

T Cell Requirements for the Rejection of Renal Allografts Bearing an Isolated Class I MHC Disparity

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Summary

This study has examined the cellular and humoral responses underlying the rejection of rat renal allografts bearing an isolated RT1A^a class I MHC disparity. RT1A^a disparate kidneys were rejected promptly by high responder RT1^u but not by low responder RT1^c recipients (median survival time 10 d and >100 d, respectively). The magnitude and phenotype of the cellular infiltrate were similar in rejecting and nonrejecting RT1A^a disparate kidneys. Paradoxically, graft infiltrating cells and spleen cells from RT1^u recipients showed minimal ability to lyse donor strain lymphoblasts in vitro, whereas effector cells from RT1^c recipients showed modest levels of cytotoxicity. Injection of RT1^u rats with MRC OX8 mAb was highly effective at selectively depleting CD8⁺ cells from graft recipients but had no effect in prolonging the survival of RT1A^a disparate grafts despite the complete absence of CD8⁺ cells from the graft infiltrate, which included numerous CD4⁺ T cells and macrophages. RT1^u, but not RT1^c, recipients mounted a strong alloantibody response against RT1A^a disparate kidneys. Immune serum obtained from RT1^u recipients that had rejected a RT1A^a disparate graft was able, when injected into cyclosporin-treated RT1^u recipients, to restore their ability to reject a RT1A^a, but not a third-party RT1^c, kidney. These results suggest that CD8⁺ cells in general and CD8⁺ cytotoxic effector cells in particular are unnecessary for the rapid rejection of RT1A^a class I disparate kidney grafts by high responder RT1^u recipients. By implication, CD4⁺ T cells alone are sufficient to cause prompt rejection of such grafts and they may do so by providing T cell help for the generation of alloantibody.

Studies of the cellular response to allografts expressing isolated class I or class II MHC disparities have contributed substantially to current understanding of the relative roles of CD4⁺ and CD8⁺ T cells in graft rejection (1-7). Adoptive transfer experiments in T cell-depleted mice bearing mutant class I or class II disparate skin grafts have shown that purified Lyt-2⁺ (CD8⁺) but not L3T4⁺ (CD4⁺) cells initiate rejection of class I disparate skin grafts, whereas L3T4⁺ but not Lyt-2⁺ cells initiate rejection of class II disparate grafts (1-3). The contribution of CD8⁺ T cells to the rejection of mouse skin grafts bearing mutant or allelic class I disparities has been confirmed by the demonstration that treatment with anti-Lyt-2 to selectively deplete CD8⁺ T cells prolongs graft survival (4, 6). Although selective depletion of CD4⁺ T cells using anti-L3T4 does not prolong the survival of skin grafts bearing an isolated class I disparity, there is evidence that in some mouse strain combinations, CD4⁺ T cells may also participate in the rejection of such grafts (6).

Most reports on the cellular effector response to isolated class I disparate tissue relate to skin graft models in the mouse.

We chose to examine the immunological response to rat kidney allografts differing at an isolated class I MHC antigen because it is likely that there are important differences in the nature of the rejection response between indirectly vascularized skin and directly vascularized organ grafts. In addition, the rat renal allograft model enabled us to make a detailed analysis of the phenotype and in vitro cytotoxic activity of cells infiltrating the grafts. The rejection of allografts bearing the genetically isolated RT1A^a class I antigen is under strict Ir gene control; the PVG RT1^c and PVG RT1^u strains are low and high responders, respectively (8, 9). Using the appropriate intra-MHC recombinant rat strains as kidney donors we were therefore able to compare the cellular and humoral response with rejecting and nonrejecting class I RT1A^a kidney grafts.

Materials and Methods

Animals. The PVG congenic and recombinant rat strains used are shown in Table 1 together with their MHC haplotypes. Animals

Table 1. MHC Haplotype of PVG Congenic and Recombinant Rat Strains

Rat strain	Haplotype	RT1 regions			Reference
		A	B/D	C	
PVG RT1 ^c	c	c	c	c	—
PVG RT1 ^a	a	a	a	a	10
PVG RT1 ^u	u	u	u	u	10
PVG R1	r1	a	c	c	11
PVG R8	r8	a	u	u	12

Animals are subsequently referred to by their haplotype name alone.

were obtained from Harlan Olac Ltd. (Bicester, Oxon, UK). Male rats (8–16 wk old) were used throughout.

Kidney Transplantation. Kidneys were transplanted into the left orthotopic site with end-to-end anastomosis of the renal artery, renal vein, and ureter (13). The procedure was performed under chloral hydrate anesthesia and ischemic times were ~25 min. For graft survival studies, the recipient's right kidney was excised 7 d after transplantation so that the continued survival of the transplanted animal was dependent on the function of the renal graft. Graft function was also monitored by performing sequential serum urea and creatinine measurements.

Cyclosporin A Treatment. Rats received 15 mg/kg of cyclosporin (a gift from Sandoz Pharmaceuticals), dissolved in olive oil, via a gastric tube on the day of transplantation, and then daily for the next 13 d.

Antibodies. The following mouse mAbs were used to label rat leukocytes: MRC OX1 (leukocyte common antigen [14]); MRC OX8 (CD8⁺ T cytotoxic/suppressor lymphocytes and NK cells [15]); W3/25 (CD4⁺ T helper lymphocytes and some macrophages [15]); MRC OX12 (rat Ig κ chains on B lymphocytes [16]); MRC OX19 (CD5 determinant on T lymphocytes and thymocytes [15]); and MRC OX21 (human C3b inactivator [17]) was used as a negative control. These mAbs were kindly provided by Dr. D.W. Mason and Prof. A.F. Williams (MRC Cellular Immunology Unit, Sir William Dunn School of Pathology, Oxford, UK). The mouse mAbs ED1, which labels most tissue macrophages, monocytes, and dendritic cells (18); R73, which recognizes a constant determinant of the rat TCR- α/β (19); and MRC OX18 and MRC OX6, which recognize polymorphic determinants of MHC class I and class II antigens, respectively (20), were obtained from Serotec Ltd., Oxford, UK.

