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CLINICAL INVESTIGATION

Immunopathology of renal allograft rejection analyzed with monoclonal antibodies to mononuclear cell markers

G. ALEX BISHOP, BRUCE M. HALL, GEOFFREY G. DUGGIN, JOHN S. HORVATH, A. G. ROSS SHEIL and DAVID J. TILLER

Department of Renal Medicine, Royal Prince Alfred Hospital, Missenden Road, Camperdown, 2050, N.S.W. Australia

Immunopathology of renal allograft rejection analyzed with monoclonal antibodies to mononuclear cell markers. The composition of the mononuclear cell infiltrate in rejecting renal allografts was determined on 96 renal biopsies and 22 nephrectomy specimens by the use of monoclonal antibodies to mononuclear cell surface markers and an indirect immunoperoxidase staining technique. During rejection the composition of the infiltrate was heterogeneous, with T cells (T11), monocytes (OKM1) and HLA-DR expressing mononuclear cells the most frequent sub-populations. B cells (B1) and activated T cells, identified by OKT10, were always in the minority. The T cells infiltrate usually included the helper/inducer (T4) and cytotoxic (T8) subclasses, which suggests that both may contribute to the mediation of rejection. Whether T4 or T8 predominated in the graft did not relate to the ratio of T4:T8 in blood, the HLA A, B or DR incompatibilities of the graft, or the immunosuppressive used. The frequency of T11, T4, T8, HLA-DR positive cells and monocytes, but not B cells, increased with the severity of rejection and was similar in biopsies from patients immunosuppressed with Cyclosporine (CSA) to those given a combination of azathioprine, prednisone and antilymphocyte globulin (AZA). Severe rejection episodes which did not respond to treatment with corticosteroids were more often characterized by a predominance of T8 over T4 cells and T cells infiltrating the glomeruli. In grafts with evidence of cellular rejection, renal tubular cells were shown to have a marked increase in their expression of HLA-DR antigens compared to normal kidneys or grafts with minimal rejection. The expression of HLA-DR antigens on graft tubular cells correlated with the presence of T cells in the interstitium and the severity of rejection, except for moderate rejection in CSA treated biopsies, in which HLA-DR expression was lower than in AZA biopsies. These immunopathological studies have demonstrated that a variety of potential effector cells exist within the graft, and several features have been identified which may assist in assessing the prognosis of the rejection episode.

The precise diagnosis of renal allograft rejection is dependent upon the identification of well described changes seen by light microscopy in biopsies [1–3]. The morphology of the rejection process is heterogeneous, which reflects the fact that allograft rejection can be mediated by a variety of immune mechanisms, including T lymphocytes or antibody, either directly or through antibody dependent, cell mediated cytotoxicity and natural killer cells [4–6]. T lymphocytes have been identified as the principal effector response [7]. More recently it has been shown that T lymphocytes of the helper/inducer subclass, as well as cytotoxic T lymphocytes, can mediate allograft destruction [8–13]. Until recently it has been thought that helper/inducer cells mediate rejection via activation of macrophages in a classical delayed-type hypersensitivity response, and that cytotoxic T cells acted directly on graft cells. However, there is now evidence that both subclasses of cells can mediate both delayed-type hypersensitivity and direct cytotoxic effects [14]. It has also been suggested that helper/inducer cells only act against cells expressing class II major histocompatibility complex (MHC) antigens and cytotoxic cells against those expressing the relevant class I MHC antigens (14, 15). The relative importance of these two effector mechanisms in the mediation of kidney allograft rejection remains unknown. It is possible that the presence of different subclasses of T cells in renal allografts may indicate that different effector mechanisms are operating and that these mechanisms may have different responses to anti-rejection therapy.

Monoclonal antibodies, which can identify mononuclear cell subpopulations, including the different subsets of T cells, have been used with immunostaining techniques to confirm that during acute renal allograft rejection the cellular infiltrate is heterogeneous [16–19]. In this study commercially available monoclonal antibodies and indirect immunoperoxidase staining were used to identify the mononuclear cell infiltrate in renal transplant biopsies and nephrectomy specimens. T cells, B cells, helper/inducer T cells, cytotoxic T cells, activated T cells and monocytes have been identified, using the reagents listed in Table 1 [20–26] (T11, T4, T8, B1, I1, Coulter Electronics Inc., Hialeah, New Jersey, USA; OKT10 and OKM1, Ortho Pharmaceutical Co., Raritan, New Jersey, USA). As we have previously observed that renal tubular cells express HLA-DR antigens during severe rejection episodes [27], the expression of these antigens by cells within biopsies was also examined. This report is an extension of previous studies where we have reported on the immunopathology of rejection [19, 27]. However, in this study a larger number of biopsies was evaluated and it was possible to analyze the diagnostic usefulness of identification of the cellular infiltrate in determining the severity, prognosis or reversibility of the rejection episode, and also a comparison of the patterns of cellular infiltrate in kidneys from patients with different HLA incompatibilities and treated with either Cyclosporine (CSA) or azathioprine, prednisone and antilymphocyte globulin (AZA).

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Table 1. Monoclonal antibodies used for indirect immunoperoxidase stains of renal biopsies

Antibody	Concentration	Specificity	Reference
T11	1:40	Pan T cell	[20]
T4	1:20	Helper/inducer T	[21]
T8	1:80	Cytotoxic/suppressor T	[22]
OKT10	1:80	Activated T, myeloid precursors	[22]
B1	1:10	B cells	[24]
OKM1	1:160	Monocytes	[23]
I2	1:160	HLA-DR non-polymorphic structure	[25]
Neuraminidase	1:20	Influenza neuraminidase	[26]

Table 2. Clinical data of patients at time of biopsy or nephrectomy

	Biopsies grade		Severity of rejection					
	Minimal		Moderate		Severe		Nephrectomy	
Treatment group	CSA	AZA	CSA	AZA	CSA	AZA	CSA	AZA
Specimens	18	5	30	17	15	11	10	12
Patients	15	5	25	16	8	11	10	12
Time since transplant								
Less than 2 weeks	5	3	6	1	5	5	1	3
15 days to 13 weeks	4	1	14	8	10	3	5	5
More than 13 weeks	8	1	10	8	0	3	4	4
Graft function								
Post-trans. oliguria	7	3	12	1	11	6	—	—
Acute deterioration	9	2	17	15	5	5	—	—
Chronic deterioration	2	0	1	1	0	0	—	—
Rejection treatment								
Less than 7 days prior	9	0	9	6	6	7	6	6
After biopsy	6	2	17	15	14	11	—	—
Course of Rejection								
Good recovery	6	2	6	12	5	2	—	—
Progressed to nephrectomy	2	1	2	5	8	7	—	—
Days to nephrectomy								
Median	—	30	—	180	36	9	—	—
Minimum	40	—	−100	3	17	7	—	—
Maximum	500	—	−180	360	72	39	—	—

