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Effect of cyclosporin A on immunoglobulin class in patients receiving blood transfusions

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Effect of cyclosporin A on immunoglobulin class in patients receiving blood transfusions. IgG antibodies detected by flow cytometry in sera from potential renal transplant recipients are associated with an increased number of rejection episodes and impaired graft function. Furthermore, cytotoxic antibodies may develop if pre-transplant blood transfusions are given to such patients. We have investigated the effect of cyclosporin A on the development of IgM and IgG antibodies detected by flow cytometry after blood transfusions in 16 previously untransfused dialysis patients. Eight (group 1) received three to five third-party blood transfusions at two weekly intervals and the remaining eight (group 2) received transfusions with concomitant cyclosporin A therapy (10 mg/kg/day). Sera obtained after each transfusion were tested using flow cytometry against lymphocytes from six normal donors. In all 462 serum/cell combinations were tested. Sera from six out of eight patients in group 1 showed IgG antibody activity following blood transfusions and none developed IgM antibodies alone. In contrast IgG antibody activity was detected in one serum sample from only one of the eight patients in group 2 ($P < 0.02$); a further three patients developed IgM but not IgG antibody activity during the transfusion protocol. IgG antibodies were found in 25/228 serum/cell combinations in group 1 but in 1/234 in group 2 ($P < 0.001$). The patient in group 2 who developed IgG antibodies in one serum/cell combination was known to have red cell autoantibodies. This IgG activity was removed by red cell absorption, suggesting that the autoreactive red cell antibody cross-reacted with lymphocytes. Cytotoxic antibodies were detected in two serum/cell combinations in group 1 alone. We conclude that the switch from IgM to IgG antibody production is abrogated by cyclosporin A. This may be one mechanism by which cyclosporin A prevents the development of cytotoxic antibodies in potential graft recipients given third-party blood transfusions.

Since Opelz et al demonstrated the beneficial effect of blood transfusions given before transplantation on renal graft survival in 1973 [1], the administration of transfusions to potential graft recipients has become routine in many transplantation centers. More recently, however, the place of elective blood transfusions has become more controversial [2–5], but a recent large study [6] shows a beneficial effect in these where at least one HLA-DR antigen was foreign to the recipient.

Unfortunately blood transfusions can also lead to the development of cytotoxic antibodies in sera from potential graft recipients. Such antibodies may react with donor lymphocytes causing a positive crossmatch test. If broad reactivity develops against lymphocytes from a high percentage of members of a normal lymphocyte panel the number of suitable kidney donors diminishes markedly. The development of broad sensitization is rare after blood transfusions alone but common in patients who are transfused after a previous pregnancy [7, 8] or failed allograft [9, 10]. The clinical problem of identifying suitable donors for the increasing number of sensitized recipients has become a potent factor against a policy of pre-transplant blood transfusions. The concomitant administration of immunosuppressive drugs such as azathioprine [11–13] or cyclosporin A [14] with donor specific transfusions [15] or third party transfusions prevented sensitization; cyclosporin A appeared the more effective drug [16].

Cyclosporin A has previously been shown to abrogate the humoral response to T dependent antigens in a rodent model [17], and in recent animal studies we have demonstrated that cyclosporin A can reduce the initial IgM response to allogenic blood transfusion and suppress the subsequent production of IgG [18].

IgG antibodies against donor lymphocytes detected by the standard crossmatch technique are clearly associated with graft failure [19, 20]. If, however, the positive crossmatch is due to IgM antibodies then successful transplantation is still possible, particularly if the IgM antibodies are autoantibodies [21–23]. Lymphocyte binding antibodies can be detected using flow cytometry and are associated with a high risk of the patient developing broadly reactive cytotoxic antibodies after further transfusions [24]. Such antibodies may represent low levels of cytotoxic anti-HLA antibodies. If present in pretransplant sera they appear to be associated with an increased incidence of impaired graft function [25, 26]. Other groups have gone further and shown an association between the presence of lymphocyte binding IgG antibodies detected against donor lymphocytes using flow cytometry and poor graft survival rates [27, 28]. The aim of the present study, therefore, is to investigate the effect of cyclosporin A on the alloantibody response to third-party blood transfusions in potential allograft recipients using flow cytometry.

