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*Original Article*

# Controlled Trial of Azathioprine and Cyclosporin to Prevent Anti-LHA Antibodies due to Third-party Transfusion

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**Abstract.** The beneficial effect of elective transfusion on renal allograft survival must be weighed against the risks of sensitisation. We report a randomised controlled trial in which patients in end-stage renal failure who were non-parous and not previously transplanted or transfused, were entered in a transfusion protocol during which one group received no drugs (controls), one received azathioprine, and one received cyclosporin. Each group was given three identical transfusions of leucocyte-enriched fresh blood at 2–3 week intervals. The transfused blood was of known HLA type and donor/recipient pairs were completely mismatched. Sensitisation rates were assessed by T and B cell cross-matches between donor and recipients and by the screening of all sera against lymphocytes from 40 random donors.

Fifty-one patients have completed the protocol, 20 in the control group, 12 in the azathioprine group, and 19 in the cyclosporin group. The sensitisation rate in the control group was 30%, occasionally of high titre, and persistent. In the azathioprine group, 25% developed anti-HLA antibodies and reactivity was of high titre and was broadly specific. Sensitisation in the cyclosporin group was 10%, was narrowly specific, reacting with only 10% of a panel, and was transient. There was no difference in graft survival between the groups. We conclude that cyclosporin therapy concurrent with third-party transfusion reduces the incidence, titre, and duration of sensitisation.

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**Key words:** Anti-HLA antibodies; Azathioprine; Cyclosporin; Transfusion

## Introduction

The beneficial effect of prior blood transfusion on graft survival rates in human renal allograft recipients was first reported in 1973 [1]. Despite early controversy this effect was confirmed in most centres, and by 1981 prior blood transfusion was considered to have a dominant effect on graft survival and elective blood transfusion prior to transplantation was widely practised [2]. With the advent of cyclosporin (CsA) there was some reduction in the magnitude of this effect, but it was still demonstrable 3 years after the general availability of this drug [3]. It was apparent, however, that the practice of elective transfusion of potential allograft recipients had an important detrimental effect. A variable proportion of such patients developed anti-HLA antibodies, which resulted in substantially increased waiting time for a cross-match negative kidney, and rendered a small proportion of these patients virtually untransplantable [4,5].

Graft survival rates in patients who had preformed anti-HLA antibodies, whether acquired through parity or transfusion, were significantly inferior to that of their unsensitised counterparts [6]. Various protocols were devised to minimise sensitisation due to elective transfusion while conserving the beneficial effect. One method of reducing sensitisation involved the use of frozen or stored blood in the expectation that storage would reduce

the number of viable leucocytes and thus its immunogenicity while preserving the beneficial effect [7]. Another strategy was the use of blood transfusion from HLA-matched donors and this approach resulted in reduced sensitisation rates, but is logistically very difficult to organise [8,9]. Blood products such as platelets were used in the hope that the absence of class II HLA gene products would result in even lower sensitisation rates, but this protocol was followed by very poor graft survival [10].

In the case of live donor transplantation a protocol was devised in which aliquots of blood from the potential kidney donor would be administered to the graft recipient [11]. It was hoped that the subsequent antibody response, if any, would be directed at the small number of mismatched HLA specificities and would not prejudice cadaveric transplantation or the use of an alternative live donor. These various donor-specific transfusion protocols resulted in sensitisation rates of 30%, and an attempt was made to reduce this by the concurrent administration of azathioprine [12]. This reduced subsequent sensitisation rates to 5%–10%, but high-titre anti-HLA antibodies still developed in parous female patients and in patients who had had prior transplantations or prior third-party transfusions [13].

Many of the transfusion protocols used up to now have suffered from design defects in that some were retrospective, few were randomised, controls used were usually historical, and prior history of parity and transfusion were not accounted for. The immunising agent used, i.e. transfused blood, was heterogeneous in the amount used, the storage methods, and the degree of HLA match between blood donor and recipient. We therefore embarked on a prospective controlled trial in which patients in end-stage renal failure would be transfused with a clearly defined amount of blood at defined intervals and with a known HLA disparity between donor/recipient pairs. The patients would be randomised to three groups, one to receive azathioprine during the transfusion protocol, one to receive cyclosporin, and a control group which would receive no drug therapy. The aim of the trial would be to examine the impact of these drug therapies on sensitisation rates and subsequent allograft survival.