Materials and Methods

Source of clinical material

Twenty two nephrectomy and 96 biopsy specimens were obtained from a total of 76 renal transplant patients who had consented to enter a randomized clinical trial that compared the efficacy of treatment with CSA as the sole immunosuppressant, to that with AZA. Details of patient management and clinical indications for performance of a biopsy or nephrectomy have been previously reported [3, 28]. Briefly, renal biopsies were performed every seven to ten days in the post-transplant oliguric phase or to diagnose the cause of deterioration in renal function. Biopsies were only performed when required to assist in the clinical management of the patient and after written consent was obtained. Clinical details of patients who had nephrectomy and biopsy specimens are shown in Table 2. HLA A, B, and DR typing was performed by the Tissue Typing Laboratory (N.S.W. Red Cross Blood Transfusion Service, Sydney, N.S.W. Australia) as described [29]. Therapy for acute rejection episodes was 1 g of methyl prednisone (Upjohn, West Ryde, Australia) given i.v. as a single bolus on three consecutive days. A good response to therapy was recorded if the serum creatinine fell and graft function remained stable for seven days. Failed response to therapy was recorded if the serum creatinine continued to rise in spite of anti-rejection therapy and did not return to its pre-rejection nadir.

Processing of renal biopsy tissue

Renal biopsy or nephrectomy material was divided and one part fixed in 5% buffered formalin and embedded in paraffin for routine light microscopic examination. The remainder was embedded in Tissue Tek II mounting medium and snap frozen in liquid nitrogen for immunoperoxidase staining, as previously described [19, 27]. Light microscopic sections were assessed for evidence of rejection, which was graded as severe, moderate or minimal, on criteria which have been previously described [2, 3]. Briefly, mild rejection was diagnosed when only local or sparse cellular infiltrate of the interstitium was present without vascular or glomerular lesions. In moderate rejection, there was more intense and diffuse cellular infiltrate with some vascular damage present, such as mild intimal proliferation. Severe rejection had changes as in moderate rejection, but they were more extensive and diffuse with more marked vascular changes, interstitial hemorrhage and areas of necrosis. The frequency of specimens in each category is listed in Table 2.

Immunoperoxidase stains

Frozen sections six microns in thickness were cut onto gelatinized slides, air dried and fixed in acetone at room temperature for ten min. before being washed in phosphate buffered saline (PBS) for five min. Sections were then incubated for 30 min. with 25 μl of monoclonal antibody diluted in 5% AB serum in PBS. The panel of monoclonal antibodies used is shown in Table 1. Slides were washed in PBS then incubated for 30 min. with 25 μl of rabbit antiserum to mouse immunoglobulin conjugated with horseradish peroxidase (Dako A/G, Copenhagen, Denmark), diluted 1:20 in 5% AB serum. After being washed in PBS, the sections were exposed for 10 min. to 1 mg/ml diaminobenzidine tetrahydrochloride (Sigma) and 0.01% H_2O_2 and 0.3% sodium azide in 0.05 M Tris buffer pH 7.6. Sections were counterstained in Mayer's hematoxylin, dehydrated and mounted.

Assessment of peroxidase stained sections

Immunoperoxidase-stained sections were assessed by two of us (GAB & BMH) without knowledge of the clinical or pathological diagnosis, and each section was scored in a semi-quantitative manner. This method is similar to that used to assess severity of light microscopic changes in transplant biopsies and gave reproducible results with a high concordance between observers. The score represented an assessment of the whole section, which was at least a 3 mm length of the needle biopsy specimen. The mononuclear cell interstitial infiltrate was scored as described previously [19]: +++, when mononuclear cells surrounded each tubule and were present both in the interstitium and between tubular cells, and when the whole tissue examined was uniformly infiltrated; ++, when there were cells surrounding tubules but few or no cells between tubular cells, and when the infiltrate was not distributed uniformly; +, when cells were sparsely scattered through the

interstitium and many tubules had no cells nearby; −, indicated that cells were absent or present only occasionally. The number of stained mononuclear cells infiltrating glomeruli was counted, and the results from 10 glomeruli were averaged. The phenotype of cells in perivascular infiltrates attached to the vascular endothelium was noted.

The extent of renal tubular cell staining for HLA-DR antigen was assessed as previously described [27]. The distribution and intensity of tubular staining for DR was graded: +++, intense staining of all tubular cells, including proximal tubule, distal tubule, loop of Henle, and collecting duct tubular cells; ++, moderate to intense staining of some tubular cells, proximal tubules were usually stained, and staining of distal tubules, loop of Henle and collecting ducts was variable; +, moderate tubular cell staining, usually confined to proximal tubules; −, no increased tubular staining, compared to non-transplant control kidney sections.

Proximal convoluted tubules were distinguished from distal convoluted tubules by their larger diameter and more columnar appearance. In biopsies with severe rejection or tubular necrosis, this distinction was often not possible.

Reproducible staining of renal biopsy material was obtained, using the indirect immunoperoxidase technique. To optimize conditions, we used sections from normal spleens as positive controls and biopsy specimens from non-transplanted kidneys with no mononuclear cell infiltrate as negative controls. The latter had been performed to investigate hematuria and proteinuria (17 were normal to light microscopy and 9 had mild mesangial disease). In normal kidneys, only occasional cells in capillaries and vessels stained with T11, T4, T8, OKT10, B, or OKM1. Staining with I2 was prominent on capillary endothelium and dendritic cells in the interstitial space, and the mesangial cells and capillary endothelium of the glomeruli. Proximal tubular cells showed no evidence of membrane staining with I2 but had a faint diffuse staining pattern concentrated at the base of the cell. There was no staining of distal tubules, collecting ducts and loop of Henle.