Production and In Vivo Treatment with mAb. Hybridoma cells secreting the antibodies MRC OX8 and MRC OX21 (a kind gift of Prof. A.F. Williams and Dr. D.W. Mason) were grown in tissue culture medium and injected intraperitoneally into pristane-primed (DBA/2 \times BALB/c)F₁ mice (Harlan Olac Ltd.) to produce ascites. The immunoglobulin content of ascites was quantified using anti-mouse IgG1 immunodiffusion plates and known mouse Ig standards (Serotec Ltd.). Antibodies were diluted in PBS to 2 mg/ml and stored at -20°C. They were administered intraperitoneally to recipient rats according to the following schedule: 3 mg on day -1, 2 mg on day 0 (day of transplant), and 1 mg on days 3, 6, and 9.

Cell Preparation and Fluorescence Analysis. Single cell suspensions of lymph nodes and spleen were prepared as described previously (21). PBL were separated by centrifugation of heparinized whole blood over 75% iso-osmolar Percoll (Sigma Ltd., Poole, UK). For

single-color fluorescence analysis, cells were incubated with the appropriate mAb for 1 h at 4°C, washed twice in Dulbecco's A+B buffer (DAB)/0.2% BSA, and incubated for 1 h at 4°C with FITC-conjugated F(ab')₂ rabbit anti-mouse Ig (Dako Ltd., High Wycombe, UK) containing 10% normal rat serum to prevent cross-reaction of the antibody with rat Ig. For two-color fluorescence analysis, the first stage was as described above. For the second stage, unbound sites of the second antibody were blocked with MRC OX21 (mouse anti-human C3b) and the cells were incubated for 30 min at 4°C with biotinylated W3/25 or biotinylated MRC OX8 (a gift from Dr. E. Bell, Department of Immunology, University of Manchester, UK) followed by a phycoerythrin-streptavidin complex (Becton Dickinson, Mountain View, CA). Cells were washed twice in (DAB)/0.2% BSA to remove excess antibodies. The labeled cells were analyzed on a FACScan flow cytometer (Becton Dickinson).

Immunohistology. Kidney tissue was snap frozen in liquid nitrogen and cryostat sections (5 μ m) were cut at -20°C onto gelatinized slides. A range of mAbs was used to label the slides using an indirect immunoperoxidase technique (22). A portion of each kidney was also fixed in formalin, embedded in paraffin wax, sectioned and stained with hematoxylin and eosin.

Morphometric Analysis of Cellular Infiltrate. The area of each immunoperoxidase-labeled tissue section infiltrated by leukocytes of a particular phenotype was determined by morphometric analysis using the point-counting technique as previously described (23). Sections were examined at a magnification of \times 400 in the presence of a microscope eyepiece graticule bearing a squared grid with 745 intersections. For each of 10 adjacent high power fields, the number of positively stained cells superimposed by an intersection was counted and the percentage area of each section occupied by cells of a particular phenotype was calculated as: $100 \times [(\text{number of positive cells under grid intersections}) / (\text{total number of grid intersections})]$.

Harvesting of Graft Infiltrating Cells. Graft infiltrating cells (GIC)¹ were harvested from kidney allografts by a nonenzymatic technique as described previously (21). In brief, the freshly excised kidney allografts were finely diced, passed through a fine stainless steel mesh and the mononuclear cells separated from the resulting cell suspension by Percoll density gradient centrifugation.

Cell-mediated Cytotoxicity Assays. GIC and spleen cells from transplanted recipients were tested for alloantigen-specific cytotoxicity and nonspecific cytotoxicity using a standard 6-h ⁵¹Cr-release assay essentially as described elsewhere (24). Alloantigen-specific cytotoxicity was assessed using ⁵¹Cr-labeled kidney donor strain and third-party Con A-transformed splenic blasts as targets. The mouse lymphoma line YAC-1 (which is susceptible to NK cell-mediated lysis) was also used as a target. Specific ⁵¹Cr release was calculated by the formula: percent specific release = $100 \times [(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})]$. Data shown are the means of triplicate determinations (spontaneous release <25% of maximum release in all experiments).

Radioimmunoassay for Anti-RT1A^a Antibodies. Antibodies against RT1A^a class I antigens were detected by a two-stage binding assay using donor strain erythrocytes and radiolabeled sheep anti-rat Ig (25). Serial dilutions of test sera in DAB/2% FCS were added, in duplicate 50- μ l aliquots, to the wells of 96-well U-bottomed microtiter plates (Flow Laboratories, Rickmansworth, UK) that had been pretreated with 2% BSA/DAB (to prevent nonspecific binding of Ig). To each well, 50 μ l of a 2% suspension of washed

¹ Abbreviations used in this paper: DAB, Dulbecco's A+B buffer; GIC, graft infiltrating cells; MST, median survival time.

Table 2. Rejection of Rat Renal Allografts Bearing Isolated MHC Subregion Disparities

Group	Donor	Recipient	Incompatibility	n*	Recipient survival [‡]	MST [§]
					<i>d</i>	<i>d</i>
1	r8	u	A ^a	6	10, 10, 10, 10, 10, 11	10
2	r1	c	A ^a	6	All > 100	>100
3	a	c	A ^a B/D ^a C ^a	5	10, 10, 10, 10, 17	10

* Number of animals in group.

† Contralateral nephrectomy performed on day 7.

§ Median survival time.

rat erythrocytes was added and the plates were incubated for 1 h at room temperature and then washed four times in DAB/FCS. Next, 100 μ l ¹²⁵I-conjugated F(ab')₂ sheep antibodies against rat Ig (Amersham International, Amersham, UK) was added (ensuring at least 50,000 cpm/well) and the plates were incubated for a further 1 h. The erythrocytes were then washed a further four times, transferred to tubes, and the cell bound radioactivity was counted.

Lymphocytotoxic Antibody Determinations. Test sera were incubated with ⁵¹Cr-labeled Con A-transformed splenic blast targets in the presence of guinea pig complement (Sera-Lab, Sussex, UK) or fresh rat serum in a cytotoxicity assay as follows. Serial dilutions of test sera in RPMI/10 mM Hepes/5% FCS were prepared in 96-well U-bottomed microtiter plates in duplicate aliquots of 50 μ l. ⁵¹Cr-labeled Con A-transformed splenic blasts at 10⁶ per ml in RPMI/Hepes/FCS were added to each well, and incubated at 37°C for 30 min. 100 μ l guinea pig complement (Sera-Lab) or fresh rat serum, appropriately diluted, were added and incubated for 1 h at 37°C. Plates were then centrifuged briefly, and 100- μ l aliquots of supernatant were transferred to tubes for counting released ⁵¹Cr. Specific ⁵¹Cr release was calculated by the formula: Percent specific release = 100 \times [(experimental release - spontaneous release)/(maximum release - spontaneous release)].

