

PROLONGED SURVIVAL OF ACTIVELY ENHANCED RAT
RENAL ALLOGRAFTS DESPITE ACCELERATED CELLULAR
INFILTRATION AND RAPID INDUCTION OF BOTH
CLASS I AND CLASS II MHC ANTIGENS

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In some animal models, notably the rat, preoperative administration of donor strain blood is sufficient to produce long-term renal allograft survival, even in the absence of adjunctive immunosuppression (1, 2). The mechanisms underlying this enhancing effect are unclear, and interpretation of relevant experiments is made difficult by the complexity of the rejection response and controversy about the relative importance of the different cellular effectors (cytotoxic T cells and delayed-type hypersensitivity [DTH]¹ responses) (3, 4). Possible mechanisms of enhancement include the elimination (5) or clonal deletion (6) of alloreactive lymphocytes, removal of passenger leukocytes (7), blocking of effector cells by antibody or antigen, and in particular, the induction of alloantigen-specific suppressor cells (or factors) (8–10).

In the rat, renal allografts undergoing unmodified rejection are characterized by a progressive and heterogeneous mononuclear cell infiltrate (11) and a striking increase within the rejecting graft of donor class I and class II MHC antigen expression (12). These events are closely related because the induction of MHC antigens is mediated, at least in part, by products (notably IFN- γ —a potent inducer of both class I and class II MHC antigens) (13, 14) released from activated infiltrating cells whereas the distribution and density of MHC expression may influence the vulnerability of the graft to the effector responses (4).

In this report we show that, although preoperative administration of donor strain whole blood increases rat renal allograft survival, it results in a rapid disappearance of graft interstitial dendritic cells and, paradoxically, an accelerated cellular infiltration of the graft. The levels of alloantigen-specific and -nonspecific cytotoxicity produced by splenocytes and graft infiltrating cells are comparable in both transfused and unmodified recipients. In addition, enhanced grafts unexpectedly show an accelerated and substantial induction, not only of donor class I but also of class II MHC antigens. These findings suggest that

This work was supported by the National Kidney Research Fund and the Western Infirmary Kidney Research Fund. Dr. Spencer is a Rank Foundation Scholar. Address correspondence to Dr. Bradley, University Department of Surgery, Western Infirmary, Glasgow G11 6NT, Scotland.

¹ *Abbreviation used in this paper:* DTH, delayed-type hypersensitivity.

preoperative blood transfusion causes sensitization of the recipient resulting in an accelerated immunological response to the allograft that is, nevertheless, rapidly and effectively suppressed by immunoregulatory mechanisms.

Materials and Methods

Animals. Inbred male rats of the DA (RT1^a) and PVG (RT1^c) strains were obtained from OLAC Ltd. (Bicester, Oxon, England) and used when 8–16 wk old.

Kidney Transplantation. Kidneys from DA strain donors were transplanted into the left orthotopic site of PVG recipients with end-to-end anastomosis of the renal artery, renal vein, and ureter (15). The procedure was performed under chloral hydrate anesthesia and ischemia times were ~25 min. Except where otherwise stated, the right kidney of the recipient was left in situ. Rats in the transfused groups received 1 ml of heparinized donor strain whole blood i.v. 7 d before transplantation.

Antibodies. The following mouse mAbs were used to identify rat leukocytes: MRC OX-1 (leukocyte common antigen [16]), MRC OX-8 (T cytotoxic/suppressor cells [Tc/s] and NK cells [17–19]), W3/25 (T helper cells [Th] and some macrophages [20, 21]), MRC OX-19 (pan T cell determinant [18]), W3/13 (T cells, neutrophils and plasma cells [22]) and MRC OX-39 (leukocytes expressing IL-2 receptor). MRC OX-39 is a mouse IgG1 antibody, raised against rat T blasts, that recognizes the IL-2 receptor on the basis that it immunoprecipitates a protein of M_r 45,000 nonreduced and 50,000 reduced, and inhibits pure IL-2 from binding to T blasts (Jefferies, W. A., D. J. Paterson, J. R. Green, M. R. Brandon, P. Corthesy, M. Puklavec, and A. F. Williams, manuscript in preparation). MRC OX-21 (human C3b inactivator [23]) was used as a negative control throughout. The following mAbs identified monomorphic MHC class I and II determinants: MRC OX-18 (rat MHC class I antigens [24]) and MRC OX-6 (rat MHC class II I/A antigen [24]). These antibodies were a kind gift of Dr. D. W. Mason and Dr. A. F. Williams (MRC Cellular Immunology Unit, Oxford). In addition, mAbs used to distinguish between donor and recipient MHC class I and II antigens were: MN4-91-6 (NDS 54), which reacts with a polymorphic determinant of MHC class I present in DA but not PVG strain rats (25), and F17-23-2, which reacts with a polymorphic determinant of MHC class II I/A antigen present in DA but not PVG rats (25, 26). These antibodies were a kind gift of Professor J. Fabre, Blond McIndoe Centre for Medical Research, East Grinstead, Sussex.

