CELLULAR REQUIREMENTS FOR RENAL ALLOGRAFT REJECTION IN THE ATHYMIC NUDE RAT

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The rejection of a vascularized renal allograft by an unmodified host is characterized by a complex series of cellular interactions in which the T lymphocyte plays a central and essential role. There has been considerable debate concerning the roles played by the phenotypically distinct $CD4^+$ and $CD8^+$ T lymphocyte subpopulations in initiating and effecting graft rejection and their relative importance in renal allograft rejection remains to be clearly defined (1–3). Adoptive transfer experiments in T cell deficient rodents have provided convincing evidence that the $CD4^+$ and, in some instances, the $CD8^+$ T cell subsets may each be able to cause rejection of skin or heart allografts (4–11). Moreover, the relative contributions of these two T cell subsets may depend on several factors including the MHC disparity between donor and host and the immune status of adoptively transferred T cells. In addition, it is likely that there are important differences in the nature of the rejection response, not only between indirectly vascularized skin and directly vascularized organ grafts, but also between different types of organ grafts. Because most reports on the role of $CD4^+$ and $CD8^+$ lymphocytes in graft rejection relate to skin or heart allograft models, we sought to determine the ability of these phenotypically distinct T cell subpopulations to reject a renal allograft. Congenitally athymic rats were chosen as the T cell–deficient recipients in preference to animals rendered T cell deficient by whole body irradiation, to ensure that radioresistant, thymically processed host cells could not participate in the rejection response and thereby complicate interpretation of the experiments. This model enabled us to determine not only the T cell requirements for kidney allograft rejection but also to make a detailed analysis of the phenotype and in vitro functional characteristics of cells infiltrating the grafts. Our results demonstrate that the $CD4^+$ but not the $CD8^+$ lymphocyte subpopulation alone is able to initiate renal allograft rejection. Rejecting grafts in recipients given $CD4^+$ T cells were heavily infiltrated by mononuclear cells derived predominantly from the athymic host. These cells displayed high levels of MHC associated (but not restricted) cytotoxicity, suggesting that extrathymically derived nonspecific cytotoxic cells may be an important effector cell population in this model of graft rejection.

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Materials and Methods

Animals. Inbred male rats of the PVG ($RT1^c$), DA ($RT1^a$), Lewis ($RT1^l$), AO ($RT1^u$), and PVGrl ($RT1A^aB^cD^cC^c$) strains were obtained from Harlan Olac Ltd. (Bicester, Oxon, U.K.). Congenitally athymic nude rats (PVG *rnu/rnu*, $RT1^c$ [12th backcross]) were obtained from Harlan Olac Ltd. and maintained in the animal facility at the University of Glasgow where they were kept in laminar flow isolators and provided with autoclaved water, bedding, and food. Congenic rats of the PVG $RT7^b$ strain were provided originally by Dr. S. V. Hunt (Sir William Dunn School of Pathology, University of Oxford, Oxford, U. K.) and subsequently by Harlan Olac Ltd. The RT7 alloantigen system comprises two allelic forms ($RT7^a$ [or RT7.1] and $RT7^b$ [or RT7.2]), which differ according to their expression of a polymorphic determinant of the leukocyte common antigen. PVG rats (euthymic and nude) express the "a" variant (PVG $RT7^a$).

Kidney Transplantation. Kidneys from DA strain donors were transplanted into the left orthotopic site of nude PVG recipients (aged 8–12 wk) with end-to-end anastomosis of the renal artery, renal vein, and ureter (12). The procedure was performed under chloral hydrate anesthesia and ischaemic times were approximately 25 min. 7 d after transplantation, the recipient's right kidney was excised 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.

Preparation of Immune Serum. Immune serum for adoptive transfer was obtained from unmodified PVG rats that had received a DA renal allograft 10 d previously. Hyperimmune serum was obtained from PVG rats that had received a DA skin graft followed by four booster injections of DA splenocytes at two weekly intervals.

Antibodies. The following mouse mAbs were used to label rat leukocytes: MRC OX1 (leukocyte common antigen [13]); MRC OX8 ($CD8^+$ T cytotoxic/suppressor lymphocytes and NK cells [14]); W3/25 ($CD4^+$ T helper lymphocytes and some macrophages [14]); MRC OX12 (rat Ig κ chains on B lymphocytes [15]); MRC OX19 (CD5 determinant on T lymphocytes and thymocytes [14]); MRC OX21 (human C3b inactivator [16]) which was used as a negative control throughout; and MRC OX6, which labels a nonpolymorphic determinant of rat MHC class II antigens (17). These mAbs were kindly provided by Dr. D. W. Mason and Dr. A. F. Williams (MRC Cellular Immunology Unit, Sir William Dunn School of Pathology). F17-23-2, against a polymorphic determinant of MHC class II IA antigens in DA but not PVG rats [18] was kindly provided by Professor J. Fabre (Blond McIndoe Centre, Queen Victoria Hospital, East Grinstead, Sussex). ED1 labels most tissue macrophages, monocytes, and dendritic cells [19] and was obtained from Serotec Ltd. Oxford, U. K. HIS 41 is a mouse IgG1 antibody raised against thymocytes of the congenic rat strain PVG $RT7^b$, that recognizes a polymorphic determinant of the leukocyte common antigen expressed on leukocytes of the PVG $RT7^b$ but not PVG $RT7^a$ rat strain (Kampinga, J., F. G. M. Krose, G. H. Pol, D. Opstelten, J. H. A. Boot, B. Roser, P. Nieuwenhuis, and R. Aspinall, manuscript in preparation). Anti-asialo GM1 was obtained from Wako Chemicals (Neuss, FRG).

Preparation and Fractionation of Lymph Node Cells. Lymph node cells (LNC)[1] were prepared from pooled cervical and mesenteric lymph nodes obtained from either normal rats or animals that had been immunized by the application of two full thickness skin allografts 14 days apart, the second of which had rejected >30 d before use. After excision, lymph nodes were diced and passed through a fine stainless steel mesh in the presence of Dulbecco's A+B medium containing 2% FCS (DAB/FCS). The resulting cell suspension was washed twice and finally resuspended in DAB/FCS.