Results

Rejection of Renal Allografts Bearing an Isolated RT1A^a Class I MHC Disparity. Rejection of RT1A^a incompatible skin and organ grafts is under strict MHC-linked immune response gene control (8, 26, 27). The PVG RT1^u strain is a high responder to class I RT1A^a incompatible grafts from the PVG R8 donor whereas the PVG RT1^c strain is a low responder to RT1A^a incompatible grafts from PVG R1 animals. This was confirmed here for renal allografts (Table 2). R8 kidneys were rapidly rejected by RT1^u recipients, which died shortly after contralateral nephrectomy (MST 10 d) with markedly raised serum urea and creatinine levels. Grafts excised 5 d after transplantation already showed histological features of severe rejection, with widespread vascular damage and associated intravascular fibrin and platelet deposition. Focal tubular necrosis and ischemia of glomeruli were present and there was an interstitial mononuclear cell infiltrate. By day 7, extensive interstitial hemorrhage was apparent and grafts had frequently undergone complete infarction. In contrast, R1

Table 3. Magnitude and Phenotype of Cellular Infiltrate and Expression of MHC Antigens in Renal Allografts

Group	Donor	Recipient	Percentage area occupied by infiltrate*				Class II MHC expression [‡]	
			MRC OX1 (L-CA)	MRC OX8 (CD8)	W3/25 (CD4)	ED1 (M ϕ)	Renal tubules	Arteriolar endothelium
1	r8	u	16 \pm 10 (100%)	6 \pm 1 (38%)	2 \pm 1 (13%)	10 \pm 6 (63%)	++	+/-
2	r1	c	18 \pm 1 (100%)	7 \pm 2 (39%)	6 \pm 2 (33%)	9 \pm 1 (50%)	++	+/-
3	a	c	52 \pm 12 (100%)	16 \pm 11 (31%)	9 \pm 6 (17%)	23 \pm 8 (44%)	++	++
4	c	c	4 \pm 1	<1	3 \pm 1	<1	+	-

Kidneys were excised on day 5 after transplantation and cryostat sections were labeled using the immunoperoxidase technique.

* The percentage area infiltrate was determined by point counting with a microscope eye piece graticule. Values are mean \pm SD of five allografts. Results in brackets represent the phenotype as a percentage of the total area of the cellular infiltrate (i.e., percent of OX1 are an infiltrate).

† Class I and class II MHC expression was determined by labeling with MRC OX18 and MRC OX6, respectively. In all allografts the vascular endothelium and renal tubules were strongly class I positive. Staining for class II MHC antigens was as shown: -, no staining; +/-, very occasional weak staining; +, weak staining; ++, strong staining.

kidneys survived indefinitely in RT1^c recipients (MST >100 d) and serum urea and creatinine levels remained normal throughout. R1 grafts showed mononuclear cell infiltration but no evidence of renal parenchymal damage. The influence of Ir gene control on renal allograft survival was not apparent when an isolated A^a class I disparity was replaced by a full haplotype RT1^a MHC disparity since low responder RT1^c animals rapidly rejected RT1^a kidneys (MST 10 d).

Magnitude and Phenotype of Cellular Infiltrate in RT1A^a Disparate Grafts. The demonstration that class I RT1A^a incompatible kidneys were rapidly rejected by RT1^u recipients but not by RT1^c recipients led us to compare the intragraft cellular responses. Our first approach was to assess the magnitude and phenotype of the cellular infiltrate within the grafts. Cryostat sections of kidney allografts were labeled with a range of mouse anti-rat mAbs by an indirect immunoperoxidase technique and infiltrates assessed by morphometric analysis (Table 3). Both rejecting R8 grafts in RT1^u recipients and nonrejecting R1 grafts in RT1^c recipients showed a diffuse interstitial mononuclear cell infiltrate with perivascular mononuclear cell aggregates. The magnitude of cellular infiltration (determined by OX1) was similar in rejecting and nonrejecting grafts. The infiltrate in class I disparate grafts was fourfold greater than that observed in grafts between syngeneic animals, but substantially less than that found in rejecting grafts bearing a full haplotype RT1^a disparity. The phenotype of the cellular infiltrate in rejecting and nonrejecting RT1A^a disparate grafts was similar. CD8⁺ cells formed a large component of the total infiltrate but the predominant cell type was the macrophage (ED1⁺). CD4⁺ and CD8⁺ cells were distributed homogeneously within the infiltrate and there was no preferential localization of either phenotype to specific areas of the graft.

Cytotoxic Repertoire of GIC and Spleenocytes in Recipients with RT1A^a Disparate Grafts. Butcher and Howard showed that high responder RT1^u rats bearing RT1A^a incompatible skin grafts develop cytotoxic T cells in the draining lymph nodes and that generation of such cells is impaired in low responder RT1^c rats (8). We predicted, therefore, that GIC from rejecting RT1A^a disparate kidneys in RT1^u recipients would, when tested in vitro, demonstrate higher levels of specific anti-donor cytotoxic activity than cells obtained from nonrejecting RT1A^a grafts in RT1^c recipients, thereby reflecting a role for cytotoxic T cells in the rejection response of these class I disparate grafts. To test this, the cytotoxic activity of GIC and spleen cells from these animals was studied in 6-h ⁵¹Cr-release assays. The results of representative experiments are shown in Fig. 1. Unexpectedly, GIC obtained from rejecting RT1A^a incompatible R8 kidneys in high responder RT1^u recipients showed minimal ability to lyse Con A blasts of the kidney donor strain. Paradoxically, GIC from nonrejecting RT1A^a disparate R1 grafts in low responder RT1^c recipients often showed modest levels of cytotoxicity against donor strain lymphoblasts. However, this cytotoxicity was not entirely donor specific, since lysis of third-party Con A blasts was also apparent. As expected, full MHC disparate rejecting RT1^a grafts in RT1^c recipients were infiltrated by cells that demonstrated high levels of donor-specific in vitro