## Materials and Methods

### *Patient Selection*

Patients were selected who were in end-stage renal failure (creatinine clearance $< 5$ ml/min), who had never been pregnant, and had never previously been transplanted or transfused. Any patients in whom the taking of immunosuppressive drugs was contraindicated were excluded. Informed consent was obtained from each patient and the trial was approved by the Hospital Ethics Committee. The patients were randomised by drawing cards into three treatment groups: one would receive three elective transfusions from a single HLA-mismatched donor at 2–3 week intervals and no concurrent therapy would be given. The second group would be given an identical series of transfusions, but would commence azathioprine 1.5–2.0 mg/kg per day 10 days prior to the first transfusion and continue until 10 days after the last transfusion. The third group would have similar transfusions and would commence CsA 15 mg/kg per day 4 days prior to the first transfusion and continue for 10 days after the last transfusion. Azathioprine dosage was adjusted as clinically indicated, depending on platelet and neutrophil counts. CsA dosage was adjusted to maintain plasma trough values as measured by high-performance liquid chromatography between 75 and 150 ng/ml [14]. Patients who required unplanned transfusions during the protocol would be withdrawn from the study.

### *Blood Product Transfused*

Each transfusion consisted of an aliquot of cell-separator residue collected during routine platelet harvesting from healthy donors of known HLA type. The residue was collected in acid citrate dextrose anticoagulant, using an Haemonetics Model 30 cell separator. It contained approximately $50–80 \times 10^9$ leucocytes/l of which 60%–70% were lymphocytes. Each specimen had a white-cell count performed on a Coulter counter, and an aliquot was adjusted in volume to contain $1.0\ (\pm 0.1) \times 10^9$ leucocytes/aliquot. Each patient received three identical aliquots from a single donor at 2–3 week intervals (Fig. 1). Donor/recipient pairs were blood-group compatible, and standard red cell cross-matches were performed. The aim was to have donor/recipient pairs completely HLA mismatched at the AB and DR loci, and in the event all had a minimum of 5 out of 6 mismatched HLA antigens. Donors bearing the HLA A2 specificity were excluded in order to avoid sensitisation against such a prevalent HLA specificity.

### *Anti-HLA Antibodies*

Serum from each recipient was collected and frozen at the time of entry and 2 weeks after each of the three transfusions. Sera were also collected at regular intervals up to the time of transplantation. Upon completion of the protocol a cross-match was performed between all frozen sera and donor T and B lymphocytes, using the long incubation NIH technique [15]. Control cross-matches between all sera and recipient peripheral blood lymphocytes were performed to look for the presence of antoantibodies. Cross-matches were performed at 4°, 22° and

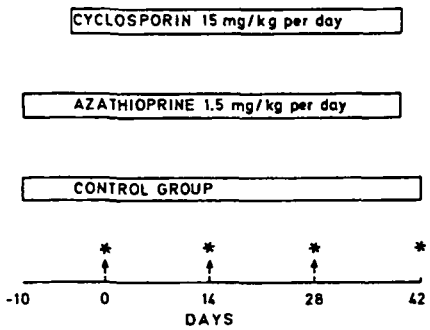


Fig. 1. Design of protocol for programmed transfusion of previously unsensitised patient. † Blood transfusion; *Serum sample for anti-HLA antibodies.

37°C. All frozen sera were screened for the presence of anti-HLA antibodies against a panel of lymphocytes from 40 individuals of known HLA type and so chosen as to embrace all known HLA specificities. All cross-match and screening procedures were performed by the same individual (CJL) who was unaware of the details of randomisation. Sensitisation was considered to have occurred if the cross-match was positive against donor T cells or if there was a 10% increment in panel reactive antibody between first and last serum samples.

### *T Lymphocyte Subsets*

Heparinised blood was collected from each recipient at entry and 2 weeks after each transfusion, and peripheral blood lymphocytes were separated on a Ficoll Hypaque gradient. Total T cells and T lymphocyte subsets were labelled using the Orthomune reagents OKT3 (CD3), OKT4 (CD4), and OKT8 (CD8) (Ortho Pharmaceutical Corp., NJ, USA). Results were analysed on an Orthocytofluorograph 50 H fluorescence-activated cell sorter (Ortho Diagnostics, Westwood Mass, USA) and results were expressed as total T-cell numbers and ratio of CD4 to CD8 positive cells.

Upon completion of the transfusion protocol, patients were entered on the transplant waiting list and post-transplant immunosuppression was with CsA and prednisolone 0.25 mg/kg per day.

### *Statistical Analysis*

Sensitisation rates in each of the treatment groups were compared to that of the controls using contingency tables and the chi-square test. Differences between the mean CD4:CD8 ratios in the groups were calculated using Student's T test, and the life table method was used for the calculation of graft survival.