LNC were depleted of lymphocyte subpopulations according to their reactivity with the mouse anti-rat mAbs MRC OX12, MRC OX8 and W3/25 using a modification of the indirect rosette depletion technique described by Mason (20). LNC were incubated with saturating concentrations of the appropriate antibodies for 1 h on ice. After washing the cells were mixed with sheep erythrocytes coated with rabbit anti-mouse Ig (Dako Ltd., High Wycombe, U. K.), which resulted in rosette formation between the sheep cells and the antibody-coated lymphocytes. To improve the purity of the depleted lymphocyte subpopulation, the

[1] *Abbreviations used in this paper:* LNC, lymph node cells; MST, median survival time.

resulting rosettes were stabilized by mixing with sheep erythrocytes coated with mouse IgG (Dako Ltd.). The rosette aggregates were separated from the unlabeled lymphocytes by layering over a Ficoll/sodium metrizoate mixture (specific gravity 1.088) and centrifuging at room temperature at 1,200 *g* for 30 min.

In all experiments, the purity of the unlabeled lymphocytes retrieved from the interface was determined either by immunoperoxidase labeling of cytocentrifuge slide preparations or by FACS analysis. The negative selection procedure resulted consistently in a T cell population contaminated with <0.1% of the unwanted T cell subpopulation and comprising >95% of the desired T cell subset.

Immunohistology. Kidney tissue was snap frozen in liquid nitrogen and cryostat sections (5 μm) were cut at −20°C onto gelatinised slides. A range of monoclonal antibodies was used to label the slides using an indirect immunoperoxidase technique (21).

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 (22). Sections were examined at a magnification of ×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 × [(number of positive cells under grid intersections)/(total number of grid intersections)].

Harvesting of Graft Infiltrating Cells. Infiltrating mononuclear cells were harvested from kidney allografts by finely dicing the excised kidney and passing it through a fine stainless steel mesh. The resulting cell suspension was washed once in Hepes-buffered RPMI/5% FCS (Gibco Ltd., Paisley, Scotland) and the mononuclear cells were separated by Percoll centrifugation. Iso-osmolar 90% Percoll (Sigma Ltd., Poole, U. K.) stock solution was prepared in Ca^{2+} and Mg^{2+} free, 10× concentrated HBSS (Gibco Ltd.) containing 10 mM Hepes buffer, and this was further diluted with single strength HBSS/10 mM Hepes to give 80% and 35% solutions of the stock 90% Percoll. The harvested graft infiltrating cells were resuspended in 80% Percoll, layered underneath 35% Percoll, and centrifuged at 500 *g* for 30 min. The mononuclear cells, free of dead cell debris and erythrocytes, were then retrieved from the interface and cell viability, assessed by trypan blue exclusion, was always >90%.

Cell-mediated Cytotoxicity Assays. Splenocyte effector cells were prepared by teasing spleens apart, with forceps, in Hepes-buffered RPMI/5% FCS. Erythrocytes were removed by hypotonic lysis and after two further washes, splenocytes were resuspended in RPMI/5% FCS. Graft infiltrating cells and spleen cells from transplanted recipients were tested for alloantigen-specific cytotoxicity and nonspecific cytotoxicity using a standard 6-h Cr-release assay essentially as described elsewhere (23). Alloantigen-specific cytotoxicity was assessed using ^{51}Cr-labeled kidney donor strain and third-party Con A–transformed splenic blasts as targets. The rat myeloma Y3 (24), which has the RT1^u MHC genotype, the mouse lymphoma line YAC-1 (both of which are susceptible to natural killer [NK] cell mediated lysis), and the mouse mastocytoma P815 (25) (which is NK resistant but susceptible to lymphokine activated killer [LAK] cells) were also used as target cells. Specific ^{51}Cr release was calculated by the formula: percent specific release = 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Data shown are the means of triplicate determinations (SD <5%; spontaneous release <20% of maximum release in all experiments).

Lymphocytotoxic Antibody Determination. Serial dilutions of test sera were incubated with ^{51}Cr-labeled spleen cells from donor strain and third-party rats in the presence of guinea pig complement (Sera-Lab, Sussex, U. K.) in a 4-h cytotoxicity assay essentially as described elsewhere (26).

Results

Ability of T Lymphocyte Subpopulations to Initiate Renal Allograft Rejection in the Athymic Nude Rat. Congenitally athymic PVG (RT1^c) recipients of fully allogeneic DA (RT1^a) kidneys were unable to reject their grafts and achieved prolonged survival (MST >79 d) with no increase in their serum urea or creatinine levels. Histological

examination of the transplanted kidney when rats were killed failed to demonstrate evidence of graft rejection.

To determine the T cell requirements in this model for initiating graft rejection, renal allograft recipients were injected (immediately after transplantation) with highly purified T lymphocyte subpopulations obtained from either normal or specifically sensitised euthymic PVG ($RT1^c$) animals. The T cell subpopulations were prepared from LNC by negative selection using the mAbs MRC OX12 plus MRC OX8 (to yield $CD4^+$ cells), or MRC OX12 plus W3/25 (to yield $CD8^+$ cells). The results of these adoptive transfer experiments are summarized in Table I.

PVG nude rats injected with $\geq 2.5 \times 10^7$ $CD4^+$ LNC (equivalent proportionally to $\geq 5 \times 10^7$ unseparated LNC) rapidly rejected their grafts with a survival time comparable to those animals receiving unseparated LNC. Compared with naive $CD4^+$ cells, specifically sensitized $CD4^+$ cells (obtained from PVG rats that had rejected two successive full thickness DA skin grafts) were several times more potent since as few as 2×10^6 sensitized $CD4^+$ cells led to rapid graft rejection (MST 10 d). Histological examination of rejecting grafts from $CD4^+$ injected recipients confirmed severe renal injury with a diffuse mononuclear cell infiltrate, widespread tubular damage and extensive papillary necrosis.