Methods

Patients

Sixteen previously untransfused dialysis patients were given a planned program of third-party transfusions between 1985 and

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1989. All patients were Caucasian and natives of Scotland. Eight patients (group 1) received between 3 and 5 units of blood at two weekly intervals. Eight patients (group 2) received transfusions with concomitant cyclosporin A (10 mg/kg/day) from four days before the first transfusion until one week after the last. Two patients in group 1 received one and three additional clinically-indicated transfusions. Each group was comprised of three women and five men, and one parous woman was included in each group. Both women had had two pregnancies; none of our patients suffered from systemic lupus erythematosus. We have since changed our policy and parous women are no longer electively transfused. Blood donors came from the North-East of Scotland and hence belonged to the same population group as the patients.

Sera and target cells

Serum samples, obtained from the 16 patients prior to each transfusion and two weeks after the last, were heat inactivated at 56°C for 45 minutes, ultracentrifuged at 100,000 × g for 60 minutes and stored at −70°C until assayed. Mononuclear leucocytes were separated from the blood of six healthy Caucasian volunteers, chosen to share HLA antigens common in the North-East of Scotland, by centrifugation with Ficoll-Hypaque. In all 462 serum/cell combinations were tested. The tissue types of the panel members were: (1) A3, B7,18, DR4,w6 (2) A1,2, B8,w62(15), DR3,4 (3) A2,11, B7,44,DR1,7 (4) A3,10(25,26), B15(63),18,DR2,4 (5) A4,w19, B14,50 DR 1,4 (6) A1,B35, w55(22), DR5. These represent over 85% of the HLA A and B antigens which occur at a frequency of greater than 10% in the blood donor population of North-East Scotland. All HLA DR specificities (1 to 7) were present in the panel.

Complement dependent cytotoxicity (CDC)

The standard long incubation, two-stage NIH microlymphocytotoxicity assay was performed using peripheral blood lymphocytes [29]. Lymphocytotoxicity was considered positive if cell kill was more than 20% above background.

Flow cytometry

The presence of lymphocyte antibodies was detected by indirect immunofluorescence and flow cytometry. Twenty-five microliters of mononuclear cell suspension were incubated with 25 μl of test serum for 30 minutes at 22°C. After two washes the cells were resuspended in 25 μl of a 1:50 dilution of the F(ab')2 fragment of a polyspecific goat antibody to human immunoglobulins conjugated to fluorescein isothiocyanate (FITC) (TAGO), and incubated for 30 minutes in the dark at 4°C. The cells were washed twice, resuspended and analyzed by flow cytometry using an EPICS C flow cytometer. The mean channel of 560 nm fluorescence intensity was determined for each test sample performed in duplicate. Results obtained were compared with the patient's pretransfusion serum and a normal male control serum. The test serum was considered positive when the mean channel shift was above the 95% confidence limits determined by 36 control sera/cell combinations compared with the patient's pre-transfusion serum.

Positive serum/cell combinations were subsequently screened for antibody activity by incubating each serum cell combination in parallel with the polyspecific antihuman FITC conjugate, a 1:20 dilution of goat antihuman IgM FITC conjugate (Sigma, Poole, Dorset, UK) and a 1:30 dilution of goat antihuman IgG FITC conjugate (Sigma).

A human IgM monoclonal antibody which binds to lymphocytes from all donors (from Dr. A. Ting and Mr. C.J. Taylor, Nuffield Department of Surgery, John Radcliffe Hospital, Oxford, UK) and an IgG preparation of serum containing multispecific lymphocyte alloantibodies were used as positive controls. The fluorescein conjugated antibodies were shown to be specific for the appropriate isotype in the flow cytometry assay.

