigenous intestinal flora representing PG to which the body is continuously exposed at high quantities. Other studies support our findings. For instance, Rantakokko et al. (31) found lower IgG levels against PG from cultured *Streptococcus pyogenes* in RA patients compared to controls. Another study showed that *in vitro* responses of B cells to PG were markedly depressed in RA patients compared to healthy controls (42), which might provide a mechanistic explanation for reduced antibody levels in RA patients.

The lower antibody levels against PG-PS in RA might be explained by the capture of IgG antibodies by inflammatory sites. With radiolabelled IgG scintigraphy, it has been demonstrated that IgG accumulates at the site of inflammation (43). Increased exudation of the radiolabelled IgG through the leaky capillary walls at the site of inflammation into the locally expanded extravascular space (43), and the chemical nature of the radiolabel (44,45) are all factors important in IgG localization. In addition, it has been shown that radiolabelled IgG binds to gram-negative and in even higher extent to gram-positive bacteria *in vivo* and *in vitro* (46,47). Furthermore it has been shown that radiolabelled IgG is able to bind to extracellular matrix components (48). Although it is unclear to what extent accumulation of PG-PS specific antibodies in inflamed joints will be reflected in the lowered serum levels, it can be hypothesized that IgG antibodies specific for PG-PS accumulate in the synovium and bind to PG located in the synovium and/or to extracellular matrix components.

Another explanation for the reduced amount of systemic IgG antibodies in RA patients is a lowered antibody response against PG, suggesting that antibodies against PG can have a protective effect against the development of RA. This hypothesis is supported by two findings. First, lower antibody levels against PG in healthy persons are correlated with RA incidence. Second, RA patients have lower antibody levels against PG than healthy controls. A possible mechanism is that more PG reaches tissues outside the mucosal sites, and is captured in the synovial tissue where it promotes proinflammatory responses (22). Increased access of PG to non-mucosal sites may be due to at least two mechanisms. First, reduced gut barrier function may result from inflammation, leading to increased bacterial translocation. Ultrastructural lesions of the gut wall have been reported in RA patients (49). Second, lower antibody levels against PG may not be sufficiently capable of eliminating the bacterial products from mucosal sites and/or the bloodstream. Antibodies that reach the mucosal lumina perform 'immune exclusion', a term coined for non-inflammatory mucosal surface protection against antigens. This function is performed by secretory IgA or IgM (S-IgA, S-IgM), but also by serum IgG antibodies (50,51).

In conclusion, the lower antibody levels against PG in RA patients suggest a local accumulation of IgG antibodies to inflammatory sites or a protective role of IgG antibodies. The protective properties of antibodies against PG can be further investigated by measuring mucosal antibody levels in feces and intestinal fluids. The correlation between synovial

fluid IgA antibodies against PG and disease activity suggesting intra-articular production of IgA is currently being further investigated by *in situ* detection of PG specific antibody forming cells in synovial tissues using labeled PG. The study implies that restricting the access of PG to non-mucosal sites may contribute to dampening RA-activity. Therapeutic agents designed to decrease mucosal permeability should therefore be investigated in *in vitro* and *in vivo* models of bacterial translocation to assess whether they might have clinical relevance.

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CHAPTER 3.2

Bacterial peptidoglycan from human spleen induces T cell proliferation and inflammatory mediators in rheumatoid arthritis patients and healthy subjects

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ABSTRACT

Peptidoglycan (PG), a component of gram-positive bacteria may be involved in rheumatoid arthritis (RA) because of its capability to induce production of proinflammatory cytokines, to induce arthritis in rodents, and its presence in antigen presenting cells in RA joints. The present study shows that physiologically relevant PG fractionated from human spleen is able to induce T cell proliferation in peripheral blood and synovial fluid samples of RA patients but the magnitude of the response did not differ from that of cells from healthy subjects. In addition, production of cytokines associated with RA (IL-1β, IL-6, IL-8, IL-10, IL-12, TNF-α) and of the matrix metalloproteinase, gelatinase B (MMP-9) was induced in blood and synovial fluid cultures of RA patients. The fact that PG, which can be found in antigen presenting cells in synovial tissues of RA patients is able to induce production of inflammatory mediators supports the hypothesis that PG plays a role in the pathogenesis of RA by influencing the inflammatory microenvironment of the joint.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by the infiltration and proliferation of macrophages and T cells and to a lesser extent of B cells, fibroblasts and dendritic cells into the synovial membrane. Within this pannus tissue, inflammation, cellular proliferation and humoral and cellular immune responses lead to release of different mediators including antibodies, cytokines and matrix metalloproteases, contributing to degradation of cartilage, bone and other connective tissue.

For many years, the most attractive concept of the pathogenesis of RA has been the model of autoimmunity in which autoreactive CD4+ T cells play an important role. The association of susceptibility to RA and disease outcome with HLA-DR4/DR1 antigens (1,2) has been regarded as the strongest argument for the T cell-dependent nature of the disease. Moreover, T cells present in the inflamed joints predominantly consist of the primed CD45RO subset, expressing early and late activation antigens (3-5). However, despite the expression of activation markers, the T cell population at the site of inflammation is in a hyporesponsive state (6,7). Furthermore, the fact that T-cell derived cytokines like IL-2, IL-4 and IFN-γ can hardly be detected in RA joints (8,9) and that T cell-directed therapies using depleting anti-CD4 mAbs, as well as IL-2-receptor immunotoxins have yielded disappointing results so far (10-12) argue against the T cell as the initiator of RA. Therefore alternative viewpoints have been suggested to explain autoimmune diseases (13). The importance of interactions between T cells and antigen presenting cells (APC) as shown by the expression of CD40L, CD28, CTLA4 by T cells and B7-1 (CD80), B7-2 (CD86) and CD40 by APC in the synovium has focussed attention on the role of antigen presenting cells in RA (14-17). A crucial role of antigen presenting cells in the pathogenesis of RA is suggested by the high expression and production of the proinflammatory cytokines IL-1β, IL-6 and TNF-α produced by monocytes/macrophages, which affects fibroblasts, chondrocytes and lymphocytes in blood, synovial fluid and synovial tissue of RA patients (18). This is supported by the promising results with anti-TNF-α or anti-TNF receptor therapies in RA patients (19) and the development of spontaneous arthritis in TNF-α mice (20,21). Furthermore, the matrix metalloproteinase gelatinase B, the expression of which is regulated by cytokines and other inflammatory mediators, has been detected in synovial fluid of RA patients (22).

The (auto)antigen(s) leading to chronic stimulation of T cells and/or macrophages are still unknown. In reactive arthritis, the triggering antigens are thought to be microbes that cause infections of the urogenital tract and the gut, such as *Chlamydia trachomatis*, *Yersinia*, and *Salmonella* species (23,24). Antigens of these bacteria have been detected in synovial cells (25-27), and T cells specific for these antigens have been found in the synovium (28,29). It can be suggested that the bacterial load in the intestine, with which we are in lifelong close contact, might be important for the induction and perpetuation of RA. This is supported by the observation that some patients with Crohn's disease and ulcerative colitis, both intestinal autoimmune diseases, may suffer from arthritis (30,31). Furthermore Crohn's-like inflammatory bowel disease and chronic inflammatory arthritis develop spontaneously in a knock-out mouse strain lacking a TNF AU-rich element, responsible for TNF mRNA destabilization and translational repression in hemopoietic and stromal cells (32).

We and others have previously hypothesized that peptidoglycan (PG) plays a role in the pathogenesis of RA (33-36). Peptidoglycan (PG) is the major component of the cell wall of gram-positive bacteria and is composed of long sugar chains of alternating N-acetyl glucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc) residues, which are interlinked by peptide bridges, resulting in a large complex macromolecular structure (37). PG isolated from the anaerobic bacterium *Eubacterium aerofaciens*, which is present in numbers over 10^9/g human feces, induces a severe and chronic arthritis in rats (38). Furthermore, PG

could be detected in antigen presenting cells in synovial tissues of RA patients using a specific antibody (39,40). PG was also isolated from sterile normal human spleen using biochemical methods (PG_{spleen}) (41,42). *In vitro* analysis showed that the latter PG fraction is able to induce production of the proinflammatory cytokines TNF-α, IL-1 and IL-6 (42), which are crucially involved in RA pathogenesis (18). Furthermore, it was shown that PG isolated from human spleen is able to bind its receptor (CD14) and to elicit T cell proliferation of CD4+ and CD8+ αβTCR+ T cells after proteolytic processing (Schrijver et al. submitted). Previously, low T cell responses against peptidoglycan-polysaccharide complexes ($PG\text{-}PS_{feces}$) isolated from human feces were analyzed in peripheral blood and synovial fluid of RA patients (43). In the current study we used PG_{spleen} because it reflects the properties of PG present in human tissues, it is structurally more intact than $PG\text{-}PS_{feces}$ and it has a 10-100 fold higher biological activity (42).

To further delineate the role of PG in the pathogenesis of RA we determined the influence of PG fractionated from sterile human spleen on T cell proliferation and production of inflammatory mediators. Therefore we assessed the production of the proinflammatory cytokines IL-1β, IL-6, IFN-γ and TNF-α, the immunoregulatory cytokine IL-10, the chemokine IL-8 and the matrix metalloproteinase gelatinase B using peripheral blood of RA patients as well as control subjects and synovial fluid samples of RA patients upon PG stimulation.

PATIENTS AND METHODS

Blood samples

Heparin blood samples of 29 RA patients fulfilling the American College of Rheumatology criteria (44) were obtained from the Zuiderziekenhuis Hospital Rotterdam. Patients received no immunosuppressive medication except for some patients receiving low dose prednisone (up to 10 mg/ day) or methotrexate (up to 30 mg /week).
RA disease activity at time of donation was determined score by an experienced rheumatologist using the DAS-28 score (45):
$0.56\sqrt{TJC} + 0.28\sqrt{SJC} + 0.7 \text{ LOG ESR} + 0.014 \text{ GH}$
TJC= number of painful joints (out of 28)
SJC= number of swollen joints (out of 28)
GH= general health score on a visual analogue scale (0-100)
27 blood samples of healthy donors were a kind gift of the Department of Epidemiology from the Erasmus University and University Hospital Rotterdam-Dijkzigt.10 blood samples were obtained from healthy volunteers. Donor features are shown in Table 1. All blood samples were processed within 2-5 hours after collection.

Synovial fluid samples

Synovial fluid samples of an inflamed joint of 14 RA patients were obtained from the Zuiderziekenhuis in Rotterdam (table 1). Synovial fluid was collected in heparin tubes. Paired blood and synovial fluid samples were collected from 10 of these patients.

Table 1. General features of the donor groups

Group	Source	Sex (m/f)	Age (years)*	Duration (months)*	DAS score*
Healthy controls	blood	12/21	60 (25-84)	n.a.	n.a.
RA patients	blood	9/21	58 (30-82)	101 (8-360)	5.0 (1.6-7.8)
	synovial fluid	3/11	63 (46-82)	66 (8-180)	5.7 (3.4-7.4)

* expressed as mean, with the range shown in parentheses
n.a.= not applicable

Proliferation assays

Peripheral blood mononuclear cells (PBMC) of all healthy donors and RA patients and synovial fluid mononuclear cells (SFMC) of 14 RA patients were isolated from heparin blood using Ficoll Hypaque (Pharmacia, Uppsala, Sweden). $2x10^6$ cells in 100 µl were cultured in 96 well plates in the presence of 100 µl PG isolated from human spleen containing 0.70 µg muramic acid (42), PG-PS from human feces containing 70 µg/ml muramic acid (46), 2 µg/ml lipopolysaccharide (LPS) (Sigma, St. Louis, MO) or 6 Lf/ml tetanus toxoid (TT) as a control recall protein antigen (RIVM, Bilthoven, the Netherlands). Cells and antigens were diluted in RPMI (Biowittaker, Verviers, Belgium) + 10% heat inactivated human serum. All antigens were analyzed in triplicates and concentrations mentioned in the result section are final concentrations. The cells were cultured during 7 days, the last 8 hours in the presence of tritiated thymidine (Amersham, Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). The cells were then harvested and the incorporated radioactivity counted in a liquid scintillation counter. The proliferative response was expressed as counts per minute (cpm) or stimulation index (SI), the ratio of mean counts per minute in the presence and absence of antigen. When SI > 3, it was concluded that proliferation had occurred.

Induction of cytokine release in whole blood or PBMC after stimulation with PG

Cytokine induction was performed in 20 blood and 9 synovial fluid samples of RA patients and in 6-10 healthy control blood samples. 12.5 µl of RPMI 1640 (Biowittaker) containing 90 ng/ml PG (muramic acid) or 25 ng/ml LPS (Sigma) were added to a polypropylene tube containing 112.5 µl blood, synovial fluid, PBMC or SFMC. The mixtures were incubated for 4 or 17 hours at 37°C in a 5% CO_2 atmosphere. Then 375 µl RPMI was added to each tube and the mixtures were centrifuged for 10 minutes at 400 *g*. The supernatants were stored at –20°C until cytokine and gelatinase B analysis.

Determination of cytokines

Cytokine production was measured by commercial capture ELISA. In a pilot study of 10 healthy donors, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p40, TNF-α, INF–γ and GM-CSF were measured in culture supernatants of 4 h and 17 h supernatants. IL-4 and GM-CSF could hardly be measured in the supernatants. After LPS stimulation, IL-1β, IL-6, IL-10, IL-12 and TNF-α reached highest levels after 4 h of stimulation and IL-8 and IFN-γ after 17 h. Therefore IL-1β, IL-6, IL-10, IL-12p40 and TNF-α production was measured in the 4 h supernatants and IL-8 and IFN-γ in the 17 h supernatants. ELISA was performed accord-

ing to the manufacturers' guidelines (Biosource, Fleurus, Belgium). Briefly, polystyrene microtiter wells (Immuno Maxisorp, Nunc, Roskilde, Denmark) were coated overnight at room temperature with monoclonal anti-cytokine antibodies followed by washing (0.9% NaCl) and 2 h of blocking (PBS/BSA 0.5%). Freshly thawed supernatants of the cell cultures and recombinant human cytokine-standards were incubated in duplicates for two hours in the presence of a biotinylated second anti-cytokine antibody, followed by washing steps, poly-streptavidin-horseradish peroxidase (CLB, Amsterdam, The Netherlands) and enzyme substrate (TMB peroxidase, (KPL, Gaithersburg, MD). Optical density was measured at 450 nm.

Determination of gelatinase B activity

Gelatinase B (MMP-9) was measured by two methods in the supernatants obtained after 4 h of stimulation. First, the quantitative gelatine zymography method was used, which is a densitrometric method that, by inclusion of appropriate standards, discriminates inducible gelatinase B (MMP-9) from constitutive gelatinase A (22,47). Quantitative determination of gelatinase activity was achieved by computerised image analysis through two-dimensional scanning densitometry. Gelatinase activity was expressed in scanning units representing the scanning area under the curves, which is an integration ratio that takes into account both brightness and width of the substrate lysis zone. Second, the gelatinolytic activity which results from the balance between enzymes and enzyme inhibitors (the so-called protease load) was measured in the samples using fluorescence-activated gelatine conversion. Both methods were described in detail earlier (22,47,48).

Statistical analysis

Statistical analysis was performed using the Mann-Whitney method to analyze differences between PG-induced proliferation and production of effector molecules between RA patients and healthy subjects. Furthermore, the Wilcoxon signed rank test method was used to analyze whether PG was able to induce cytokine production. Linear regression was performed to determine relationships between disease activity or disease duration with T cell proliferation and cytokine production. p values < 0.05 were considered significant.

RESULTS

Proliferation of PBMC after stimulation with PG

To examine whether proliferation of T cells against PG was increased in RA patients versus healthy subjects, 29 RA patients and 33 age-matched controls were examined for the proliferative response against PG_{spleen} and $PG\text{-}PS_{feces}$. LPS and TT were used as positive controls. The results show (figure 1) that after 7 days of culture, T cell proliferation was elicited by PG_{spleen} in 21 of the 29 RA patients. Twenty-five of 33 healthy donors reacted against PG_{spleen}. The median of the SI values was 6.9 for RA patients and 6.7 for healthy subjects. The proliferative response against $PG\text{-}PS_{feces}$ was lower compared to PG_{spleen} despite the 100-fold higher concentration used.

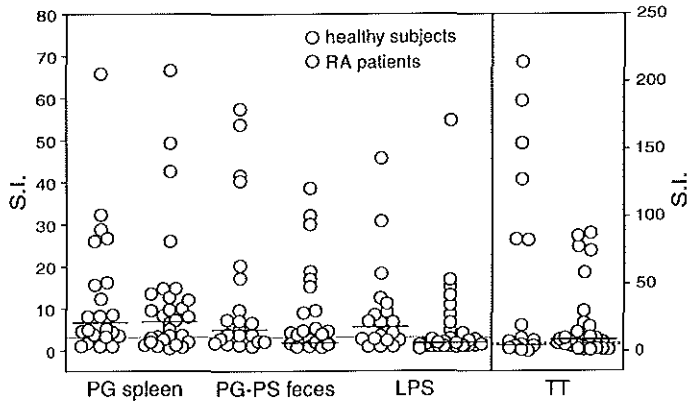

Figure 1. PG isolated from sterile human spleen induces proliferation of peripheral blood mononuclear cells (PBMC) in both RA patients and healthy subjects. PBMC isolated from 29 RA patients and 20 healthy controls were incubated during 7 days with PG_{spleen}, PG_{feces}, LPS or TT. Results are indicated as stimulation index (SI). No differences were observed between RA patients and healthy controls after stimulation with both PG sources. LPS and TT induced proliferation were decreased in RA patients, but these effects did not reach significance. The horizontal line indicates the cut off point for proliferation (SI=3).

The proliferative response against LPS and TT was lower in RA patients than in healthy donors, but this difference did not reach significance.

Proliferation of SFMC after stimulation with PG

Synovial fluid was obtained from 14 RA patients. Mononuclear cells were isolated from both samples using Ficoll. In only two of the synovial fluid samples a proliferative response against PG_{spleen} could be measured (figure 2). After stimulation with $PG\text{-}PS_{feces}$, LPS and TT none, 3 and 2 patients out of 14 did respond, respectively.

When the proliferative responses of 10 samples of synovial fluid mononuclear cells of the four analyzed antigens were compared with paired peripheral blood cells, responses were significantly lower, despite the use of identical cell numbers and stimulation conditions (data not shown).

Relationships between proliferation and disease activity or disease duration

Correlation analysis was performed to determine the possible relationships between proliferation and RA disease activity or disease duration as scored at the time of sampling. No correlations were found for any of the four antigens examined.

Cytokine production in peripheral blood after stimulation with PGspleen

To investigate whether PG is able to stimulate production of cytokines involved in RA, production of IL-1β, IL-6, IL-8, IL-10, IL-12, INF-γ and TNF-α was measured after whole blood cell stimulation of 20 RA samples and 10 healthy donor samples. The samples were stimulated with PG_{spleen} and LPS served as a positive inducer. Results of RA and healthy donor samples stimulated with medium and PG_{spleen} are shown in figure 3.

These results show that without stimulation, concentrations of all analyzed cytokines were

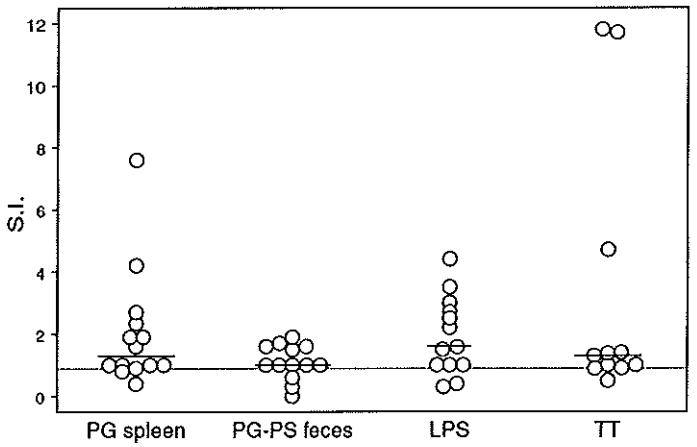

Figure 2. PG_{spleen} induces proliferation of RA synovial fluid mononuclear cells (SFMC). SFMC isolated from 14 RA patients were cultured during 7 days with PG_{spleen}, PG_{feces}, LPS and TT. Results are indicated as stimulation index (SI). Proliferation of SFMC of 2 patients was observed after PG_{spleen} stimulation. After stimulation with PG_{feces}, LPS and TT none, 3 and 2 out of 14 patients responded respectively. The horizontal line indicates the cut off point for proliferation (SI=3).

higher for RA patients compared to healthy subjects. Because this higher concentration was mostly due to 5-7 high responding patients the difference between control subjects and RA patients was only significant for IL-10.

In healthy subjects, PG_{spleen}-induced production of IL-1β, IL-6, IL-8, IL-10, IL-12 and TNF-α was significantly increased compared to spontaneous production. In the RA patients a significant increase in the production of IL-1β, IL-6, IL-8, IL-10, and TNF-α was observed.

Because cytokine levels in unstimulated samples of RA patients were very high, probably due to cytokines already present in the plasma, mononuclear cells were isolated to measure newly secreted cytokine. Table 2 shows that after control stimulation with medium alone all cytokine levels were low except IL-8. After PG stimulation a significant increase was observed for all cytokines except for IL-8, IL-12 and IFN-γ.

Table 2. PG_{spleen} induces production of cytokines by peripheral blood mononuclear cells

Cytokine	Medium	PG_{spleen}
IL-1β	3 (0-233)	217 (13-1763)*
IL-6	30 (0-1418)	1602 (84-4118)*
IL-8	2938 (372-3962)	3293 (567-3779)
IL-10	2 (0-42)	80 (0-280)*
IL-12	0 (0-444)	1 (0-2470)
IFN-γ	0 (0-0)	0 (0-0)
TNF-α	13 (0-7002)	2864 (0-13263)*

results are shown as median cytokine production (pg/ml) with range in parentheses

* $p < 0.01$

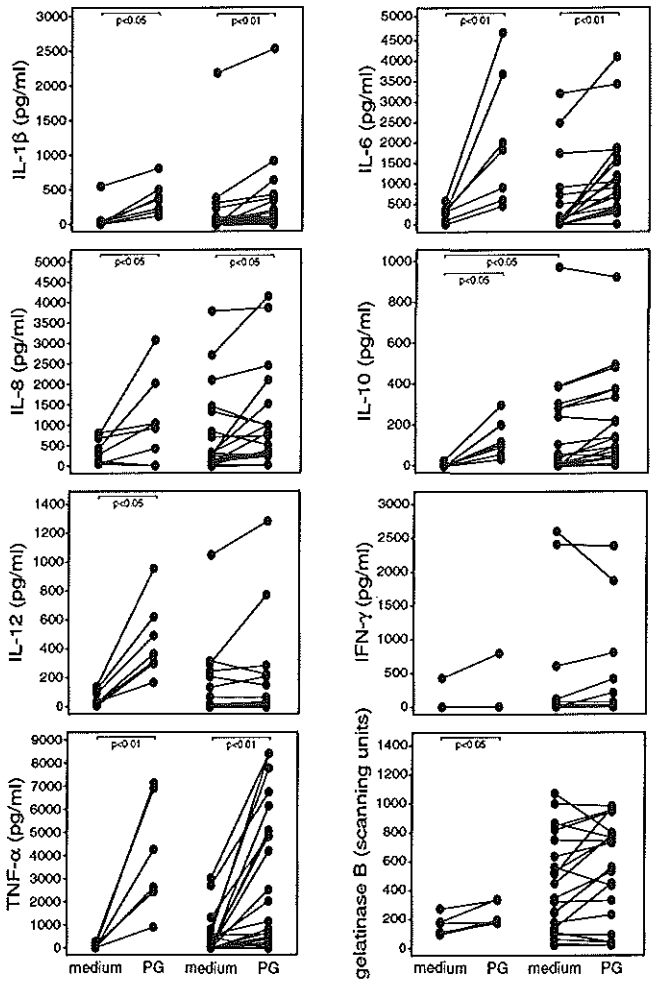

Figure 3. PG_{spleen} induces cytokine production in healthy subjects and RA patients. Whole blood cell samples of 20 RA patients and 10 healthy controls were stimulated with and without PG_{spleen}. Production of IL-1β, IL-6, IL-8, IL-10, IL-12 TNF-α and IFN-γ was assessed by ELISA. Levels of all cytokines analyzed were higher in unstimulated samples of RA patients compared to healthy controls, which was significant for IL-10. PG_{spleen} was able to induce significantly production of IL-1β, IL-6, IL-8, IL-10 and TNF-α in both healthy subjects and RA patients. IL-12 was only significantly induced in healthy subjects.