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 monoclonal antibodies was used to label the slides using a two-layer immunoperoxidase technique (21).

Quantitative 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 (27, 28). Sections were examined at a magnification of 400 in the presence of a microscope eyepiece graticule engraved with a squared grid bearing either 121 intersections (1 mm apart) or 745 intersections (0.5 mm apart). For each high power field, the number of positively stained cells superimposed by an intersection was counted and the area of the field occupied by cells of a particular phenotype was calculated as: percent area of infiltrate = $100 \times (\text{number of positive grid intersections}) / (\text{total number of grid intersections})$. All sections stained with MRC OX-8, W3/25, MRC OX-19, W3/13, and MRC OX-39 were counted using a 745-point graticule, as were some sections stained with MRC OX-1 or a mixture of MRC OX-8 and W3/25. However, in the latter two cases, slides with very heavy infiltrates were, for convenience, counted using the 121-point graticule. For all sections, the total number of points observed was well in excess of that required to maintain the accuracy of the point-counting technique (27). All slides were assessed by two observers who had no prior knowledge of the time point or the group from which the kidney had been harvested. Each observer counted 10 adjacent high power fields of the kidney cortex and the results were expressed as the mean of the results of the two observers. There was close agreement between the two observers for all the antibodies used as shown by the correlation coefficient (r) and by the mean percent difference (D) between the two observers, where

$D = 100 \times [(\text{area infiltrate, observer A}) - (\text{area infiltrate, observer B})] / [(\text{area infiltrate, observer A}) + (\text{area infiltrate, observer B})]$. Values of r and D for individual antibodies were as follows: MRC OX-1, $r = 0.99$, $D = 6.7\%$; MRC OX-8, $r = 0.98$, $D = 10.9\%$; W3/25, $r = 0.85$, $D = 16.0\%$; MRC OX-19, $r = 0.93$, $D = 14.3\%$; W3/13, $r = 0.95$, $D = 8.6\%$; MRC OX-39, $r = 0.97$, $D = 15\%$ and (OX8 + W3/25), $r = 0.98$, $D = 11.1\%$. Because no attempt was made by the two observers to count the same area of the tissue section, the close correlation between two observers indicated that the area counted was representative of the whole section.

Harvesting of Graft Infiltrating Cells. Infiltrating mononuclear cells were harvested from kidney allografts using enzyme digestion (hyaluronidase and collagenase) as previously described (29).

Cytotoxicity Assays. 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 (29). Alloantigen-specific cytotoxicity was assessed using ^{51}Cr -labeled DA (RT1^a) Con A-transformed splenic blasts as targets and nonspecific cytotoxicity was assessed using the rat myeloma Y3 (30), which has the RT1^a MHC genotype and is susceptible to NK activity. Specific ^{51}Cr release = $100 \times [(\text{experimental release}) - (\text{spontaneous release})] / [(\text{maximum release}) - (\text{spontaneous release})]$. Data shown are the means of quadruplicate determinations (SD <5%; spontaneous release <20% of maximum release in all experiments).

Kidney Homogenates. The methods used for preparation of tissue homogenates and quantitative analysis of MHC antigens were essentially those of Milton et al. (12). Homogenates were prepared from normal DA kidneys, and from DA renal allografts from both enhanced and untreated recipients. Kidneys were excised, snap frozen in liquid nitrogen, crushed, and stored as powder at -70°C . Homogenates were prepared by pooling five kidneys in each group for each study day and mechanically homogenising them with a Teflon pestle at 4°C in the presence of Tris-buffered saline containing 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 2.5 mM iodoacetamide to inhibit proteolysis. The washed homogenates were frozen at -70°C until use.