Control sections were included in the staining of each specimen and were incubated with a mouse monoclonal antibody to influenza neuraminidase. These had no membrane or cytoplasmic staining of cells, confirming that the technique used identified the specific epitopes of the monoclonal antibody used rather than non-specific or FC receptor staining.

Identification of peripheral blood lymphocyte subsets

Immunofluorescent staining of peripheral blood lymphocytes using OKT4, OKT8 and OKT3 (Ortho Diagnostics, Raritan, New Jersey, USA) was performed as described by Cosimi et al [30]. Fluorescent labelled sheep anti-mouse Ig (Silenus Laboratories, Dandenong, Australia) was used as the second phase reagent. Fluorescent cells were counted on a Spectrum III Cytofluorograph (Ortho Instruments, Westwood, Massachusetts, USA) as described [30]. There was no difference in the percentage of lymphocytes labelled by OKT4 and T4 (mean ± SD, 36.6 ± 7.7% vs. 37.2 ± 8.1%, $P = 0.52$ in paired Student's t test) or by OKT8 and T8 (30.1 ± 14.4% vs. 27.9 ± 15.0%, $P = 0.20$), when these reagents were compared in blood samples from eight renal transplant recipients.

Statistical Analysis

Analysis of the ranked data was by Brandt-Snedecor 4 × 2 contingency table analysis with the ranks −, +, ++ and +++ corresponding to the four rows of the contigency table. Using this technique, two frequency histograms could be compared to give a χ^2 with three degrees of freedom. Groups of numerical data were compared during Student's t test.

Results

T lymphocytes were the most numerous infiltrating cells in 84 of the 96 biopsies, and in 14 of the 22 nephrectomies. Monocytes were the next most numerous population of infiltrating cells, and were the most numerous cells in the infiltrate in seven nephrectomy and six of the biopsy specimens. B lymphocytes were observed in a minority of the biopsies and were never seen to comprise a major component of the infiltrate. T4 lymphocytes were the major T cell subset in five nephrectomy and 27 biopsy specimens. T8 lymphocytes were the major subset in six nephrectomy and 18 biopsy specimens. All other specimens had a similar frequency of T4 and T8 cells. OKT10 cells and B lymphocytes represented the minority of cells in all specimens. HLA-DR was identified on the majority of infiltrating cells and there was increased expression of HLA-DR on tubular cells during rejection.

T and B lymphocytes in infiltrates

T lymphocytes preponderated over B lymphocytes in all specimens. Even in severe cases of rejection, few B lymphocytes were present. The T cell infiltrate was widely scattered throughout the interstitium, between tubular cells and in clusters surrounding medium sized vessels. The extent of the infiltrate depended upon the severity of rejection, with only scattered foci of cells in the interstitium in cases of mild rejection. Foci of T cells were found around vessels and some glomeruli; these were often seen in mild to moderate rejection, when the interstitial infiltrate was sparse or patchy, as well as in severe rejection and nephrectomy specimens. In biopsies with moderate or severe rejection and nephrectomy specimens, T cells, but not B cells, were seen lining the intima and infiltrating the adventitia but were not in the media of medium-sized vessels.

B lymphocytes were found as focal infiltrates, usually associated with perivascular T lymphocyte infiltrates. B cells were infrequent, or absent in the interstitial infiltrate, between tubular cells, on the intima of vessels or in glomeruli. Many biopsies with severe rejection had virtually no B cell infiltrate.

Frequency histograms of the incidence of T and B lymphocytes are shown in Figure 1. Comparison of the CSA and AZA therapy groups with the same histological grade of rejection showed no significant difference between the two groups in the extent of infiltration with either T or B lymphocytes. Pooled results of the CSA and AZA data showed there was a significant increase in the extent of T lymphocyte infiltrate from minimal to moderate rejection ($P < 0.0005$) and from moderate to severe rejection ($P = 0.04$). There was no significant difference in the extent of T cell infiltration in nephrectomies compared to the severe biopsy group. T lymphocytes were observed infiltrating the glomerulus (Fig. 2) and there were increasing numbers of glomerular T lymphocytes with increasing severity of rejection

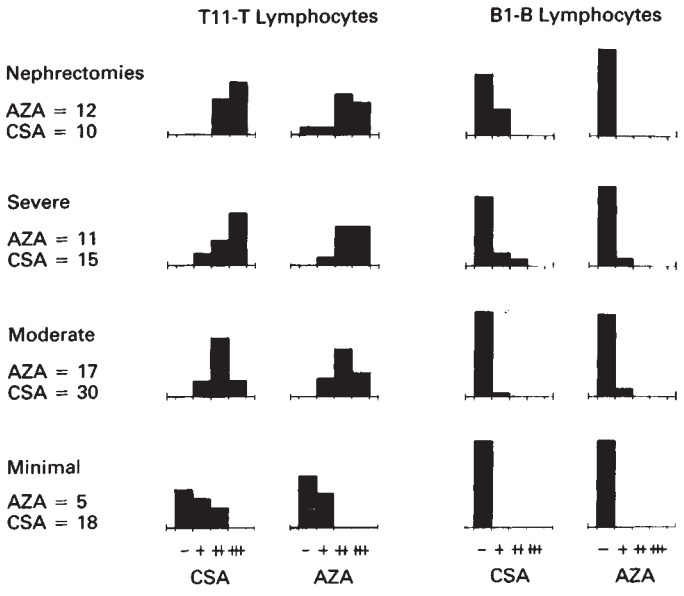

Fig. 1. *Histograms showing the number of specimens that had a different extent of infiltrate.* Data is shown for extent of infiltrate, identified by T11 or the B1 monoclonal antibodies. Results were divided so that patients treated with Cyclosporine (CSA) were compared to a combination of azathioprine, prednisone and antilymphocyte globulin (AZA). Extent of infiltrate is scored from − to +++ as described in **Materials and Methods.** T lymphocytes predominate in all specimens observed.