In contrast to the ability of $CD4^+$ cells to cause rejection, neither unsensitized nor specifically sensitized $CD8^+$ lymphocytes were effective when injected into athymic recipients. As shown in Table I, administration of 1.3×10^7 sensitized $CD8^+$ cells (equivalent proportionally to $>10^8$ unseparated LNC) failed to cause re-

TABLE I

Ability of Negatively Selected $CD4^+$ and $CD8^+$ T Cell Subpopulations to Initiate Rejection in PVG Nude Recipients of DA Renal Allografts

Number of cells	T cells injected*	n‡	Recipient survival	MST§
			d	*d*
0	None	4	75,¶ 79,¶ >100, >100	>79
5×10^7	LNC‖	4	9, 10, 10, 15	10
10^7	$CD4^+$	7	9, 10, 83, >100, >100, >100, >100	>100
2.5×10^7	$CD4^+$	3	9, 10, 13	10
5×10^7	$CD4^+$	5	9, 10, 10, 11, 21	10
2×10^6	$CD4^+$ (sensitized)**	4	9, 10, 10, 11	10
10^7	$CD4^+$ (sensitized)	3	8, 9, 9	9
10^7	$CD8^+$	4	60,¶ 76,¶ >100, >100	>76
2.6×10^7	$CD8^+$	2	>100, >100	>100
1.3×10^7	$CD8^+$ (sensitized)	6	>100, >100, >100, >100, >100, >100	>100

* LNC subpopulations were prepared by negative selection using the mAbs MRC OX12, MRC OX8, and W3/25 in a rosette depletion procedure. Athymic PVG rats received a DA renal allograft and were immediately reconstituted with PVG LNC or subpopulations. Contralateral nephrectomy was performed on day 7.

‡ Number of animals in group.

§ Median survival time.

‖ Unseparated LNC typically comprise 41% B cells, 10% $CD8^+$ cells, and 46% $CD4^+$ cells on FACS analysis.

¶ Animals were killed/died with normal serum urea and creatinine and no evidence of graft rejection on histological examination.

** LNC from PVG rats that had been immunized by two DA skin grafts, 14 d apart.

jection; the MST was >100 d and there was no increase in serum urea or creatinine. Histological examination of longstanding grafts from $CD8^+$ injected recipients revealed essentially healthy kidneys although some grafts showed occasional small foci of mononuclear cells in the cortex and medulla with adjacent atrophic tubules. Significant glomerular or vascular damage was not observed.

Immunohistological Observations in Nonrejecting and Rejecting Renal Allografts. The demonstration that PVG nude recipients of a renal allograft could be induced to reject their grafts rapidly by injection of $CD4^+$ T lymphocytes prompted an investigation of the intragraft cellular mechanisms concerned. Our first approach was to assess the magnitude and phenotype of the cellular infiltrate in rejecting renal allografts from athymic recipients that had received purified $CD4^+$ T cells. 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 II). Nonrejecting grafts from unmodified PVG nude animals showed only a mild leukocyte infiltrate with very few $CD5^+$ T cells or MRC $OX8^+$ cells. Most infiltrating cells had a phenotype consistent with that of macrophages ($W3/25^+$, MRC $OX19^-$, or $ED1^+$). In contrast, rejecting kidneys from animals injected with either unseparated LNC or purified $CD4^+$ cells showed an intense cellular infiltrate comprising not only an increased number of macrophages, but also a substantial increase in the number of cells with a phenotype (MRC $OX8^+$, MRC $OX19^-$) consistent with rat NK cells. The frequency of $CD5^+$ cells remained extremely low. Thus, although $CD4^+$ T cells are sufficient to initiate renal allograft rejection, this is associated with a cellular infiltrate consisting predominantly of cells whose phenotype suggests they are derived from the athymic recipient.

We next determined more directly the relative contributions of donor- and host-derived leukocytes to the cellular infiltrate in rejecting grafts. PVG nude allograft recipients were injected immediately after transplantation with $CD4^+$ LNC from histocompatible PVG $RT7^b$ congenic rats that express the epitope of the leukocyte

TABLE II

Immunohistological Observations in Kidney Allografts from PVG Nude Recipients

Group	Cells injected	Percentage area infiltrate* MRC OX1 (L-CA)	MRC OX19 (T cells)	MRC OX8 ($T_{c/s}$ + NK)	W3/25 (T_h + Mø)	ED1 (Mø)
1	None	14 ± 4	<1	1 ± 1	9 ± 3	6 ± 2
2	5×10^7 PVG $CD4^+$ ($W3/25^+$, $OX12^-$, $OX8^-$)	26 ± 5	2 ± 1	8 ± 1	13 ± 2	15 ± 1
3	5×10^7 unseparated PVG LNC	26 ± 5	2 ± 1	9 ± 2	16 ± 2	17 ± 1

PVG nude rats received a DA renal allograft. Animals in groups 2 and 3 were injected immediately afterwards with negatively selected $CD4^+$ LNC and unseparated LNC, respectively. Nonrejecting (group 1) and rejecting (groups 2 and 3) kidneys were excised on day 7. Cryostat sections were labeled using the immunoperoxidase technique and percentage infiltrate determined by point counting with a microscope eye piece graticule.

* Data are mean ± SD of five grafts.

common antigen, labeled by the mAb HIS 41, not found on the PVG RT7^a hosts. The specificity of HIS 41 for PVG RT7^b is demonstrated in Fig. 1, *a* and *b*, which shows strong staining of PVG RT7^b lymph node cells but no staining of PVG nude lymph node cells. As seen in Fig. 1 *c*, rejecting renal allografts in PVG nude hosts injected with PVG RT7^b CD4$^+$ cells showed a heavy leukocyte infiltrate when labeled with MRC OX1 (in keeping with the results already described in Table II). However, labeling with HIS 41 (Fig. 1 *d*) confirmed that the majority of the infiltrate (consistently >80% by morphometric analysis) was derived from the host rather than from the T lymphocyte inoculum.