Quantitative Analysis of Donor Class I MHC Antigens. 80 μl of tripling dilutions of each kidney homogenate were incubated with 80 μl of a predetermined dilution of donor-specific anti-class I antibody MN4-91-6 representing conditions of target antigen excess in the assay system. After incubation for 1 h at 4°C the supernatants were assayed for the presence of unbound antibody using an indirect radioactive binding assay (31). Duplicate 25- μl samples of supernatant were incubated with 25 μl of DA erythrocytes at $10^9/\text{ml}$ (class I target) for 1 h at 4°C , which were then washed twice and incubated with 100 μl of ^{125}I -labeled sheep anti-mouse F(ab')₂ (Amersham International, Aylesbury, UK) at saturating concentrations for 1 h at 4°C . The cells were then washed and the cell bound radioactivity measured in a gamma counter (LKB Instruments, London).

Quantitative Analysis of Donor Class II MHC Antigens. The donor-specific anti-class II antibody F17-23-2 was unsuitable for absorption analysis because, although it worked well for immunohistologic analysis, its affinity did not appear to be sufficient to prevent high free antibody concentrations even in the presence of antigen excess in the absorption analysis (25). Instead, polyspecific PVG anti-DA spleen serum was the source of anti-class II antibodies, and the specificity of the assay derived from the use of purified DA class II (I/A) MHC antigen as the target. As in the class I analysis, tripling dilutions of kidney homogenates were incubated with a predetermined dilution of antibody, in this case the polyspecific PVG anti-DA spleen serum. After incubation for 1 h at 4°C , the supernatants were assayed for the presence of unbound anti-DA class II antibody using a radioactive binding assay. 25- μl samples of supernatants were placed in 96-well polyvinyl chloride (PVC) plates (Dynatech, Billingshurst, Sussex), which had been previously coated with the purified DA class II antigen. After incubation for 1 h the wells were washed and incubated for a further 1 h with 50 μl of ^{125}I -labeled rabbit F(ab')₂ anti-mouse F(ab')₂, which is highly crossreactive (>80%) with rat F(ab')₂ and has been described elsewhere (12). The wells were again washed, then cut out individually and the bound radioactivity measured in a gamma counter.

Results

In the DA into PVG rat strain combination, administration of 1 ml of donor strain whole blood 7 d before renal transplantation produces active enhancement with prolonged graft survival, whereas unmodified recipients rapidly reject their grafts within a few days of transplantation. In an initial experiment (during which contralateral nephrectomy was performed at the time of transplantation), five transfused allograft recipients all survived for >100 d and blood urea measurements at day 5 and thereafter weekly remained low (<20 mM) in all animals. By contrast the individual survival times in eight unmodified allograft recipients were 7, 7, 7, 7, 7, 8, 8, and 10 d, with a mean day 5 blood urea of 44 mM (range, 32–54 mM), increasing thereafter until death.

Kinetics of Leukocyte Infiltration

The results of the morphometric analysis of cryostat sections labeled with MRC OX-1 (leukocyte-common antigen) are shown in Fig. 1*a*. Day 1 renal allografts showed a minimal cellular infiltrate, but thereafter both rejecting DA kidneys from unmodified PVG recipients and nonrejecting kidneys from transfused recipients showed a progressive mononuclear cell infiltrate. As can be seen, cellular infiltration occurred more rapidly in the enhanced than in the rejecting grafts, and day 3 enhanced grafts had a mean percent area leukocyte infiltrate of 31% ($\pm 8\%$) compared with that of 11% ($\pm 3\%$) in rejecting grafts ($p < 0.05$). However, by day 5, the cellular infiltrate in rejecting grafts had increased rapidly to reach levels similar to, or in excess of, those seen in day 5 enhanced allografts. Day 7 rejecting allografts were too severely damaged (frequently they had undergone complete infarction) for meaningful immunohistologic analysis but in day 7 enhanced grafts the level of infiltrate was similar to that at day 5. Enhanced allografts were also examined at day 100 after transplantation (three grafts studied) and showed only a minimal (<5%) leukocyte infiltrate. Comparison of the distribution of the leukocyte infiltrate in rejecting and enhanced grafts showed a similar pattern. Initially, the infiltrate was predominantly focal and largely perivascular, and subsequently it progressed to a diffuse interstitial, peritubular and periglomerular infiltrate but with a notable absence of infiltrating cells within the glomeruli. In contrast to allografts, DA isografts examined at days 1, 3, and 5 after transplantation showed only a sparse cellular infiltrate, which when assessed by morphometric analysis, did not exceed 5% in any of the grafts examined (results not shown).