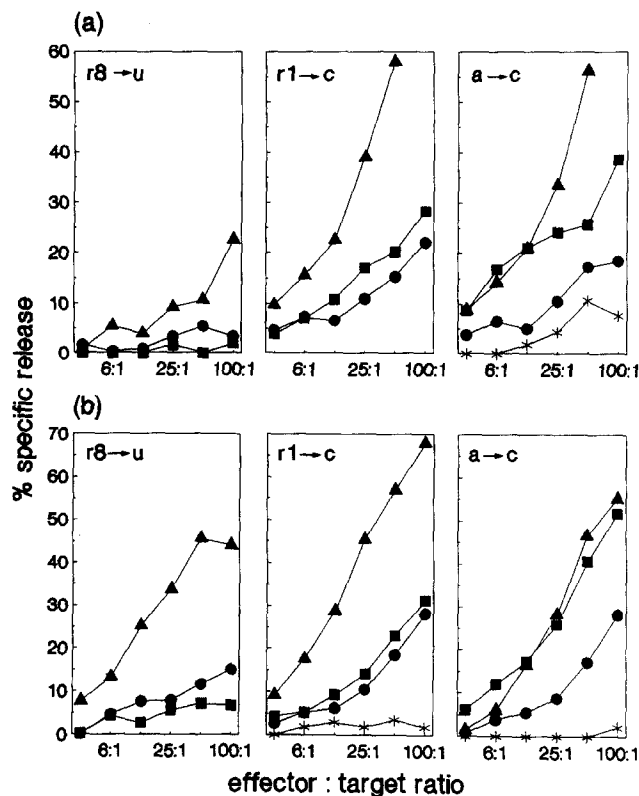


Figure 1. Cytotoxicity of graft infiltrating cells (a) and spleen cells (b) from renal allograft recipients 5 d after transplantation. GIC were recovered by mechanical disaggregation of excised kidneys ($0.5\text{--}2 \times 10^7$ cells obtained per graft) and together with spleen cells were used as effectors in 6-h ⁵¹Cr-release assays. The results show typical curves for lysis of targets by cells obtained from RT1^u recipients bearing rejecting R8 grafts, RT1^c recipients bearing nonrejecting R1 grafts, and RT1^c recipients bearing rejecting RT1^a grafts. Targets are represented as follows: donor strain Con A blasts (■), third-party (RT1^b) Con A blasts (●), syngeneic Con A blasts (*), and YAC-1 (▲). For each strain combination, at least six separate experiments were performed with similar results.

cytotoxic activity. This last result is important since it confirms that the specific cytotoxic activity of GIC, when it is present, can be readily detected under the in vitro conditions used in these experiments. GIC from all grafts were able to lyse the NK-susceptible target YAC-1 but levels of cytotoxicity were lowest in rejecting RT1A^a incompatible grafts.

The cytotoxic activity of spleen cells from the graft recipients was also tested and showed the same general pattern as that already described for GIC. As before, the most notable observation was the inability of effector cells, from RT1^u recipients bearing a rejecting RT1A^a disparate R8 graft, to show significant levels of specific killing of donor lymphoblasts (donor specific lysis was <10% in all of 10 recipients tested).

Effect of Anti-CD8 (MRC OX8) Antibody on Renal Allograft Rejection. The finding that class I incompatible R8 kidneys were rapidly rejected by high responder RT1^u recipients but that the graft infiltrate, when tested in vitro, showed little ability to lyse donor strain target cells led us to question

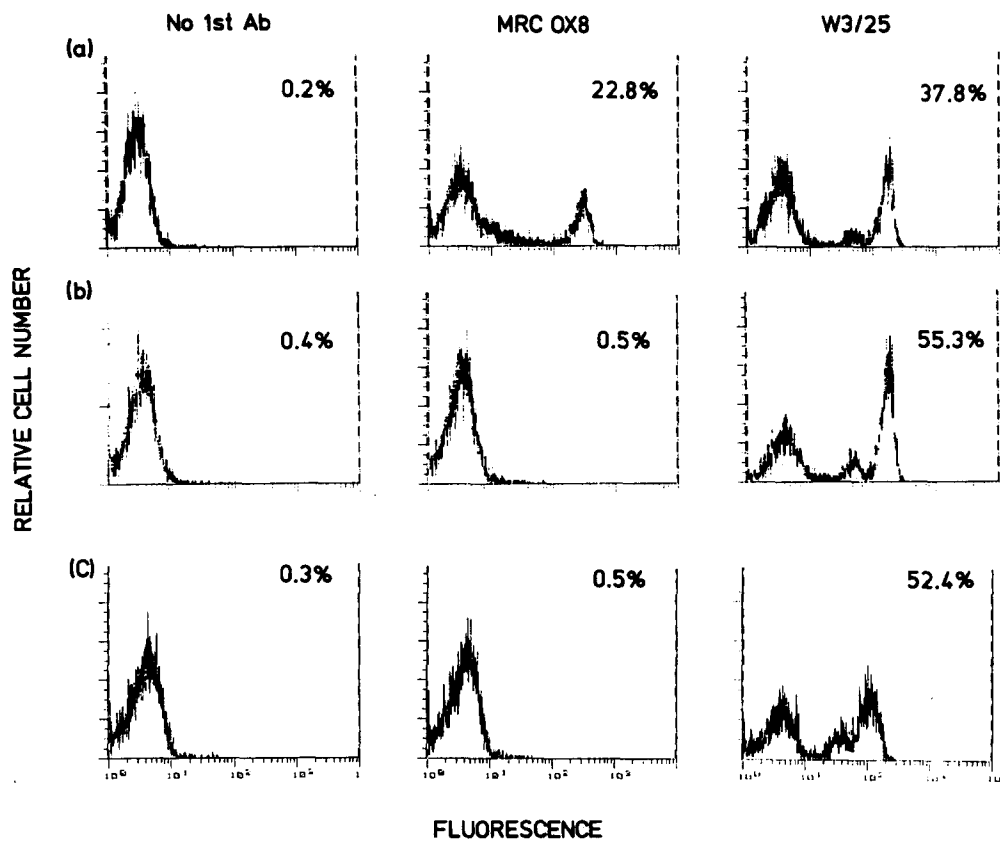


Figure 2. FACS profiles of PBL from normal RT1^u rats (row a) and RT1^u rats 6 d (row b) or 14 d (row c) after starting in vivo treatment with MRC OX8 (according to the schedule described in Materials and Methods). PBL were stained with FITC anti-mouse Ig alone, MRC OX8 and FITC anti-mouse Ig, or W3/25 and FITC anti-mouse Ig. FACS analysis of LNC gave comparable results.