Class II MHC Expression in Rejecting Kidneys after Reconstitution with CD4$^+$ T Cells. Class II MHC expression was detected by immunoperoxidase staining with F17-23-2, which labels DA but not PVG class II MHC antigens, thereby allowing unequivocal determination of donor class II expression, and also with MRC OX6, which labels a monomorphic class II MHC antigen determinant. The distribution of class II MHC expression in normal DA kidney (indicated by labeling with F17-23-2), is shown in Fig. 2 *a*. There was strong staining of interstitial dendritic cells, weak staining of some renal tubular cells, but no staining of glomeruli or vascular endothelium. Rejecting DA kidneys from CD4$^+$ reconstituted PVG nude rats showed strong staining of donor class II MHC antigens on the renal tubular cells from day 3 onwards. However, even by day 7 after transplantation, there was little convincing evidence of class II MHC antigen expression by the renal vascular endothelium. As shown in Fig. 2 *b*, arteriolar endothelium remained largely negative. This latter observation is in marked contrast to previous studies of rejecting DA kidneys in euthymic PVG recipients where vascular endothelium stained strongly for class II MHC antigens from day 5 onwards (22). Therefore, the ability of CD4$^+$ T cells to initiate renal allograft rejection in the nude rat does not appear to be critically dependent on the induction of detectable class II MHC antigens on the vasculature of the graft.

Cytotoxic Repertoire of Effector Cells from PVG Nude Rats Bearing DA Renal Allografts. To investigate further the cellular mechanisms whereby injection of CD4$^+$ T cells was able to initiate renal allograft rejection in PVG nude rats, the in vitro cytotoxic repertoire of graft infiltrating cells and splenocytes from these animals was studied in 6-h ^{51}Cr-release assays.

Infiltrating mononuclear cells obtained from rejecting allografts 7 d after transplantation showed high levels of cytotoxicity against Con A blasts of the kidney donor strain (Fig. 3 *a*). However, this cytotoxicity was not allospecific since the infiltrating cells also showed high levels of lysis against all third-party allogeneic Con A blasts tested. Despite this nonspecific activity, cytotoxicity appeared to be directed against MHC-associated determinants because the infiltrating cells lysed PVGrl Con A blasts (which differ from the nude host at only the RT1A class I locus), but had minimal lytic activity against syngeneic PVG Con A blasts. Infiltrating cells from rejecting renal allografts also showed high levels of cytotoxicity against the NK-susceptible targets Y3 and YAC-1 and against the reputedly NK resistant but LAK susceptible target P815 (Fig. 3 *b*). Attempts were made to examine the cytotoxic repertoire of graft infiltrating cells obtained from nonrejecting renal allografts in PVG nude rats that had not received CD4$^+$ T cells. Because of the reduced mononuclear cell infiltrate in nonrejecting grafts (cell yield $<2 \times 10^6$ per graft), only a limited anal-