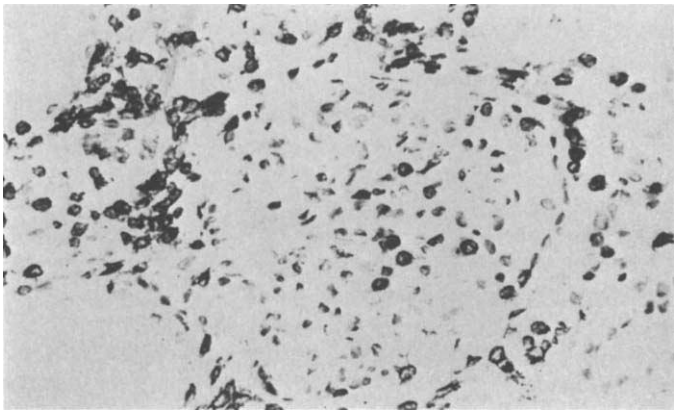

Fig. 2. *Photomicrograph of a renal allograft biopsy showing severe acute cellular rejection.* An indirect immunoperoxidase stain using monoclonal antibody T11 identifies T lymphocytes which have a dark rim of stain. T lymphocytes are seen infiltrating the glomerulus, as well as scattered in a diffuse periglomerular infiltrate.

(Fig. 3). There were significantly more glomerular T cells in biopsies with severe compared to moderate rejection (P = 0.002), and between nephrectomy and severe biopsies (P = 0.05). No B cells were found in glomeruli of transplant kidneys. No T or B cells were found in the glomeruli of control non-transplant kidneys.

Helper/inducer (T4) and cytotoxic/suppressor (T8) T lymphocyte subsets

Both T4 and T8 cells were present in large numbers in all biopsies with moderate and severe rejection. However, their

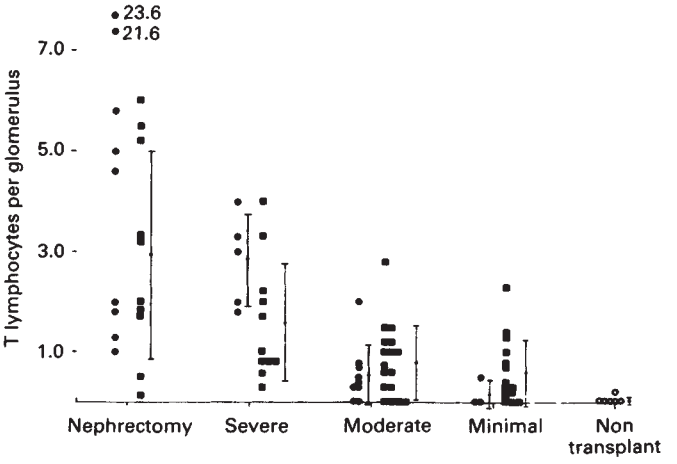

Fig. 3. *Intensity of glomerular T lymphocyte infiltrate with severity of rejection.* Glomerular T lymphocytes were counted and the average number of T cells in 10 glomeruli is shown. The extent of glomerular T cell infiltrate increases with severity of rejection. Symbols are: ■ CSA-treated patients; ● AZA-treated patients; ○ Non-transplant patients. Error bars show mean ± SD.

distribution in the infiltrate was quite different. T4 cells were concentrated in the focal infiltrates, often in a perivascular or periglomerular location. They were usually the minority of cells in the interstitium, and were less often found between tubular cells, in the glomeruli, or attached to the intima of vessels. In some biopsies, a diffuse staining with T4 was seen which could not be removed with repeated washing, and was present in sections that gave clear membrane staining with other reagents. This widespread staining may represent a release of the T4 epitope into the plasma. The T8 cells had a much more widespread distribution. They were the preponderant mononuclear cells in the interstitium, between tubular cells, in glomeruli and on the intima of vessels, but were the minority in focal cellular infiltrates.

Frequency histograms of the extent of infiltration with T lymphocyte subsets is shown in Figure 4. There was an increase in the infiltrate of both T4 and T8 cells with increasing severity of rejection. Comparison of the AZA and CSA treatment groups with similar histological grades of rejection showed a preponderance of T4 compared to T8 only in biopsies with moderate rejection in the CSA treated patients. In neither treatment group did T8 predominate over T4. No difference in the incidence of either subset was seen in the AZA or the other CSA biopsy groups. No significant differences in the infiltrate of T4 compared to T8 cells was observed when the data from both treatment groups were pooled.

In biopsies with moderate or severe rejection, both T4 and T8 cells were found in glomeruli and along the intima of vessels. In the glomeruli, T4 cells predominated over T8 in only five of the 49 biopsies that had a T cell infiltrate, compared to the predominance of T8 over T4 in 19 biopsies. Both cell types were found in the capillary loops and mesangium of the glomeruli.

Relationship of T4, T8 ratios in blood and graft

Thirty-three biopsies with moderate or severe rejection had peripheral blood T4:T8 ratios performed on the day of biopsy. The blood ratio showed no correlation with the relative pre-

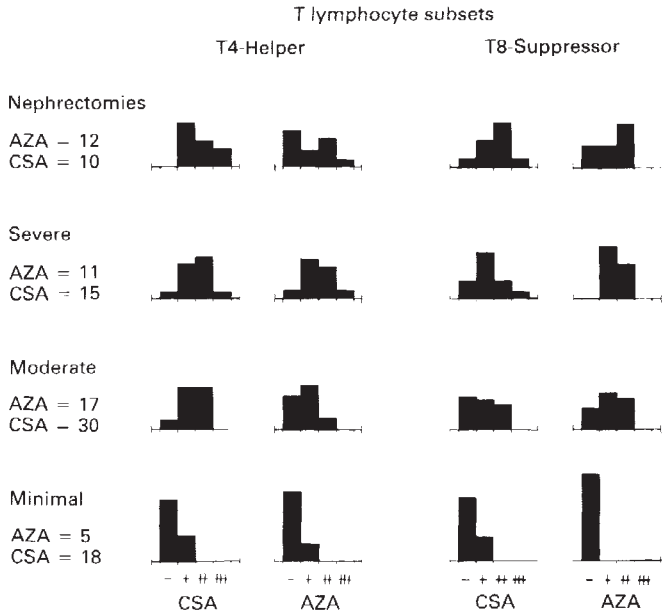

Fig. 4. *Histograms showing the number of specimens that had a different extent of infiltrate.* Data compares T4 and T8 monoclonal antibodies and patients treated with CSA or AZA. Extent of infiltrate is scored from − to +++ as described in Materials and Methods. With comparable severity of rejection, a similar extent of infiltrate for each subset was observed, except when biopsies from CSA patients with moderate rejection were observed. These biopsies showed significantly greater T4 cells than T8 cells ($P = 0.05$, Brandt-Snedecor contingency table analysis).