Table 1. Antibodies detected by complement dependent cytotoxicity (CDC) and flow cytometry (FC) during the blood transfusion protocol; results expressed as number of positive serum/cell combinations

Patient	FC total	IgM	IgG	CDC
Group 1				
1	10	2	10	—
2	2	2	1	—
3	4	2	3	—
4	6	—	6	2
5	2	—	2	—
6	3	—	3	—
7	—	—	—	—
8	—	—	—	—
	27	6	25	2
Group 2				
1	3	3	—	—
2	3	3	—	—
3	4	4	—	—
4	3	—	1	—
5	—	—	—	—
6	—	—	—	—
7	—	—	—	—
8	—	—	—	—
	13	10	1	—

Red cell absorption

One serum sample which was positive in flow cytometry was obtained from a patient with previous known red cell autoantibodies. This sample was tested for autoantibodies using the sensitive papain layer enzyme technique before and after absorption with group O red cells. Equal volumes of serum, 0.1% papain-cystein and a 3 to 5% red cell suspension from each of eight normal donors were layered in a precipitin tube and incubated for one hour at 37°C. The development of microscopic agglutination was accepted as positive. Simultaneously absorbed and unabsorbed sera from three other patients which were positive in flow cytometry were screened for the presence of red cell autoantibodies.

Statistical analyses were performed using χ^2 or Fisher's exact test.

Results

Development of cytotoxic antibodies during the transfusion protocol

Only one patient (No. 4 in group 1) developed cytotoxic antibodies (Table 1) against two of the six target cells. The antibodies developed after the second and third transfusions in sera from a woman who had two previous pregnancies.

Table 2. Time course of IgM and IgG antibody development during the blood transfusion protocol

Patient	BT[a]	1[b]	2	3	4	5
Group 1						
1	8	—	—	—	IgM/IgG	IgM/IgG
2	3	—	—	IgM/IgG	—	—
3	6	—	—	IgM	IgM/IgG	IgG
4	3	—	IgG	IgG	—	—
5	4	—	—	IgG	IgG	—
6	5	—	—	—	IgG	IgG
7	5	—	—	—	—	—
8	5	—	—	—	—	—
Group 2						
1	3	—	IgM	IgM	—	—
2	5	—	IgM	IgM	IgM	—
3	5	—	IgM	IgM	IgM	IgM
4	5	IgG	—	—	—	—
5	5	—	—	—	—	—
6	4	—	—	—	—	—
7	5	—	—	—	—	—
8	5	—	—	—	—	—

[a] Number of administered blood transfusions (BT)
[b] Antibody development after blood transfusion 1 respectively 2, 3, 4, 5

Development of lymphocyte binding antibodies detected by flow cytometry

All 462 serum/cell combinations were screened for lymphocyte antibodies by flow cytometry using the polyspecific anti-human immunoglobulin as a secondary antibody. None of the patients including the two previously pregnant women developed antibodies before receiving blood transfusions. Six of eight patients in group 1 developed such antibodies in 27 serum/cell combinations during the transfusion protocol (Table 1). Only four patients in group 2 developed antibodies which were present in 13 of the serum/cell combinations tested. This difference in positive serum/cell combinations between the two groups was statistically significant ($P < 0.02$, χ^2 analysis).

Development of IgM and IgG antibodies during blood transfusion administration

Six of the eight patients in group 1 developed IgG antibodies during the course of the protocol (Table 1); IgG activity occurred mainly after the third and fourth transfusion (Table 2). In one patient the development of IgG antibodies was preceded by IgM antibody production (patient 3) indicating that we could demonstrate the switch from IgM to IgG antibody production following the allogeneic stimulus. In four sera from three patients in this group IgM and IgG antibodies were detected. Thus both IgM and IgG activity was present in sera from patients given blood transfusions without cyclosporin A.