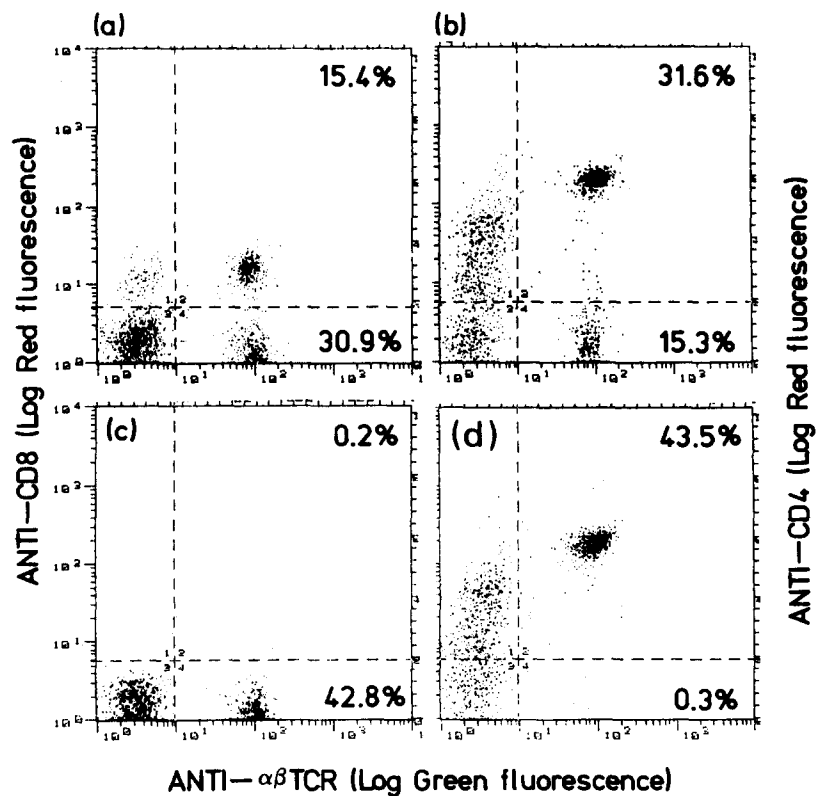


Figure 3. FACS analysis of PBL from normal RT1^u rat (a and b) and RT1^u rat 5 d after starting treatment with MRC OX8 (c and d).

whether CD8 effector cells play an essential role in the rejection of RT1^a disparate grafts. We therefore specifically depleted CD8⁺ cells from RT1^u rats, by *in vivo* treatment with the mAb MRC OX8, and then examined their ability to reject RT1^a kidneys.

FACS analysis (Fig. 2), together with immunohistology (results not shown), indicated that after MRC OX8 treatment was started, (according to the protocol described in Materials and Methods), CD8⁺ cells were undetectable in the blood, lymph nodes, and spleen of RT1^u rats for at least 14 d. This was not due to masking of the CD8 antigen by antibody coating since mouse Ig was not detectable on the surface of lymphoid cells. Loss of CD8⁺ cells was associated with a corresponding increase in the relative frequency of residual CD4⁺ T cells and MRC OX12⁺ B cells. Confirmation that MRC OX8 treatment caused depletion of CD8⁺ cells rather than modulation of the CD8 antigen was obtained by dual staining with R73 (which labels the TCR- α/β) and either MRC OX8 or W3/25 (Fig. 3). After *in vivo* treatment with MRC OX8, the CD8⁺ TCR- α/β ⁺ cell population was completely eliminated and all residual TCR- α/β ⁺ cells coexpressed the CD4 antigen.

Although MRC OX8 treatment depleted CD8⁺ cells from RT1^u rats, it had no effect on their ability to reject RT1^a class I disparate renal allografts (Table 4). Survival times for graft recipients given MRC OX8 were the same as those for recipients treated either with MRC OX21 (control mAb) or left untreated (MST 10 d in all groups). Rejection in MRC OX8-treated recipients was accompanied by mononuclear cell infiltration of the graft together with a T cell-dependent anti-RT1^a cytotoxic alloantibody response (similar to that found in untreated recipients, results not shown). The CD8⁺ infiltrate previously observed in rejecting RT1^a disparate grafts from untreated recipients was completely absent from rejecting grafts in MRC OX8-treated animals (Fig. 4, *a* and *b*). Much of the residual infiltrate comprised macrophages, as shown by labeling with ED1 (Fig. 4 *c*). There were also numerous T cells within the graft, as shown by expression of the TCR- α/β (Fig. 4 *d*). Two-color FACS analysis of the harvested GIC confirmed that this cell population consisted exclusively of TCR- α/β ⁺ CD4⁺ T cells (results not shown).

Alloantibody Levels in Recipients Bearing Class I Incompatible Grafts. It is known to have a strong influence not only on graft survival but also on the production of T-dependent anti-RT1^a alloantibody (9). This influence was readily apparent in the present experiments. High responder RT1^u recipients developed a strong anti-RT1^a antibody response to R8 kidney grafts as detected by a two-stage binding assay using donor strain erythrocytes (Fig. 5). In contrast, low responder RT1^c recipients showed a minimal antibody response to class I incompatible R1 grafts.

When the sera from grafted animals were assayed for cytotoxic antibody, RT1^u recipients showed a progressively increasing anti-RT1^a antibody response detectable from day 3 after transplantation, whereas cytotoxic antibody in RT1^c recipients was barely detectable on any day after transplantation (Fig. 6). The cytotoxic antibody in RT1^u recipients was specific for the RT1^a haplotype since it failed to lyse third-party RT1^c target cells (results not shown). The high levels of cytotoxic antibody in the serum of RT1^u graft recipients were initially demonstrated by assays in which guinea pig serum was used as a source of complement. Fresh syngeneic RT1^u rat serum was also effective as a complement source in these assays (Fig. 7). In contrast, RT1^c rat serum was not only ineffective but when mixed with RT1^u serum appeared to inhibit its effectiveness as a complement source. The explanation for this unexpected difference is unclear and the phenomenon is currently being investigated further in our laboratory although one possibility is polymorphism in class III MHC gene products between the *u* and *c* MHC haplotypes.