Cytokine production in synovial fluid after stimulation with PG_{spleen}

Cytokine production was induced and measured in supernatants of whole synovial fluid mononuclear cells and of isolated mononuclear cells of 9 RA patients. The results show that all cytokines analyzed were present in the synovial fluid. After stimulation with PG the production of IL-1β, IL-6, IL-8 and TNF-α was increased (table 3).

When mononuclear cells were isolated, high concentrations of IL-6, IL-8, and TNF-α were measured without further stimulation, indicating that activated mononuclear cells spontaneously produced these cytokines. After PG stimulation, IL-1β, IL-8, IL-10 and TNF-α were

produced at significantly higher levels, indicating that PG can enhance production of these cytokines in SFMC.

Table 3. PG spleen induces production of proinflammatory mediators in synovial fluid (SF) samples

Cytokine	Medium		PG_{spleen}	
	whole SF	SF mononuclear cells	whole SF	SF mononuclear cells
IL-1β	9 (0-59)	7 (0-14)	36 (0-556)*	121 (65-168)*
IL-6	789 (0-9452)	133 (2736-3453)	1286 (0-9443)*	496 (444-2761)
IL-8	498 (34-1295)	3003 (2736-3453)	1530 (960-3570)*	3438 3233-3866)*
IL-10	49 (0-733)	2 (0-16)	150 (0-678)	86 (32-133)*
IL-12	320 (0-2543)	0 (0)	348 (0-2918)	0.5 (0-1.5)
IFN-γ	0 (0-8089)	0 (0)	0 (0-8132)	0 (0-12)
TNF-α	279 (0-3638)	952 (0-4080)	2147 (0-9099)*	8668 (6182-15697)*
gelatinase B	719 (612-2122)	n.d.	607 (70-2003)	n.d.

data shown as median cytokine production (pg/ml) and median gelatinase B production (scanning units) with range in parentheses, n.d. = not done
*$p<0.05$

For 9 patients paired samples of peripheral blood and synovial fluid were analyzed. Concentrations of the different cytokines in unstimulated samples were comparable in peripheral blood compared to synovial fluid except for IL-10, IFN-γ and IL-12 for which cytokine concentrations were higher in synovial fluid compared to peripheral blood but these differences were not significant (data not shown).

Gelatinase B production in whole blood and synovial fluid after stimulation with PG_{spleen}
Quantitative analysis of gelatinase B production showed that in blood cells, gelatinase B concentration was higher, although not significant, in RA patients compared to healthy subjects, suggesting a higher spontaneous release of gelatinase B in the peripheral blood of RA patients (figure 3). Furthermore it was shown that PG induces production of gelatinase B. In blood samples of healthy subjects this induction was significant (figure 3). In synovial fluid samples the induction of gelatinase B by PG did not reach significance (table 3). Analysis of the kinetics of the dose dependent induction of gelatinase B by PG showed that gelatinase B was induced after 4 h of incubation (data not shown). To evaluate whether this induction resulted in an increase of gelatinase B activity all samples were also analyzed by fluorescent activated substrate conversion. None of these samples showed net gelatinase B activity.

Relationships between cytokine production and disease activity or disease duration
To examine the possible relationships between (PG-induced) cytokine and gelatinase production and RA pathology, linear regression was performed for cytokine production separately for both peripheral blood and synovial fluid and disease activity and disease duration. Although cytokine production by unstimulated cells showed a positive relationship with disease activity in both blood and synovial fluid samples with most cytokines, in particular with IL-6 and TNF-α, no significant correlation could be observed (data not shown).

DISCUSSION

The present study shows that PG fractionated from sterile human spleen is able to induce T cell proliferation in peripheral blood and synovial fluid of RA patients, and that PG is able to induce production of the inflammatory mediators IL-1β, IL-6, IL-8, IL-10, TNF-α as well as gelatinase B in peripheral blood and synovial fluid cells. No differences were observed between RA patients and healthy subjects for T cell proliferation and cytokine production induced by PG.

In about 75 % of RA patients and control donors proliferation of PBMC against PG could be observed. This indicates that no apparent disturbances of specific T-cell proliferation exists against PG in RA patients compared to healthy donors. The lower proliferative response against PG-PS_{feces} in both groups even with a 100-fold higher concentration does support the importance for intact PG structure in the induction of T cell proliferation because PG_{spleen} contains intact peptide bridges which are almost absent in PG-PS_{feces}. In addition, there are also no indications that, at the site of inflammation, proliferation after stimulation with PG contributes to pathogenesis, because in only 2 of the 14 RA patients analyzed, proliferation was observed. Because T cell proliferation in synovial fluid mononuclear cells is very low after stimulation with the positive control stimuli LPS and TT, it can be suggested that the method used was not optimal. The mean ratio of antigen presenting cells (i.e monocytes/macrophages) over T cells was 1 :1.4 in the synovial fluid as shown by morphology analysis of cytospins of the synovial fluid cells (data not shown). In peripheral blood this ratio is approximately 1 to 5 implying that many more potentially responsive T cells are present in the wells. Another explanation for the lack of synovial fluid T cell proliferation is that most synovial fluid T cells, despite the expression of CD45RO, are hyporesponsive upon stimulation by anti-CD3 and anti-CD28 (7). A defect in T-cell-receptor mediated signaling at the level of tyrosine phosphorylation has been suggested (6).

Cytokine induction by PG was measured using two different cell populations, whole blood or synovial cells and isolated mononuclear cells. The whole blood cell culture technique has proved to be quite reproducible (49-51) and to closely reflect the *in vivo* situation. However, because in some RA patients, cytokine concentrations present in unstimulated samples were considerably high, further enhancement by PG on cytokine production was difficult to examine. Therefore we also used Ficoll purified mononuclear cells to measure newly secreted cytokines and not cytokines already present in the plasma.

A number of cytokines known to play a role in the pathogenesis of RA was analyzed. The proinflammatory cytokines IL-1β, IL-6, IL-12 and TNF-α are able to activate T and B cells, and are major effectors of cartilage and bone destruction (18,52). The chemokine IL-8 is a potent attractor of leukocytes to the inflamed joint and may contribute to inflammation and the formation of the pannus (53,54). IL-8 has also been shown to activate neutrophils to secrete gelatinase B (47). Although IL-10 has proinflammatory properties like B cell activation, in RA, IL-10 has predominantly anti-inflammatory properties such as inhibition of TNF-α and IL-1 production (55). Therapy with IL-10 has resulted in clinical improvement

of RA (56). Although the expression of IFN-γ is not high in RA synovia, cytokine profile analysis of T cells from synovial fluid has shown that these cells mainly produce IFN-γ compared to IL-4. This indicates that a Th1-pattern of T cell reactivity occurs, which may contribute to delayed hypersensitivity responses (57).
Both in blood and synovial fluid of RA patients, higher levels of IL-6, IL-8, IL-10, IL-12, IFN-γ and TNF-α were present in unstimulated samples compared to healthy subjects. This is consistent with earlier reports (18,58,59). Production of IL-1β, IL-6, IL-8, IL-10 and TNF-α was enhanced after PG_{spleen} stimulation in both healthy donors and RA patients by cells in peripheral blood and synovial fluid. An enhancement of IL-12 after PG_{spleen} induction was only found in whole blood cell stimulations of healthy subjects. No production of IFN-γ induced by PG_{spleen} could be demonstrated. This is not surprising as IFN-γ is mainly produced by T cells and T cell stimulation by PG_{spleen} reached highest levels after 7 days of culture instead of 17 hours. In accordance with this, cytokine production measured in PBMC stimulations of healthy donors at day 7 showed that high amounts of IFN-γ (137-399 ng/ml) were produced and no IL-4 (data not shown).
Gelatinase B is detected in synovial fluid (22) and is expressed in the synovial tissues of RA patients (60,61), where it plays an important role in connective tissue destruction by degrading components of the extracellular matrix (62). This study shows that PG_{spleen} is able to induce production of this enzyme. Whether this enzyme is induced directly or indirectly through e.g. IL-8 or TNF-α, which is known to induce gelatinase B (22,63,64), has to be further analyzed using specific antibodies. The kinetic analysis of gelatinase B expression is in favor, however, of a direct effect of PG. The lack of net gelatinase B activity in the samples can be explained by the presence of natural and possibly induced inhibitors of gelatinase B activity (65).

To determine if the biological properties of PG are directly involved in the pathogenesis of RA, a relationship between cellular proliferation, cytokine/gelatinase B production and disease activity or disease duration was analyzed using correlation analysis. For most cytokines a trend could be observed that higher cytokine production was positively related with disease activity and negatively related with disease duration. These trends did not reach significant levels.

The question remains which role PG plays in the pathogenesis of RA. The main indication for a role of PG in RA is the presence of PG in antigen presenting cells in synovial tissues (40) suggesting that bacterial antigens, ubiquitously present at that mucosa, gain access to the joints, probably carried by phagocytic antigen presenting cells in the bloodstream (66). The distribution of PG might be increased by ultrastructural gut lesions (67) or a lowered mucosal surface protection provided by antibodies against PG (Schrijver et al submitted). Although this study did not identify disturbances in the T cell response against PG, the results do show that PG is able to induce production of effector molecules crucially involved in RA. Based on these results we hypothesize that PG is involved in the pathogenesis of RA by inducing cytokines and gelatinase B. First, the production of these cytokines and gelatinase B can lead to destruction of the joint tissue. Second, cytokines might allow autoreactive T cells to overcome self-tolerance to certain autoantigens. Recent studies have shown that various infectious and proinflammatory antigens, such as LPS, a functional PG analogue,

are capable of reversing the tolerant state of CD4+ (68) and CD8+ (69) positive T-cells in the presence of specific (auto) antigens by production of cytokines stimulating T-cell proliferation and survival (70,71). Cytokines may also be involved indirectly in the breaking of tolerance to autoantigens. T cells might be exposed to autoantigens upon destruction of joint tissue by cytokines leading to activation of these T cells. Autoantigens involved in this process are cartilage antigens like collagen and cartilage protein HC-gp39 to which T cell reactivity has been found in the synovium (72,73). Thus, inflammatory activity within the RA joint may be dependent on factors governing redistribution of PG to non-mucosal sites. Therapeutic intervention restricting the access of PG to non-mucosal sites may therefore be of clinical benefit to patients. This can be examined using *in vivo* bacterial translocation models.

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Chapter

4

BACTERIAL PEPTIDOGLYCAN STIMULATES INFLAMMATION *IN SITU*

Chapter 4.1

Pararosaniline fixation for detection of costimulatory molecules, cytokines and specific antibody
Journal of Histochemistry and Cytochemistry, in press

Chapter 4.2

Antigen-presenting cells containing bacterial peptidoglycan in synovial tissues of rheumatoid arthritis patients coexpress costimulatory molecules and cytokines
Submitted for publication

Chapter 4.3

Bacterial peptidoglycan promotes immune reactivity in the central nervous system in multiple sclerosis
Submitted for publication

Chapter 4.1

Pararosaniline fixation for detection of costimulatory molecules, cytokines and specific antibody

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ABSTRACT

Integral immunohistochemical analysis of immune responses in frozen sections requires that in addition to constitutively expressed membrane CD markers, less stable determinants can be reliably visualized. Therefore, we compared the commonly used acetone fixation method with pararosaniline fixation for six determinant categories. These categories included selected constitutively expressed markers, inducible costimulatory molecules, pro- and anti-inflammatory cytokines (including the novel cytokine IL-18, also known as IGIF and IL-1γ), antigen-specific antibody in plasma cells, bacterial peptidoglycan and lysosomal acid phosphatase activity.

Human spleen and mouse spleen activated by agonistic anti-CD40 antibody or TNP-Ficoll immunization were analyzed in parallel with brain tissue from multiple sclerosis (MS) patients and marmoset monkeys with experimental autoimmune encephalomyelitis (EAE), an animal model for MS. Fixation with pararosaniline resulted in better morphology of all tissues and inhibited endogenous alkaline phosphatase activity in brain tissue. Most determinants could be reliably detected. Staining sensitivity and intensity were markedly increased for selected determinant-tissue combinations, e.g. for IL-4 in human spleen and CD40 in human and mouse spleen.

These data show that pararosaniline is a useful alternative to acetone, resulting in superior morphology and specific staining for selected determinant-tissue combinations. This provides additional flexibility for in situ analysis of immune reactivity.

INTRODUCTION

Chronic inflammatory diseases such as rheumatoid arthritis (RA) and multiple sclerosis (MS) are generally believed to result from the activity of autoreactive CD4+ T cells. The formation and activity of lesions caused by infiltrating mononuclear cells are complex processes that are incompletely understood, and this also holds true for the cellular effector mechanisms that might be responsible for the tissue damage that leads to temporary and

cumulative clinical symptoms. Such effector functions include local (auto)-antigen presentation, (auto)-antibody production, secretion of pro- and anti-inflammatory cytokines and activity of enzymes involved in proteolysis and nitric oxide production (1,2). It is therefore crucial to analyze inflammatory lesions functionally *in situ* by conventional and novel immunohistochemical methods to provide an integral view of ongoing cellular activities. Relevant parameters are the localization of antigen possibly involved in inflammation (e.g. bacterial peptidoglycan (PG) in RA). PG may be involved in the pathogenesis of chronic inflammation, particularly in RA (3,4). With a monoclonal antibody against PG (Mab 2E9) antigen presenting cells (APC) containing PG can be detected in synovial tissues (5,6). Furthermore the integrity of antigen binding sites of antibodies present in the cytoplasm of plasma cells can be evaluated (7-9). This was studied by detection of antibody forming cells specific for 2,4,6-trinitrophenyl (TNP) in mice immunized with TNP-Ficoll (10).

The identification of mononuclear cell subsets (e.g. T, B and APC, granulocytes) by markers such as IgM, IgG, CD14 and HLA-DR or enzyme activity (lysosomal acid phosphatase in macrophages) can be used to examine the presence of B cells and antigen presenting cells that can contribute to pathogenesis of RA and MS by producing antibodies and cytokines or enzymes. Functional activation profiles can be identified by strongly regulated costimulatory molecules such as CD40, CD40L, CD80 (B7-1) and CD86 (B7-2) (11,12) which have been claimed to play an important role in the inflammatory process of both MS (13,14) and RA (13-16). Other important activation markers in immunological responses are intracytoplasmic soluble cytokines (17,18) like the anti-inflammatory cytokines IL-4 and IL-10, the proinflammatory cytokines TNF-α, IFN-γ, and the novel cytokine IL-18, also known as interferon gamma inducing factor (IGIF) and IL-1γ (19-21).

To identify all these relevant parameters involved in inflammation by immunohistochemistry, adequate fixation of frozen sections is an essential requirement. The main function of fixation is to preserve morphology of the tissue which is often best accomplished by strong fixation. The disadvantage of strong fixation is that the structure and integrity of the parameters are changed resulting in a poor quality of the staining pattern. The optimal fixation condition, therefore, consists of a compromise between these contradictory requirements, and may differ depending on the nature of the antigen (22).
Pararosaniline has previously been shown to have good fixation properties for frozen tissue sections without affecting antigenicity. This was concluded from staining for constitutively expressed markers in mouse lympho-hemopoietic organs in which acetone fixation was compared with pararosaniline fixation (22).

The aim of this study was to determine whether pararosaniline has advantages over acetone fixation for detection of less stable determinants such as bacterial antigen, intracellular cytokines, co-stimulatory molecules and specific antibody located in plasma cells. Expression of the markers was examined in different types of organ tissues (spleen, brain) from three species (human, mouse and monkey). Tissues were activated either by chronic inflammation or by *in vivo* administration of TNP-Ficoll (a thymus independent type 2 antigen) or agonistic anti-CD40 antibody.

MATERIALS AND METHODS

Human and animal tissues

All mouse markers (except CD40) were examined in spleens of BALB/c mice immunized intravenously with 20 μg TNP-Ficoll, a polysaccharide thymus independent type 2 antigen, which evokes IgM and IgG antibody forming cells, expression of CD40L (CD154), and several cytokines four days after injection (10). CD40 detection was performed on C57Bl/6 spleen in which CD40 expression was upregulated by i.v. injection of 100 μg agonistic anti-CD40 antibody (FGK-45) (23,24). At 96 h after injection the mice were sacrificed and spleen tissue was removed.

To study expression of costimulatory molecules and cytokines in tissue affected by chronic autoimmune inflammation, brain tissue from marmoset monkey EAE (*Callithrix jacchus*) was used. The monkeys were bred at the Biomedical Primate Research Center (BPRC) and used under conditions approved by Dutch laws on animal experimentation. EAE was induced essentially as described previously (25-27). Per animal 20 mg of human myelin (28) in PBS was emulsified with an equal volume of complete Freund's adjuvant, containing 3 mg/ml of *Mycobacterium tuberculosis* (H37 RA strain). Intracutaneous injections of 150 μl were divided over four sites on the dorsal axillary and inguinal regions. Animals were sacrificed during active disease periods and brain tissue was obtained.

Expression of human markers was determined in human spleen without evidence of infection or inflammation. Two human spleens were removed after damage to the spleen occurred during surgery for a stomach carcinoma.

To study expression of costimulatory molecules and cytokines in human tissue affected by chronic autoimmune inflammation, brain tissue of MS patients was used for determination of all cytokines and costimulatory molecules expected to be expressed in lesions. Two human MS brain tissue specimens were obtained at autopsy with short post mortem intervals from the Netherlands Brain Bank in Amsterdam, The Netherlands (coordinator Dr. R. Ravid).

Tissue processing

All tissues were snapfrozen in liquid nitrogen in aluminum containers, which were stored at –80°C. Six micron frozen sections were cut on a cryostat and thaw-mounted on slides precoated with 0.1% gelatin (Merck, Darmstadt, Germany) and 0.01% chromium potassium sulfate (Merck). The sections were kept overnight in a box with humidified atmosphere after which they were air dried at room temperature for 1 h and stored in a box with silica until use.

Fixatives

Acetone (purity < 99.5%) (Fluka, Chemie AG, Buchs, Switzerland) supplemented with 0.5% H_2O_2 (from a 30% stock solution) was used at RT.

Pararosaniline was prepared as described by Burnstone (29) with minor modifications (22). Four percent (w/v) pararosaniline (Sigma Chemical Co., St. Louis, MO) was dissolved in 2 M HCL by heating at 70°C. After the pararosaniline was dissolved the solution was filtered and 1 ml of this solution was mixed with 1 ml 4% (w/v) $NaNO_2$ in distilled water. After

precisely 1 min this reaction mixture was dissolved in 200 ml milliQ water (Millipore, Bedford, MA PF+ system, resistance > 18 MΩ). This solution can be used for at least up to 1 month when stored at 4°C. Pararosaniline and solutions containing this chemical should be handled with precaution in view of their potential carcinogenicity.

Table 1. Fixation procedures

Acetone			Pararosaniline		
Treatment	Duration (min)	Temperature	Treatment	Duration (min)	Temperature
acetone with 0.5% H_2O_2*	10	RT	pararosaniline	2	RT
air dry	1	RT	wash in PBS	5	RT
			0.5% H_2O_2 in PBS*	10	RT
wash in PBS-Tween	5	RT	wash in PBS-Tween	5	RT
primary reagent	overnight	4°C	primary reagent	overnight	4°C

* 0.5% H_2O_2 obtained from a 30% stock solution.

(Immuno)histochemistry

Sections were fixed according to the different protocols (table 1). The slides were next washed with PBS and then with PBS/0.05% (v/v) Tween 20 (Fluka) and incubated with the first reagent (antibody, antigen-enzyme conjugate (TNP-AP) or enzyme substrate solution) in PBS/1% BSA/0.05%Tween (HPLC-grade, fraction V, Sigma) (table 2) overnight at 4°C (or 30 min at 37°C for the acid phosphatase substrate). After washing the slides twice with PBS/0.05% Tween, the sections were incubated with the appropriate secondary reagent (PBS/1%BSA/0.05% Tween/1% normal human serum) for 30 minutes at RT (table 2). For some stainings the slides were then washed twice with PBS/Tween and incubated for 30 min with the tertiary reagent (table 2). After the slides were rinsed twice in PBS, they were washed in 0.05 M NaAc buffer pH 5.0 for 5 minutes (except for the TNP-AP (alkaline phosphatase staining). Horseradish peroxidase activity (HRP) was revealed by incubating the slides for 15 min in substrate containing 1.5 ml of 3-amino-9-ethylcarbazole (AEC, Sigma) dissolved in N,N-dimethylformamide (DMF, Sigma) (17 mg/ml) and 30 µl H_2O_2 30% in 60 ml of NaAc buffer pH 5.0, followed by a washing step in PBS. The use of AEC results in a bright red precipitate. The histochemical revelation of TNP-AP staining was performed with Fast Blue Base (FBB) substrate after rinsing the slides in 0.1 M TRIS-HCL pH 8.5. FBB substrate was prepared as follows. Sixteen mg FBB (Sigma) was added to 400 µl 2M HCL and 400 µl 4% $NaNO_2$ and was mixed for 1 min before adding to 65 ml of 0.1 M Tris-HCL pH 8.5. Then 8 mg Naphtol-AS-MX-phosphate (Sigma) was dissolved in 400 µl of DMF and added to the solution. Finally 100 µl of 1 M levamisole (Sigma) was added to the solution to inhibit endogenous alkaline activity. The slides were incubated in the substrate for 45 min. The slides were washed in NaAc buffer pH 5.0 or 0.1 M TRIS-HCl pH 8.5.