Phenotypic Analysis of the Cellular Infiltrate

Fig. 1 also shows the results of the phenotypic analysis of the cellular infiltrate in rejecting and enhanced allografts. Serial sections labeled with the different monoclonal antibodies were assessed by morphometry and the results are expressed as the percentage area infiltrate for each antibody. Day 3 enhanced allografts showed a heavier infiltrate of MRC OX-8⁺ cells (Tc and most NK cells) than day 3 rejecting grafts (Fig. 1*b*) but by day 5, the MRC OX-8⁺ infiltrate in rejecting grafts had increased markedly and was greater than that in day 5 enhanced allografts (21% \pm 3% vs. 11% \pm 1%, $p < 0.01$). See also Fig. 2*A* and *B*. The percentage area infiltrate in day 7 enhanced grafts was similar to that seen at day 5.

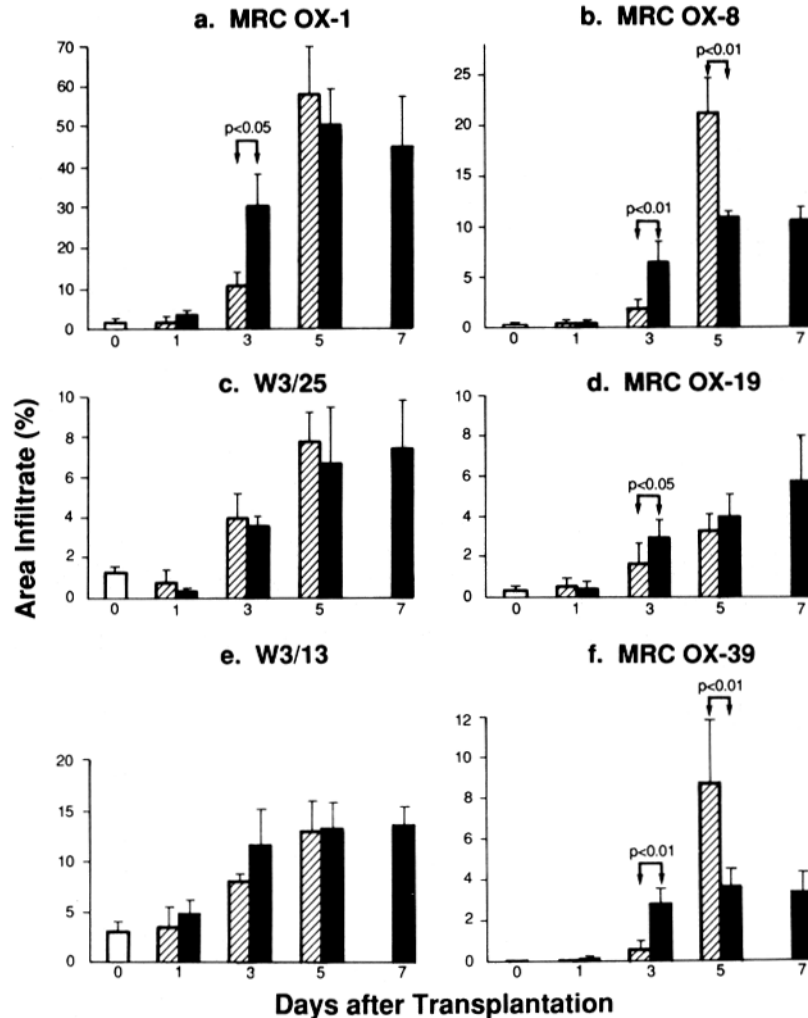


FIGURE 1. Percent labeled cellular infiltrate in normal DA kidney (*open bars*), in DA renal allografts from unmodified PVG recipients (*hatched bars*), and actively enhanced allografts from transfused recipients (*solid bars*). Cryostat sections were labeled with MRC OX-1 (*a*), MRC OX-8 (*b*), W3/25 (*c*), MRC OX-19 (*d*), W3/13 (*e*), and MRC OX-39 (*f*). Percent infiltrate was determined by point counting with a microscope eyepiece graticule. The results are the mean \pm SD of five allografts examined at each time point. Statistical analysis was performed using the Mann-Whitney U test.