Passive Transfer Experiments. To determine whether alloantibody played a role in the rejection of RT1^a disparate kidney grafts by RT1^u recipients, a series of passive transfer experiments was undertaken (Table 5). Immune serum for passive transfer was obtained from RT1^u rats bearing a rejecting R8 kidney graft 5, 6, or 10 d after transplantation. Intravenous injection of immune serum into low responder RT1^c recipients of R1 kidneys did not cause graft rejection (MST >50 d, no increase in serum urea or creatinine). This result did not, however, exclude a role for cytotoxic alloantibody in the rejection of RT1^a disparate grafts by high responder RT1^u recipients because, as already noted, RT1^c rat serum (in contrast to RT1^u serum) was not effective as a

Table 4. Inability of MRC OX8 Treatment to Prevent Rejection of RT1^a Disparate Renal Allografts

Group	Donor	Recipient	mAb treatment*	n	Recipient survival [†]	
					d	d
1	r8	u	MRC OX8	6	9, 9, 10, 10, 10, 10	10
2	r8	u	MRC OX21 (control)	4	10, 10, 10, 10	10

* Recipient rats were treated *in vivo* with either MRC OX8 or MRC OX21 (control). mAbs were given intraperitoneally on days -1, 0, 3, 6, and 9 as described in Materials and Methods.

† Contralateral nephrectomy performed on day 7.

‡ Median survival time.

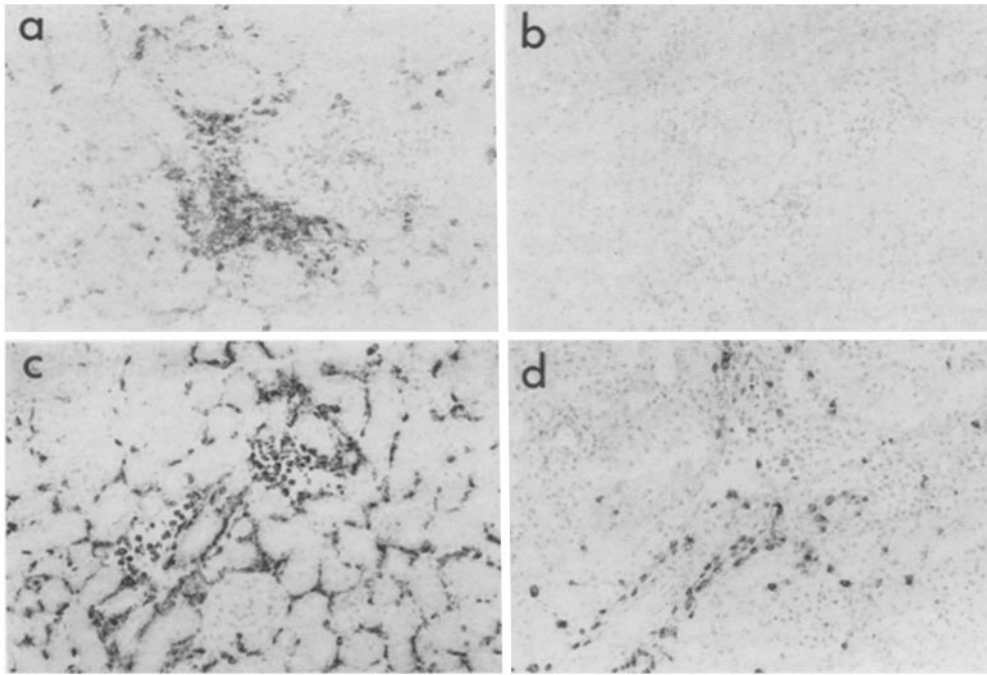


Figure 4. Immunohistological analysis of the leukocyte infiltrate in rejecting RT1A^a disparate R8 kidneys removed at day 5 from RT1^u recipients. Cryostat sections were labeled with mAbs using the immunoperoxidase technique, lightly counterstained with hematoxylin and photographed using a green filter to enhance the contrast of the peroxidase reaction product. All sections $\times 160$. (a) Rejecting R8 kidney from unmodified RT1^u recipient, labeled with MRC OX8. Note perivascular aggregate and scattered CD8⁺ infiltrate. (b-d) Rejecting R8 kidney from RT1^u recipient treated in vivo with MRC OX8; (b) labeled with MRC OX8, note complete absence of CD8⁺ cells; (c) labeled with ED1, note the relatively large number of macrophages; and (d) labeled with R73 (anti-TCR- α/β), note perivascular and interstitial T cell infiltrate.

complement source for assaying the *in vitro* cytotoxicity of RT1^u anti-RT1A^a antibody. The ability of immune serum to cause renal allograft rejection in cyclosporin-treated RT1^u recipients was therefore tested (Table 5). Treatment of RT1^u rats with cyclosporin for 14 d prevented them from rejecting

R8 kidney grafts (MST > 50 days) and also abrogated their antibody response to these grafts (results not shown). Injection of day 10 (but not day 5/6) immune serum restored the ability of cyclosporin treated RT1^u rats to promptly reject a R8 kidney (MST 9 d). This effect of immune serum was

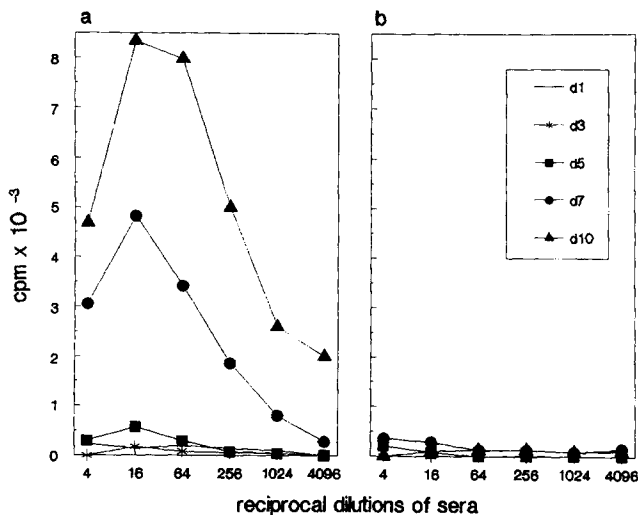


Figure 5. Antibody response to RT1A^a disparate renal allografts. Serum was obtained on days 1, 3, 5, 7, and 10 after renal transplantation, titrated against donor strain erythrocytes, and binding was detected with ¹²⁵I sheep anti-rat Ig. (a) RT1^u recipients of R8 renal allografts and (b) RT1^c recipients of R1 renal allografts. Values shown are mean of four graft recipients.