All slides were counterstained with hematoxylin (Mayer's) and washed for 10 minutes in tapwater after which they were embedded in Kaiser's glycerin/gelatin (Merck).

Table 2. Reagents used for the detection of different determinants

Primary reagent (supplier)	Detects	Secondary reagent	Tertiary reagent
PRIMATE			
Enzyme substrate			
Acid phos. substrate	Acid phos. activity		
Antibody			
179-1.1-PO (TNO-PG)	IgM	-	-
343-4.1-PO (TNO-PG)	IgG	-	-
L234-bio (Coulter clone)	HLA-II	AV-PO (Sigma)	-
MY-4 (Coulter Clone)	CD14	HoαM-bio (Vector)	AV-PO
5D12 (Tanox Pharma BV)	CD40	HoαM-bio	AV-PO
24-31 (Dr. RJ Noelle)	CD154/CD40L	HoαM-bio	AV-PO
M24 (Tanox Pharma BV)	CD80/B7.1	HoαM-bio	AV-PO
1G-10 (Tanox Pharma BV)	CD86/B7.2	HoαM-bio	AV-PO
MD-2 (Dr. P v/d Meide)	IFN-γ	HoαM-bio	AV-PO
B-S10 (Instruchemie)	IL-10	HoαM-bio	AV-PO
Anti-IL-4 (Genzyme)	IL-4	HoαM-bio	AV-PO
61E71-bio (Dr. P v/d Meide)	TNF-α	AV-PO	
IGIF-bio (R&D systems)	IL-18/IGIF/IL-1γ	SAV-PO (Jackson)	
2E9-bio (Dr. MP Hazenberg)	Peptidoglycan (PG)	SAV-PO	
MOUSE			
Enzyme substrate			
Acid phos. Substrate	Acid phos. activity		
Antibody			
226aKappa + 210887 (Dr. HFJ Savelkoul)	IgM + IgG	RαRa-bio (Dako)	AV-PO
M5/114 (ATCC)	HLA-II	RαRa-bio	AV-PO
F4/80 (Dr. PJM Leenen)	F4/80)	RαRa-bio	AV-PO
3/23 (Instruchemie)	CD40	RαRa-bio	AV-PO
MR-1 (Dr. RJ Noelle)	CD154/CD40L	RαRa-bio	AV-PO
01941D 1G20 (Pharmingen)	CD80/B7.1	RαRa-bio	AV-PO
GL1 1196 (ATCC)	CD86/B7.2	RαRa-bio	AV-PO
11B11-bio (Dr. HFJ Savelkoul)	IL-4	AV-PO	
SXC-1-bio (BPRC)	IL-10	AV-PO	
DB-1-bio (BPRC)	IFN-γ	AV-PO	
2E9-bio (Dr. MP Hazenberg)	PG	SAV-PO (Jackson	
Antigen-enzyme			
TNP-AP	αTNP-antibody	-	-

Abbreviations: acid phos. (acid phosphatase), AV-PO (avidin peroxidase), HoaM-bio (horse-anti-mouse-biotin), SAV-PO (streptavidin-peroxidase), RaRa-bio (Rabbit-anti-Rat-biotin) Suppliers: TNO-PG, Leiden, The Netherlands; Coulter Clone, Hialeah, FL; R.J. Noelle, Darmouth College,Lebanon, NH; Tanox Pharma BV, Amsterdam, The Netherlands; Dr. P. van der Meide, BPRC, Rijswijk, the Netherlands; R&D systems, Abingdon, United Kingdom; Sigma Chemical Co., St. Louis, MO;Dr. P.J.M. Leenen/Dr. H.F.J. Savelkoul/Dr. M.P. Hazenberg, EUR, Rotterdam, The Netherlands; Instruchemie, Hilversum, The Netherlands; Genzyme, Cambridge, MA; Pharmingen, San Diego, CA; ATTC, Rockville, MA; Dako, Glostrup, Denmark; Vector Lab., Peterborough, UK; Jackson Immunoresearch, Westgroove, PA

Evaluation and quantitation of (immuno)histochemistry
Staining on two sections of each tissue was performed for each marker. All sections were evaluated for the following parameters; tissue morphology, sensitivity of the procedure as defined by number of positive cells, staining intensity per cell, and background endogenous enzyme activity (HRP and AP). The evaluation was performed by light microscopy by two independent observers blinded to the fixation procedure.

RESULTS

Morphology
To assess the preservation of tissue and cell morphology after the different fixation procedures, the tissue sections were stained using the entire staining procedure but with omission of the primary reagent. As described previously (22), after acetone fixation the morphology was suboptimal for mouse and human spleen. The distinction between red and white pulp was not very clear, and the distinction between individual cells was not optimal, especially in the white pulp (figure 1,a and b). The morphology of monkey EAE brain was reasonable after acetone fixation except for the mononuclear cell infiltrates in which the individual cells were not clearly discernible. Morphology of MS brain was good in unaffected parts but suboptimal in demyelinating parts of the tissue.
In all tissues (except for MS brain) the morphology improved after fixation with pararosaniline. For MS brain tissue there was no difference between the two fixatives.
After pararosaniline fixation, both densely populated areas such as the splenic white pulp and less densely populated areas (red pulp) of the spleen and infiltrates of EAE brain showed better morphology. At low power magnification, the distinction between spleen white and red pulp could be made much more easely after pararosaniline fixation compared to acetone. In addition, the distinction between individual cells and determination of cell type based on the basis of morphology was much easier after pararosaniline fixation compared to acetone fixation. After pararosaniline fixation the counterstaining of the nuclei with hematoxylin was more intense compared to acetone fixation.

Endogenous enzymatic activity
To determine whether endogenous peroxidase or AP activity could be inhibited by pararosaniline or acetone fixation, all tissues were stained with omission of the primary antibody and the histochemical revelation of HRP and AP-activity was performed with AEC substrate and Fast Blue Base substrate respectively. Endogenous peroxidase activity was routinely inhibited by incubation with 0.05% H_2O_2 and endogenous AP activity with levamisole added to the substrate.
In none of the tissues endogenous peroxidase staining could be observed after acetone or pararosaniline fixation. In human and mouse spleen endogenous AP activity was completely blocked after both fixation procedures. In contrast, EAE and MS brain tissue showed a difference between the fixation procedures. After acetone fixation, endogenous AP activity was present in the endothelial cells of the blood vessels in MS brain and monkey EAE brain.

After pararosaniline fixation this endogenous AP activity was completely abolished (figure 1, c and d).

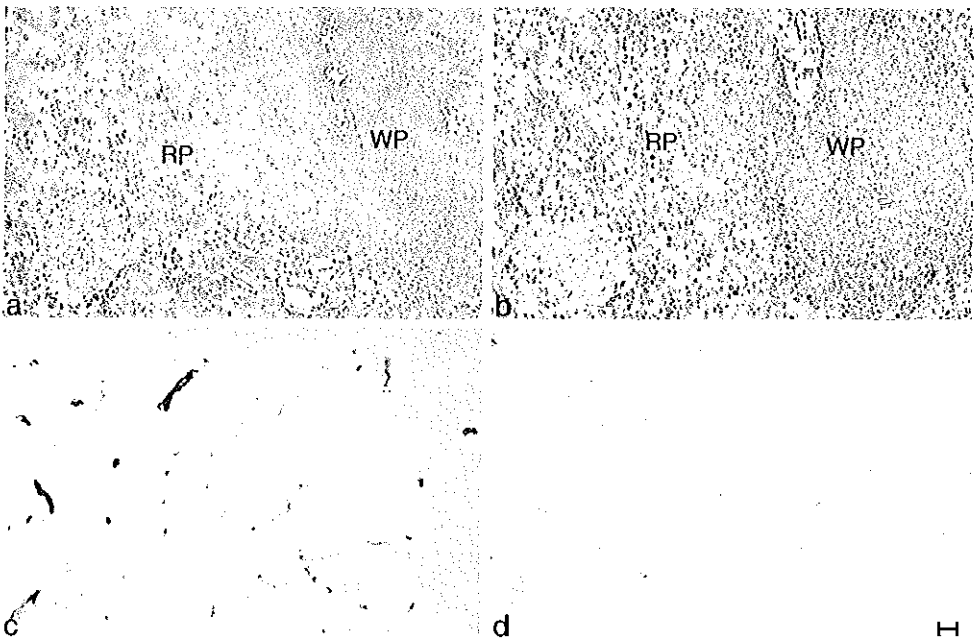
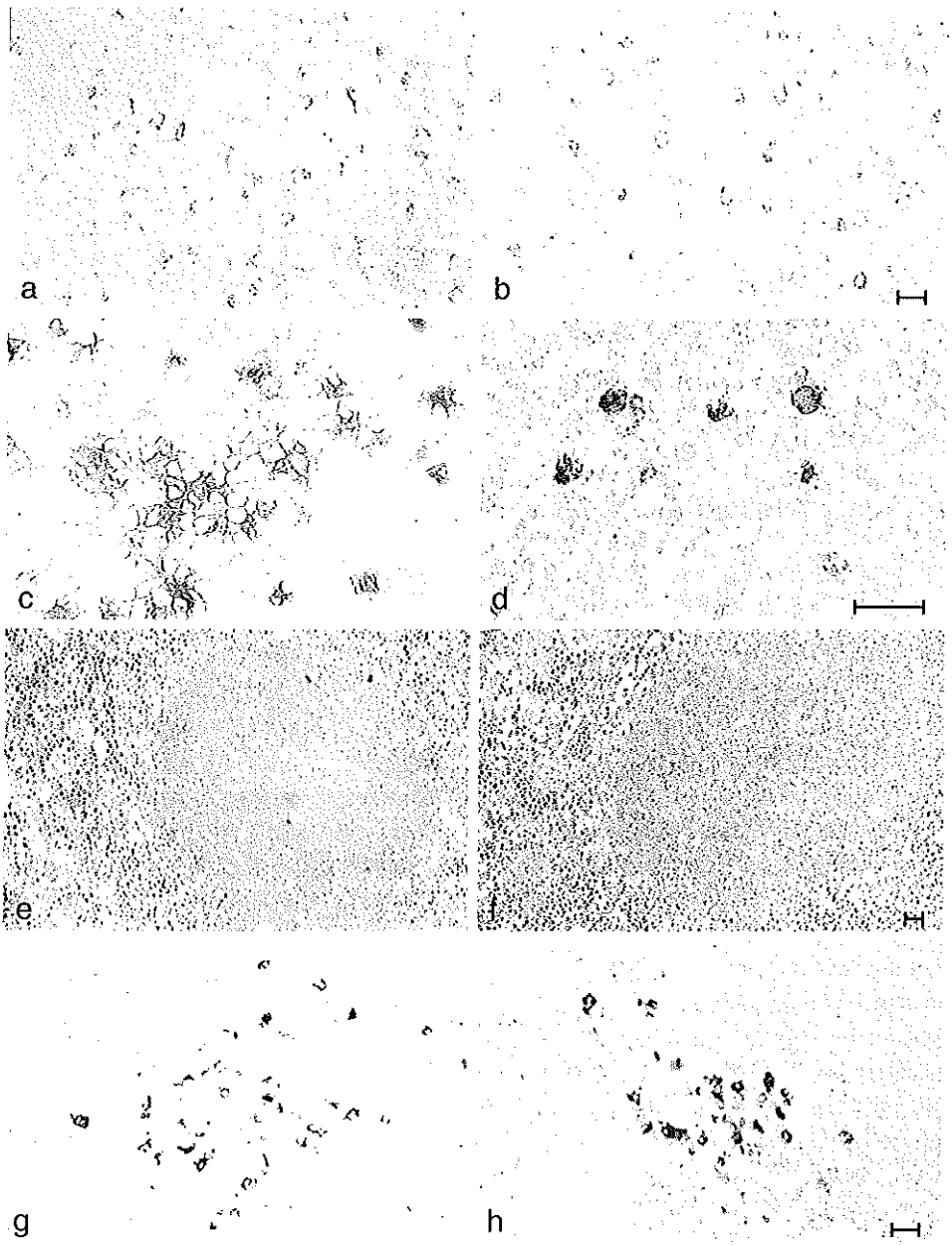


Figure 1. Morphology and endogenous alkaline phosphatase activity after acetone and pararosaniline fixation. Morphology of human spleen tissue strongly improved after pararosaniline (b) fixation compared to acetone fixation (a). After pararosaniline fixation the distinction between red (RP) and white pulp (WP) is much clearer and hematoxylin counter staining is stronger. Endogenous alkaline phosphatase activity of endothelial cells of EAE brain could be observed after acetone (c). After pararosaniline fixation endogenous alkaline phosphatase activity was totally inhibited (d). Scale bar represents 20 μm for all figures.

Preservation of determinants

To examine the effect of acetone and pararosaniline on antigen preservation, detection of a broad range of different determinants on various tissues was analyzed. The antigen preservation after the fixation method was analyzed by two parameters. First the number of cells positive for the antigen was counted and second the staining intensity on a per cell basis was determined. The semi-quantitative results are shown in table 3. The staining properties were dependent on the combination of marker and tissue used. Many of the markers showed the same staining pattern after both fixation procedures. However, some markers showed considerably different staining patterns for the two fixation procedures. For example, pararosaniline is preferable for detection of CD40 in all tissues. For other markers the difference between the fixatives is restricted by the tissue used. For example, for IL-10 detection pararosaniline fixation is preferable for marmoset brain but not for human MS brain. In addition, in the six categories of markers described above there is no general rule as to which fixative is preferable. For example, higher numbers of IL-4 producing cells were detected with pararosaniline in human spleen, but no IL-18 could be detected. The general

impression is that for human and mouse spleen tissue, pararosaniline is the better fixative. The staining of the cells is more discrete compared to acetone with which the staining is more diffuse, with some color precipitate outside the cell. Therefore it is sometimes difficult to determine which individual cell is positive for the determinant under investigation, which may lead to an overestimation of the number of positive cells after acetone fixation. Especially with markers present on a high number of cells, such as CD14 and HLA-DR, the staining intensity of the cells was higher after pararosaniline fixation.



A disadvantage of pararosaniline fixation is that no positive cells could be observed for IFN-γ and IL-18 in human spleen and for CD86 in MS brain.
Figure 2 shows some selected stainings representing the different categories of determinants used in the study. Lysosomal acid phosphatase activity can be detected histochemically in all macrophages present in the EAE brain tissue. Pararosaniline fixation resulted in similar staining intensity compared to acetone fixation (figure 2, a and b). IL-4 is expressed in the red pulp of human spleen. The staining pattern is more discrete and stronger after pararosaniline compared to acetone fixation (figure 2, c and d). CD40 expression in mouse spleen injected with the agonistic anti-CD40 antibody is weak on B cells in the white pulp and strong on some macrophages in the red pulp. After pararosaniline the staining of the B cells is more intense compared to acetone fixation. (figure 2, e and f). Plasma cells containing intracellular antibody specific for TNP were detected in TNP-Ficoll immunized mice. Anti-TNP plasma cells were present in groups of 10-50 cells located in the outer pals and terminal arterioles of the spleen. Staining patterns did not differ between the two fixation procedures indicating that pararosaniline does not affect the integrity of antigen binding sites, but morphology of the tissue strongly improved after pararosaniline fixation (figure 2, g and h).

DISCUSSION

The present data clearly demonstrate that pararosaniline is a permissive and mild fixative for frozen sections, which can result in improved immunohistochemical staining compared to acetone, depending on the combination of the marker and the tissue under study.
The purpose of this study was to identify improved fixation conditions for the study of immune responses *in situ*. Integral immunohistochemical analysis of immune responses requires that in addition to constitutively expressed membrane CD markers, strongly regulated determinants such as bacterial antigen, intracellular cytokines, costimulatory molecules, enzyme activity and antigen specific plasma cells can be visualized reliably. Although some studies have reported that instable determinants such as cytokines are detected reliably in paraffin embedded tissue (30), most studies of strongly regulated determinants employ frozen sections because efficient detection of these markers remains difficult in paraffin embedded tissues.

Figure 2. Antigen preservation of different markers after acetone and pararosaniline fixation. Acetone (left column) and pararosaniline (right column) fixed sections of non infiltrated part of EAE brain (a,b), human spleen (c,d) and mouse spleen (e,f,g,h). Macrophages containing lysosomal acid phosphatase were located throughout the section (a,b). No difference of staining pattern was found between the two fixatives. IL-4 producing cells (c,d) were mostly located in the red pulp of the spleen. After pararosaniline fixation, staining intensity per cell was improved compared to acetone in which the staining was more diffuse. CD40 was expressed on B cells and some macrophages (e,f). After pararosaniline fixation expression of CD40 on B cells was much stronger compared to acetone fixation. Plasma cells specific for TNP were analyzed in TNP-Ficoll injected mice (g,h). Groups of 10-50 cells were detected in the outer pals and terminal arterioles of the spleen. Pararosaniline fixation did not affect integrity of antigen binding sites of antibodies but morphology was strongly improved. Scale bars represent 20 μm for both left and right colomn figures.

Table 3. Staining intensity per cell (A) and number of positive cells (B) for six determinant categories analyzed in different tissues

	Human spleen				Human MS-brain				Marmoset EAE-brain				Mouse spleen			
	Acetone		Pararosaniline		Acetone		Pararosaniline		Acetone		Pararosaniline		Acetone		Pararosaniline	
Cell surface markers	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
IgM	+	+	++	+	+	±	++	±	+	±	+	±	++	++	+	++
IgG	±	+	±	±	±	±	±	±	-	+	+	+	++	++	+	++
HLA-DR	+	+++	+	+++	±	++	+	++	+	+	++	+	n.a.		n.a.	
F4/80	n.a.		n.a.		n.a.		n.a.		n.a.		n.a.		+	++	+	++
CD14	±	++	++	++	+	+	+	+	+	+	+	+	n.a.		n.a.	
Costimulatory molecules																
CD40	±	+	+	+	±	+	+	+	±	+	±	+	±	+	+	+
CD40L	+	+	±	±	±	±	±	±	+	±	±	±	++	++	++	+
CD80/B7-1	±	±	+	±	±	+	±	+	±	±	±	±	±	±	+	±
CD86/B7-2	+	+	+	±	±	+	-	-	±	±	+	±	±	+	+	+
Cytokines																
IL-10	++	+	++	±	+	+	±	±	±	±	+	±	+	±	+	±
TNF-α	+	++	++	+	+	++	±	++	+	±	+	±	+	±	+	±
IFN-γ	+	±	-	-	+	+	+	±	+	±	+	±	+	+	±	+
IL-4	±	+	+	+	+	±	±	±	±	±	+	±	+	+	±	+
IL-18	+	+	-	-	±	±	±	±	±	+	+	+	n.a.		n.a.	
Bacterial antigen																
Peptidoglycan	±	±	++	±	n.a.		n.a.		+	±	+	±	+	+	+	+
Enzyme substrate																
Acid phosphatase	+	+++	+	+++	+	+	+	+	+	++	+	++	++	+++	+	++
Antigen detection																
anti-TNP	n.a.		n.a.		n.a.		n.a.		n.a.		n.a.		++	+	++	+

evaluation of number of positive cells: - no positive cells, +/- 0-5% of cells positive, + 5-10% of cells positive, ++ 10-50% of cells positive, +++ > 50% of cells positive BALB/c mice were immunized with TNP-Ficoll, CD40 expression was detected in C57Bl/6 mice injected with agonistic anti-CD40 antibody (FGK-45)
n.a.: not applicable

A major disadvantage of the use of frozen tissues is the frequently poormorphology of cryosections. Morphology of the tissues is strongly dependent on the fixative used. Good morphology is achieved with paraformaldehyde, formaline and gultaraldehyde which are able to crosslink proteins. A major disadvantage of these fixatives is the alteration of tertiary structure by crosslinkage, resulting in alteration of antigenic structures of the tissues (31). In contrast, acetone, which precipitates proteins, is a reliable fixative for detection of different markers but morphology is often poor (32).

To improve morphology while maintaining staining intensity we compared the most commonly used mild acetone fixation with pararosaniline. Pararosaniline is a hexazonium salt, that contains three reactive diazonium groups and therefore, in theorie must be able to crosslink proteins, which was shown previously to yield a better morphology of mouse tissue compared to acetone without affecting immunogenicity (22). In the present study detection of six categories of determinants was analyzed for different tissues. Human and mouse spleens were used because the spleen is an important secondary lymphoid organ in which a wide diversity of immune responses takes place. Therefore most of the markers examined are expressed in these organs. We also evaluated expression of these determinant categories in inflamed tissues of MS patients and EAE affected marmoset monkeys because it is expected to be chronically increased and because good morphology is very important in these activated tissues.
Antigen preservation was analyzed by examination of staining intensity per cell and number of positive cells. In general the antigen preservation after pararosaniline fixation did not differ from that of acetone fixation. Staining of antigen specific antibodies and acid phosphatase could be detected reliably in all tissues implying that these determinants are well preserved. The preservation of strongly regulated molecules such as costimulatory molecules and cytokines differed between the two fixation procedures. The restriction of pararosaniline fixation is that for a few determinant-tissue combinations, the number of cells positive for the determinant is lower compared to acetone fixation. In these cases, acetone fixation would be preferable. In all other cases fixation with pararosaniline is preferable over acetone fixation for four reasons. First, morphology is strongly improved after pararosaniline fixation, which results in a better judgment of the location of positive cells. Second, endogenous AP activity could be completely inhibited after pararosaniline fixation. This is especially important when double stainings are performed with revelation of both peroxidase and AP enzymatic activity (8). Third, in some cases staining intensity after pararosaniline fixation improved leading to a more discrete staining pattern compared to acetone fixation where sometimes color precipitate is seen outside the cell. Especially when high numbers of cells are positive for the determinant this will lead to a better distinction between positive cells. Fourth, hematoxylin counter staining of the tissue was much stronger compared to acetone fixation leading to an improved distinction between adjacent cells.

In conclusion, pararosaniline fixation gives a better morphology than acetone fixation and inhibits endogenous AP activity in brain tissue. Antigen preservation in general does not differ between the two fixatives for a wide range of determinants. When staining protocols are established for new and existing reagents pararosaniline is an easy and cost effective

alternative fixation for frozen sections. For studying a new marker-tissue combination it is recommended to analyze acetone and pararosaniline fixation routinely side by side to determine the optimal fixation procedure.