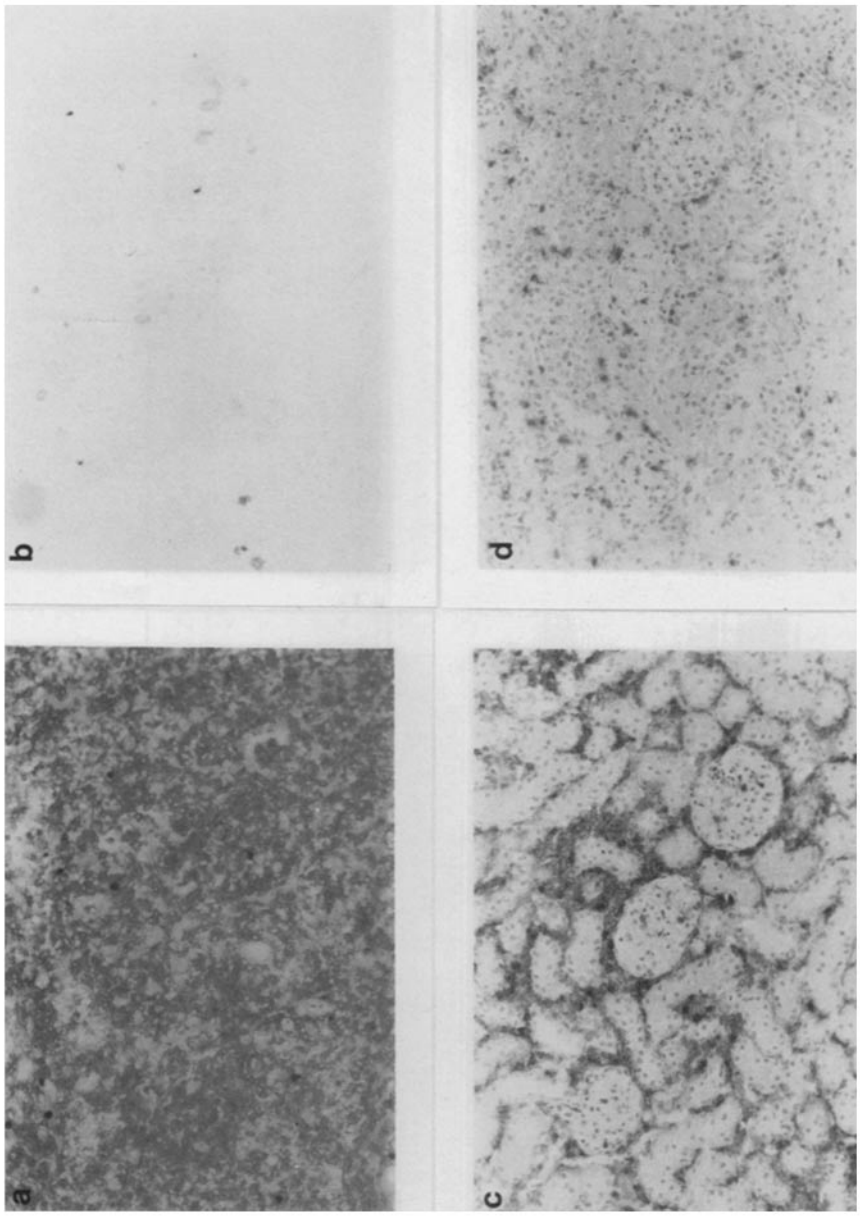

FIGURE 1. Immunohistological analysis of origin of leukocyte infiltrate in rejecting renal allografts. Cryostat sections were labeled with mAbs using the immunoperoxidase technique and photographed using a green filter to enhance the contrast of the peroxidase reaction product. Specificity of mAb HIS 41 for PVG $RT7^b$ and not PVG nude leukocytes was confirmed by labeling sections of lymphoid tissue from unmodified animals in the absence of counterstain. (*a*) Cervical lymph node from PVG $RT7^b$ showing strong staining of leukocytes; (*b*) cervical lymph node from PVG nude showing absence of staining. Occasional eosinophils with endogenous peroxidase are visible in both sections. The same pattern of staining was seen in other lymphoid organs examined. Rejecting DA renal allografts were obtained at day 7 from PVG nude recipients injected with 5×10^7 PVG $RT7^b$ $CD4^+$ T cells for immunohistological analysis. Sections were lightly counterstained with hematoxylin. (*c*) Labeled with MRC OX1 (LCA). Note heavy mononuclear cell infiltrate; (*d*) labeled with HIS 41 (detecting inoculum derived leukocytes only). Note majority of infiltrate is host derived. (*a–d*) ×160.

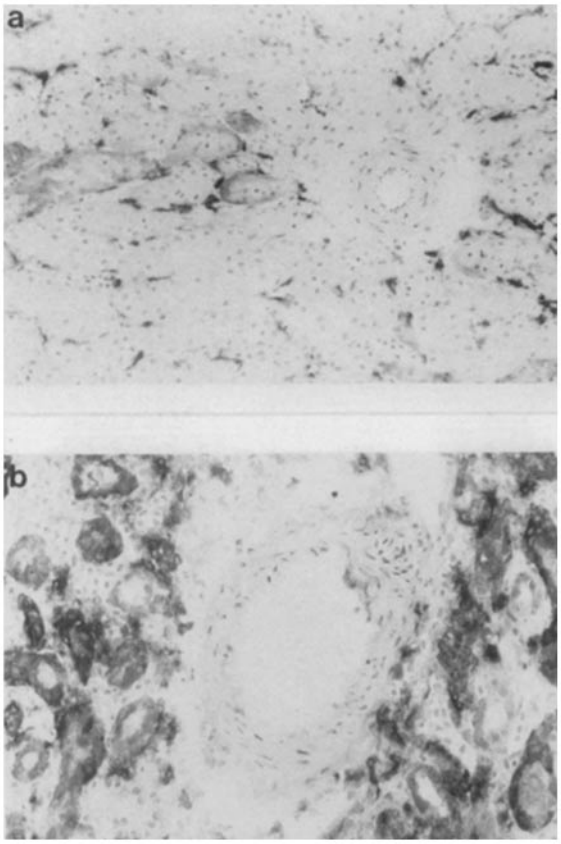

FIGURE 2. Immunohistological studies of class II MHC antigen expression in normal and in rejecting DA kidneys. Sections were labeled with mAb F17-23-2 against donor but not recipient class II MHC antigens. (*a*) Normal DA kidney. Note strong staining of interstitial dendritic cells and weak staining of interstitial dendritic cells and weak staining of some proximal tubules (× 160). (*b*) Rejecting DA renal allograft obtained at day 7 from PVG nude given 5 × 10^7 PVG CD4$^+$ T cells. Note strong staining of renal tubular cells but absence of staining of arteriolar vascular endothelium (× 320).

ysis could be undertaken. However, the results of two separate experiments using infiltrating cells from pooled kidney allografts showed no cytotoxicity against kidney donor strain Con A blasts (<5% cytotoxicity at E/T ratio 50:1, results not shown).

The cytotoxic repertoire of spleen cells obtained at day 7 from CD4$^+$ reconstituted allograft recipients and from nonreconstituted recipients was also examined.

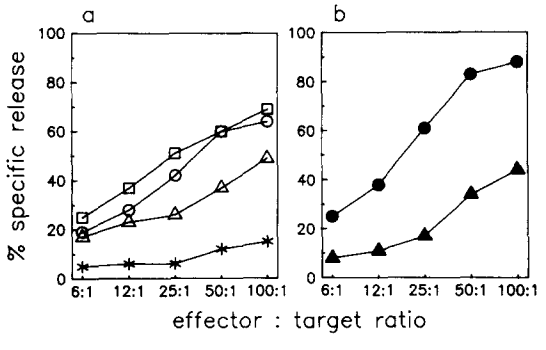

FIGURE 3. Cytotoxicity of graft infiltrating cells. Nude PVG rats (two to three animals per experiment, total of four separate experiments) were given a DA renal allograft and immediately reconstituted with 5 × 10^7 CD4$^+$ PVG lymphocytes. Seven d later the grafts were excised, pooled, and the infiltrating cells were recovered by mechanical disaggregation of the kidneys. The harvested cells (~10^7 per graft) were used as effectors in 6-h ^{51}Cr-release assays. Results show typical curves for lysis of allogeneic Con A blasts (*a*) and nonspecific targets (*b*). Targets are represented as follows: DA (□), PVGrl (○), Lewis (△), PVG (*), Y3 (●), P815 (▲).

The cytotoxic activity of spleen cells from these two groups was identical (Fig. 4 *a–c*). Spleen cells showed high levels of cytotoxicity against allogeneic Con A blasts, not only of kidney donor strain but also of third-party strains. In addition, spleen cells from both $CD4^+$ reconstituted and nonreconstituted recipients showed high levels of nonspecific cytotoxicity against the NK-susceptible targets Y3 and YAC-1 and against the LAK susceptible target P815. Because spleen cells from nonreconstituted nude recipients were shown, unexpectedly, to lyse allogeneic target cells a series of experiments was performed to determine the in vitro cytotoxic characteristics of spleen cells from unmodified, nontransplanted PVG nude rats.