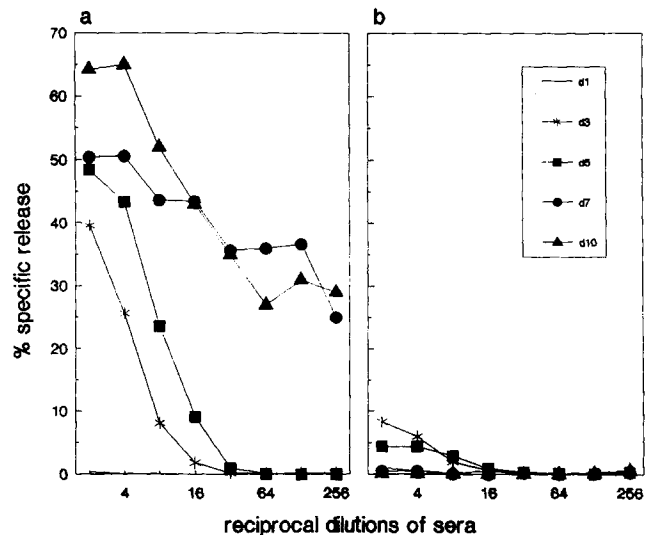


Figure 6. Cytotoxic antibody response to RT1A^a disparate renal allografts. Serum was obtained on days 1, 3, 5, 7, and 10 after renal transplantation and assayed against ⁵¹Cr-labeled donor strain Con A blasts in the presence of guinea pig complement. (a) RT1^u recipients of R8 renal allografts. (b) RT1^c recipients of R1 renal allografts. Values shown are mean of four graft recipients.

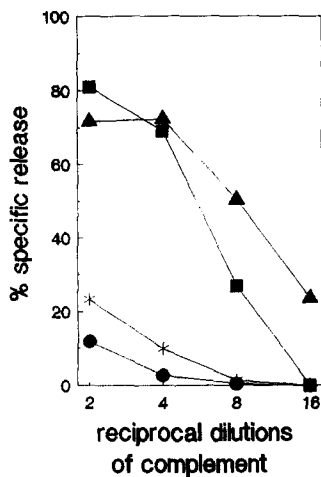


Figure 7. Complement-mediated cytotoxicity of ⁵¹Cr-labeled R8 Con A blasts by RT1^u anti R8 immune serum (day 5 after transplant). The assay was performed in the presence of guinea pig complement (▲), fresh RT1^u (■), RT1^c (●), or RT1^u + RT1^c (50:50 mixture) rat serum (*).

allospecific since injection of anti-RT1A^a immune serum into cyclosporin-treated RT1^u rats bearing a RT1^c kidney did not cause graft rejection (MST > 50 d). The histopathological appearance of class I disparate grafts rejected in the presence of passively transferred immune serum was broadly similar to that of RT1A^a disparate grafts undergoing rejection in unmodified RT1^u recipients. Damage to the graft vasculature was a major feature and neutrophil margination and infiltration (a characteristic feature of hyperacute rejection) was not apparent.

Discussion

Rat renal allografts bearing an isolated RT1^a class I disparity are rejected promptly by high responder RT1^u but not

by low responder RT1^c recipients. In this report we have shown that class I-restricted cytotoxic CD8⁺ effector cells are not necessary for the rejection of RT1A^a disparate kidney grafts by RT1^u recipients. This finding for class I disparate kidney grafts is of interest because most reports on the rejection of class I disparate skin or heart allografts in the rodent have emphasized the importance of the CD8⁺ T cell subset. Experiments in mice have shown that Lyt-2⁺ T cells play a major role in the rejection of class I disparate skin grafts, whereas L3T4⁺ cells may, depending on the strain combination, be unnecessary (1-6). Similarly, using a rat model, Lowry et al. (7) reported an absolute requirement for CD8⁺ cells in the rejection of class I disparate heart grafts by acutely irradiated rats, and in addition, suggested that this related to their role as cytotoxic effector cells. Moreover, congenitally athymic rats of low responder RT1^c haplotype can only be induced to reject RT1A^a class I disparate skin grafts by the adoptive transfer of both CD4⁺ and CD8⁺ T cells (28).

It has been recently shown that low responder RT1^c and high responder RT1^u rats have a similar frequency of anti-RT1A^a T cytotoxic precursor cells and that the frequency of such cells increases in RT1^u but not RT1^c recipients of a class I RT1A^a disparate cardiac allograft (29). In the present study it is notable, therefore, that GIC harvested from rejecting RT1A^a class I disparate kidney grafts showed minimal *in vitro* cytotoxicity towards donor strain lymphoblasts. Since in this and previous studies (23), we were able to demonstrate readily, the presence of substantial levels of specific cytotoxicity in rejecting grafts differing from their host at the entire (class I and class II) MHC locus the apparent absence of significant cytotoxicity in RT1A^a class I disparate grafts led us to question whether class I-restricted cytotoxic effector cells played a necessary role in their rejection. According to the orthodox view, class I-restricted cytotoxic cells would bear the CD8⁺ phenotype (30). We therefore tested

Table 5. Passive Transfer Experiments

Group	Donor	Recipient	Treatment	n	Recipient survival*	
					d	d
1	r1	c	Normal serum	3	All >50	>50
2	r1	c	Day 5/6 immune serum [‡]	4	All >50	>50
3	r1	c	Day 10 immune serum	4	All >50	>50
4	r8	u	Cyclosporin [§] + normal serum	6	All >50	>50
5	r8	u	Cyclosporin + day 5/6 immune serum	6	All >50	>50
6	r8	u	Cyclosporin + day 10 immune serum	5	9, 9, 9, 9, 10	9
7	c	u	Cyclosporin + day 10 immune serum	4	38, >50, >50, >50	>50
8	c	u	None	4	9, 10, 10, 10	10

* Contralateral nephrectomy on day 7.

[‡] Immune serum was obtained from RT1^u recipients bearing rejecting R8 kidneys 5, 6, or 10 d after transplantation. After heat inactivation (56°C for 30 min) it was stored at -20°C and microfuged before use. 1 ml of immune serum (or normal RT1^u serum) was given to graft recipients by i.v. injection on days 1, 2, 3, 4, and 5 after transplantation.