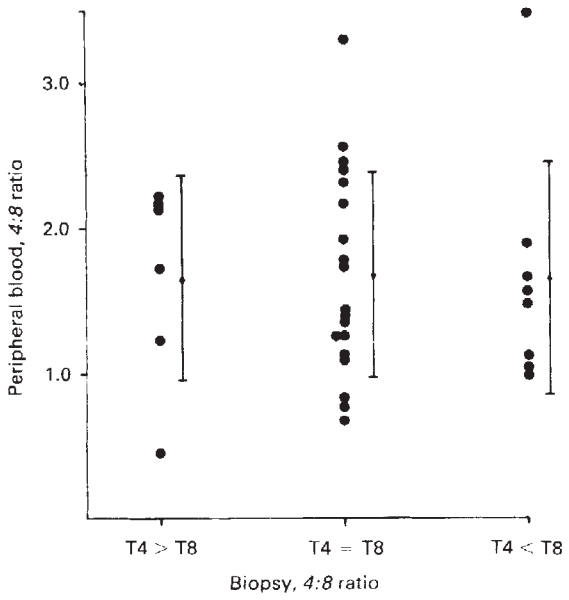

Fig. 5. *Comparison of the ratio of T4 lymphocytes to T8 lymphocytes in blood and graft with all samples taken on the same day.* Blood T4:T8 ratios are grouped according to whether the corresponding biopsy had a preponderance of the T4 lymphocytes. T4 and T8 lymphocytes were present in equal numbers; T8 lymphocytes were preponderant. No significant difference was seen in the blood T4:T8 ratio between any of the groups, indicating that blood T4:T8 ratio does not reflect graft T4:T8 ratio.

dominance of T4 or T8 in the rejecting graft, in that the blood T4:T8 ratio exceeded one for seven of eight biopsies in which T8 cells were present in greater numbers than T4, and for 16 of 19 where the frequency of T4 and T8 in the biopsy was similar (Fig. 5). Thus there appeared to be a relatively greater influx of T8 than T4 cells from blood into the graft in the majority of cases. The blood T4-T8 ratio exceeded one in five of the six biopsies where T4 predominated over T8.

Relationship of T4 and T8 infiltrate to HLA match

If T4 cells act against class II MHC antigens and T8 against class I MHC antigens, differences in the infiltrate may be expected in grafts matched for one class of MHC antigens and not the other. In 26 biopsies, with severe or moderate rejection from 19 patients with no HLA-DR mismatches but one or more HLA-A or B mismatches, there was an infiltrate of T4 in 23 and of T8 in 20 specimens. In the six biopsies from five patients with no mismatches for HLA-A B, there was a T4 infiltrate in five, and a T8 infiltrate in two. The latter two also had no HLA-DR mismatch. These results showed that no significant preponderance of one subset of T cells was demonstrable in biopsies from grafts with different HLA matches. There were also no significant differences in the pattern of infiltrate of T cells, B cells, OKT10 cells or monocytes with different HLA mismatches.

Lymphocyte activation markers

In an attempt to assess the proportion of the mononuclear cell infiltrate which was activated, two monoclonal antibodies were used. I2, which recognizes HLA-DR, reacts with T cells when they are activated, and also with normal B cells, monocytes and macrophages. I2 not only stained the majority of infiltrating mononuclear cells, including nearly all the cells in foci, but also stained renal tubular cells in rejection. Comparison of the AZA and CSA treatment groups showed no difference in the degree of expression of HLA-DR on infiltrating mononuclear cells. When AZA and CSA data were pooled, there was a significant increase in interstitial HLA-DR from minimal to moderate ($P < 0.001$), and from moderate to severe ($P = 0.05$), but there was no increase from severe biopsies compared to nephrectomy specimens (Table 3).

OKT10 identified a proportion of, but not all activated T cells, plasma cells and null cells. OKT10-staining lymphocytes were found in small foci of five to ten cells which were scattered in the interstitium. They were not found in the large foci, in vessel walls or in the glomeruli. There was no difference in the incidence of OKT10 when the AZA and CSA treatment groups were compared. Analysis of pooled AZA and CSA data showed a significant increase in OKT10 lymphocytes from minimal to moderate ($P = 0.05$) and an upward trend in the nephrectomy compared to the severe biopsy group ($P = 0.08$). However, many biopsies with moderate and severe rejection or nephrectomy specimens had no infiltrate of OKT10 cells (Table 3).

Monocyte infiltrate

Monocytes were defined by the monoclonal antibody OKM1, which reacts with all peripheral blood monocytes [23] but which does not identify all infiltrating monocytes and macrophages [17]. Monocytes were found to be scattered through the interstitium, in glomeruli and the intima of vessels. When the CSA and AZA treatment groups were analyzed separately, in the

Table 3. Infiltrate in kidney allografts

Severity of rejection	Total	OKM1$^+$ (%)					Type of cellular infiltrate OKT10$^+$, %					Interstitial I2$^+$, %				
		−	+	++	+++	*P*	−	+	++	+++	*P*	−	+	++	+++	*P*
Nephrectomy	22	27	32	9	32		41	32	27	0		0	19	33	48	
						0.025					0.08					NSD
Severe	26	58	17	21	4		71	9	5	5		0	21	62	17	
						NSD					NSD					0.05
Moderate	47	60	30	10	0		73	22	5	0		9	45	32	14	
						0.05					0.05					0.001
Minimal	23	90	0	10	0		100	0	0	0		65	31	4	0	

Percentage of specimens in each rejection category, showing extent of cellular infiltrate graded from − (minimal evidence of infiltrate) to +++ (extensive infiltrate). Biopsies were graded as minimal, moderate or severe rejection on the basis of conventional histologic criteria. Cell populations examined were monocytes (OKM1), activated lymphocytes (OKT10) and HLA-DR positive interstitial influence (interstitial I_2). The percentage of specimens showing moderate or heavy infiltrate increased with severity of rejection.