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CHAPTER 4.2

Antigen-presenting cells containing bacterial peptidoglycan in synovial tissues of rheumatoid arthritis patients coexpress costimulatory molecules and cytokines

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by intima lining hyperplasia and massive infiltration of the synovial sublining by antigen presenting cells (APC), lymphocytes and plasma cells. Peptidoglycan (PG), a major cell wall component of gram-positive bacteria, which is abundantly expressed in all mucosa, is believed to be involved in the pathogenesis of RA, because of its capability to induce production of proinflammatory cytokines and to induce arthritis in rodents. Furthermore, PG can be detected in APC in RA joints but little is known about the role of these cells in RA. In the current study the presence and immune competence of PG-containing cells in synovial tissues of 14 RA and 14 osteoarthritis (OA) patients was analyzed *in situ*. Using immunohistochemistry, we examined the coexpression of phenotypic markers, costimulatory molecules and cytokines by PG-containing cells. PG was detected in both RA and OA synovial tissues, with a tendency towards higher numbers of PG-containing cells in RA. PG-containing cells were mainly macrophages but also some dendritic cells contained PG. A high percentage of PG-containing cells in both RA and OA synovial tissues coexpressed HLA-DR. CD40, CD80, and CD86 expression by PG-containing cells was higher in RA compared to OA, implying that the activation state of PG-containing cells is higher in RA compared to OA. Furthermore, PG-containing cells coexpress cytokines which modulate inflammatory reactions, especially TNF-α, IL-6, and IL-10. The results suggest that PG-containing cells contribute to inflammation in RA within the joint microenvironment.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease with primary manifestations in the synovial tissue. The synovium becomes thickened due to synovial cell proliferation, infiltration by inflammatory cells and impaired apoptosis. The infiltrating cells mainly comprise T lymphocytes, macrophages, and plasma cells but also other cell types such as dendritic cells infiltrate the synovial membrane (1). The etiopathogenesis of RA is still not understood. Several lines of evidence support an important role of T lymphocytes (2) and antigen presenting cells (APC) in the pathogenesis of RA (3-5).

The (auto)antigen(s) leading to chronic stimulation of T cells and/or macrophages are still unknown. In reactive arthritis, the triggering antigens are thought to be microbes that cause infections of the urogenital tract or the intestine, such as *Chlamydia trachomatis*, *Yersinia* or *Salmonella* species (6,7). Antigens of these bacteria have been detected in synovial cells (8-10), and T cells specific for these antigens are present in the synovium (8,11,12). Because RA is a chronic disease, it can be suggested that the bacterial load in the intestine with which we are in lifelong close contact is important for the induction and perpetuation of RA. This is supported by the observation that some patients with Crohn's disease and ulcerative colitis, both intestinal autoimmune diseases, suffer from attacks of joint inflammation (13,14). Furthermore, inflammatory bowel disease mimicking Crohn's disease and chronic inflammatory arthritis develop spontaneously in knockout mice lacking a TNF AU-rich element, which is responsible for TNF mRNA destabilization and translational repression in hemopoietic and stromal cells (15).

We and others have previously hypothesized that peptidoglycan (PG) plays a role in the pathogenesis of RA (16-18). Peptidoglycan (PG) is the major component of the cell wall of gram positive bacteria and is composed of long sugar chains of alternating N-acetyl glucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc) residues, which are interlinked by peptide bridges, resulting in a large complex macromolecular structure (19). PG isolated from the anaerobic bacterium *Eubacterium aerofaciens* which is present in numbers > 10^9/g human feces induces a severe and chronic arthritis in rats (20). PG could also be isolated from sterile normal human spleen using biochemical methods (21,22). *In vitro* analysis showed that this PG fraction is able to induce production of the proinflammatory cytokines TNF-α, IL-1 and IL-6 (21) which are crucially involved in the pathogenesis of RA (23).
When PG plays a role in RA, the presence and persistence of PG in the synovia of RA patients may be a prerequisite. With a monoclonal antibody (Mab) 2E9, PG could be detected in 8 out of 10 RA synovia and in only 2 out of 20 non-rheumatoid synovial tissue samples (24). In addition, it was shown that the PG-containing cells were macrophages and dendritic cells as defined by coexpression with CD68 (macrophage marker), RFD1 and L25 (dendritic cell markers). Other studies using PCR techniques have detected bacterial DNA in RA synovia (25,26) suggesting bacterial involvement in RA.
To investigate whether PG contributes to inflammation in the synovial tissues we analyzed whether the PG-containing cells produced cytokines and coexpressed costimulatory molecules in a panel of synovial tissues of RA and osteoarthritis patients (OA). OA is a degener-

ative disease of the joints characterized by a non-infectious chronic inflammatory condition. The OA patients in the present study were selected on the basis of clinically inflamed joints secondary to OA. Furthermore, we analyzed the cellular phenotype of the PG-containing cells with an extended panel of APC markers (CD14, CD68, CD83, CD11b). Cytokines analyzed were IL-6, IL-10, TNF-α and IFN-γ (23). CD80 (B7.1), CD86 (B7.2), CD40 and HLA-DR were analyzed to determine the involvement of PG-containing cells in antigen presentation and costimulation.

PATIENTS AND METHODS

Patients and biopsies

Synovial biopsies of 14 RA patients and 14 inflammatory OA patients were investigated. All RA patients had active arthritis and fulfilled the American College of Rheumatology criteria for RA (27). Patient features are shown in table 1.

Table 1. Patient features

Patients	M/F	Age (years)	RF+/RF-	CRP	Disease duration (months)
Rheumatoid arthritis	4/10	59 (41-80)	11/3	48 (7-118)	123 (8-300)
Osteoarthritis	1/13	75 (69- 81)	3/11	14 (5-60)	-

age, CRP (C-reactive protein level) and disease duration are indicated as mean and range in parentheses

Biopsies were taken from the knee joint of the patients with a Parker Pearson needle (26). The tissue samples were snap frozen together in Tissue-Tek OCT (Miles, Elkhart, IN) by immersion in methylbutane (-70°C) and stored in liquid nitrogen. Cryostat sections (5 µm) were placed on adhesive glass sides (Starfrost, Knittelgläser, Germany), air-dried overnight, fixed in acetone for 10 minutes, and stored at –80°C until immunohistochemical analysis was performed.

Immunohistochemistry

Procedures used here have been reviewed in detail previously (28,29). The sections were allowed to warm to room temperature and washed with PBS after which endogenous peroxidase activity was inhibited using 0.5 % H_2O_2. The sections were washed between all steps with PBS/0.05% (v/v) Tween 20 (Fluka, Chemie AG, Buchs, Switzerland). The sections were incubated with the first antibody (table 2) diluted in PBS/T/1% BSA (HPLC-grade, fraction V, Sigma) overnight at 4°C. The secondary and tertiary reagents diluted in PBS/1%BSA/1% normal human serum were incubated during 60 minutes at room temperature (table 2). Biotinylated anti-PG (Mab 2E9) diluted in PBS/T/1% BSA was incubated overnight at 4°C followed by an incubation with streptavidin-peroxidase (Jackson Immunoresearch, Westgroove, PA) during 60 minutes. Histochemical detection of alkaline phosphatase activity was performed with Fast Blue Base (FBB) substrate after rinsing the sections in 0.1 M Tris-HCL pH 8.5. FBB substrate was prepared as follows. 16 mg FBB

(Sigma) was added to 400 µl 2M HCL and 400 µl 4% $NaNO_2$ and mixed during 1 min before adding to 65 ml of 0.1 M Tris-HCL pH 8.5. Then 8 mg Naphtol-AS-MX-phosphate (Sigma) was dissolved in 400 µl of DMF and added to the solution. Finally, 100 µl of 1 M levamisole (Sigma) was added to the solution to inhibit endogenous alkaline activity. The sections were incubated in the substrate during 45 min. After alkaline phosphatase activity relevation, horseradish peroxidase activity (HRP) was revealed by incubating the sections during 15 min in substrate containing 1.5 ml of 3-amino-9-ethylcarbazole (AEC, Sigma) dissolved in N,N-dimethylformamide (DMF, Sigma) (17 mg/ml) and 30 µl H_2O_2 30% in 60 ml of NaAc buffer pH 5.0, followed by a washing step in PBS. The use of AEC results in a bright red precipitate. All sections were washed in tap water during 10 minutes after which they were embedded in Kaiser's glycerin/gelatin (Merck, Darmstadt, Germany). Sections from reactive human tonsils were included to provide internal positive control tissue. In control sections, the primary antibody was omitted or irrelevant antibodies were applied. An anti-sheep-red blood cells Mab (IgG3) was used as a negative control for the Mab against PG.

Table 2. Reagents used for the detection of various determinants

			Reagent	
Marker	Clone	Expressed by	secondary	tertiary
CD3	A452	pan T cells	GαR-AP	RαG-AP
CD11b	aMac-1	monocytes, macrophages, granulocytes, NK cells	RαRa-AP	GαR-AP
CD14	MY-4	monocytes, granulocytes	RαRa-AP	GαR-AP
CD15	VimD5	granulocytes	RαRa-AP	GαR-AP
CD68	KP-1	monocytes, macrophages	RαRa-AP	GαR-AP
CD40	5D12	macrophages, B cells	RαRa-AP	GαR-AP
CD80/B7.1	M24	macrophages, dendritic cells, B cells	RαRa-AP	GαR-AP
CD86/B7.2	1G10	monocytes, macrophages, dendritic cells, B cells	RαRa-AP	GαR-AP
CD83	HB15a	dendritic cells	RαRa-AP	GαR-AP
HLA-DR	L234	macrophages, dendritic cells	RαRa-AP	GαR-AP
IL-4	anti-IL-4	T cells, mast cells	RαRa-AP	GαR-AP
IL-6	anti-IL-6	T/B cells, macrophages, endothelium, fibroblasts	RαRa-AP	GαR-AP
IL-10	B-S10	T cells, monocytes, macrophages	RαRa-AP	GαR-AP
IFN-γ	MD-2	T cells, NK cells	RαRa-AP	GαR-AP
TNF-α	MC16	monocytes, macrophages, T cells	RαRa-AP	GαR-AP

Abbreviations: AP (alkaline phosphatase), GαR (Goat-anti-Rabbit), RαG (Rabbit-anti-Goat) RαRa (Rabbit anti-Rat), RαM (Rabbit-anti-Mouse). Suppliers: CD3, CD68 (Dako, Glostrup, Denmark) CD14, CD15, HLA-DR (Coulter Clone, Hialeah, FL) CD40 (Tanox Pharma BC, Amsterdam,:The Netherlands) CD83 (Immunotech, Westbrook, ME), CD80, CD86 (Innogenetics N.V., Gent, Belgium) anti-IL-4, anti-IL-6 (Genzyme, Cambridge MA) anti-IL-10 (Instruchemie, Hilversum, The Netherlands). GαR-AP (DAKO), RαRa-AP, RαG-AP (Jackson Immunoresearch, Inc., Westgroove, PA).

Evaluation and quantification of (immuno)histochemistry

Labeling for the relevant markers was scored by two independent observers using light microscopy. 2-3 tissue sections were analyzed per synovium to determine the number of PG-containing cells per mm^2. Single labeling of the other markers was analyzed on a five-point scale: 0 represented no or minimal staining, whereas a score of 4 was assigned to patients with the most abundant staining. All areas of each biopsy were taken into account when examining sections. The scoring system which has been described before, is calibrated for each marker and has been developed previously by examining an extended series of rheumatoid synovial tissues (1,30). Because some cell types or cytokines are present more abundantly than others, the evaluation of each requires a different sensitivity level. The scoring system of 0-4 therefore refers to different numbers of cells stained positive for each marker, e.g. for CD68 a score of 4 represents massive infiltration by macrophages compared to CD15 for which a score of 4 might be assigned for only a limited number of cells. Double labeling of these markers with PG-containing cells was analyzed by counting the PG positive cells and the number of cells expressing both PG and the secondary marker. Results are shown as the percentage of all PG-containing cells coexpressing the analyzed marker.

Statistical analysis

The Mann-Whitney or Student t-test method was used, dependent on the distribution of the data, to determine whether the number of PG-containing cells differed between RA and OA synovial tissues and whether the percentage of double labeling for a particular marker differed between RA and OA synovial tissues.

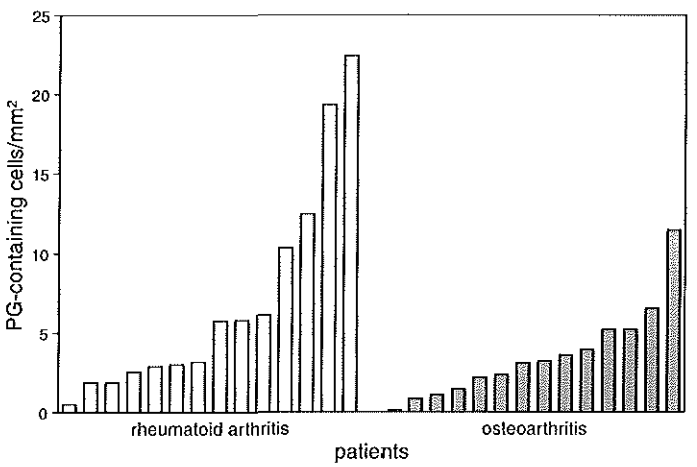

Figure 1. PG containing cells in synovial tissues of RA and OA patients. Immunohistochemistry was performed on synovial tissues of 14 RA and 14 OA patients using an antibody raised against PG isolated from human feces. Results are shown as the number of positive cells/mm^2 for each patient. Samples are ordered according to increasing numbers of PG containing cells.

RESULTS

Number of PG-containing cells in synovial biopsies
The number of PG-containing cells was analyzed in 14 RA and 14 OA synovial tissues. Total numbers of PG-containing cells were counted and expressed as number of PG-containing cells per mm^2, as shown in figure 1.

All RA and OA synovia expressed PG-containing cells except for one OA synovium which was completely negative for the presence of PG. The mean number ± standard deviation of PG positive cells was 7.0± 6.8 for the RA synovia and 3.6±2.7 for the OA synovia but this difference did not reach statistical significance. The intensity of cytoplasmic staining of cells containing PG was high (figure 2a) in both RA and OA. The PG-containing cells were usually located throughout the whole section but could also be found in clusters in infiltrated areas (figure 2b).

Different cell types and expression of costimulatory molecules and cytokines
The expression of the different markers used to functionally characterize PG-containing cells were semiquantitatively analyzed using a four-point scale. As shown in table 3, the expression of all costimulatory molecules and cytokines tended to be higher in RA compared to OA, which was significant for CD3, CD80, CD86, IL-6, IL-10 and TNF-α as defined by the student-t test.

Table 3. Phenotypic markers, costimulatory molecules and cytokines expressed in RA and OA synovium

Category	Marker	RA	OA
Phenotypic marker	CD3	2.0 ±1.0	0.9 ±0.7*
	CD11b	1.2 ±1.0	0.7 ±0.6
	CD14	2.0 ± 0.6	1.0 ± 0.8
	CD15	2.3 ±1.2	1.2 ±0.7
	CD68	2.4 ±0.9	1.3 ±1.0
	CD83	1.6 ± 1.1	0.6 ± 0.8
Costimulatory molecule	HLA-DR	2.6 ±0.7	1.8 ± 0.8
	CD80	2.1 ±0.8	0.9 ± 0.9**
	CD86	2.4 ±0.7	1.3 ±1.0*
	CD40	1.9 ± 0.8	1.3 ±0.9
Cytokine	IL-6	2.7 ±0.8	1.4 ±0.9*
	IL-10	2.6 ±1.0	1.3 ±0.8*
	TNF-α	2.1 ±1.0	0.6 ±0.7**
	IFN-γ	1.8 ±1.0	1.5 ± 0.9

Values are expressed as the mean +/- SD of a semi-quantitative four point scale, * $p < 0.05$, ** $p < 0.01$

Identity of PG-containing cells
To determine the phenotype of PG-containing cells, coexpression of PG with CD3, CD14, CD11b, CD15, CD68 and CD83 was determined. The results are shown as the percentage

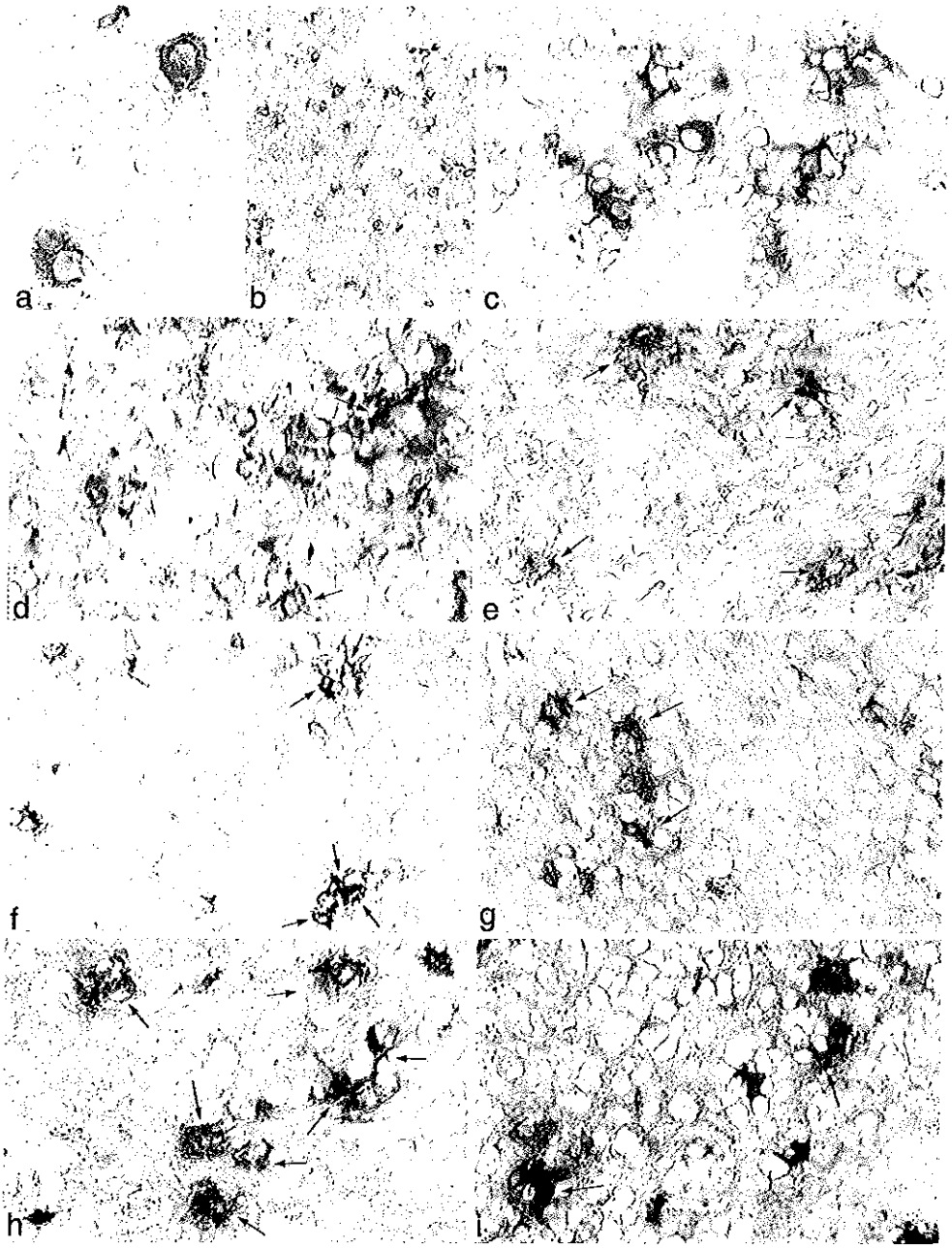

Figure 2. PG is localized in antigen presenting cells expressing costimulatory molecules and cytokines. PG (red cells) can be detected in synovial tissues of RA patients in the cytoplasm of cells distributed throughout the tissue (a) or clustered (b). Double labeling with different markers (blue cells) showed that PG-containing cells are often found within CD3+ T cell infiltrates (c). Furthermore, PG is often found in cells expressing CD68 (d) and HLA-DR (e). Coexpression was also found for CD86 (f) and CD40 (g), both costimulatory molecules. In addition, it was shown that PG containing cells can coexpress the proinflammatory cytokines TNF-α (h) and IL-6 (i). Arrows point to cells expressing both PG and the analyzed marker.

of coexpression by all PG-containing cells (figure 3a). The results show that T lymphocytes and granulocytes (CD3+, CD15+ respectively) do not contain PG, and that PG is present in both immature CD14+ and mature CD68+ (figure 2d) macrophages and only in low numbers of CD11b+ macrophages and CD83+ dendritic cells. PG-containing cells were often found within T cell infiltrates as shown with double labeling with CD3 (figure 2c). Only minor differences could be observed between the phenotype of PG-containing cells in RA and OA synovia.

Expression of costimulatory molecules by PG-containing cells
To examine whether the PG-containing cells coexpress molecules involved in antigen presentation, the presence of PG was analyzed simultaneously with HLA-DR, CD80, CD86 and CD40 expression. The percentages of all PG-containing cells coexpressing the different markers are shown in figure 3B. The majority of PG-containing cells do express HLA-DR (figure 2e) and a high number of cells also express CD80, CD86 (figure 2g), and to a lesser extent CD40 (figure 2f). The expression of CD80, CD68, and CD40 on PG-containing cells is less frequent in OA synovia compared to RA synovia, which is significant for CD80 ($p < 0.05$), suggesting that these cells have lower costimulatory activity in vivo.

Cytokine production by PG-containing cells
To asses whether PG-containing cells produce cytokines involved in the pathogenesis of RA, coexpression of PG with the cytokines IL-6, IL-10, TNF-α and IFN-γ was analyzed. In figure 3C the percentage of PG-containing cells expressing the different cytokines is shown. Especially TNF-α (figure 2h) and IL-6 (figure 2i) are coexpressed by a high number of PG-containing cells. Although coexpression of PG was found with IL-10, IL-10 containing cells were also often detected directly juxtaposed with PG- containing cells. Such colocalization was also found for IFN-γ. The coexpression of TNF-α, IL-6 and IL-10 by PG-containing cells was significantly (respectively $p < 0.05$, $p < 0.05$, $p < 0.01$) higher in RA synovial tissues compared to OA synovial tissues.