Both rejecting and enhanced grafts showed a progressive accumulation of W3/25⁺ cells (Th and some macrophages). However, the absolute percent area infiltrate, which was the same in both rejecting and enhanced allografts (Fig. 1 *c*) was consistently less than that observed when corresponding sections were labeled with MRC OX-8 (see also Fig. 2 *C* and *D*). Cryostat sections were also labeled with a mixture of MRC OX-8 and W3/25 antibodies. The percent area infiltrate determined in these sections was very similar to that calculated as the sum of MRC OX-8⁺ and W3/25⁺ cells for each section (results not shown), indicating

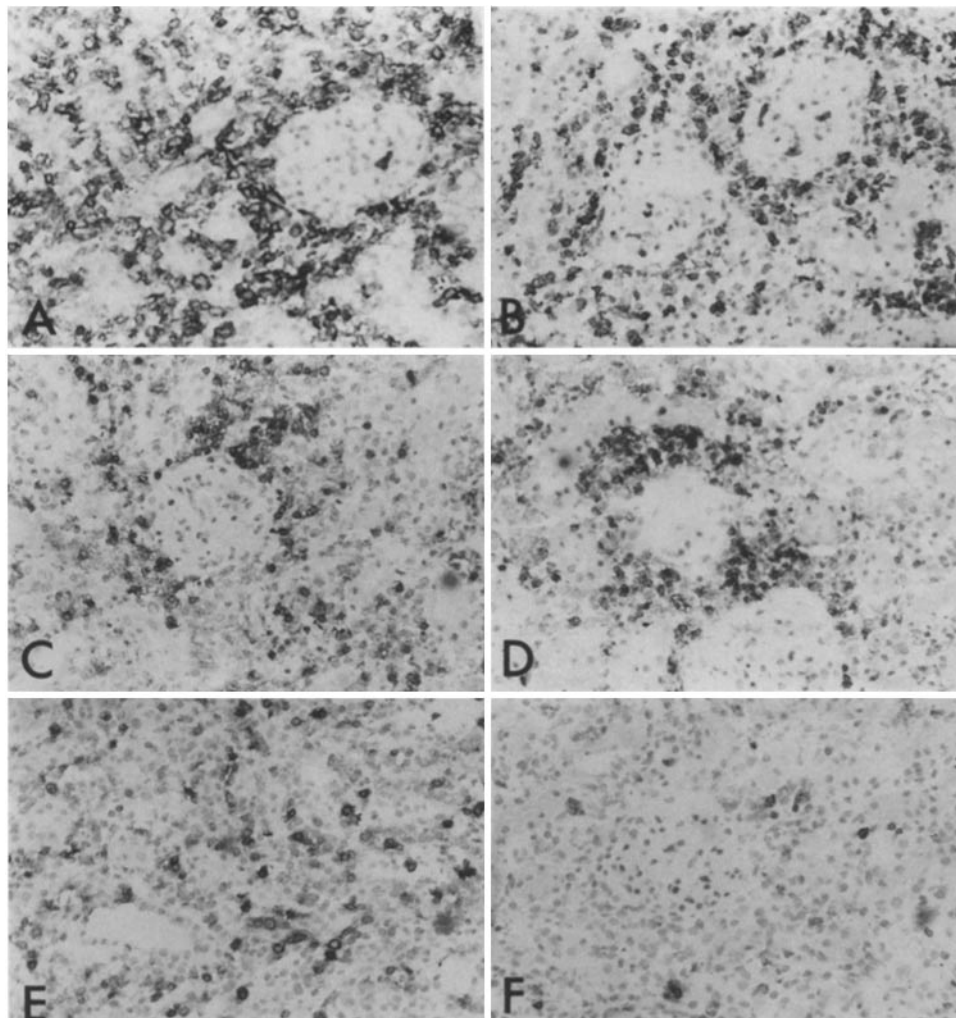


FIGURE 2. Immunohistologic studies in rejecting and actively enhanced DA renal allografts removed at day 5 from PVG recipients. Cryostat sections were labeled with mAbs using the immunoperoxidase technique. Sections were lightly counterstained with hematoxylin and photographed using a green filter to enhance the contrast of the peroxidase reaction product. All sections $\times 250$ (A) Rejecting and (B) enhanced renal allografts labeled with MRC OX-8. Note heavier infiltrate in rejecting graft. (C) Rejecting and (D) enhanced allografts labeled with W3/25 showing similar levels of infiltration. (E) Rejecting and (F) enhanced allografts labeled with MRC OX-39. Note relative absence of IL-2 receptor-positive cells in enhanced graft.