Table 4. Comparison of infiltrates of T4$^+$ and T8$^+$ cells on the basis of subsequent response of rejection to corticosteroid therapy

Severity of rejection	Response to cortico-steroids	Total	T4$^+$, %				Significance *P*	T8$^+$, %			
			−	+	++	+++		−	+	++	+++
Severe	Yes	7	0	29	42	29	0.09	57	29	14	0
							0.09 0.01				
	No	18	11	50	39	0	0.5	0	56	33	11
Moderate + Severe	Yes	33	12	48	33	7	0.15	30	33	37	0
							0.6 0.07				
	No	21	20	47	33	0	0.4	5	52	33	10

AZA group but not the CSA, there was a significant increase in the incidence of monocytes in nephrectomies compared to the severe rejection group ($P = 0.04$). Pooled data on CSA and AZA (Table 3) showed there was a significant increase in the nephrectomized group compared to the severe biopsy group ($P = 0.025$), which was due mainly to the increase in the AZA treatment group. There were further increases from minimal to moderate and to severe. The increase in monocytes from minimal to moderate was the only significant difference ($P = 0.05$).

Difference in biopsies which did or did not respond to rejection treatment with corticosteroids

Results of biopsies graded as moderate or severe from patients who had been given corticosteroid therapy immediately subsequent to biopsy, were grouped according to whether they had responded to therapy with return to stable renal function ($N = 35$), or whether they had not responded to therapy ($N = 21$). There was a tendency for T8 lymphocytes to be more numerous in biopsies from patients who did not respond to therapy ($P = 0.07$). Analysis of severe rejection biopsies alone showed a significant ($P = 0.01$) increase in T8 lymphocytes in those patients who did not respond to treatment ($N = 18$), compared to those patients whose rejection did respond to treatment ($N = 7$) (Table 4). There was a corresponding trend to a lesser degree of T4 lymphocyte infiltrate in patients who did not respond to treatment, compared to responders ($P = 0.09$) (Table 4). When the severe and moderate groups were pooled and the T4 and T8 subsets were compared in each patient, it was found that the T4 subset predominated in 12 of 35 responders, whereas the T8 subset predominated in only six. In the patients with non-response to rejection treatment, T8 predominated in seven of 21 patients, whereas the T4 predominated in five patients. Although there is an overlap between groups, the data shows that if there is no T8$^+$ cell infiltrate, the rejection should respond to corticosteroids. If there is intense (+++) T8$^+$ cell infiltrate, there is a high chance that rejection will not respond to corticosteroid therapy. Glomerular T cells in biopsies from patients who responded to therapy ($N = 21$) showed significantly fewer glomerular T cells than those who did not respond ($N = 13$) ($P = 0.05$, Fig. 6).

Effect of time after transplant on mononuclear cell infiltrate

Biopsy and nephrectomy findings were grouped into three classes according to time elapsed since transplant: early, within two weeks of transplant ($N = 29$); mid, 15 days to 13 weeks post transplant ($N = 50$); or late, more than 13 weeks post transplant ($N = 38$). The only significant differences were the presence of more T lymphocytes ($P = 0.001$) and OKT10 cells ($P = 0.03$) in the mid group than the early biopsies. However, these differences were not significant when the biopsies with only moderate and severe rejection were analyzed. In early rejection episodes, T4 cells exceeded T8 cells in nine biopsies, whereas T8 cells exceeded T4 cells in four biopsies. In the mid-group T4 cells were the minority in 12 biopsies and the majority of the infiltrate in 10 biopsies. The pattern of T4 cells versus T8 cells in the late group was similar to the early group. There were more T8 lymphocytes in the mid-group than the early group ($P = 0.03$), but not the late group.

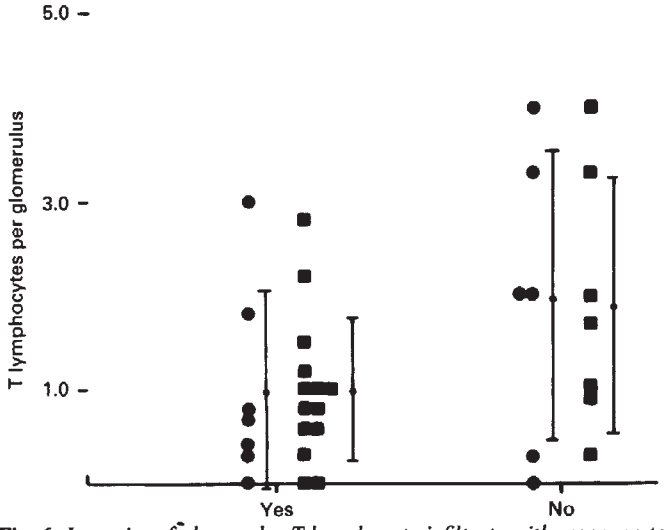

Fig. 6. *Intensity of glomerular T lymphocyte infiltrate with response to steroid therapy.* Pooled data for AZA and CSA showed a significant increase in the number of glomerular T lymphocytes in those patients who did not respond to steroid therapy (Student's t test). Symbols are: ■ CSA-treated patients; and, ● AZA-treated patients.

Change in infiltrate from biopsy to nephrectomy

Eleven of the 22 nephrectomy specimens had had a biopsy in the preceding four weeks. The only significant difference between these paired samples was that monocytes were more frequent in the nephrectomy specimens ($P = 0.03$).

Expression of HLA-DR by renal tubular cells

The extent of expression of HLA-DR by tubular cells increased with the severity of rejection (Table 5). The severe rejection biopsies had more HLA-DR antigen expression than the moderate rejection biopsies ($P = 0.04$) and the moderate biopsies showed more than the minimal ($P = 0.003$). However, nephrectomies did not show significantly greater staining for tubular HLA-DR than did the biopsies with severe rejection. There was increased expression of HLA-DR on tubular cells with increasing severity of rejection in both CSA and AZA treatment groups. The biopsies with moderate rejection in the CSA treatment patients showed significantly less staining than AZA with moderate rejection ($P = 0.025$). To determine whether the lesser expression of tubular HLA-DR in moderate CSA biopsies was due to biopsies having CSA nephrotoxicity, rather than rejection, we divided these biopsies into those whose clinical course suggested that they had concomitant CSA nephrotoxicity ($N = 11$) and those who had no evidence of nephrotoxicity ($N = 5$). There was no difference in expression of HLA-DR on the tubular cells ($P = 0.4$). Biopsies with severe and moderate rejection showed no difference in the degree of HLA-DR expression between groups with or those without HLA-DR incompatibilities. In all but two of the 59 specimens with marked HLA-DR expression (+++ or ++) on tubular cells there was an associated interstitial T cell infiltrate. These two included a patient with chronic vascular rejection and another who had been successfully treated for acute cellular rejection five days prior to biopsy. A significant T4 and T8 infiltrate was also present in the majority of biopsies, with marked HLA-DR expression on tubular cells. However, 13 of the 59 biopsies had an infiltrate of only T4 and 9 of 59 only had T8.