DISCUSSION

This study shows that PG-containing cells are present in both RA and OA synovial tissues and that they have a more activated phenotype in synovial tissues of RA patients than OA patients. The number of PG-containing cells tended to be higher in RA compared to OA, but this difference did not reach significance. In all RA synovia PG-containing cells could be detected. These results are in accordance with earlier findings (31) where PG could be found in 8 out of 10 RA synovia. In that particular study a completely different reference group, synovia obtained by arthroscopy after sport injuries, was used in which only limited numbers of PG-containing cells could be observed. To detect PG in synovial tissues, Mab 2E9 was used which is raised against a pure fraction of peptidoglycan-polysaccharides isolated from normal human feces (31). The specificity of this Mab has been extensively investigated and confirmed. First, inhibition studies using ELISA show that 2E9 recognizes cell wall fragments, muramyl dipeptide and lysozyme-solubilized cell walls of different gram-

positive bacteria (31). These experiments suggest that the Mab binds to the glycan backbone of PG since muramic acid forms part of the structure recognized. Second, spleen sections of germ-free rats and of neonatal rats in which the gut flora has not been established yet display no PG-containing phagocytes, whereas spleens of adult rats do. In addition, intravenous injection of PG into neonatal rats results in binding of 2E9 to phagocytes in spleen sections (31). Third, immuno-electron microscopy shows that Mab 2E9 binds to cell walls of *Staphylococcus epidermidis* surviving intracellularly in macrophages around infected

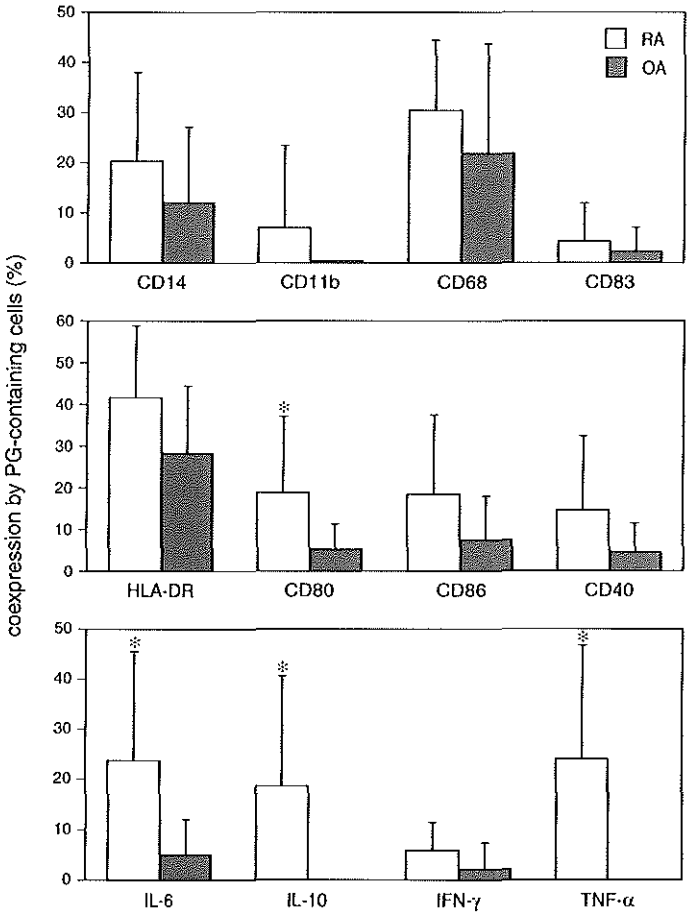

Figure 3. Antigen presenting cells containing PG are more activated in RA synovial tissues compared to OA synovial tissues. Using immunohistochemistry, double labeling was performed with the anti-PG antibody and various subset markers and functional markers of antigen presenting cells. Results are indicated as mean percentage of PG containing cells expressing the analyzed marker + standard deviation (SD). PG is present in both macrophages (CD14/CD11b/CD68) and dendritic cells (CD83) (A). These antigen presenting cells are capable of antigen presentation by the expression of HLA-DR. PG containing cells in RA synovia have a more activated phenotype as shown by the higher percentage of PG containing cells expressing CD40, CD80 and CD86 (B). Expression of cytokines by PG containing cells was also higher in PG containing cells of RA and OA patients (C). * Indicates significantly higher percentage of double labeled cells in RA synovial tissues compared to OA synovial tissues as defined by the Mann-Withney method.

biomaterial. Fourth, 2E9 displays no detectable reactivity against more than ten thousand random dodecapeptides and eight thousand tripeptides in Pepscan approaches (32), confirming this Mab has very low crossreactivity with non-PG epitopes (Slootstra J.W. , Meloen R.H. unpublished). The present study shows that the PG-containing cells are mainly present in macrophages and only in a small fraction of dendritic cells. PG could not be detected in T cells, as expected. The absence of PG in granulocytes, despite their expression of the PG-receptor CD14, can be explained by the fact that N-acetylmuramyl-L-alanine amidase (NAMLAA) is present in granulocytes which is able to degrade PG in joint action with lysozyme (33,34). NAMLAA is not present in monocytes which might result in increased persistence of PG in these cells (33). The more frequent coexpression of CD14 by PG-containing cells in RA synovial tissues compared to OA synovial tissues suggests that these cells are relatively immature and have probably infiltrated more recently compared to the cells in OA synovial tissues. The high percentage of PG-containing cells expressing HLA-DR shows that these macrophages are able to present antigenic peptides to T cells. This is consistent with the expression of costimulatory molecules as CD80, CD86 and CD40 which are required for optimal T cell activation (35). CD86 has been thought of as an important costimulatory molecule in RA. It has been shown that CD86 is more abundantly expressed in RA than CD80 in RA (36,37), and anti-CD86 was a potent inhibitor of a mixed lymphocyte reaction by RA dendritic cells (38,39). In contrast to these earlier studies in the present study both CD80 and CD86, although in lower numbers compared to RA, are also expressed in OA synovial tissues, which can be explained by differences in patients selection. The OA patients in the present study were selected on the basis of clinically inflamed joints secondary to OA. CD40 and its ligand CD40L (CD154) have also been described as important molecules in the pathogenesis of RA (40,41). In the synovial tissues of RA patients of the current study, CD40 was mostly expressed in infiltrates. The percentage of PG-containing cells expressing CD80, CD86 and CD40 is higher in RA compared to OA suggesting a more activated phenotype. Cytokines are believed to play an important role in the pathogenesis of RA (1,23). Especially the proinflammatory cytokines like IL-1, IL-6 and TNF-α are abundantly expressed in RA (1,42,43) and are able to activate T and B cells but can also induce cartilage and bone destruction (44). Immunoregulatory cytokines like IL-10 produced mainly by macrophages, are also expressed in high concentrations in RA synovial tissues (45,46). The role of IL-10 in RA is mainly anti-inflammatory as shown by *in vitro* experiments and by the possible clinical improvement upon IL-10 treatment in RA patients (47). The detection of the T cell derived immunoregulatory cytokine IL-4 has been difficult up to now in contrary to the detection of the proinflammatory T cell derived cytokine IFN-γ (48). Therefore it is hypothesized that RA results mainly from a T helper 1 response. In the present study we analyzed whether PG-containing cells coexpress these cytokines. It was shown that the expression of IL-10 and TNF-α was higher in the RA synovia compared to OA. Simultaneous labeling of PG and IFN-γ resulted in only a few double labeled cells. Instead, IFN-γ positive cells could often be found directly adjacent to PG-containing cells. This suggests that interactions might occur between PG-containing cells and IFN-γ positive cells, probably T cells. This was confirmed by the presence of PG-containing cells within infiltrates of CD3+ cells.

Coexpression with PG was found mainly with IL-6, IL-10 and TNF-α. The percentage of

coexpression of IL-6, IL-10 and TNF-α by PG-containing cells was significantly higher in RA synovia compared to OA synovia. As PG isolated from sterile human spleen is able to induce these cytokines *in vitro* it can be hypothesized that PG contributes to the cytokine production in the synovial tissues (21).

The presence of PG, an antigen ubiquitously expressed at the musosal sites, in synovial tissues suggests that PG derived from the mucosal sites is able to circulate through the bloodstream in monocytes (49,50) and can enter the synovial tissue. The distribution of PG may be dependent on two factors. First, reduced gut barrier function may result from inflammation, leading to increased bacterial translocation. Ultrastructural lesions of the gut wall have been reported in RA patients (51). Second, antibodies against PG may not be sufficiently capable of eliminating the bacterial products from mucosal sites and/or the bloodstream. Antibodies that reach the mucosal lumina perform immune exclusion and immune elimination to protect the mucosal surface against (bacterial) antigens. This is performed by secretory IgA or IgM (S-IgA, S-IgM), but also by serum IgG antibodies (52,53). It has been shown that RA patients have significantly reduced systemic IgG antibody levels against PG (Schrijver et al. submitted) implying that lowered antibody responses against PG occur, may lead to increased translocation of PG in the circulation.

An increased circulation of PG-containing cells in combination with the preference of mucosal macrophages to home to the synovial sites (54) may lead to infiltration of PG-containing cells into the synovium. Once infiltrated into the synovium, PG-containing cells may contribute to inflammation. It has been shown that monocytes recruited from the blood are more relevant for synovial inflammation and destruction than local proliferation of macrophages (55). The presence of PG-containing cells in both RA and OA synovia raises the question whether the migration of PG is a primary or secondary event in RA and OA. OA is a degenerative disease of the articular cartilage believed to be primary non-inflammatory, but a secondary synovitis is often observed. (56,57). The OA synovia analyzed in the present study display also signs of inflammation as shown by the infiltration of T cells and expression of CD40, CD80, CD86 and cytokines. It can be hypothesized that infiltration of PG-containing cells due to primary events promotes secondary inflammatory events in OA. Because PG-containing cells possess a more activated phenotype in RA compared to OA it can be suggested that PG plays a more prominent role in RA. Whether PG is involved in the initiation of RA is difficult to determine because the clinical debut of RA already represents a chronic phase of the disease (1,58). However, the ability of PG to induce arthritis in rodents clearly supports this hypothesis. PG might contribute to inflammation by the production of cytokines and the activation of T cells. Recently, it has been shown that T cell tolerance to autoantigens can be broken by additional stimuli such as LPS (59-61). It can be hypothesized that PG, which is a functional analog of LPS, activates autoreactive T cells specific for different cartilage antigens such as collagen (62) or HC-gp39 (63,64).

In conclusion, PG is observed in synovial tissue from especially RA patients. The activation state of the PG-containing cells is higher in RA patients compared to OA patients, suggesting a contribution of PG in the inflammatory process. To investigate whether PG is involved in the pathogenesis of RA, the effect of restriction of PG access to extra-intestinal sites can be investigated in animal models.

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Chapter 4.3

Bacterial peptidoglycan promotes immune reactivity in the central nervous system in multiple sclerosis

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ABSTRACT

Microbial antigens such as lipopolysaccharide can breach peripheral T-cell tolerance in experimental animal models, contributing to development and perpetuation of autoimmune disease. Peptidoglycan, a major component of the gram-positive bacterial cell wall, is a functional lipopolysaccharide analogue with potent proinflammatory properties and is conceivably a mediator of sterile inflammation. We here demonstrate that peptidoglycan is present within antigen presenting cells in the brain of multiple sclerosis patients. These cells have macrophage and dendritic cell characteristics, and are immunocompetent as evidenced by coexpression of inflammatory cytokines and costimulatory molecules. In addition, intrathecal, plasma cells specific for peptidoglycan are present in multiple sclerosis brain, and antibodies binding peptidoglycan are present in cerebrospinal fluid during active disease. Peptidoglycan can thus contribute to T- and B-cell activity during brain inflammation without a requirement for local bacterial replication.

INTRODUCTION

According to prevailing dogma, multiple sclerosis (MS) is caused by autoreactive CD4+ T cells specific for myelin components with a proinflammatory T helper phenotype (1). As healthy donors have similar numbers of myelin-reactive T cells as patients, additional factors are clearly needed to promote (auto)-reactivity of T cells within the central nervous system (CNS) (2). Recent studies in several experimental models including the experimental autoimmune encephalomyelitis (EAE) model for MS have shown that various infectious and proinflammatory agents such as lipopolysaccharide (LPS) are capable of reversing the tolerant state of CD4+ and CD8+ T cells (3-6). Activation of these cells is crucially dependent on the presence of both the stimulatory agent and the specific (auto)-antigen (5). Research on inflammatory properties of bacterial antigens in brain tissue has shown that bacteria injected into the brain parenchyma are able to induce inflammatory responses

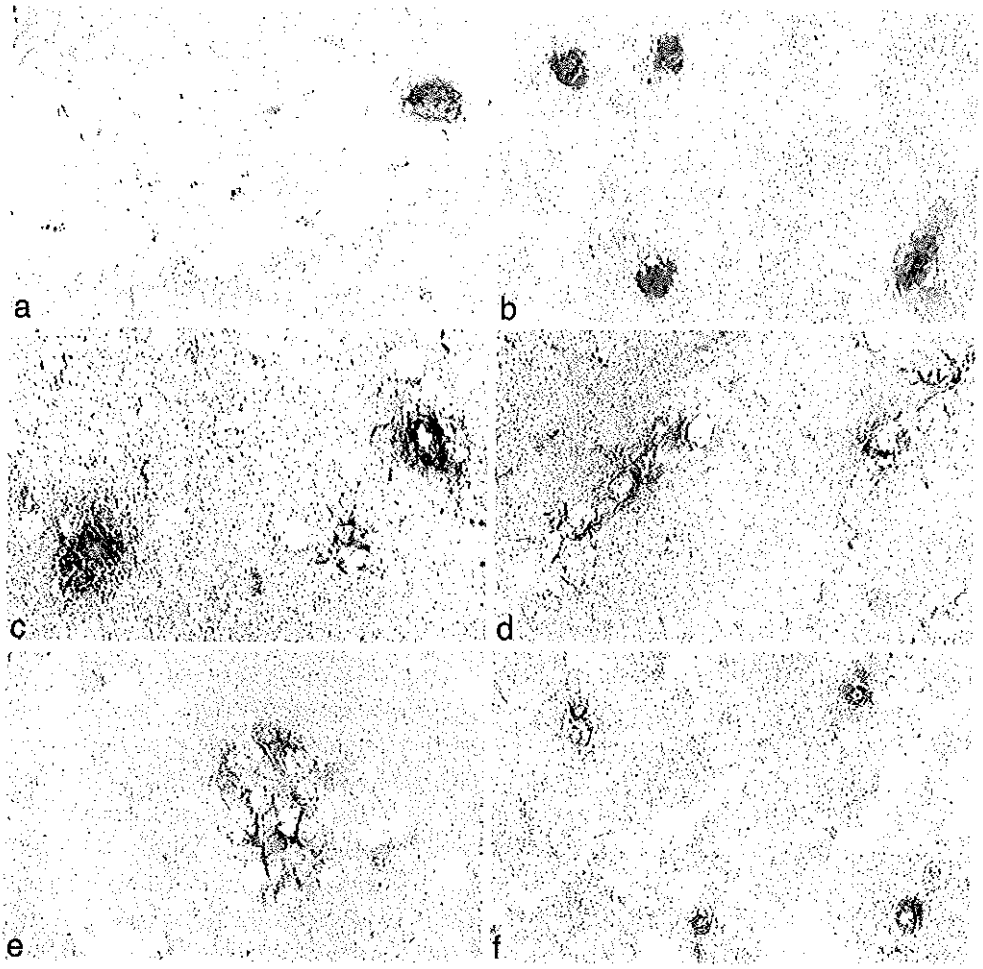

Figure 1. Peptidoglycan promotes immune reactivity in brain tissue of multiple sclerosis patients. Peptidoglycan is present in the white matter of multiple sclerosis patients on the edge of infiltrates or active lesions (a), or distributed in normal appearing white matter (b). Co-expression was found with CD80 (c) and colocalization with other cells expressing IL-10 (d). Both coexpression and colocalization was found with TNF-α (e). Using biotin-labeled PG, antibody forming cells specific for PG could be detected in MS brain tissue (f + inlay). (Original magnification 630x, f: 400x, inlay 630x)

only after peripheral sensitization of T cells (7). However, there is no clear evidence to date that bacterial agents or components derived thereof, such as LPS, are functionally involved in initiation or perpetuation of MS disease activity. In addition, studies attempting to link infectious agents to (autoimmune) inflammation are often based on the premise that replication occurs within the affected organ (8), but this is not necessarily the case.

Peptidoglycan (PG) is a major constituent of the cell wall of gram-positive bacteria, which are abundantly present at all mucosa, and most prominently in the gut as part of the normal microbial flora (see (9) for review). PG is composed of long sugar chains of alternating

N-acetyl glucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc) residues, which are interlinked by peptide bridges, resulting in a large complex macromolecular structure (10). PG is only a very minor constituent of the cell wall of gram-negative bacteria. PG can be regarded as a functional analog of LPS and both molecules activate innate immune defence mechanisms (11). First, both molecules use CD14 on monocytes /macrophages and granulocytes as their cellular receptor. Second, the intracellular signaling pathways employed by both molecules are highly similar if not identical (9,12,13), engaging the recently identified Toll-like receptor (14-16). Third, both LPS and PG induce strong proinflammatory responses *in vitro*, including production of cytokines such as IL-1 and TNF-α (12,17,18). LPS and PG are complex macromolecules which are difficult to digest by phagocytes (19). Macrophages lack amidase (N-acetyl muramyl L-alanine amidase (NAMLAA)) expression (20,21) which is required for full degradation of PG in joint action with lysozyme (22). This resistance to degradation explains at least in part the persistence of microbial cell walls, which may be a pivotal factor in chronic (autoimmune) inflammation (8).

Antigen presenting cells containing PG are present in the joints of patients with rheumatoid arthritis, possibly contributing to the local inflammatory environment (23,24). Interestingly, PG retaining proinflammatory capacity such as the induction of TNF-α and IL-1, can be fractionated from sterile human spleen (17,25,26), suggesting that continuous redistribution of PG occurs from the mucosa to the secondary lymphoid organs as well as to other anatomical sites in the absence of sepsis and without requirement for local bacterial replication. This is fully consistent with the presence of circulating leukocytes carrying PG in human healthy subjects (27) and distinct patient categories (28).

In view of the strong influx of macrophages into the CNS of MS patients facilitated by severely impaired blood-brain barrier function (29), we hypothesized that phagocytic antigen presenting cells carrying PG gain access to the CNS and can locally promote immune reactivity of T and B cells. We here show that PG-containing antigen presenting cells are present in human MS brain tissue and that they express costimulatory molecules and immunomodulatory cytokines. In addition, antibody forming cells specific for PG are present in human MS brain tissue and patients with active disease have antibodies against PG in the cerebrospinal fluid. These findings imply that PG promotes immune reactivity in the brain.

METHODS

Patient samples

Human brain tissue taken at autopsy was provided by the Netherlands Brain Bank in Amsterdam (Coordinator Dr. R. Ravid). Average post-mortem delay was 6 hours (range 4-9) for MS patients and 8 hours (range 6-10) for control patients. No abnormalities could be detected at autopsy in the brain tissues of these subjects passed away by different causes like cardiac arrest, cancer and pneumonia.

Cerebrospinal fluid samples of 10 MS patients and 10 control patients were obtained from

the Department of Neurology of the Erasmus University and University Hospital Rotterdam-Dijkzigt. Cerebrospinal fluid samples of relapsing-remitting MS patients were taken prior to immuno-modulating therapy during a relapse of MS. Control subjects were patients presented with severe headache for which no cause could be determined.

Immunohistochemistry

Immunohistochemical procedures used for detection of cellular subsets, antigens, antigen specific plasma cells, costimulatory molecules and cytokines in frozen sections have all been described in detail previously (30). Antibodies used were: CD14 (MY-4, Coulter Clone, Hialeah, FL), CD68 (KP-1, DAKO, Glostrup, Denmark), CD83 (M43, Immunotech, Westbrook, ME), CD1a (DAKO), CD3 (DAKO), HLA-DR (L234, Coulter Clone), CD80 (M24, Innogenetics N.V., Gent, Belgium), CD86 (1G10, Innogenetics), CD40 (5D12, Tanox Pharma BV, Amsterdam, The Netherlands), IL-1α (Vmp 18), IL-1β (Vhp20), IL-4 (Genzyme, Cambridge, MA), IL-6 (Genzyme), IL-10 (B-S10, Instruchemie, Hilversum, The Netherlands), IL-12 (PharMingen, San Diego, CA), IFN-γ (MD-2, a kind gift of Dr. P. van der Meide, BPRC, Rijswijk, The Netherlands), TNF-α (MC16, BPRC). To detect specific antibody forming cells, peptidoglycan-polysaccharide isolated from human feces (31) was labeled with biotin (32,33). Sections from reactive human tonsils were included on each individual object slide to provide internal positive control tissue. Sections were evaluated by two independent observers blinded to the staining procedure.

Monoclonal antibody against peptidoglycan (2E9)

2E9 is a murine monoclonal antibody Mab (IgG3) raised against a pure fraction of peptidoglycan-polysaccharides isolated from normal human feces (34). The specificity of this Mab has been extensively investigated and confirmed. First, inhibition studies using ELISA show that 2E9 recognizes cell wall fragments, muramyl dipeptide and lysozyme-solubilized cell walls of different gram-positive bacteria (34). These experiments suggest that it binds to the glycan backbone of PG since muramic acid forms part of the structure recognized. Second, spleen sections of germfree rats and of neonatal rats in which the gut flora has not been established yet display no PG-containing phagocytes, whereas spleens of adult rats do. In addition, intravenous injection of PG into neonatal rats results in binding of 2E9 to phagocytes in spleen sections (34). Third, immuno-electron microscopy shows that Mab 2E9 binds to cell walls of *Staphylococcus epidermidis* surviving intracellularly in macrophages around infected biomaterial. Fourth, 2E9 displays no detectable reactivity against more than ten thousand random dodecapeptides and eight thousand tripeptides in Pepscan approaches (35), confirming this Mab has very low crossreactivity with non-PG epitopes.
An isotype-matched control antibody (NS7, IgG3) of irrelevant specificity (sheep red blood cells) was used throughout the studies and displayed no binding activity in brain tissue of MS patients or control subjects.

Statistical analysis

The number of PG-containing cells and plasma cells binding PG present in MS brain tissue and control brain tissue were compared using the Mann-Whitney rank-sum test.
A p value < 0.05 was considered to be significant.

PG ELISA

To measure anti-PG antibodies in cerebrospinal fluid, an ELISA, extensively validated and used before, was performed as described (36). Briefly, 50 µl of peptidoglycan-polysaccharides (10 µg/ml) was coated overnight at 50°C onto 96-well polystyrene microtiter plates. 100 µl of 1:2, 1:4 and 1:8 dilutions of cerebrospinal fluid were incubated during 1 hour at 37°C. As detecting antibody, peroxidase conjugated rabbit anti-human IgM, IgG or IgA (Jackson Immunoresearch, Inc., Westgroove, PA) was used during 1 hour at 37°C. The development of the colorimetric assay took place at 37°C for 30-45 min after the addition of 100 ml of ortho-phenylenediamine/H_2O. The reaction was stopped by 4 M H_2SO_4 and the optical density was measured at a wavelength of 492 nm with a Titertek Multiskan (Flow Laboratories, Irvine, Scotland). Between all incubation steps the plates were washed three times with PBS-0.02% Tween and cerebrospinal fluid and conjugates were diluted in PBS-0.2% Tween.

RESULTS

PG-containing cells are present in MS brain tissue

The presence of PG was analyzed in cryo-sections of brain white matter of MS patients and control subjects using a monoclonal antibody against PG (34). The numbers of PG-containing cells were significantly higher in MS brains compared to control patients (figure 2) ($p=0.001$). In MS patients, PG-containing cells were mainly present on the edge of active lesions (37)(figure 1a), as defined by Oil-red O staining for myelin breakdown products ingested by macrophages. Furthermore, PG-containing cells were present around blood vessels, or scattered in the normal appearing white matter (figure 1b). Only rarely could PG-containing cells be found within the fully demyelinated foci forming the center of lesions. To further analyze the role of PG-containing cells in inflammation of the brain, double labeling was performed on brain tissue of MS patients selected for high numbers of PG-containing cells, to determine the phenotype and activation state of the PG-containing cells.

PG is present in macrophages and dendritic cells

To determine the phenotype of PG-containing cells, coexpression of PG with CD68, CD83, CD14, 27E10, CD1a and CD3 was analyzed. Figure 3 shows that PG is mostly present in macrophages expressing CD68 and dendritic cells expressing CD83. CD14, which is the cellular receptor for both PG and LPS present on relatively immature phagocytes, was expressed by 17% (range 0-27%) of the PG-containing cells (38). The majority of the PG-containing cells (mean 62%), and in 2 out of 9 patients even all of these cells (range 33-100%) expressed 27E10, an acute stage inflammatory macrophage marker characteristic for active MS lesions (39). Coexpression of CD1a was analyzed since it is concievable that PG being composed of carbohydrates is presented by the CD1a antigen presenting molecule. However, only very few PG-containing cells coexpressed CD1a. PG could not be detected in T cells (CD3).