In contrast, three patients in group 2 showed IgM activity in their sera during the transfusion protocol (Table 1) and none developed activity. Antibodies in these patients developed after the second transfusion, that is, earlier than those in group 1 (Table 2). Three positive serum/cell combinations from two different patients in this group possessed neither IgM nor IgG activity, suggesting that such antibodies might belong to another immunoglobulin class. The IgM antibodies detected did not bind to autologous lymphocytes. Only one patient out of eight in group 2 who received cyclosporin A along with transfusions developed IgG antibodies, in contrast to six of eight patients in group 2. This difference was statistically significant ($P < 0.02$, Fisher's exact test). Furthermore IgG antibodies occurred in only one out of 234 serum/cell combinations in group 2 but in 25 of 228 in group 1 ($P < 0.001$, χ^2 analysis). The patient in group 2 who developed IgG antibodies after the first transfusion had previously been known to possess IgG red cell autoantibodies. After absorption with pooled erythrocytes both the red cell and lymphocyte binding IgG antibody activity disappeared. In contrast, three sera which contained IgG lymphocyte antibodies obtained from patients in group 1 with no red cell autoantibodies continued to be active after a similar absorption procedure.

Clinical outcome

Of the eight patients in group 1, four have received a cadaver donor transplant; all grafts failed within two months, and three patients developed IgG antibodies during the course of the study. In contrast all grafts from the four transplanted patients in group 2 are functioning between two and four years after transplantation. One of the patients developed IgM antibodies during the study, the others showed no detectable antibody activity.

Discussion

This study shows that cyclosporin A given with allogeneic blood transfusions permitted an IgM response to transfusion in three of eight patients but prevented the switch from IgM to IgG production; IgG lymphocyte antibodies were detected only in one patient in this group. In contrast, six out of eight patients who received transfusions alone developed IgG antibodies.

The place of elective blood transfusions in the preparation of patients for transplantation is one of the main controversies in current transplantation research. One study [30] concluded that 'one year cadaveric graft function in cyclosporin A treated patients was independent of blood transfusion history'. The authors, however, did show a significant ($P < 0.05$) improvement in transplant survival in transfused compared with untransfused cyclosporin A treated patients, although the transfusion effect was less than in patients treated with azathioprine and prednisolone ($P < 0.01$). There are difficulties in comparing survival rates in the two groups as 77% of patients treated with azathioprine and prednisolone were transplanted before those given cyclosporin A; the study therefore represents an historical comparison. Furthermore the proportion of patients in each group who received a second or subsequent graft was not stated. A multicenter study of over 15,000 first graft recipients transplanted in 1984 and 1985 failed to show a beneficial effect of transfusions on graft survival in either cyclosporin A or azathioprine and prednisolone treated patients [2]. There were, however, only 1033 patients in the untransfused group and over 14,000 in the transfused group, and the author, Opelz, was cautious in interpreting these data. He suggested that non-transfused recipients might have had higher baseline immunosuppression and more aggressive treatment of early rejection.

Subsequently a study of over 10,000 transplants performed between 1984 and 1987 showed a significant improvement in overall transplant survival in transfused patients [6]. When the results were analyzed in relation to HLA-DR matching there was a significant beneficial effect of blood transfusion except

where none of the donor HLA-DR antigens were foreign to the recipient. Further data [31] showed a significant ($P < 0.01$) improvement in one year transplant outcome in transfused patients and a higher incidence of severe rejection (a rise in creatinine over 4 mg%) in untransfused recipients. Opelz who collated the largest body of data stated that 'there can be no question that the transfusion effect indeed exists' and suggests that to abandon elective transfusion at present would be premature; he advocated a prospective multicenter trial [2]. Such a trial should examine not only crude survival data but also the number of rejection episodes requiring expensive treatment and an increased hospital stay. It remains prudent, therefore, to determine ways of preserving the possible benefits of transfusion while preventing sensitization in those awaiting transplantation.