Discussion

These studies confirm the heterogeneous composition of the mononuclear cell infiltrate in rejecting allografts which has been previously described in animal models, as well as in clinical organ transplant material [4–12]. Our studies also confirm that T cells are the principal infiltrating cell [4–13], and that there is also a large monocytic cell accumulation in rejecting tissue [7, 31]. The intensity of the infiltrate of these two populations increases with increasing severity of rejection and is greatest in nephrectomy specimens with irreversible rejection. These findings parallel the standard histopathological analysis in which a poor prognosis is determined by the intensity of the cellular infiltrate, the presence of interstitial hemorrhage, vascular rejection and glomerulitis [1–3]. Our studies confirm that although severe irreversible rejection usually has an intense interstitial infiltrate, this feature is also seen in kidneys with rejection that is reversible with corticosteroid treatment. We observed that in irreversible rejection, the infiltrate tended to have a predominance of T8 over T4 cells compared to biopsies with reversible rejection that had a predominance of T4 cells. In the mild and moderate rejection episodes and those occurring in the first two weeks after transplant, which were easily reversed with corticosteroid therapy, the cellular infiltrate was concentrated around vessels, rather than in the interstitium, and that this infiltrate was predominantly T4. This finding concurs with that of Tufveson et al [32] in nephrectomy transplant specimens who also reported that T4 predominates in the mononuclear cell infiltrate and is concentrated around vessels. However, our findings do not support Von Willebrand's finding that their patients who had fine needle aspirate biopsies in which T4 predominated did not respond to corticosteroids [33]. This may be because aspirate samples do not totally reflect the diffuse infiltrate in the graft. In severe rejection which did not respond to steroids, and rejection occurring later than two weeks after transplant, the perivascular infiltrate of T4 cells was found, but in addition there was an interstitial infiltrate in which T8 cells predominated in a small series of biopsies. Severe and corticosteroid resistant rejection was also accompanied by infiltrates of T cells in glomeruli and along vascular endothelium. Both rejection glomerulitis and vascular rejection have been assumed to be humoral, rather than cell mediated [1, 2]. The finding of T cells in these sites raises the possibilty that T cell mediated effector mechanisms also contribute to these changes. Both T4 and T8 cells were found in the vascular interstitium and in glomeruli, where they were localized in the glomerular capillary walls, as well as the mesangium. Glomerular T cell infiltrates increased with the severity of rejection, suggesting that they may simply be a manifestation of a more widespread infiltrate of all areas of the allograft. We propose that a widespread interstitial infiltrate of T8 cells and glomerular T cell infiltrate are poor prognostic signs, which could be used to complement the classical light microscopic changes in the establishment of a poor prognosis and response to corticosteroid therapy. It remains to be evaluated whether the use of these parameters to identify patients with a high risk of failing to respond to

Table 5. HLA-DR expression on renal tubular cells

Severity of rejection	CSA treated intensity 12, %					CSA vs. AZA significance	AZA treated intensity 12, %					Significance *P*
	Total	−	+	++	+++		Total	−	+	++	+++	
Nephrectomy	10	0	10%	10%	80%	NSD	11	0	0	18%	82%	0.6
Severe	15	0	13%	27%	60%	NSD	10	0	0	20%	80%	0.03
Moderate	28	14%	36%	28%	21%	0.025	16	0	6%	38%	56%	0.003
Minimal	18	50%	33%	11%	6%	NSD	5	40%	0	20%	40%	

HLA-DR antigen expression by renal tubular cells in biopsies and nephrectomies of patients treated with CSA, compared to patients treated with AZA. Extent of HLA-DR expression by tubular cells is rated from − (no expression) to +++ (most tubular cells express HLA-DR). Percentage of specimens at each level of expression is shown. Extent of HLA-DR expression for pooled CSA and AZA results showed significant increases with increasing severity of rejection. Comparison of CSA and AZA groups showed no difference in tubular HLA-DR antigen expression with minimal or with severe rejection, but a significant reduction in the CSA group with moderate rejection.

corticosteroid therapy can be used to institute alternate anti-rejection treatment.

These studies did not help to resolve the controversy over the relative roles played by cytotoxic T cells and T cells of the helper/inducer subclass in the mediation of rejection [6, 8–13]. In vitro models of the alloimmune response identified the cytotoxic T cell as the principal effector T cell [34, 35]. However, adoptive transfer experiments in rodents have contradicted the in vitro models of the alloimmune response by demonstrating that helper/inducer cells, rather than cytotoxic T cells, effect graft rejection [8–10]. More recent adoptive transfer studies have confirmed that cytotoxic T cells can also effect rejection in vivo [11, 12]. Whatever the relative importance of these two responses in the mediation of graft destruction, our studies show that during rejection, a large number of cells of both phenotypes are present within the graft, thus leaving open the possibility that tissue destruction is mediated by effector responses from both subsets of cells. The T8 subpopulation increases in rejection episodes that occur later than two weeks compared to immediate post-transplant episodes. Comparison of blood T4:T8 ratio to the corresponding T4:T8 ratio in the graft suggests there is usually a relatively greater influx of T8 cells. The preponderance of T8 cells in severe rejection episodes appears to be a poor prognostic sign and this may be due to T8 mediated responses being more resistant to the effects of corticosteroids than T4 mediated responses. Support for this possibility is the observation that corticosteroids have a greater effect on the number of T helper cells than cytotoxic suppressor cells in systemic lupus erythematosus (SLE) patients [36] and in CSA treated renal transplant patients (Hall et al, unpublished data).