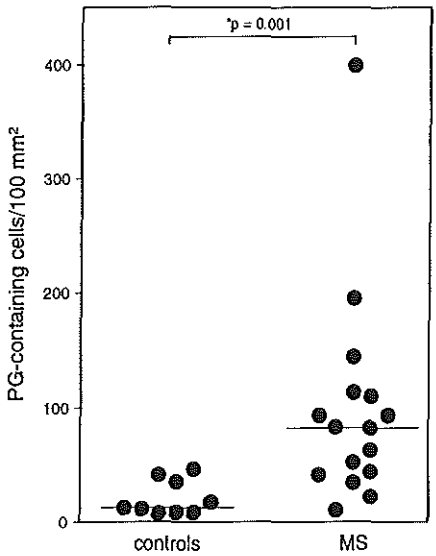

Figure 2. Peptidoglycan is present in the white matter of brain tissue of multiple sclerosis patients. Immunohistochemistry was performed on brain tissue of 17 multiple sclerosis patients and 10 control subjects using Mab 2E9 raised against PG isolated from human feces. The median (horizontal bar) of the number of positive cells was 82/100 mm^2 in multiple sclerosis patients compared to 12/100 mm^2 in the control subjects. * p=0.001 using the Mann-Whitney rank-sum test.

PG-containing cells coexpress costimulatory molecules

The capability of PG-containing cells to present antigen was examined by double staining for HLA-DR, CD80/B7-1, CD86/B7-2 and CD40, which are centrally involved in (co)-stimulation of CD4+ T cells. Coexpression with PG occurred for all four molecules to varying extents, in particular for CD80 (figure 1c), suggesting that PG-containing cells are mainly functionally competent, active antigen presenting cells (figure. 3).

Peptidoglycan containing cells coexpress different cytokines

To asses whether PG-containing cells can contribute to CNS inflammation in MS by producing cytokines, double labeling was performed for proinflammatory cytokines (IL-1α and β, IL-6, IL-12, TNF-α, IFN-γ) and immunoregulatory cytokines (IL-4, IL-10). Except for IL-1α, all cytokines analyzed were expressed by PG-containing cells, to varying extents. Of the proinflammatory cytokines, coexpression was most frequently found for TNF-α (mean 44% of PG-containing cells, range 9-100%). In one patient, all PG-containing cells produced the strongly proinflammatory and myelotoxic cytokine TNF-α. In addition, TNF-α was also often strongly expressed by cells directly adjacent to PG-containing cells (figure 1e). Coexpression of PG was also found for IL-β (mean 30%, range 14-52%), IL-6 (mean 11%, range 0-22%), IL-12 (mean 18%, range 0-65%) and IFN-γ (mean 11%, range 10-73%), although the latter cytokine was mostly expressed by cells directly adjacent to PG-containing cells. Immunoregulatory cytokines were prominently produced by PG-containing cells. IL-4 was detected in 58% (range 13-93%) of PG-containing cells. IL-10 was mainly pro-

duced by cells directly juxtaposed with PG-containing cells (figure 1d), but coexpression was found in 40% (range 0-83%) of all PG-containing cells.

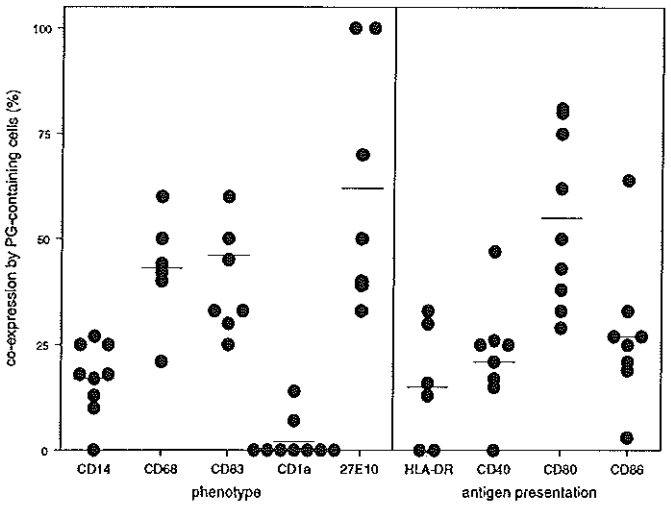

Figure 3. Peptidoglycan is present in macrophages and dendritic cells capable of antigen presentation. Using immunohistochemistry, double labeling was performed with the Mab 2E9 raised against peptidoglycan (PG) isolated from human feces and various subset markers and functional markers of antigen presenting cells. PG is present in both macrophages (CD68/27E10) and dendritic cells (CD83), which are capable of antigen presentation and costimulation as reflected by the expression of HLA-DR, CD80 and CD86. Horizontal bars represent mean numbers of PG-containing cells coexpressing the analyzed marker.

Antibody forming cells specific for peptidoglycan are present in MS brain tissue

Healthy human subjects have systemic IgM, IgG and IgA levels against PG (36), reflecting continuous exposure to gram-positive bacteria at the mucosa. To determine whether intrathecal antibodies against PG were produced in MS brain tissue, plasma cells specific for PG were visualized in situ using biotin-labeled PG (32,33). Plasma cells specific for PG could be detected in 7 out of 13 MS patients and in 2 out of 7 controls (figure 4). The numbers of plasma cells specific for PG were limited in control patient tissues and 3 of the MS patients, however 4 MS patients had high numbers of PG-specific antibody forming cells. In MS patients, the number of antibody forming cells against PG was directly correlated with the number of PG-containing cells ($p < 0.01$). Antibody forming cells specific for PG were scattered throughout the tissue (figure 1f) and could often be found nearby blood vessels. Pre-incubation of tissue sections with unlabeled PG resulted in a dose dependent reduction of the number of PG-binding cells, confirming the specificity of staining. Double labeling for IgM plus IgG and PG-biotin confirmed that PG-binding cells were indeed plasma cells containing high levels of cytoplasmic immunoglobulin.

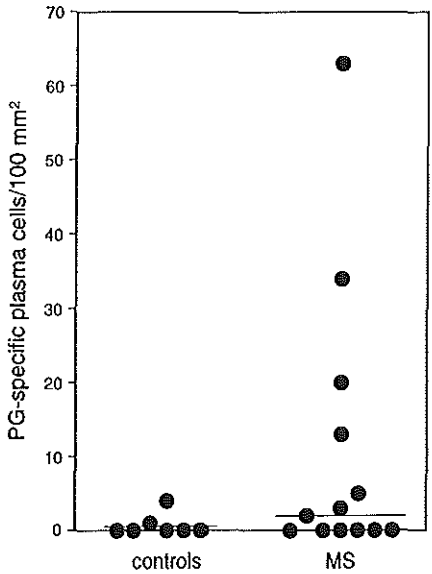

Figure 4. Peptidoglycan-specific plasma cells are present in brain tissue of multiple sclerosis patients. Using biotin-labeled PG, antibody forming cells could be detected in 7 of 13 multiple sclerosis (MS) patients and only in 2 of 7 control subjects. In four of the MS patients relatively high numbers of antibody forming cells could be detected. Horizontal bars represent the median of the number of PG specific plasma cells in both groups.

Antibodies specific for peptidoglycan are present in cerebrospinal fluid of patients with active MS

To confirm our finding of intrathecal production of antibodies against PG *in situ* in MS autopsy brain tissue, ELISA was used to determine IgM, IgG and IgA antibody levels against PG in cerebrospinal fluid samples.

Samples taken during relapse from relapsing-remitting MS patients not treated with immunosuppressive drugs were compared to samples from patients with severe headache for which no cause could be determined. In none of the control patients anti-PG antibodies could be detected. Using conservative cut off values, 6 out of 10 MS patients displayed considerable levels of anti-PG antibodies of the IgG and/or IgA isotypes (figure 5).

DISCUSSION

The present study shows that the gram-positive cell wall antigen peptidoglycan, abundantly present at all mucosal surfaces, is present in antigen presenting cells in the central nervous system of multiple sclerosis patients. Here, it contributes to inflammation by secretion of cytokines modulating T-cell function and stimulating antibody formation in the central nervous system of multiple sclerosis patients.

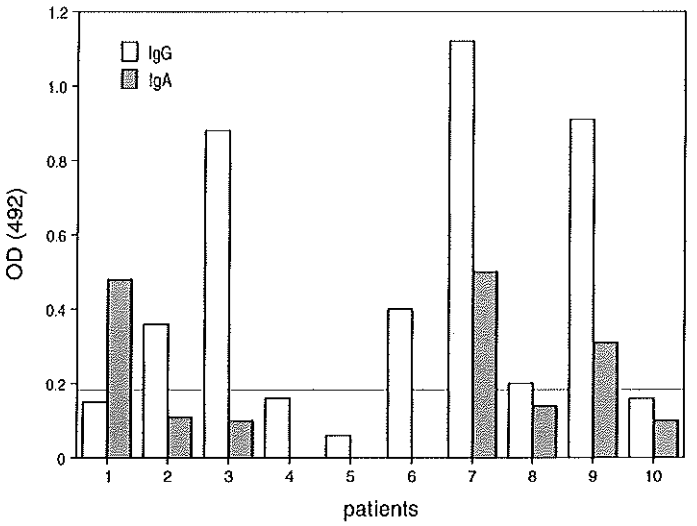

Figure 5. IgA and IgG antibodies against PG are present in cerebrospinal fluid samples of multiple sclerosis patients. Using ELISA IgM, IgG and IgA levels against peptidoglycan (PG) were measured in cerebrospinal fluid samples (CSF) of 10 relapsing-remitting multiple sclerosis (MS) patients taken during a relapse and of 10 control patients with severe headache for which no cause could be determined. Using a CSF dilution of 1:2 anti-PG IgG and/or IgA antibodies could be detected in 7 of 10 MS patients. In none of the control patients antibodies against PG could be detected. The broken line at 0.19 shows the mean OD + 3SD of the control patients. IgM was not detected in any of the fluids analyzed.

The presence of bacterial peptidoglycan in CNS tissue sheds a different light on the search for infectious agents involved in MS, where the emphasis has been on identification of viruses (e.g. HHV-6) and not on bacteria (40,41). Recently, *Chlamydia pneumoniae* and specific antibodies have been detected in the cerebrospinal fluid of MS patients (42). In that particular study and others the implicit assumption often is that local replication of viruses or bacteria needs to occur. In sharp contrast, we show that peptidoglycan stimulates CNS-inflammation in the apparent local absence of viable bacteria and replication, implying that this antigen is a microbial mediator in sterile inflammation. Thus, our data support novel emerging insights that persistence of bacterial cell wall antigens can contribute to autoimmune diseases (8).

Studies of autopsy brain material, including MS and Alzheimer patients, routinely and consistently reveal a partial overlap of pathological characteristics between patient and control subjects tissues (43,44). For example, activated macrophages can be found in control subject tissues (45). Since quantitative differences and the less outspoken expression of these pathological characteristics in control tissues are widely recognized differential parameters, this overlap does not in any way prevent clear interpretation of autopsy material studies (discussed by (43)). Furthermore, in the current report, cerebrospinal fluid from MS patients with disease relapse was also evaluated, confirming the *in situ* presence of PG-specific plasma cells.

Entry of peptidoglycan containing cells into the brain

Recent studies have shown that leukocytes can enter the blood-brain barrier and brain parenchyma under normal circumstances, most likely as a part of routine immune surveillance (reviewed in (46)). This may explain why low numbers of PG-containing cells can occasionally be found in brain tissue of control patients. A few days after brain trauma such as contusion, massive infiltration of mononuclear cells into the brain occurs, including lymphocytes and antigen presenting cells (macrophages and dendritic cells) (47). This indicates that in the absence of known infectious or autoimmune insults, inflammatory infiltrates can develop. The high number of PG-containing cells we find in MS brain tissue is likely due to the severely impaired blood-brain barrier function, leading to recruitment of high numbers of macrophages. It is highly unlikely that PG gains access to the brain in the post-mortem phase due to bacterial translocation over mucosal barriers, because PG is present in the cytoplasm of antigen presenting cells, necessitating active ingestion of PG. In addition, antibody forming cells specific for PG could be found in the brain tissue. Furthermore, ongoing experiments in EAE models in the marmoset monkey (48,49) and the SJL/J mouse (50) show that PG-containing cells and specific plasma cells can be detected in perivascular infiltrates in tissues processed immediately after sacrifice. We currently favor the interpretation that access of PG-containing cells to the CNS is a stochastic process, influenced by the activation state of the cells affecting the expression profile of adhesion molecules. We therefore predict that PG-containing cells will occur in inflammatory diseases of the CNS other than MS. In that sense, there are currently no grounds to assume a single antigen-disease link, which is consistent with novel views on polymicrobial involvement in chronic inflammatory disease (8).

Intrathecal antibody synthesis against peptidoglycan

Plasma cells producing antibody specific for PG were also present *in situ* in MS brain. This demonstrates that intrathecal antibody production against PG occurs. To confirm this finding, cerebrospinal fluid of MS patients and control patients was analyzed, showing that none of the control patients had specific antibody titers, whereas the majority of MS patients had detectable specific IgG and IgA levels. The specificity and pathological relevance of plasma cells in the brain and oligoclonal immunoglobulins in MS has been highly obscure thus far (see (51)). Our finding that PG evokes specific intrathecal antibody production *in situ* identifies a hitherto unknown specificity of local plasma cells.

Peptidoglycan is present in functionally competent antigen presenting cells

Characterization of PG-containing cells showed that macrophages containing PG were mostly mature, while only a minority had an immature phenotype. The persistence of PG in macrophages can be explained by the fact that amidase can not be detected in macrophages (21). High expression of amidase and its lytic action in granulocytes may explain why PG cannot be detected in these cells. The high percentage of 27E10 expression by PG-containing cells indicated that PG is present in active lesions (39), of which 27E10 is a reliable marker. This is consistent with the concept that antigen presenting cells mature upon uptake of antigen and migration (52), where migration through vascular endothelium is a crucial step promoting functional maturate and loss of surface markers such as CD14 (53).

CD14+ monocytes can also maturate into dendritic cells, a transition enhanced by bacterial stimuli (54), which may explain the presence of PG in a limited number of dendritic cells.

The expression of HLA-DR, CD40, CD80 and CD86 by PG-containing cells indicates that these cells are potent stimulators of antigen-specific T-cell activation. In addition, PG-containing cells synthesized cytokines involved in regulation of inflammatory activity (e.g. IL-1β, IL-6, IL-12, TNF-α, IFN-γ, IL-4 and IL-10), demonstrating that they produce soluble mediators directly affecting T-cell proliferation, activation and survival (4,55). PG isolated from sterile human spleen is capable of inducing IL-1, IL-6, TNF-α (26) by macrophages *in vitro*, and ongoing experiments show that also IL-10, IFN-γ and IL-12 and gelatinase B are induced upon PG stimulation *in vitro*. This confirms that PG can indeed be responsible for the cytokine production by the PG-containing cells in the CNS.

Peptidoglycan and T-cell tolerance

We hypothesize that PG in the CNS contributes to breaching T-cell tolerance and promotes T-cell activity by antigen presentation and induction of cytokine production (3-6). This is supported by the expression of HLA-DR, costimulatory molecules and cytokines by PG-containing cells in MS brain. It has been shown in animal models that inflammatory processes created by bacterial infections or LPS lead to activation of autoreactive CD4+ cells (3) or CD8+ cells (5) which otherwise die by apoptosis. The major mechanism leading to breaking of tolerance is cytokine induction by infectious agents leading to T-cell survival (4,56). This view is supported by the finding that LPS induces EAE in mice injected with myelin basic protein (MBP)-specific T cells through induction of IL-12 (6). Therefore, the presence and persistence of PG in brain tissue may promote loss of tolerance breakdown against autoantigens present in the brain like MBP, proteolipid protein (PLP) and others.

Perspective

In conclusion, this study shows that immunocompetent PG-containing cells are able to reach the brain in MS and promote immune reactivity, by expression of costimulatory molecules and cytokines and by stimulating intrathecal antibody formation. Redistribution of PG from mucosal sites to the brain depends on the intricate interplay between several factors such as the permeability of the intestine, activity of enzymes specifically involved in PG degradation (21), and mucosal and systemic antibody responses against PG (Schrijver et al., submitted). Restricting the access of PG to non-mucosal sites may therefore be of clinical benefit to patients, as this may lead to lower levels of inflammation.

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Chapter

5

GENERAL DISCUSSION

CHAPTER 5

General discussion

Peptidoglycan (PG) is a bacterial cell wall antigen present in large amounts in gram-positive bacteria and only in small amounts in gram-negative bacteria. PG is composed of alternating N-acetyl glucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc) long sugar chains, which are interlinked by peptide bridges resulting in a large complex macromolecular structure (1). PG and lipopolysaccharide (LPS) of gram-negative bacteria bind both to CD14 on human cells and have similar biological properties such as induction of cytokines and B and T cell activation (2).
Rheumatoid arthritis (RA) and multiple sclerosis (MS) are both chronic inflammatory diseases, characterized by infiltration of T cells, antigen presenting cells (APC) and plasma cells. The inflammatory process in both diseases is the result of interactions between (auto)antigens, cells of the immune system and resident cells of the diseased tissue (3). Crucial mediators in the inflammatory process are cytokines, chemokines and matrix metalloproteinases (4-6). In both RA and MS, the antigen(s) involved in this process have not been identified. Particularly in RA there are indications that PG may play a role in the pathogenesis of the disease, such as the ability of PG to induce arthritis in rats and the presence of PG-containing cells in synovial tissues of RA patients (7-9). In this thesis the role of PG in RA and MS is investigated. In this chapter the results from the previous sections are discussed in the context of the possible role of PG derived from the mucosa in the inflammatory events occurring in RA and MS.

Peptidoglycan present in human tissues
A prerequisite for the hypothesis that PG is involved in inflammatory diseases is the presence and persistence of bacterial cell wall products in relevant human tissues. Although detection of PG is technically difficult, different studies have described the detection of PG in fluids like urine (10) and synovial fluid (11), and also in leukocytes (12,13). Using a monoclonal antibody which recognizes peptidoglycan-polysaccharides (PG-PS), these antigens could also be detected in APC in tissues such as spleen and RA synovia (14-16).
In order to obtain more information on the molecular structure of PG in human tissues and to analyze its biological activities *in vitro*, we purified PG from sterile human spleen (14). A macromolecular carbohydrate-rich fraction was obtained after several purification steps. The presence of PG in this fraction was confirmed by the detection of MurNAc, a unique component of PG using an aminosugar/amino acid analysis method. To further investigate the structure and biological properties of the purified PG, the isolation procedure was optimized (chapter 2.1). The presence of PG in the highly purified fraction obtained was confirmed using three different independent methods: ELISA using the monoclonal antibody against PG-PS (17), the colorimetric muramic acid method (18) and detection of MurNAc by aminosugar/amino acid analysis (14). Colorimetric muramic acid detection showed that PG present in human spleen was mainly intact i.e. the stem peptides were still linked to the

aminosugar. This suggests that the PG fraction isolated from sterile human spleen had not been degraded by N-acetylmuramyl-L-alanine amidase (NAMLAA) which hydrolyses the lactamide bond linking the MurNAc of the aminosugar chains to the L-alanine end of the peptide chain (19).

Biological activities of peptidoglycan in human tissues

The effect of PG on T cells, B cells and monocytes/macrophages has previously been studied using PG isolated from cultured bacteria. These studies have shown that PG is able to induce the production of the proinflammatory cytokines IL-1β, IL-6 and TNF-α by monocytes. This activity was dependent on CD14 binding (20-23), the cellular receptor for PG and LPS. These studies also showed that PG is able to induce polyclonal antibodies (17,24) and to induce T cell proliferation dependent on monocytes (25). T cell clones specific for a PG subunit disaccharide tetrapeptide were able to induce a delayed type hypersensitivity response (DTH) (26,27). In the present thesis we showed that PG present in human spleen is still biologically active. Using bioassays and ELISA it was shown that PG isolated from sterile human spleen tissue is able to induce TNF-α, IL-1 and IL-6 dependent on CD14 during cell culture (chapter 2.1). In addition to cytokine production, PG isolated from human spleen is able to induce proliferation of T cells from healthy subjects (chapter 2.2). This is consistent with earlier findings showing activation of T cells by PG stimulation (25). In those studies it was hypothesized that PG induces polyclonal T cell proliferation, but the identity of the T cells and the nature of the activation mechanism remained unknown. In chapter 2.2 we show that the T cell proliferation is dependent on binding of PG to CD14. Recently it has been shown that signaling after PG or LPS binding to CD14 is mediated by Toll-like receptors forming a complex with CD14 since CD14 is a GPI–anchored molecule that does not traverse the cell membrane (28). PG, processed and presented by APC, is able to induce proliferation of mainly αβCD4+ and also some αβCD8+ T cells (chapter 2.2). This suggests that PG act as a polyclonal T cell activator. The inhibition of proliferation by anti-HLA-I and anti-HLA-II antibodies together with the proliferation peak at day 7-8, however, suggests an antigen-specific T-cell response. This is consistent with earlier findings that T cell activation by PG is dependent on monocytes (25). The proliferation of CD8+ T cells in response to PG has to be further analyzed because this is not common for exogenous antigens. However, recent studies have shown that CD8+ T cells can be activated by exogenous antigens which are able to reach intracellular compartments for loading onto class I molecules (29-31). Furthermore, the nature of the antigen presentation and what role HLA molecules play in this process have to be determined in detail. This is of interest because PG is not a classical T cell dependent protein antigen for which antigen processing and presentation has been studied extensively (32). Recent reports have shown that both CD4+ and CD8+ T cells can be stimulated by glycopeptides (33-35), as PG may yield after processing. Electron microscopy using the monoclonal antibody (Mab) 2E9 specific for PG-PS can be used to investigate the intracellular trafficking, processing and presentation of PG in APC and the epitopes involved in T cell activation (36). To further investigate epitopes involved in the activation of T cells by PG, T cell clones against PG need to be raised and their specificity and T cell receptor usage analyzed.

Importance of structure of peptidoglycan

From chapters 2.1 and 2.2 it is clear that the structure of PG is important for its ability to stimulate cytokines and T cell proliferation. PG from human spleen was 10-100 times more active than PG isolated from human feces in which most stem peptides were not bound to MurNAc. This suggests that the biological activity of PG is dependent on the peptides linked to the MurNAc molecule. This is consistent with an earlier study from our department in which it was shown that both lysozyme and most crucially NAMLAA degradation of PG in cell wall fragments of *Eubacterium aerofaciens* resulted in total inhibition of its capacity to induce arthritis (37). The persistence of PG with peptides still linked to MurNAc in human spleen tissue suggests that PG in spleen is able to escape NAMLAA degradation. This may be due to the fact that NAMLAA is not expressed in macrophage type APC in which intact PG can be found (16,19). In addition, processing of PG by the macrophage-like cell line RAW264 resulted in the detection of glycopeptides containing alanine suggesting that no cleavage of the stem peptide from muramic acid by NAMLAA had occurred (38).