that few of the infiltrating cells expressed both the W3/25 and MRC OX-8 antigens simultaneously.

The percent labeled infiltrate using MRC OX-19 (pan T cell) gave unexpectedly low results (Fig. 1d) for both rejecting and enhanced allografts, with values which were far exceeded by the sum of MRC OX-8⁺ and W3/25⁺ cells. This discrepancy is undoubtedly due, at least in part, to the presence of large numbers of NK cells (MRC OX-8⁺, MRC OX-19⁻) and/or macrophages (W3/25⁺, MRC

OX-19⁻) but may also be a result of weakly labeled MRC OX-19⁺ cells, which were not apparent using the immunoperoxidase staining technique. The pattern of labeling with W3/13 (T cells, neutrophils, and plasma cells) was similar to, but greater than that seen for MRC OX-19 (Fig. 1*e*).

Staining with MRC OX-39 showed a more rapid appearance of IL-2 receptor-positive cells in enhanced grafts (Fig. 1*f*) but by day 5 the percent area infiltrate was higher in the rejecting than in enhanced grafts ($9\% \pm 3\%$ vs. $4\% \pm 1\%$, $p < 0.01$). See also Fig. 2*E* and *F*.

Cytotoxicity Assays

Graft infiltrating cells and spleen cells were harvested 5 d after renal transplantation and tested for specific and nonspecific cytotoxicity in a 6-h chromium-release assay. Specific cell lysis was assessed using DA Con A blasts as the target cells, and Fig. 3*a* shows the results of a typical experiment. Both graft infiltrating cells and splenocytes from unmodified recipients demonstrated cytotoxic activity against the specific target although the levels of lytic activity were modest and, in the case of graft infiltrating cells, a marked "prozone" effect was commonly seen. Cells obtained from the graft and spleen of transfused recipients also demonstrated significant specific cytotoxicity and the levels of lysis seen were similar to those observed in cells obtained from unmodified recipients. In five separate experiments the values for specific cytotoxicity produced by splenocytes from unmodified vs. transfused recipients (at E/T ratio of 100:1) were 18% vs. 16%, 20% vs. 23%, 30% vs. 26%, 19% vs. 22%, and 25% vs. 26%. Similarly, values for the specific cytotoxicity produced by graft infiltrating cells from unmodified vs. transfused recipients (at E/T ratio of 50:1) were 22% vs. 15%, 13% vs. 13%, 14% vs. 20%, 17% vs. 29% and 21% vs. 24%. In additional experiments, in which "third-party" Lewis (RT1^b) strain Con A blasts were used as the targets, there was minimal cytotoxic activity, indicating that the cytotoxic activity against DA Con A blasts was due to alloantigen specific cytotoxic cells rather than nonspecific effector cells. It was notable that the levels of alloantigen specific cytotoxicity observed in the above experiments were consistently lower (twofold or more) than the levels found in identical experiments carried out on unmodified renal allograft recipients in other rat strain combinations (e.g., DA into Lewis [RT1^b]).

Nonspecific cell lysis was assessed using the rat myeloma Y3 as the target. Graft infiltrating cells and splenocytes from both unmodified and transfused recipients showed nonspecific cytotoxicity (Fig. 3*b*). The levels of lytic activity were similar in both untreated and transfused recipients and within each experiment were usually greater than those seen against the alloantigen-specific target. In five separate experiments the values for nonspecific cytotoxicity produced by splenocytes from unmodified vs. transfused recipients (at E/T ratio of 100:1) were 32% vs. 40%, 29% vs. 37%, 70% vs. 74%, 30% vs. 39%, and 47% vs. 45%. Similarly, values for the nonspecific cytotoxicity produced by graft infiltrating cells from unmodified vs. transfused recipients (at E/T ratio of 100:1) were 15% vs. 15%, 23% vs. 19%, 25% vs. 22%, 15% vs. 22%, and 30% vs. 26%.