Cytotoxic Repertoire of Cells from Normal Nontransplanted PVG Nude Rats. In these experiments the cytotoxic activity of splenocytes from PVG nude rats was compared with that of euthymic animals. As expected, spleen cells from unmodified PVG nude rats showed high levels of nonspecific activity against the NK-susceptible targets Y3 and YAC-1 and the levels of lysis usually exceeded those of spleen cells from euthymic PVG rats (Fig. 5 *a* and *b*). In addition, spleen cells from PVG nude animals showed a significant lysis of the LAK susceptible target P815, whereas this target was relatively resistant to spleen NK cells from euthymic PVG rats (or other rat strains tested, e.g., Lewis and AO). An unexpected but consistent finding was that spleen cells from unmodified PVG nude rats also showed high levels of cytotoxicity against a range of allogeneic Con A lymphoblasts, despite the absence of prior sensitisation and the inability of spleen cells from euthymic rats to lyse such targets (Fig. 5 *c* and *d*). Spleen cells from PVG nude rats were unable to lyse syngeneic PVG Con A blasts and their ability to lyse allogeneic Con A blasts was not mitogen related since a similar pattern of reactivity was observed against ^{51}Cr-labeled allogeneic splenocytes (results not shown). To determine whether the unrestricted cytotoxic activity of PVG nude spleen cells was mediated by a single effector population or was due to a polyclonal response to several different specificities, a series of cold target inhibition assays were performed (Table III). Homologous cold targets were generally better inhibitors of cytotoxicity than heterologous cold targets when low ratios

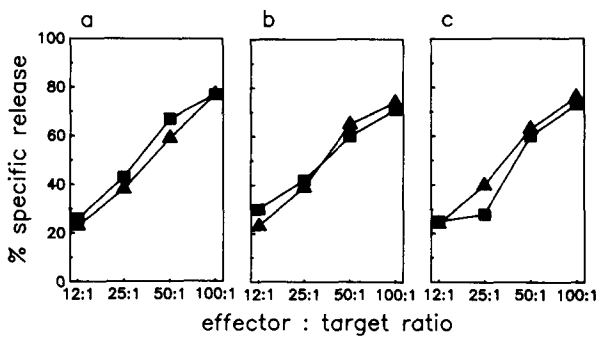

FIGURE 4. Cytotoxicity of spleen cells 7 d after transplantation. Nude PVG rats were given a DA renal allograft and were left unreconstituted (nonrejecting [▲]) or were given an injection of 5×10^7 $CD4^+$ syngeneic lymphocytes immediately after transplantation (rejecting [■]). Seven d later, spleen cells from these animals were tested for in vitro cytotoxicity in 6-h ^{51}Cr release assays. The results shown represent one of three experiments which gave the same result, against DA Con A blasts (*a*), Lewis Con A blasts (*b*) and Y3 targets (*c*). (In these experiments, spleen cells from euthymic PVG animals were also used as effectors and showed no lysis of either DA or Lewis Con A blasts.)

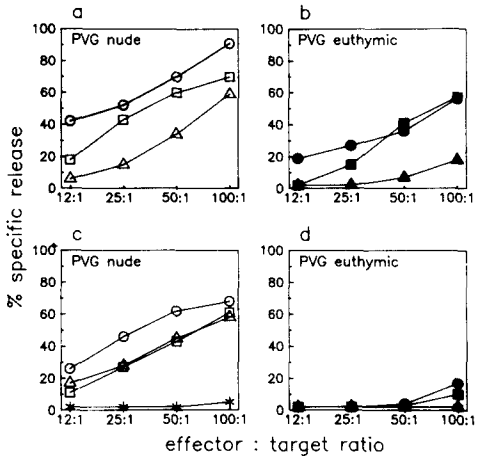

FIGURE 5. Cytotoxic activity of spleen cells from PVG nude rats (*a*) and euthymic PVG rats (*b*) against the Y3 rat myeloma (O, ●), the mouse lymphoma YAC-1 (□, ■) and the NK-resistant, LAK-sensitive P815 cell line (△, ▲). Cytotoxic activity of spleen cells from PVG nude rats (*c*) and euthymic PVG rats (*d*) against splenic Con A blasts prepared from the allogeneic rat strains DA (O, ●), AO (□, ■) and Lewis (△, ▲) and against syngeneic PVG (*) splenic blasts. This experiment was repeated on five occasions with the same result. The effector cells used in the experiment shown were macrophage depleted using cabonyl iron and the assay was performed in the presence of 5% FCS. Similar results were obtained in experiments in which macrophage depletion was omitted or where 5% FCS was replaced by heat inactivated rat serum from either PVG nude or euthymic PVG rats.

of unlabeled to labeled targets were used. However, higher ratios of heterologous unlabeled targets were clearly able to inhibit ^{51}Cr release by a variety of targets. These results suggest that the PVG nude spleen contains multiple effector cell populations that have different specificities, but that individual effector cells may also recognize common determinants on the different targets studied.

Additional experiments were performed to investigate whether in vitro treatment of PVG nude splenocytes with anti-asialo GM1 plus complement affected their subsequent in vitro cytotoxic activity. As shown in Fig. 6, this treatment effectively eliminated cytotoxic activity not only against the NK-susceptible targets (Y3 and YAC-1), but also against allogeneic Con A blast targets, indicating that the allogeneic cytotoxicity was mediated by cells expressing the asialo GM1 glycolipid.

These in vitro functional studies suggest that adoptive transfer of $CD4^+$ lymphocytes to PVG nude allograft recipients results in the recruitment of widely alloreactive, host-derived mononuclear cells into the renal allograft.

Alloantibody Responses in Allograft Recipients. The rejection of DA renal allografts by PVG nude rats invariably correlated with the presence of a detectable cytotoxic alloantibody response. Thus, nonreconstituted graft recipients and those receiving $CD8^+$ T cells had no detectable cytotoxic alloantibodies up to 100 d after transplantation, whereas recipients injected with 5×10^7 $CD4^+$ T cells demonstrated a brisk, donor-specific cytotoxic alloantibody response (median day 7 titer of 1/64, results not shown). Nevertheless, evidence that alloantibody alone was not sufficient to cause graft rejection was provided by adoptive transfer experiments using serum but not cells. PVG nude recipients of DA renal allografts, which were injected on days 1–5 after transplantation with 0.5 ml hyperimmune serum (see Materials and Methods section), or with 0.5 ml serum from euthymic PVG recipients of a DA renal allograft, failed to reject their grafts (MST >100 d, no increase in serum urea or creatinine, $n = 6$) despite the persistence of detectable levels of circulating cytotoxic antibody for several days after serum transfer.