[§] Cyclosporin was given daily (15 mg/kg) by gavage for 14 d after transplantation.

the effect of depleting CD8⁺ cells from RT1^u rats on their ability to reject an RT1A^a disparate kidney. Injection of the mAb·MRC OX8 is highly effective at specifically depleting CD8⁺ cells from treated rats (31–33). The loss of CD8⁺ cells is accompanied by a functional loss of specific alloreactive cytotoxicity and a marked reduction in NK cell activity (many rat NK cells are CD8⁺). In the present study, the MRC OX8 treatment schedule completely depleted CD8⁺ cells from the peripheral blood and lymphoid tissue of RT1^u rats for at least 2 wk after starting treatment. However, MRC OX8-treated RT1^u rats showed no impairment in their ability to reject RT1A^a renal allografts despite the complete absence of CD8⁺ GIC from the rejecting kidneys. Therefore, whereas CD8⁺ T cells may or may not contribute to the rejection of RT1A^a disparate kidneys in unmodified RT1^u recipients, their participation is not essential for the rejection of such grafts.

Since rodents deficient in both CD4⁺ and CD8⁺ lymphocytes are unable to reject allografts, our results suggest that the CD4⁺ T cell subset is both able and sufficient to induce rejection of class I disparate renal allografts. Moreover, CD4⁺ cells appear to be essential for RT1^u rats to reject RT1A^a disparate kidneys promptly, since we have recently shown that depletion of CD4⁺ T cells by *in vivo* treatment with the mAbs MRC OX35 plus MRC OX38, given according to a previously described protocol (34), prevents rejection for at least several weeks (Porteous, C., E.M. Bolton and J.A. Bradley, manuscript in preparation).

Because CD4⁺ T cells are class II restricted, they would not be expected to recognize allo-class I MHC molecules directly, although exceptional CD4⁺ T cell clones have been described that are lytic towards class I MHC targets (35, 36), and therefore direct recognition of class I molecules by CD4⁺ cells cannot be completely discounted. However, the contemporary view is that class I-restricted CD4⁺ T cells recognize allo-class I antigen that is processed and presented in the context of self class II MHC. With reference to the present experiments this implies that RT1^u CD4⁺ T cells recognize A^a antigen that has been processed, either by donor or host antigen-presenting cells, and is presented in the context of RT1B/D^u class II MHC molecules.

The question arises as to the mechanism whereby CD4⁺ T cells activated in this way are able to mediate rejection of class I disparate kidney grafts. Numerous CD4⁺ T cells were identified within rejecting kidneys in MRC OX8-treated recipients, and in principal, they could mediate graft damage by recruiting and activating nonspecific cellular effectors in a classical DTH reaction. However, neither the immunohistological nor the functional comparison of GIC in rejecting and nonrejecting class I disparate kidneys supported this suggestion. Although macrophages were a major component of the cellular infiltrate in rejecting class I disparate grafts, they were also present in similar numbers in nonrejecting grafts. In addition, GIC harvested from nonrejecting grafts showed greater levels of *in vitro* cytotoxicity against target cells susceptible to NK cell-mediated lysis.

It is not possible, from these observations alone, to completely exclude a role for DTH in mediating rejection of class

I disparate kidney grafts. However, there is convincing evidence, at least in the case of skin allografts, that the tissue destruction accompanying rejection is exquisitely specific (37, 38), implying that either antigen-specific effector T cells or else alloantibody are responsible for graft rejection.

In a vascularized allograft, the microvasculature is likely to be a critical target of the effector responses (39, 40) and the early vascular injury followed by ischemia and hemorrhage in the rejecting class I disparate renal allografts in the present experiments points to the vascular endothelium as being the major target of the rejection process. It is interesting to speculate that the requirements for an antigen-specific effector cell could be fulfilled by a class II-restricted CD4⁺ T cell able to recognize allo-class I peptides, presented in the context of self class II MHC by donor endothelial cells. Although damage of vascular endothelium by syngeneic antigen-specific CD4⁺ effector cells has a precedent in the rat model of experimental allergic encephalomyelitis (41), the suggestion that a CD4⁺ effector T cell is directly responsible for the rejection of class I disparate kidney grafts is made less likely by the observation that the vascular endothelium in these rejecting grafts remains largely class II-negative (see Table 3).

The results of the present experiments are most consistent with the notion that CD4⁺ T cell-dependent alloantibody plays a decisive role in the rejection of RT1A^a disparate kidney grafts by high responder RT1^u recipients. Antibody could, in principal, mediate tissue damage through antibody-dependent cellular cytotoxicity or by complement activation, resulting in endothelial activation and injury, release of kinins and vasoactive peptides, and activation of the coagulation cascade. Indirect evidence that alloantibody may play a role in rejection was provided by the close correlation between the development of a strong RT1A^a antibody response and graft rejection in RT1^u recipients. The histopathological appearances of rejecting RT1A^a kidneys were also consistent with antibody-mediated damage of the graft microvasculature since the endothelial injury occurred in the absence of significant infiltration of the vessel walls by mononuclear cells. Direct evidence that circulating anti-RT1A^a antibody was capable of causing renal allograft damage *in vivo* was provided by the demonstration that passive transfer of immune serum was able to restore the ability of cyclosporin-treated RT1^u recipients to reject RT1A^a disparate but not third-party RT1^c kidney grafts. Moreover, the histopathological appearance of these rejecting kidneys was similar to that seen in RT1A^a disparate grafts undergoing rejection in unmodified RT1^u recipients. The inability of passively transferred anti-RT1A^a immune serum to cause rejection of RT1A^a disparate kidney grafts in low responder RT1^c rats is intriguing and may be attributed in part to the apparent differences in complement activity between the RT1^u and RT1^c rat strains.

The role of alloantibody in acute rejection is controversial and it is generally accepted that cellular rather than humoral effector mechanisms are responsible for rejection of allogeneic grafts by unsensitized recipients. Adoptive transfer experiments in acutely irradiated rats have shown that purified T cells are able to restore rejection of both fully allogeneic (42,

43) and class I disparate (7) heart grafts in the absence of detectable circulating cytotoxic antibodies in the recipient. The results reported here for RT1A^a class I disparate kidney allografts are therefore of interest because they suggest that

CD4⁺ T cells play an important role in the rejection of such grafts and that their role may be to provide T cell help for the generation of antibody mediated effector mechanisms.

We are grateful to Drs. A. M. Campbell and W. Cushley (Department of Biochemistry, University of Glasgow) for help with FACS analysis; and to Dr. D. W. Mason (MRC Cellular Immunology Unit, Oxford) and Dr. A. M. Mowat (Department of Immunology, University of Glasgow) for helpful discussions and comments on the manuscript.

This work was supported by the Western Infirmary Kidney Research Fund.

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Received for publication 26 March 1990 and in revised form 26 July 1990.

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