We have previously demonstrated in a rat model [18] that cyclosporin A could diminish but not abolish the primary IgM response to blood transfusions; the switch to IgG production, however, was completely abrogated. The results of the animal study and the present clinical study are therefore broadly similar.

Cyclosporin A acts not only on T lymphocyte responses [17, 32] but can also affect B cells directly [33]. B lymphocyte activation can be achieved by either a T cell independent or dependent mechanism. The response to a T dependent antigen depends on the production of lymphokines by activated T helper lymphocytes. These lymphokines are essential for the proliferation and differentiation of B lymphocytes into antibody secreting cells [34]. Cyclosporin A modifies the activation and abrogates the proliferation of T helper cells; the secretion of T cell derived cytokines, including interleukin 2, which influence the humoral response is thus diminished [35].

In neonatal and adult mice thymectomy alters the IgG but not the IgM response to H-2 antigens [36], suggesting that the IgM response to histocompatibility antigens is T cell independent. Furthermore, IgM production from T cell-depleted, EB virus-stimulated human lymphocytes in vitro is not affected by cyclosporin A, whereas the development of an IgG response is inhibited [37]. Although we are cautious about extrapolation from experimental studies, these results may explain why cyclosporin A did not suppress the IgM response to blood transfusions in three of our patients. A primary IgG response is highly dependent upon T helper cells, in particular the T cell-derived cytokine interleukin 4, which inhibits the production of IgM and promotes the switch from IgM to IgG1 production [38]. Cyclosporin A blocks the production of interleukin 4 [39], which may explain the suppression of the antibody switch in patients in the present study receiving cyclosporin A along with transfusions.

One patient previously known to have IgG red cell antibodies in group 2 developed IgG antibodies after the first transfusion. Lymphocyte binding activity was removed by absorption of the serum with red cells, suggesting that it was due to cross reactivity of the red cell autoantibodies with lymphocytes.

Although our primary aim was to determine the effect of cyclosporin A given with transfusion on the isotype of antibodies produced, we also noted the outcome in the small number of patients who received a transplant. All of those who received transfusions alone had grafts which failed in less than two months and most showed IgG activity against panel lymphocytes. The grafts of those given cyclosporin A with transfusions, however, are all functioning at least two years later and one patient developed IgM activity during the study period. It would be valuable to confirm these results in a larger study.

Scornik and his colleagues have demonstrated that IgG antibodies detected using flow cytometry are associated with a high risk of developing cytotoxic antibodies after further blood transfusions [24]. They therefore may be an early indicator of sensitization. Such antibodies when present against donor lymphocytes are associated with prolonged primary graft dysfunction, a greater number of rejection episodes requiring treatment with additional therapy including OKT3 and ATG, and a higher creatinine level three months after transplantation [25, 26]. Furthermore a positive crossmatch detected using flow cytometry is associated with poor graft outcome in patients with preformed cytotoxic antibodies (PRA >10%) [6] and in those receiving a second or subsequent graft [27, 28].

The relevance of IgM antibodies, however, is less clear. Several studies have demonstrated that successful renal transplantation can be performed across a positive crossmatch due to cytotoxic IgM antibodies [21–23] even if they are alloreactive; the significance of IgM antibodies against donor lymphocytes detected only using flow cytometry, however, is unknown. Clearly many factors influence the ultimate fate of a transplant, but our results taken together with evidence from the literature may indicate that IgG antibodies were detected using flow cytometry or even graft failure. The presence of IgM antibodies, however, appears to have little influence on graft outcome.

In conclusion, we have shown that cyclosporin A can abrogate the switch from IgM to IgG antibody production in patients receiving third-party blood transfusions. The administration of cyclosporin A with blood transfusions may prevent the formation of antibodies which are associated both with development of sensitization and subsequent impaired graft function.

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