## Results

These were first analysed after 12 patients had completed all three arms of the protocol. Because at that time three patients in the azathioprine group had become sensitised, two of them with high-titre multispecific antibodies, it was considered unethical to randomise further patients to this arm. One patient who was randomised to the CsA group required transfusion for anaemia prior to his first programmed transfusion and he was therefore withdrawn. Fifty-one patients have completed the protocol, 20 in the control group, 12 in the azathioprine group and 19 in the CsA group (Table 1). Three patients in the azathioprine group required dosage reductions due to neutropenia. All patients in the CsA group experienced side-effects such as anorexia, nausea and paraesthesiae, but withdrawal of therapy was not necessary in any case. Trough CsA values as measured by HPLC ranged from 67 to 375 ng/ml ($150 \pm 94$ mean + SD), and 35% required dosage adjustment to keep concentrations within the target range.

**Table 1.** Sensitisation rates and outcome of transplantation in transfused patients

| | Controls | Azathioprine | Cyclosporin |
|---|---|---|---|
| Patient number | 20 | 12 | 19 |
| Sensitisation to donor | 6 | 3 | 2 |
| PRA > 10% | 6 (30%) | 3 (25%) | 1 (7.6%) |
| PRA > 30% | 5 | 2 | 0 ($P < 0.05$) |
| Transplanted | 11 | 10 | 14 |
| Functioning | 6 | 8 | 12 |

PRA, panel-reactive antibody

### *Anti-HLA Antibodies*

None of the patients had anti-HLA antibodies at the time of entry to the study. Six patients developed antibodies directed at donor lymphocytes in the control group, three in the azathioprine group, and two in the CsA group (Fig. 2). Differences were just short of statistical significance. Donor-specific sensitisation in the CsA group was unusual in that it was narrowly specific (anti-B18 in one patient, anti-B7 and B14 in the other), was of low titre, and was transient, lasting 3 weeks in one patient and 3 months in the other. Two patients had low-titre autoantibodies at the start of the protocol and these did not change throughout the study.

Antibody responses directed at more than 10% of a panel of lymphocytes developed in six of the control patients (30%), in three of the azathioprine-treated patients (25%) and in one of the CsA patients (7.6%). The

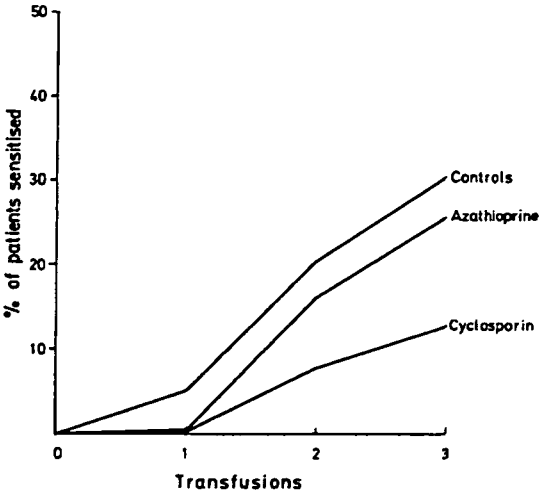


Fig. 2. Time course of anti-donor responses in the three transfused groups.

difference between the control and CsA groups just reached statistical significance; $P=0.05$. However, panel reactivity in the control group ranged from 20% to 84%, whereas it ranged from 7% to 19% in the CsA-treated group. Thus the difference between the two groups is greater if compared for the emergence of antibody directed at a larger proportion of the panel: e.g. antibody directed at 30% of the panel CsA 0, controls 5, ($0.01<P<0.05$). Of the three patients who developed anti-HLA antibodies in the azathioprine group, two had high-titre, broadly reactive antibody reacting with 90% and 60% of the panel respectively.

### *T Lymphocyte Subsets*

There were no significant changes in total T-cell numbers throughout the study. There was a modest rise in the CD8 positive subset and a resultant decline in mean CD4:CD8 ratios from 2.3 to 2.03 in the control group, 2.1 to 2.01 in the azathioprine group, and 2.61 to 2.17 in the CsA group. None of the changes reached statistical significance.

### *Transplantation*

Eleven patients have been transplanted in the control group, ten in the azathioprine group and 14 in the CsA group. All patients received cadaveric grafts. Actuarial graft survival at one year is 56% in the control group, 80% in the azathioprine group and 85% in the CsA group. Differences do not reach statistical significance. The poor graft survival in the control group is not a reflection of excess losses due to graft rejection, but is a reflection of three patients deaths in that cohort, one from CMV pneumonia, one from pneumocystis pneumonia, and one patient who developed renal-vein thrombosis in his graft and was not subsequently dialysed. A fourth graft loss in the control group was for technical reasons, and only one of these grafts was lost due to irreversible rejection.