Biological relevance of the presence of peptidoglycan in healthy tissues

As described above, our studies show that PG is present in human tissues and is still biologically active in healthy subjects (chapter 2). The question remains whether the presence of PG in human tissues have a biological role. Recent papers have advanced the hypothesis that continuous exposure to microbes is necessary to educate the immune system (39). This is supported by studies using germfree laboratory animals which have smaller lymphoid organs and reduced serum Ig levels (40). Furthermore, in these germfree animals, macrophage functions are impaired as the result of decreased chemotaxis and phagocytosis (41,42). Therefore, it has been hypothesized that the continuous encounters of an individual with the indigenous bacterial flora, including its PG, keep the immune system at a sufficient level of activation. The presence of bioactive PG in human spleen tissue may result in a continuous immune response keeping the immune system alert to react to other antigens. In addition, PG may have effects on the maturation of APC. It has been argued that continuous exposure to bacterial antigens is important in the regulation of T-helper 1 (Th1) versus T-helper 2 (Th2) CD4+ T cells (39). Therefore it has been hypothesized that modern vaccines and hygiene may disturb the balance between the activation of Th1 and Th2 cells, which may lead to allergy or autoimmune diseases (43,44), but more evidence for this hypothesis is needed. Studies in Fisher rats show that these animals are not susceptible to arthritis induction with cell wall fragments under conventional circumstances but do develop a severe arthritis after injection of bacterial cell wall fragments under germfree conditions (45,46). These studies suggest that T cell tolerance is at least partly generated and maintained by cross-talk of the immune system with the bacterial flora.

Peptidoglycan in rheumatoid arthritis

To investigate whether immune responses induced by PG are disturbed in RA patients, antibody levels specific for PG, and the PG-induced cytokine production and the T cell proliferation were compared between RA patients and healthy subjects (chapter 3).

Systemic antibody levels against PG

Several studies have reported that PG is able to induce a polyclonal antibody formation (24,47). Circulating IgM, IgG and IgA against PG-PS isolated from human feces could be detected in all healthy humans (17,48). In earlier studies focusing on antibodies against PG or PG-PS, increased levels were found in RA patients as compared to healthy controls (49). In contrast, two studies showed that decreased levels of PG-specific antibodies were present in RA patients and activation of B cells and plasma cells by PG was decreased in RA patients compared to healthy controls (50,51). In these studies PG was isolated from bacterial cultures. Because we hypothesize that PG derived from the normal bacterial flora is involved in RA, we considered it of more relevance to study antibody levels against PG derived from the mucosal sites. PG-PS isolated from human feces was mainly used in our studies because the amount of PG isolated from human spleen was limited (chapter 3.1). In this PG-PS fraction also some polysaccharide chains like rhamnose are present. Healthy females had significantly lower antibody levels compared to healthy males, and in females the amount of anti-PG-PS antibodies was inversely related with age. Although there is no evidence of causal linkage, it is of interest that the lower antibody levels against PG in females as compared to males correlate with the 2-3 fold higher general incidence of RA in females and that the lower antibody levels at higher age parallel the increasing incidence of RA with age (52,53). Furthermore, both females and males with RA had significantly lower levels of IgG anti-PG-PS compared to age and sex-matched controls. The same phenomenon was also observed in patients with early RA, i.e. with a diagnosed disease period of less than 12 months. Based on these results we hypothesize that anti-PG-PS antibodies regulate the translocation of bacterial products from the mucosa to non-mucosal sites.

Translocation of PG into non-mucosal sites

Translocation of bacterial products can occur via transport by M cells in the intestine or after intestinal injury. M cells, which are present in follicle-associated epithelium, are specialized epithelial cells that have few microvilli and abundant vesicles with few lysosomal enzymes (54-56). After transport by M-cells, the antigens are thought to be processed and presented by macrophages in the Peyer's patches resulting in stimulation of T and B cells. IgA produced during this process becomes dimerized and is transported into the gut lumen preventing further translocation of the bacteria or bacterial antigens (57,58). IgG produced locally in the mucosa but also systemically aids significantly in the protection against infections by contributing to immune exclusion or immune elimination (59). Therefore the lower levels of IgG antibodies specific for PG in the serum might also lead to increased translocation of PG to extraintestinal sites.

Another mechanism which might lead to increased distribution of PG into extraintestinal sites is intestinal injury. There are some indications that the intestinal permeability is increased in RA patients (60,61), but it cannot be excluded that this effect is due to the use of non-steroidal anti-inflammatory drugs (NSAID) (62,63). In addition, fasting periods followed by a vegetarian diet have been shown to have beneficial effects on the course of RA (64-66). Such diet results in changes in the fecal flora suggesting an involvement of the intestinal flora in RA.

The hypothesis that bacterial translocation might be involved in the pathogenesis of RA is

supported by the fact that bacterial overgrowth of the small bowel causes joint inflammation in humans (67-69) and in animal models (70). In these animal models inflammation could be inhibited by mutanolysin, which is a peptidoglycan degrading enzyme similar to lysozyme, suggesting that PG is involved in the inflammation (71,72). Using these bacterial overgrowth models, the importance of absorption of PG from the mucosal sites and the role of antibodies specific for PG herein can be further investigated. For example, the animals can be treated with other PG-degrading enzymes like lysozyme and NAMLAA and with monoclonal antibodies specific for PG like 2E9 or serum antibodies. Another approach is to increase the permeability of the intestine in animals susceptible to arthritis like Lewis rats and to determine whether these animals spontaneously develop arthritis or are protected by higher levels of serum antibodies specific for PG (73,74).

Antibodies against PG in synovial fluid

Because it has been shown that PG is located in the synovial tissues of RA patients, possibly due to decreased immune exclusion of PG, it is important to know whether PG present in human tissues is able to induce or contribute to inflammation. We showed that antibodies specific for PG were present in the synovial fluid of RA patients (chapter 3.1). Therefore antibodies against PG in synovial fluid may contribute to tissue damage in RA by forming immune complexes (75). The presence of these antibodies in synovial fluid can be explained by at least three mechanisms. First, the antibodies may be derived from the bloodstream by passive diffusion. Second, these antibodies may be captured in the joint as a result of local inflammation. With radiolabeled IgG scintigraphy, it has been demonstrated that IgG accumulates at the site of inflammation. This may be due to increased exudation of the radiolabeled IgG through the leaky capillary walls at the site of inflammation into the locally expanded extravascular space (76). Third, intra-articular production of the antibodies may occur. Because the level of IgA antibodies present in the synovial fluids correlates significantly with disease activity, we hypothesized that one or both of the latter two mechanisms occur. Up till now there is no indication that intraarticular production of antibodies against PG occurs because we were unable to detect plasma cells specific for PG in synovial tissues using labeled PG in immunohistochemistry, but this has to be further evaluated (I.A. Schrijver, unpublished data).

Inflammatory capacities of peptidoglycan in RA patients

In chapter 2.2 we show that T cell proliferation can be induced by PG, but PG-induced T cell responses of peripheral blood mononuclear cells (PBMC) did not differ between RA patients and healthy humans (chapter 3.2). Locally, in the inflamed joints, only limited proliferation of synovial fluid mononuclear cells was observed. This suggests that disturbances in specific T cell proliferation induced by PG do not play a prominent role in the pathogenesis of RA. However, T cell activation by PG can still contribute to the inflammatory process as PG-containing APC, which are abundantly present in synovial tissues of RA patients and not in healthy subjects, may stimulate proliferation of T cells locally in the synovial tissues. Other important mediators in the inflammatory events in RA are cytokines and matrix metalloproteinases (4-6). In chapter 3.2 we show that PG is able to induce IL-1β, IL-6, IL-8, IL-10, IL-12, TNF-α and the metalloproteinase gelatinase B in blood cells and synovial

fluid cells. These cytokines are most likely produced by monocytes or macrophages because a purified monocyte fraction also produced IL-1β, IL-6 and TNF-α (unpublished results). In addition, the cytokines were induced during a short incubation period (4 –17 hours) and production of cytokines by T cells requires more time for activation of the T cells by APC. Therefore, production of cytokines by T cells would require a longer culture period. IL-4 could not be detected in the PG stimulated samples, but IFN-γ was detected after 3 days of culture (unpublished results).

We hypothesize that the cytokines present in peripheral blood and synovial fluid of RA patients are at least in part induced by PG. The induction of these cytokines by PG might result in activation of other cells leading to destruction of joint and cartilage tissues. *In vivo,* the production of cytokines may be different between RA patients and healthy subjects, dependent on the localization of PG and on the nature and amounts of cytokines induced by PG.

There are several examples of polymorphisms occurring within cytokine genes, some of which are associated with disease susceptibility or clinical course (77-80). Polymorphisms within cytokine genes have also been described for arthritic disorders (79,81,82). Whether the induction of cytokine production by PG is dependent on particular cytokine polymorphisms has to be evaluated. These studies have to be performed under strict conditions because cytokine production is highly dependent on the culture conditions and circumstances and time point the blood sample is taken (83).

PG present in RA synovial tissues promotes inflammation

To investigate whether PG is also able to activate the immune system *in vivo*, *in situ* analysis of PG-containing cells was performed on human tissues. For this purpose PG-containing cells were characterized using immunological markers. Because detection of these markers, e.g. cytokines and costimulatory molecules, is difficult in paraffin-embedded tissues, cryosections had to be used. The disadvantage of the use of cryosections is their relatively poor morphology. In chapter 4.1 we show that the mild fixative pararosaniline is an alternative for acetone fixation because it preserves antigenicity of the tissues and strongly improves the morphology.

To study PG localization and function *in situ* we used an antibody raised against PG isolated from human feces. The specificity of this Mab (2E9) has been extensively investigated and confirmed. First, inhibition studies using ELISA showed that 2E9 recognizes cell wall fragments, muramyl dipeptide and lysozyme and NAMLAA-solubilized cell walls of different gram-positive bacteria. These experiments suggest that 2E9 binds to the glycan backbone of PG since the presence of muramic acid is necessary for recognition by 2E9. Secondly, spleen sections from germfree rats and from neonatal rats in which the gut flora has not been established yet display no PG-containing phagocytes, whereas spleens of adult rats do. In addition, intravenous injection of PG into neonatal rats results in binding of 2E9 to phagocytes in spleen sections (15). Thirdly, immunoelectron microscopy shows that Mab 2E9 binds to cell walls of *Staphylococcus epidermidis* surviving intracellularly in macrophages after an experimental *in vivo* infection (36). Fourthly, 2E9 displays no detectable reactivity against more than ten thousand random dodecapeptides and eight thousand tripeptides in Pepscan approaches (R.J. Slootstra R.H., Meloen R.H, unpublished data), confirming that

this Mab has very low cross-reactivity with non-PG epitopes (84).
PG-containing cells are present in synovial tissues of RA and osteoarthritis (OA) patients (chapter 4.2) (16). In both RA and OA synovia the PG-containing cells were mainly macrophages (CD68+, CD14+) and only a minority of the cells were dendritic cells (CD83+). PG could not be detected in granulocytes, which in principle are able to bind PG by the CD14 receptor. This may be explained by the fact that granulocytes contain NAMLAA and are therefore able to degrade PG, while monocytes do not, leading to persistence of PG in these cells (19). Although PG is present in both RA and OA synovia, the cells containing PG have a higher expression of more activated phenotype in RA as compared to OA. This was shown by the expression of CD80, CD86 and CD40, costimulatory molecules critical to the activation of T cells (85-87), and the expression of different cytokines, in particular TNF-α and IL-6, which are both involved in RA (4). These data suggest that PG-containing cells are able to interact with T cells and to induce cytokines not only *in vitro* but also in the synovial tissues of RA patients. The presence of PG-containing cells in both RA and OA synovial tissues shows that the presence of PG is not restricted to the synovial tissues of RA patients. Because PG-containing cells circulate in the bloodstream they can reach all tissues (11,12). As mucosal leukocytes can preferentially home to synovia (88,89), this may enhance the distribution of PG-containing cells to synovia. T cells activated in the mucosal sites may also home to these tissues, which might explain why some people suffering from Crohn's disease and ulcerative colitis may also experience attacks of joint inflammation (90). In these diseases it is assumed that the intestinal flora plays an important role as specific T cell responses towards bacterial antigens of the resident flora occur and disease flare-ups are enhanced by increased gut permeability (91-93).

Peptidoglycan in multiple sclerosis
Because it has been shown that PG is present in different tissues, even in healthy subjects, it can be hypothesized that PG may be involved in other chronic inflammatory diseases besides RA. Therefore we studied whether PG also occurs in the inflammatory lesions in the brains of MS patients. The brain tissue is a special site for the immune system because it is protected by the blood-brain barrier. Therefore the presence of PG in brain tissue with intact blood-brain barrier seems unlikely. Up till now there is little evidence that bacterial antigens are involved in the pathogenesis of MS, but a few studies report the capability of PG to induce inflammation in brain tissues of animal models (94-96). There are also some indications that patients with Crohn's disease can have brain lesions similar to MS (97,98). Chapter 4.3 shows that PG-containing cells can be detected in MS brain tissue in significantly higher numbers than in control brain tissue. Interestingly, low numbers of PG-containing cells were also observed in brain from patients who died from non-neurological diseases. This illustrates that the protection of the brain tissue from non-brain cells is not as strict as has been thought previously. Recent papers have shown that after brain contusion massive cellular infiltrates including macrophages and dendritic cells can be found in the brain tissue (99). Therefore it can be hypothesized that minor brain damage can also cause infiltration of leukocytes, among which PG-containing cells.
The PG-containing cells present in the brain tissue of MS patients possess an activated phenotype as shown by the expression of CD40, CD80 and CD86 and the expression of TNF-α,

IL-1, IL-6, IL-12 and IL-10. The expression of HLA-DR molecules by PG-containing cells was low. This may be due to the fact that, in general, HLA-DR expression in brain tissue is low, presumably to prevent T cell responses and inflammation.
In MS brains, PG may contribute to the inflammation by the induction of intrathecal specific antibody production. In 60% of the patients, antibodies and plasma cells specific for PG could be detected in both the cerebrospinal fluid and the brain tissue. The presence of antibodies in the cerebrospinal fluid may be partly due to leakage from the blood but the presence of plasma cells specific for PG within the brain tissue shows that antibodies specific for PG are also produced intrathecally. The presence of plasma cells specific for PG in MS confirms that PG induces specific immunological activity and suggests that antibodies against PG may be involved in the local pathology. The existence of antibodies against PG in the cerebrospinal fluid identifies a novel specificity of the immunoglobulins at this site and may partly be responsible for the occurrence of oligoclonal immunoglobulin bands in the cerebrospinal fluids of MS patients (100). To confirm this, additional experiments have to be performed using blotting techniques.
Induction of experimental autoimmune encephalomyelitis (EAE) by PG will provide further evidence for the involvement of PG in MS. In ongoing experiments, PG has been detected in the central nervous system of mouse and marmoset EAE. Oral administration of PG may exacerbate EAE in these models (101,102) as has also been shown with LPS in collagen induced arthritis (103).

A role of PG in "autoimmunity"?

The studies described in this thesis show that PG is present in different tissues undergoing chronic inflammation of presumably autoimmune origin, and that PG is able to contribute to immune reactivity. The question that remains is how PG contributes to the development and pathogenesis of RA and MS. In other words, is PG able to promote autoimmunity in susceptible individuals? Both RA and MS are considered to be CD4+ T cell mediated diseases of the Th1 type (104,105). This means that T cells play a crucial role in the initiation and perpetuation of the disease. It is believed that these T cells react against autoantigens as a result of the lack of self-tolerance. However, in recent years it has become apparent that self-tolerance is not complete, not even in healthy humans (106). Many healthy individuals have both T-cell mediated and antibody-mediated reactivity to a number of self-proteins like major basic protein (MBP) or cartilage antigens (107,108). In most individuals regulatory T cells and possibly regulatory APC keep this self-reactivity under control (109). These regulatory T cells are able to inhibit responses in a non-antigen specific way which is mediated by the production of IL-10 and TGF-β (110). These cells probably induce an equilibrium of proinflammatory and inhibitory activities. When this balance becomes disturbed, development of (auto)immunity may be the result.
We propose that PG may promote (auto)immunity by one of the following mechanisms.

PG specific T cell responses

First, T cell responses against PG present in human tissues may result in tissue damage. The studies in this thesis show that PG is able to induce activation of T cells but the specificity of these T cells has to be further evaluated. The proliferation of T cells against PG in the joints

of RA patients was very low. This suggests that at least in an advanced stage of RA this mechanism of PG induced autoimmunity in the affected organ itself is unlikely. Whether PG can stimulate T cells in the brain in MS or EAE has to be investigated.

Molecular mimicry

The second mechanism by which PG may induce autoimmunity is molecular mimicry. It has been hypothesized that infectious agents are able to elicit T cell or antibody responses against epitopes which are more or less similar to epitopes present in normal tissues (111,112). These T cells or antibodies can subsequently cause damage to the individual's tissues. Because PG is able to induce both T cell and antibody responses, this phenomenon can occur at both levels. Thus far, there is no evidence supporting this hypothesis. For example, the antibody 2E9 which recognizes PG does not cross-react with cartilage and a panel of 18,000 different peptides (84) (J.W. Slootstra, R.H. Meloen, personal communication), suggesting that for the 2E9 epitope of PG, molecular mimicry with peptides does not readily occur. Also other *in vivo* studies do not support the hypothesis that cross-reactivity between bacterial epitopes and cartilage is the underlying mechanism in RA (113,114).

Danger model

Third, PG may fit in the 'Danger model' postulated by Matzinger (115,116). The described mechanisms for autoimmune diseases are all based on the self-non-self-model of the immune system. The 'Danger model' suggests that immune responses are initiated by a 'danger' signal. Danger signals induce T cell activation irrespective of whether the antigen is self or non-self. Danger signals are induced upon injury of cells and may activate neighboring APC by induction of costimulatory molecule expression by these cells. PG therefore may act as a danger signal by inducing cytokines, which lead to tissue destruction and APC activation. According to Matzinger (auto)immunity might be due to an ongoing infection in the target organ. The effector cells clear the pathogen but they also damage the target organ in the process. Because PG is always abundantly present at the mucosal sites and circulates through the bloodstream in leukocytes (12,13), this may reflect a continuous exposure to an infectious antigen. Although PG fits into this model it is difficult to explain why PG present in normal spleen and liver does not cause T cell activation and tissue damage.

Breaking of tolerance for autoantigens

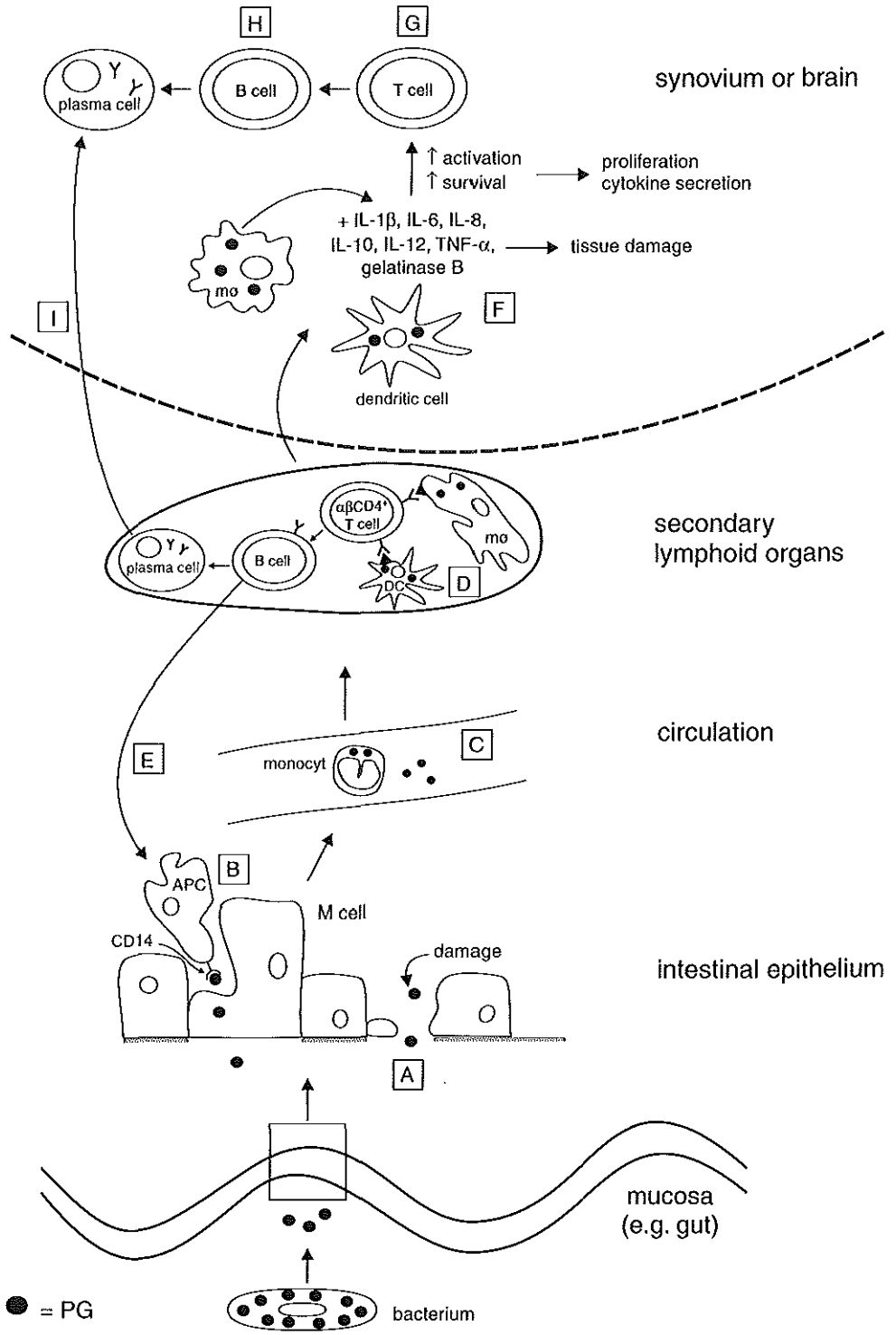
A fourth mechanism by which PG might induce (auto)immunity is a variant of the danger model. Infectious agents like viruses or bacteria, but also bacterial cell wall fragments like LPS, have been shown to break tolerance to certain autoantigens (117,118). This means that autoreactive T cells need an additional stimulus such as LPS or PG to become activated. The breaking of tolerance to certain autoantigens can occur by different mechanisms. First, when the infectious agents cause damage of the tissue, exposure of usually 'cryptic' antigens may be increased. T cells might recognize these antigens as non-self and proliferate and react to these antigens. Secondly, an additional stimulus such as LPS is able to activate autoreactive T cells leading to pathology. This mechanism probably depends on the LPS-induced production of particular cytokines. These cytokines activate T cells but are also able to promote the survival of the autoreactive T cells by inhibiting apoptosis (119,120).