The mechanism of action of corticosteroids on the T8 and T4 mediated rejection response is not precisely defined, although it is known that corticosteroids cause lymphocytopenia and monocytopenia by inhibiting cell migration and inhibiting release of lymphokines from activated T cells [37], and as a consequence have a profound effect on delayed-type hypersensitivity cellular responses. Corticosteroids do inhibit the generation and cytolytic capacity of cytotoxic T cells in vitro [37, 38] and thus would be expected to have some action on cytotoxic cells in vivo. However, it cannot be excluded that cells already infiltrating the graft are refractory to the effect of corticosteroids and that their sole effect is to inhibit migration of more mononuclear cells into the graft. It has also been reported that some T cell functions are relatively corticosteroid-resistant in some patients and not others [39], and a similar variation in responsiveness may occur for T4 and T8 mediated rejection.

Our studies suggest that the identification of a diffuse and intense T cell infiltrate in which T8 cells predominate, and which is associated with a glomerular T cell infiltrate, may indicate a need for alternate rejection therapy. At present this may be antithymocyte globulin. However, the use of monoclonal antibodies with reactivity for one T cell subclass, such as the T8 cells, also needs to be assessed.

Our observations failed to show that T4 and T8 cells react against different HLA antigen classes in that neither was the sole infiltrating subset in grafts mismatched for only Class I or Class II MHC antigens. In the six grafts examined from patients with no incompatibility for HLA A and B, the four that had HLA-DR incompatibility only had T4 cells in the infiltrate, which would be consistent with a T4 mediated response against HLA-DR. The other two patients had no mismatches for HLA-DR and these were the biopsies which had T8 cells in the infiltrate, which would be consistent with the observation in rodents that responses to non-MHC transplantation antigens are usually mediated by cytotoxic T cells, rather than delayed-type hypersensitivity responses [15]. A large number of cases with no class I MHC incompatibilities need to be examined to see if these trends are real.

In this study T4 cells often predominated in the infiltrate during rejection and were always a major component of this infiltrate. This differs from previous studies of renal transplant rejection in which the T8 subpopulation was found to predominate [16–18]. Part of the explanation for these differences may be the different monoclonal antibodies used to identify the helper/inducer subset. In this report T4 was used, which is known to stain some monocytes and macrophages as well as the helper/inducer subclass of T cells [40]. In the reports of Platt et al [16] and Hancock et al [17, 18] the OKT4 reagent was used, and this reagent has been shown to give less intense staining of both the T cell subset and monocytes [19, 32]. The finding of large numbers of T4 and T8 cells as well as monocytes and macrophages during rejection episodes identifies all elements required for the mediation of graft damage by delayed type hypersensitivity and direct T cell cytotoxicity responses. Whether these subpopulations include activated effector cells, and the cells' specific reactivity against graft alloantigens, has not been shown in these studies or other reported studies, and has been very difficult to determine with the currently available monoclonal antibodies. Thus the proportion of the infiltrate

which has specific reactivity, compared to that which represents a nonspecific component, cannot be assessed. OKT10 failed to stain any cells in the majority of specimens even in nephrectomies and biopsies with severe rejection, and thus was not found to be a suitable reagent with which to identify activated T cells. I2 stained nearly all infiltrating cells in severe rejection, as well as graft cells. This widespread expression of HLA-DR antigens by all cells within the graft may reflect the presence of lymphokines which indiscriminately induce expression of class II antigens on all cells within the graft, including infiltrating cells and renal tubular cells. Experiments have shown that cells with specificity not directed to the graft antigens do accumulate in rejecting tissue, and similar nonspecific accumulation of cells is likely to occur in clinical rejection episodes [40]. Alternatively, it may reflect the high proportion of activated T cells and monocytes/macrophages in the infiltrate. Distinguishing specific alloantigen reactive cells from cells with no specific reactivity is not feasible without monoclonal antibodies which react either against activated T lymphoblasts or idiotypes of alloreactive T cells.

These studies did demonstrate that there was no difference in the type of cellular infiltrate in patients immunosuppressed with Cyclosporine compared to those immunosuppressed with azathioprine, prednisone and antilymphocyte globulin, even though Cyclosporine has a mechanism of immunosuppression of the rejection response quite different from azathioprine and ALG [41]. The distinction of rejection from nephrotoxicity in CSA treated patients is often difficult, and we have previously described the criteria we use to distinguish rejection [2]. The only significant difference was the lower intensity of expression of HLA-DR antigens on tubular epithelial cells in the CSA treated patients compared to the AZA treated patients with moderate rejection. This difference could not be accounted for on clinical outcome by CSA toxicity being confused with rejection. There was also no difference in the intensity of T cell infiltrate in the CSA biopsies. It is known that induction of the HLA-DR antigens on epithelial and endothelial cells is dependent upon gamma interferon released by activated T cells [42–44] and CSA has been shown to inhibit T cell production of this lymphokine [45]. The smaller increase in expression of HLA-DR antigens in the CSA treated patients may be due to the effect CSA has on inhibiting release of lymphokines by activated T cells [45].

The demonstration that tubular cells express class II MHC antigens during rejection has several important implications. First, T4 mediated responses are thought to be restricted to act against cells carrying class II antigens [13, 14]. Thus in normal renal tissue their potential targets would be restricted to endothelial cells, dendritic cells and some mesangial cells. Because renal tubular cells acquire class II MHC antigens during rejection, they also would be vulnerable to T4 effector responses. Even in grafts where there is no HLA-DR incompatibility, there is increased expression of class II antigens. In these circumstances the T4 response may be acting against minor transplantation antigens, but to be effective the target cells must also express the relevant class II antigen. It would appear from these studies that expression of HLA-DR antigens is an index of immune inflammation in the graft rather than HLA incompatibility, in the same way as self tissue expresses class II antigens in cell mediated immune responses such as primary biliary cirrhosis and thyroiditis [43, 44]. A consequence of this increased expression of class II antigens is that many cells in the graft may acquire the capacity to act as antigen presenting cells, and thus initiate a rejection response. Such changes make attempts to reduce the immunogenicity of a graft by removal of passenger leucocytes futile [46]. Whether or not increased expression of class II MHC precedes or is a prerequisite of T cell mediated cell destruction remains to be answered.

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Reprint requests to Dr. Bruce M. Hall, Department of Renal Medicine, Royal Prince Alfred Hospital, Missenden Road, Camperdown 2050, N.S.W. Australia

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