TABLE III

Cold Target Cell Competition Experiments

Exp.	Labeled target	Ratio of unlabeled: labeled	Percentage inhibition by unlabeled targets			
			DA	LEW	Y3	YAC-1
1	DA	80:1	[93]	71	31	ND
	(55%)*	40:1	[82]	47	22	ND
		20:1	[71]	24	29	ND
	LEW	80:1	71	[86]	75	ND
	(65%)*	40:1	60	[71]	38	ND
		20:1	72	[69]	38	ND
	Y3	80:1	52	75	[88]	ND
	(48%)*	40:1	25	35	[90]	ND
2	DA	80:1	[95]	60	ND	43
	(56%)*	40:1	[88]	21	ND	0
		20:1	[76]	25	ND	0
		10:1	[60]	13	ND	0
	LEW	80:1	75	[93]	ND	57
	(54%)*	40:1	48	[90]	ND	21
		20:1	24	[88]	ND	2
		10:1	24	[70]	ND	6
	YAC-1	80:1	43	38	ND	[84]
	(75%)*	40:1	24	17	ND	[63]
		20:1	21	27	ND	[53]
		10:1	9	11	ND	[23]

Cold target inhibition assays were performed in which unlabeled target cells, added to the wells in standard in vitro cytotoxicity assays, competed with the labeled targets for lysis by effector cells prepared from the spleens of PVG nude rats. The ratio of effector cells to labeled target cells was 100:1 throughout. Results (expressed as percent inhibition of cytotoxicity [mean of triplicate determinations]) are shown for two representative experiments. Boxed results are those for homologous cold targets. In both experiments, control wells containing unlabeled PVG splenocyte "filler" cells were set up and inhibition was <10% in all cases.

* Percent cytotoxicity at E/T ratio of 100:1 in absence of unlabeled competitor targets.

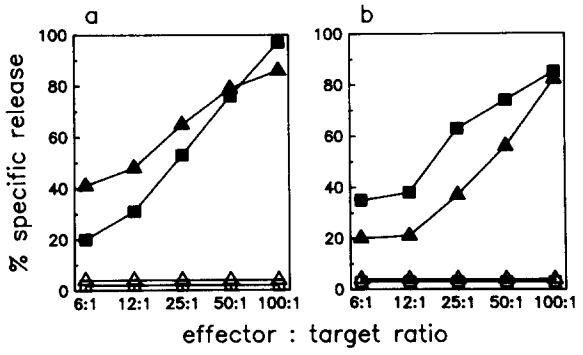

FIGURE 6. Effect of anti-asialo GM1 and complement. Spleen cells from unmodified PVG nude rats were incubated with guinea pig complement alone (*closed symbols*) or with anti-asialo GM1 and complement (*open symbols*). (*a*) Cytotoxic acitivity against Y3 (□, ■) and YAC-1 (△, ▲). (*b*) Cytotoxic activity against DA Con A blasts (□, ■) and Lewis Con A blasts (△, ▲). This experiment was repeated with the same result.

Discussion

We have shown in this report that adoptively transferred $CD4^+$ T cells are able to cause rejection of fully allogeneic renal allografts in the nude rat. This finding is in accordance with the ability of $CD4^+$ T cells to cause rejection of skin allografts in nude or ATXBM rats (5, 8) and to reject heart and renal allografts in acutely irradiated rats (7, 27). Our results also demonstrate that the $CD4^+$ subpopulation alone is able to carry immunological memory for renal allograft rejection since cells from immunized donors were severalfold more potent (on a cell for cell basis) than those obtained from naive animals. Whether or not the greater potency of sensitized $CD4^+$ cells is due to an increase in the number of specifically alloreactive $CD4^+$ cells transferred or to an increase in their functional capability was not determined.

In contrast to $CD4^+$ cells, lymphocytes of the $CD8^+$ phenotype were unable, by themselves, to initiate renal allograft rejection even when obtained from specifically sensitized animals. This result for kidney allografts is consistent with the failure of naive $CD8^+$ T cells to effectively reject skin (8) or heart (7) allografts in T cell–deficient rats, but contrasts with the ability of sensitized $CD8^+$ T cells to mediate heart allograft rejection in acutely irradiated rats (6, 9). Furthermore, recent experiments using mutant mice with isolated class I differences have shown that adoptive transfer of $CD8^+$ cells alone is sufficient to cause skin graft rejection in ATXBM (10) or nude recipients (11). The reason for these apparent differences in the autonomy of $CD8^+$ cytotoxic cells is unclear. However, with regard to the mouse skin graft experiments it may be relevant that mutant class I differences generally elicit a strong alloreactive response when compared with that provoked by allelic class I differences, and with respect to the present experiments, it is necessary to note that the PVG rat is a "low responder" to DA class I MHC (10, 28, 29).

The ability of $CD4^+$ T cells to initiate renal allograft rejection in the nude rat demonstrates that thymically processed $CD8^+$ T cells are not essential for graft rejection, but this does not necessarily exclude a role for specific cytotoxic T cell lysis by the injected cells. Although the majority of the cellular infiltrate in rejecting grafts was shown to originate from the host, it is possible that the relatively small number of inoculum-derived $CD4^+$ T cells present throughout the rejecting kidney were able and sufficient to cause cytotoxic damage. Since $CD4^+$ T cells that have cytolytic activity are generally (30, 31), although not exclusively (32), activated by and lytic towards class II MHC antigens, it is notable that the vascular endothelium within rejecting grafts (commonly regarded as a major target in the rejection response against a vascularized allograft) showed little expression of class II MHC antigens. However, the renal tubular cells were strongly class II–positive and would therefore be potential targets for class II–restricted cytotoxic T cell lysis.