In animal models it has been shown that inflammatory reactions caused by bacterial infections or LPS lead to the activation of autoreactive CD4+ cells (117) or CD8+ cells (118) which otherwise go into apoptosis. This mechanism is supported by the finding that LPS induces EAE in mice injected with MBP-specific T cells through induction of IL-12 (121). Therefore, the presence and persistence of PG in brain tissue may promote loss of tolerance against autoantigens present in the brain like MBP, proteolipid protein (PLP) and others. Similarly, in RA autoreactive T cells against collagen (122,123), HC-gp39 (124) and other joint antigens may be activated by PG.
Whether PG is involved in the development of RA and MS by breaking tolerance to certain autoantigens has to be further evaluated. This can be performed in T-cell receptor (TCR) transgenic mice with autoreactive T cells (125,126). TCR-transgenic mice expressing a TCR specific for MBP spontaneously develop EAE dependent on the environment (127). The role of PG in this process can be analyzed by injecting PG into these mice. Furthermore, it can be hypothesized that in germfree animals EAE will not spontaneously develop due to lack of additional stimuli. When administration of PG to such animals leads to EAE induction this would provide further evidence for the role of PG in EAE.

Contribution of PG to RA and MS is dependent on different factors
The studies of this thesis show that PG at least in part contributes to the inflammation in RA and MS. To what extent PG damages the host depends on different predisposing factors. First, PG has to gain access to the synovium or central nervous system. As shown in this thesis, synovia of RA and brains of MS patients contain more PG-containing cells than the same tissues of controls. The number of these cells will depend on the accessibility of the synovium and the permeability of the blood-brain barrier. Furthermore, recurrent infections, intestinal permeability, enzymatic activity of PG degrading enzymes and the composition of the intestinal flora will influence the amount of circulating PG. Specific antibodies against PG may help retain the PG inside the mucosal tissues (chapter 3.1). Once present in the synovium and central nervous system, immune reactivity of PG will depend on genetic factors like certain cytokine polymorphisms and the ability of a person to react to self-antigens. The latter will mainly depend on HLA-restriction. In RA it has been shown that HLA-DR4 molecules are potent binders of cartilage peptides (128). Therefore HLA-DR4 positive individuals probably have higher frequencies of potentially autoreactive T cells or autoreactive T cells with higher avidity to the relevant antigenic peptides. These suggestions imply that the presence of PG in tissues is not sufficient to promote chronic inflammation but that this is also dependent on other environmental factors and on genetic factors.

Peptidoglycan promotes inflammation in RA and MS: a hypothesis
The studies in this thesis show that PG might be one of the external factors in the development and perpetuation of RA and MS. In what way PG can be involved in the pathogenesis of RA and MS is summarized by the following hypothesis (figure).
PG, which is abundantly present in the bacterial flora at all mucosal sites, is able to pass the mucosal epithelium either via M cells or by intestinal injury (figure A)(70). Whether released PG can pass the mucosal epithelium or translocates with the whole bacterium is not known. Macrophages or dendritic cells lying adjacent to the M cells may ingest the whole

Peptidoglycan promotes inflammation in RA and MS: A hypothesis (explained in text)



bacteria, or PG using the CD14 receptor (B). Transport of PG to the extraintestinal sites can occur by two mechanisms: by monocytes in the bloodstream or by free circulating PG (C). The presence of PG in leukocytes has been confirmed by the detection of muramic acid in these cells (12,13) but up till now no evidence has been postulated that free PG is circulating in the blood. The leukocytes travel through the body and pass the lymph nodes and the spleen. In these secondary lymphoid organs immune responses against PG may take place (D). It has been shown that PG is able to induce proliferation of mainly αβCD4+ T cells. This is dependent on interaction with CD14 and processing and presentation of the antigen by HLA. Furthermore, B cells may be activated as IgM, IgG and IgA antibodies specific for PG can be found in the serum of healthy donors. These immune responses can also occur in the Peyer's patches (E). When the level of serum IgG specific for PG decreases, extraintestinal sites may be increasingly exposed to PG. When PG-containing cells (macrophages or dendritic cells) lacking NAMLAA that renders PG less inflammatory are trapped in tissues like synovial tissue and brain, PG can induce the production of cytokines and gelatinase B (F).

In this thesis we show that macrophages exposed to PG *in vitro* are able to produce IL-1β, IL-6, IL-8, IL-10, IL-12, TNF-α and gelatinase B. Whether PG is also able to induce cytokine production by dendritic cells has to be further evaluated. The production of these inflammatory mediators can have two different effects: first, direct tissue damage can occur, also resulting in an increased expression of cryptic antigens that activate T cells. Second, the production of cytokines activates and influences the survival of autoreactive T cells (119,120). In RA these autoreactive T cells are specific for autoantigens like heat shock proteins (129), collagen (122,123) and HC-gp39 (124,128), explaining the existence of T cell reactivity against these antigens in RA joints (G). In MS, the T cell tolerance for autoantigens such as MBP, MOG and PLP (107,108) can be broken. In addition, B cells may be activated locally inside the brain or synovial tissue. In the latter, germinal center-like structures have been found indicating the local formation of memory B cells (130,131) (H). In addition, plasma cells specific for PG can be redistributed to these tissues (I). The production of cytokines, matrix metalloproteinases, and antibodies by the various types of activated cells will lead to damage of cartilage and bone in RA and myelin of the central nervous system in MS as shown in figures 3 and 4 of chapter 1, the general introduction.

In this hypothesis, PG contributes to inflammation by first activating macrophages, which subsequently leads to the activation of T cells and /or B cells. Whether the development of RA in animal models by PG is dependent on all these cell types can be analyzed in different knockout mice. Recently it has been shown that arthritis can be induced by collagen in DBA/1 mice lacking mature T and B cells, suggesting an important role of macrophages and dendritic cells or resident cells of the synovium (132). Whether initiating events like joint injury, synovial inflammation or breakdown of the blood-brain barrier have to occur before PG can reach the synovial tissues or brain tissue has yet to be established. This can be investigated by monitoring the presence of PG-containing cells and the biologic activity of PG during the initiation and perpetuation of the inflammation. Because clinical signs of RA and MS usually become evident only long after the initiation of the disease process, this cannot be studied in humans. Therefore, animal models have to be used. The most

appropriate arthritis model for this purpose is arthritis induced in rodents by bacterial cell wall fragments. The presence of PG in the brain tissues can be monitored in both mouse and marmoset monkey EAE. Other models like collagen induced arthritis (133) or spontaneous models for arthritis like TNF-α transgenic mice (134,135) are suitable models for the determination of the role of PG derived from the mucosal sites. To analyze the trafficking and immune reactivity of PG the use of germfree animals is recommended because PG injected into these animals can be monitored as these animals do not have PG from the mucosal flora.

Concluding remarks

This thesis shows that PG present in healthy and chronically inflamed tissues is able to promote immune reactivity. We show that PG present in sterile spleen tissue has potent effects on T cells, B cells and macrophages, including the induction of proliferation, synthesizing antibodies and producing cytokines and matrix metalloproteinases. Furthermore, we show that PG is present in higher numbers of APC and with a more activated phenotype in inflamed tissues compared to non-inflamed tissues. A unique finding reported in this thesis is the presence of PG in the brain tissues, which is usually considered to be protected by the blood-brain-barrier. These bacterial antigens may promote immune reactivity to MBP and other relevant antigens in the brains of MS patients.

These results, together with previous studies from our group showing that PG isolated from bacteria from the intestinal flora or ileostoma feces induces arthritis in a rat model, support the hypothesis that PG plays a central role in the pathogenesis of chronic inflammatory diseases. Crucial questions in this hypothesis are whether PG is the initiating agent in the development of the disease or only enhances the disease, whether PG is able to induce MS like disease in animal models and whether PG is able to break tolerance to certain autoantigens. If additional research provides further evidence that PG plays an important role in the pathogenesis of (auto)immune diseases, intervention studies should be envisaged. Intervention in PG-induced autoimmunity may be performed by restricting PG to the musocal sites by an appropriate diet, by administration of NAMLAA, antibodies specific for PG, or antibiotics which inhibit PG synthesis.

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SUMMARY

Rheumatoid arthritis (RA) and multiple sclerosis (MS) are both chronic inflammatory diseases of unknown origin. CD4+ T cells are believed to play an important role, but antigen presenting cells (APC) also contribute significantly to the pathogenesis of both diseases. This is mainly due to the production of cytokines and matrix metalloproteinases. (Auto)antigens responsible for the activation of CD4+ T cells and/or macrophages are still not unequivocally identified.
Peptidoglycan (PG), a major cell wall component of gram-positive bacteria, which is abundantly present in all mucosa, is a candidate antigen. PG is composed of long sugar chains of alternating N-acetyl glucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc) residues, which are interlinked by peptide bridges, resulting in a large complex macromolecular structure. Because of its capability to induce production of proinflammatory cytokines, to induce arthritis in rodents and its presence in APC in RA joints it is believed to play a role in the pathogenesis of RA.

In the present thesis we investigated whether PG is involved in the pathogenesis of RA and MS. *In vitro*, PG isolated from conventional bacterial cultures can induce secretion of pro-inflammatory cytokines by human monocytes, indicating that PG may be involved in immune responses against infections by gram-positive bacteria. To investigate the biological activity of PG present in human tissues, we developed an improved method to isolate PG present in APC from sterile human spleen tissue. Biochemical analysis demonstrated that PG isolated from human spleen was largely intact. Human whole blood cells were able to produce the proinflammatory cytokines TNF-α, IL-1 and IL-6 after stimulation with PG isolated from human spleen (chapter 2.1). We also investigated whether PG isolated from human. Furthermore, PG isolated from human spleen is able to elicit a proliferative response of CD4+ and CD8+ T cells after binding to CD14 on monocytes, followed by intracellular processing and presentation mediated by HLA-class II and I (chapter 2.2).

Disturbances in the immune response against PG may be related to the pathogenesis of RA. Therefore we analyzed antibody levels against PG and induction of cytokine production and T cell proliferation by PG in RA patients and healthy controls. A reduced systemic IgG response against PG derived from the gut flora was associated with RA, suggesting an antibody-mediated protection against the spreading of PG over non-mucosal sites (chapter 3.1). T cell proliferation and cytokine production induced by PG did not differ between RA patients and healthy controls, but PG was able to induce cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12, IFN-γ, TNF-α and the matrix metalloproteinase, gelatinase B (MMP-9), all associated with RA (chapter 3.2).

To determine whether these *in vitro* activities of PG are also operational *in vivo*, immunohistochemical studies were performed. To detect unstable immunological markers, fixation

agents have to be used which often result in suboptimal morphology. Fixation with pararosaniline resulted in better morphology of all tissues and inhibited endogenous alkaline phosphatase activity in brain tissue while maintaining antigenicity (chapter 4.1). PG could be detected in APC in RA joints but little is known about the function of these cells. In chapter 4.2 it is shown that PG is present in both macrophages and dendritic cells and that these cells coexpress HLA-DR, CD40, CD80 and CD86, suggesting that these cells are immunocompetent APC. Furthermore PG containing cells coexpressed cytokines such as IL-6, IL-10 and TNF-α.

Because it can be hypothesized that PG also plays a role in other chronic inflammatory diseases than RA, we determined the presence of PG in MS (chapter 4.3). We showed that PG-containing cells are present in APC in MS brain tissues. Similar to RA, these cells coexpressed costimulatory molecules and cytokines. In addition, the presence of plasma cells specific for PG in MS brain and anti-PG antibodies in cerebrospinal fluid from MS patients suggests that PG is able to induce antibody production intrathecally in MS.

These data suggest that PG may translocate from the mucosa to the bloodstream. This may be dependent to little enzymatic degradation of PG, increased intestinal permeability or relatively low antibody levels against PG. After translocation, PG is distributed to non-mucosal tissues carried by leukocytes in the bloodstream. When PG is captured in synovial or brain tissues, production of cytokines and metalloproteinases may lead to damage of the tissues but also to the breakdown of tolerance to certain autoantigens. Therefore PG may promote inflammation in RA and MS.

Our studies identified PG as a bacterial modulator of inflammation (cytokines, matrix metalloproteinases), but also of specific adaptive immunity (T cell proliferation, anti-PG antibodies). The presence of PG in APC coexpressing different activation markers in inflamed tissues of RA and MS patients suggests that PG may play a role in these diseases. The precise mechanisms of action and whether PG is involved in the initiation or perpetuation of the disease should be further elucidated. When the role of PG is further established, interfering with access of PG to non-mucosal sites may be a novel approach to anti-inflammatory therapy.

SAMENVATTING VOOR NIET-INGEWIJDEN

Het immuunsysteem beschermt het lichaam tegen vreemde indringers zoals bacteriën en virussen. Cruciaal hierin is het samenspel van drie verschillende soorten cellen, de antigeen-presenterende cellen, de T cellen en de B cellen. Antigeen presenterende cellen zijn in staat om vreemde indringers (antigenen) 'op te eten' en deeltjes hiervan op hun oppervlak te brengen. De T cellen kunnen deze deeltjes herkennen waardoor ze geactiveerd worden. Deze geactiveerde T cellen kunnen dan de geïnfecteerde cel vernietigen of activeren zodat het schadelijke antigeen opgeruimd wordt. Daarnaast kunnen T cellen ook B cellen activeren om uit te rijpen tot plasmacellen die antistoffen (eiwitten) maken die de vreemde antigenen kunnen binden en onschadelijk maken. In sommige gevallen raakt deze immuunrespons uit balans en richt het immuunsysteem zich niet alleen tegen bacteriën en virussen, maar tegen structuren van het lichaam zelf. De ziekten die hierdoor ontstaan worden auto-immuunziekten genoemd. Voorbeelden zijn reumatoïde artritis (RA), beter bekend als reuma, en multiple sclerose (MS). RA en MS worden gekenmerkt door chronische ontstekingen in respectievelijk de gewrichten en het centrale zenuwstelsel (ruggenmerg en de hersenen). Echter, de precieze oorzaak van beide ziekten is nog steeds onbekend. Acute vormen van artritis, zoals reactieve artritis, worden veroorzaakt door bacteriële infecties van onder andere de darmen en urinewegen. Omdat RA een chronische ziekte is, wordt verondersteld dat bacteriën die zich permanent op de slijmvliezen van de darm en luchtwegen bevinden, een rol kunnen spelen in het in stand houden van de ontstekingen.
Een belangrijk bestanddeel van bacteriën is peptidoglycaan (PG). PG bevindt zich in de celwand van bacteriën en is in staat om een ontstekingsreactie op gang te brengen. Dit wordt onder andere veroorzaakt door het aanzetten van de productie van cytokinen door antigeen presenterende cellen. Cytokinen zijn oplosbare signaaleiwitten die belangrijk zijn in ontstekingen. Omdat PG aangetoond kan worden in gewrichten van RA patiënten en artritis kan veroorzaken in ratten wordt dit antigeen in verband gebracht met RA.

Het doel van het onderzoek dat beschreven is in dit proefschrift, is om te onderzoeken of PG een rol zou kunnen spelen in chronische ontstekingen, zoals RA. Daarvoor is het belangrijk om vast te stellen of het PG zoals het zich bevindt in weefsels in het lichaam, nog steeds biologisch actief is en niet onschadelijk is gemaakt door enzymen. In hoofdstuk 2.1 is een methode beschreven om PG te isoleren uit menselijk weefsel. Dat het PG uit menselijk weefsel nog steeds actief is blijkt uit het feit dat het in staat is om de productie van ontstekingsbevorderende cytokinen TNF-α, IL-1 en IL-6 te stimuleren (hoofdstuk 2.1) en T cellen kan activeren. (hoofdstuk 2.2).

De bijdrage van PG aan RA zou veroorzaakt kunnen worden door een gestoorde immune reaktie tegen PG. Dit is onderzocht in hoofdstuk 3. Uit hoofdstuk 3.1 blijkt dat RA patiënten een verlaagde hoeveelheid antistoffen gericht tegen PG in hun bloed hebben. Dit zou erop kunnen duiden dat RA patiënten een verlaagde bescherming hebben tegen de verspreiding van PG vanuit de slijmvliezen. De stimulatie van cytokine productie en T cel activatie door

PG is niet verschillend tussen RA patiënten en gezonde controles (hoofdstuk 3.2) maar uit dit hoofdstuk kwam wel naar voren dat PG in staat is om de productie van belangrijke cytokinen in het ontstaan van RA te stimuleren.

De experimenten in deze studies zijn allemaal '*in vitro*' experimenten. Dit wil zeggen dat we het effect van PG bestudeerd hebben buiten de situatie in het lichaam om. Om de rol van PG in het ontstaan van chronische ontstekingen ook '*in vivo*' te bestuderen, zijn immunohistochemische technieken uitgevoerd op menselijk weefsel (hoofdstuk 4). Met immunohistochemie worden in plakjes weefsel, in dit geval synovia uit de gewrichten van RA patiënten, cellen aangetoond met behulp van specifieke antistoffen. Belangrijk bij deze methode is dat de cellen goed gefixeerd blijven en dat de vorm en structuur van deze cellen niet verandert. In hoofdstuk 4.1 wordt een fixatie methode beschreven waarbij dit het geval is. In hoofdstuk 4.2 is onderzocht in wat voor cellen PG zich bevindt in synovia van RA patiënten. Hieruit blijkt dat PG zich voornamelijk bevindt in antigeen presenterende cellen. Dat deze cellen geactiveerd zijn, blijkt uit de aanwezigheid van moleculen op het oppervlak van deze cellen die betrokken zijn bij de interactie tussen T en B cellen, en de expressie van cytokinen door de PG-bevattende cellen. Dit betekent dat deze cellen wellicht aktief betrokken zijn bij de ontstekingsreactie in de gewrichten.

Omdat PG in verschillende weefsels gedetecteerd kan worden, is het mogelijk dat PG ook in andere chronische ontstekingen dan RA een rol speelt. Daarom is een studie uitgevoerd waarbij in hersenweefsel is onderzocht of daar PG aanwezig is (hoofdstuk 4.3). De hersenen nemen voor het immuunsysteem een bijzondere plaats in omdat de immuunreakties daar zoveel mogelijk onderdrukt worden om weefselschade te voorkomen. Daarom is er een zogenaamde bloed-hersen-barrière die de toegang van vreemde antigenen en cellen in de hersenen zoveel mogelijk voorkomt. In dit proefschrift is voor het eerst aangetoond dat in hersenweefsel van MS patiënten antigeen presenterende cellen aanwezig zijn die PG bevatten. Net als in RA zijn deze cellen geactiveerd. Verder zijn PG-specifieke plasmacellen en antistoffen tegen PG aangetoond in de hersenen van MS patiënten. Dit betekent dat bij MS PG immunologisch actief is in de hersenen.

Gehypothetiseerd kan worden dat verspreiding van PG vanuit de slijmvliezen kan leiden tot de aanwezigheid van PG in andere weefsels. In deze weefsels, zoals synovia en hersenen, zou dit kunnen leiden tot ontstekingsreacties als gevolg van de productie van cytokinen. Dit kan er onder meer toe leiden dat T cellen het lichaam zelf aanvallen, wat in RA leidt tot schade in de gewrichten en in MS tot schade aan het centraal zenuwstelsel. De aanwezigheid van PG zou dus een rol kunnen spelen in chronische ontstekingen zoals RA en MS.

Kort samengevat, PG is aanwezig in organen van waar chronische ontstekingen plaats vinden, en heeft de eigenschappen om zulke ontstekingen te induceren en/of in stand te houden. Of de rol van PG cruciaal is in de ontwikkeling van chronische ontstekingen zoals RA en MS, of dat het alleen een bijdrage levert aan zulke processen, dient onderzocht te worden met behulp van patiëntenmateriaal en in proefdiermodellen die de ziekte nabootsen.

ABBREVIATIONS

AEC	3-amino-9-ethylcarbazole
APC	Antigen presenting cells
AP	Alkaline phosphatase
AV-PO	Avidin peroxidase
BCG	Bacillus Calmette-Guérin
BPRC	Biomedical Primate Research Center
CNS	Central nervous system
CSF	Cerebrospinal fluid
CII	Type II collagen
DAS	Disease activity score
DMF	N,N-dimethylformamide
DTH	Delayed type hypersensitivity response
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent-assay
ENA	Epithelial cell derived neutrophil activating factor
ESR	Erythrocyte Sedimentation Rate
FBB	Fast Blue Base
FPLC	Fast performance liquid chromatography
GlcNAc	N-acetyl glucosamine
GM-CSF	Granulocyte-colony-stimulating factor
GPI	Glycosylphosphatidylinositol
GRO	Growth regulated oncogen
HC-gp39	Human cartilage glycoprotein 39.
HHV-6	Human herpes virus 6
HPLC	High performance liquid chromatography
HoaM-bio	Horse-anti-mouse-biotin
HRP	Horseradish peroxidase activity
HSP	Heat shock proteins.
IGIF	Interferon gamma inducing factor
IL	Interleukin
IFN	Interferon
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
MAbs	Monoclonal antibodies
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
M-CSF	Macrophage colony stimulating factor
MCP	Monocyte chemotactic protein

MDP	Muramyl dipeptide
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
MurNAc	N-acetyl muramic acid
NO	Nitric oxide
NAMLAA	N-acetylmuramyl-L-alanine amidase
NSAID	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
OD	Optical density
PBMC	Peripheral blood mononuclear cells
PDGF	Platelet-derived growth factor
PG	Peptidoglycan
PG-PS	Peptidoglycan-polysaccharide
PLP	Proteolipid protein
RA	Rheumatoid arthritis
RaRa-bio	Rabbit-anti-Rat-biotin
RANTES	Regulated upon Activation, Normal T cell Expressed, and presumably Secreted
RF	Rheumatoid factor
ROS	Reactive oxygen species
RP	Red pulp
SAV-PO	Streptavidin-peroxidase
SD	Standard deviation
SI	Stimulation index
S-IgA, S-IgM	Secretory IgA, Secretory IgM,
SF	Synovial fluid
SFMC	Synovial fluid mononuclear cells
TAL	Transaldolase
TCR	T-cell receptor
TGF-	Transforming growth factor
Th1	T helper 1
Th2	T helper 2
TT	Tetanus toxoid
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TNP	2,4,6-trinitrophenyl
WP	White pulp

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Cursussen

Stralingscursus 4B, Leiden
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Oxford examination in English as a foreign language, Rotterdam
Introductory course of the Postgraduate School Pathophysiology of growth & differentiation, Rotterdam/Leiden
Advanced course on Immunology, Rotterdam
Advanced course on growth and differentiation of the lympho-hematopoietic system, Rotterdam /Leiden
Advanced course on Oncogenesis and Tumorbiology, Leiden

Onderwijs activiteiten

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PUBLICATIONS

Van Langevelde P, Van Dissel JT, Ravensbergen E, Appelmelk BJ, Schrijver IA, Groeneveld PH (1998) Antibiotic-induced release of lipoteichoic acid and peptidoglycan from *Staphylococcus aureus*: quantitative measurements and biological reactivities.
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