## Discussion

Since the discovery of the beneficial effect of prior blood transfusion on renal allograft, survival attempts have been made to devise transfusion protocols that would preserve the beneficial effect while avoiding the risk of sensitisation. This has not been a simple task, since the mechanism by which the blood transfusion exerts its beneficial effect is not known. It appears to reside in the cellular components of the blood, since transfusion of plasma alone, at least in experimental animals, is not effective [16]. Furthermore, techniques used to reduce the number and viability of leucocytes in the blood appear to reduce both sensitisation rates and the beneficial effect [17,18]. Prior transfusion with platelets alone in the human results in poor graft survival rates [10] so it seems reasonable to assume that both the beneficial and sensitising effects reside in the leucocyte component.

The use of HLA-matched or partly matched blood donors is one method by which sensitisation can be reduced, but it requires the availability of a vast bank of HLA typed blood donors [8,9]. Transfusions from a prospective kidney donor is a practical proposition in live donor transplantation, but although resulting in high graft survival rates it results also in sensitisation of 30% of recipients [11]. The use of azathioprine concurrent with donor-specific transfusion results in much lower sensitisation rates, but final graft survival rates have been invariably compared to historical controls [11,12]. Significant rates of sensitisation occurs even with azathioprine treatment in parous females and patients who have received prior third-party transfusions [13]. Finally, the data from donor-specific transfusion protocols are not strictly applicable to third-party transfusion and cadaveric transplantation, where the mismatch between recipient and both blood and kidney donor is greater and where the mechanism of the transfusion effect might well be different.

The above study attempts to address this question using a clearly defined patient population who would receive prospective transfusions of a defined blood product of known HLA type. The patient population was so chosen that anamnestic responses to previous pregnancies, transfusions or transplantation would not confuse the interpretation of any anti-HLA responses encountered. The blood product chosen was cell-separator residue from platelet donors and was basically a leucocyte-enriched, platelet poor, fresh blood transfusion. The number of leucocytes per transfusion was defined and is

equivalent to the number in 200 ml of fresh blood, which is the amount used in many transfusion protocols. Because all donors were of known tissue type it was possible to assess the specificity of any antibody responses, whether directed at class I or class II HLA antigens, and also to assess their titre and persistence.

The immunosuppressants chosen were partly on the basis of previously published donor-specific transfusion protocols, where azathioprine appeared to be effective in reducing sensitisation. CsA was chosen on the basis of its known immunosuppressive effects and its ability to reduce T-cell-dependent antibody responses in experimental animals [19]. Furthermore, it was unlikely to have any serious side-effects, since the patients chosen were effectively anephric.

The sensitisation rate of 30% in the control group is precisely the level encountered in most non-immunosuppressed donor-specific transfusion protocols. Responses were directed at both B and T cells, and antibody levels declined over a 1-year period. Two of the patients developed high-titre broadly reactive antibody and neither has subsequently been transplanted. In the azathioprine-treated group the sensitisation rate of 25% is hardly different from the control group. Moreover, two of the patients developed high-titre broadly reactive antibody, and though it has proved possible to transplant them both using well-matched kidneys, one of the grafts was rapidly lost due to irreversible rejection. Because of these events it was not considered ethical to proceed with this arm of the trial, having entered 12 patients.

The CsA-treated arm proved most interesting in that the incidence of antibody response to donor was only 10%, the responses were specific, being directed at one or two of the transfused mismatched antigens, and more importantly they were of low titre and short-lived. Because of the highly specific responses, reactivity with the panel of lymphocytes was low and never exceeded 20% of the panel. Thus, the chance of early transplantation was not seriously prejudiced. Both patients who mounted short-lived anti-donor responses were transplanted within months of completing the protocol, and both grafts are functioning well.

The mechanism by which CsA might depress the antibody response is not clear. Experimental evidence suggests that CsA is a potent inhibitor of T-cell-dependent antibody responses and, because of its relatively selective effect on T-helper cells, tends to push the immune response into a suppressor mode [20,21].

There was a modest increase in CD8-positive cells in the CsA-treated group, with a corresponding decrease in CD4:CD8 ratios. Specific suppressor cells, if present, are likely to be a very small proportion of that population, so it is hardly suprising that the observed increase in CD8-positive cells was modest. A more sophisticated test of suppressor-cell function would be necessary to prove that the mechanism involved was one of cell-mediated suppression.

Recent evidence suggests that the magnitude of the beneficial effect of prior blood transfusion of recipients is declining [22]. Elective transfusion is still practised in most transplant units, however. This study suggests that CsA therapy concurrent with elective transfusion protocols will reduce the incidence and duration of sensitisation without prejudice to allograft survival rates.

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