Adoptively transferred $CD4^+$ T cells might alternatively cause renal allograft rejection in the nude rat through the generation of host effector pathways dependent on the "helper" activity provided by the $CD4^+$ T cell inoculum. In principle, these effector pathways could include extrathymically derived cytotoxic cells, activated macrophages, complement activation, and antibody-dependent cellular cytotoxicity (ADCC). Rejecting renal allografts in $CD4^+$ reconstituted nude rats were heavily infiltrated by mononuclear cells, the majority of which (>80% of the total infiltrate) were derived from the athymic host, and many of which had the MRC $OX8^+$, MRC $OX19^-$ phenotype. Congenitally athymic rats possess significant, albeit re-

duced, numbers of MRC OX8$^+$ cells (33) and it is very likely, although not yet formally proven, that the MRC OX8$^+$ cells in the rejecting renal allografts are derived from the athymic host and not from contaminating CD8$^+$ T cells in the CD4$^+$ inoculum. Large numbers of MRC OX8$^+$, MRC OX19$^-$ cells have also been observed in rejecting skin allografts in ATXBM rats (5) and rejecting heart allografts in acutely irradiated rats (7) restored with CD4$^+$ T cells alone. Rat NK cells may express the CD8$^+$ antigen and it is likely that the MRC OX8$^+$ cells in rejecting allografts are of the NK lineage. Since the nonrejecting renal allografts in nonreconstituted nude rats contained very few MRC OX8$^+$ cells, this lymphoid population may represent a potential effector cell population in graft rejection.

Nude mice have been shown to possess lymphoid precursors that can be induced, in vitro, to develop alloantigen-specific cytotoxicity (34–36). Although we were able to demonstrate the presence of host-derived lymphoid cells in rejecting grafts, such cells failed to exhibit donor-restricted cytotoxicity. Mononuclear cells harvested from rejecting allografts in CD4$^+$ T cell–reconstituted nude rats showed high levels of in vitro cytotoxicity against allogeneic but not syngeneic targets and were also lytic for NK and LAK susceptible targets. The presence, in rejecting kidney grafts, of cells that were nonspecifically cytotoxic towards donor strain targets implicates an extrathymically derived lymphoid cell population as a likely effector cell in this model of renal allograft rejection. The cytotoxic repertoire of mononuclear cells obtained from rejecting grafts was similar to that shown by spleen cells and lymph node cells from unmodified nude rats. The nature of the nonspecific cytotoxic cells present in the lymphoid tissue of nude rats (and in rejecting kidney allografts after CD4$^+$ inoculation) remains to be fully elucidated. Their expression of the asialo GM1 glycolipid and their ability to lyse NK target cells are consistent with their classification as NK cells. Interestingly, they are able to recognize MHC-associated target antigens and, as shown by cold target inhibition, they appear to be a heterogeneous effector population with a range of specificities, but also recognizing some putative shared target antigen determinants on the different allogeneic and NK target cells studied.

The in vitro characteristics shown by extrathymically derived cytotoxic cells in nude rats bear similarity to the in vivo phenomenon of allogeneic lymphocyte cytotoxicity (ALC) (37), whereby allogeneic lymphocytes are rapidly destroyed when administered intravenously to unprimed nude (and, to a lesser extent, euthymic) rats. ALC appears to be due to "NK-like" cytotoxic cells, recognizing antigens encoded within the MHC (38), and can be specifically blocked in in vivo cold target inhibition studies (39). Further experiments are in progress to determine the nature of the extrathymically derived cytotoxic effector cells present in the lymphoid tissue of nude rats and within rejecting allografts in CD4$^+$ restored recipients. One possibility is that they are extrathymically derived T-like cells lacking the TCR-α/β but with rearranging genes encoding the TCR-γ/δ; interestingly, a broadly alloreactive T γ/δ cell line has recently been derived from alloimmunized nude mice and shows MHC-associated but not MHC-restricted cytotoxicity (40).

Finally, a role for alloantibody in CD4$^+$ mediated renal allograft rejection must also be considered. Graft rejection after CD4$^+$ reconstitution correlated with a detectable alloantibody response. It is generally accepted that antibody does not play an essential role in acute allograft rejection in the rat (41, 42) and the failure in the

present experiments to cause rejection by transfer of immune serum alone invokes an essential role for cellular effector mechanisms in rejection. However, it cannot be discounted that ADCC-mediated damage caused by alloantibody and extrathymically derived nonspecific killer cells may be involved and this possibility is worthy of further study.

Summary

This study has examined the ability of adoptively transferred $CD4^+$ and $CD8^+$ T cells to mediate rejection of a fully allogeneic DA renal graft in the PVG nude rat. Transfer, at the time of transplantation, of naive $CD4^+$ T cells caused rapid graft rejection and primed $CD4^+$ cells were several times more potent. In contrast, naive or specifically sensitized $CD8^+$ cells were entirely ineffective at mediating renal allograft rejection. Whereas nonrejecting grafts showed only a mild cellular infiltrate, rejecting grafts in $CD4^+$ reconstituted animals showed a substantial infiltrate and many of the infitrating cells had a phenotype (MRC $OX8^+$, MRC $OX19^-$), consistent with NK cells. Experiments using a mAb (HIS 41) against an allotypic determinant of the leukocyte common antigen confirmed that the majority (>80%) of the cellular infiltrate in rejecting grafts derived from the host rather than from the $CD4^+$ inoculum. Infiltrating mononuclear cells, obtained from rejecting allografts 7 d after transplantation in $CD4^+$-injected PVG nude hosts, showed high levels of in vitro cytotoxicity against not only kidney donor strain Con A blasts but also third-party allogeneic Con A blasts, as well as against both NK and LAK susceptible targets. When splenocytes from nontransplanted nude PVG rats were tested in vitro they also demonstrated high levels of lytic activity against both NK and LAK susceptible targets as well as allogeneic Con A blasts, which were not susceptible to lysis by spleen cells from euthymic rats. These findings suggest that injected $CD4^+$ cells may cause renal allograft rejection by the recruitment of extrathymically derived, widely alloreactive cells into the kidney in this model of graft rejection.

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