In two experiments, alloantigen specific cytotoxicity and nonspecific cytotoxicity were examined after depletion of macrophages from the effector cells by

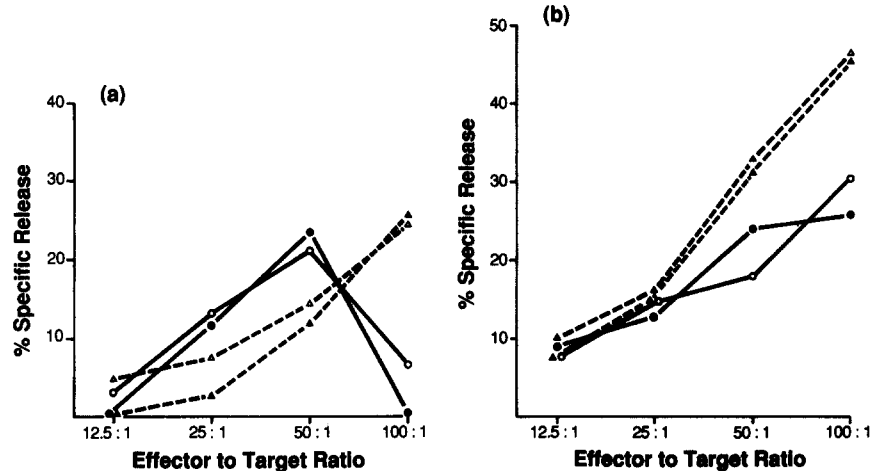


FIGURE 3. (a) Cytotoxic activity of cells recovered at day 5 from renal allograft (*open circles*) and spleen (*open triangles*) of unmodified recipient and from the renal allograft (*solid circles*) and spleen (*solid triangles*) of transfused recipient, against the alloantigen-specific target (DA Con A blasts). (b) Nonspecific cytotoxic activity of cells from day 5 renal allograft (*open circles*) and spleen (*open triangles*) of unmodified recipient and from renal allograft (*solid circles*) and spleen (*solid triangles*) of transfused recipient, against the Y3 rat myeloma target.

incubating them with carbonyl iron powder, at 37°C for 30 min and then removing the iron particles and phagocytic cells with a strong magnet. This procedure did not reduce either the alloantigen specific or nonspecific cytotoxic activity, indicating that the observed cytotoxicity was mediated by lymphocytes.

Class I MHC Antigen Expression

Immunohistology. Class I MHC expression was detected by immunoperoxidase staining with MN4-91-6, which labels DA but not PVG class I antigens (therefore allowing unequivocal determination of donor class I expression), and also by MRC OX-18, which labels a monomorphic class I MHC antigen determinant. The distribution of class I MHC antigens in normal DA kidney (indicated by labeling with MN4-91-6) is shown in Fig. 4A. There was strong staining of interstitial areas (including dendritic cells and possibly capillary endothelium) and arteriolar and venous endothelium, moderate staining of glomeruli, weak staining of the cortical tubules, and occasional patchy staining of medullary tubules. The same distribution of class I MHC antigens in normal DA kidney was also observed with MRC OX-18. DA renal allografts from both unmodified and transfused PVG recipients (five kidneys from each group for each time point were examined) showed rapid and marked induction of donor class I MHC antigens on all structures. Increased tubular staining was apparent as early as day 1, and by day 3 both rejecting (Fig. 4B) and enhanced grafts (Fig. 4C) showed strong staining of all tubular cells and glomeruli. These changes persisted in day 5 rejecting, day 5 enhanced, and day 7 enhanced allografts. In day 100 enhanced allografts (three kidneys examined), class I MHC expression had reverted to normal except that the donor strain interstitial staining was not apparent. When the above sections were labeled with MRC OX-18, a similar pattern of class I MHC expression was seen, except that, in addition, there were

large numbers of positively labeled infiltrating cells and, in particular, in day 100 enhanced allografts, there was a normal complement of recipient strain dendritic cells (MN4-91-6⁻, MRC OX-18⁺). DA isografts examined at days 3, 5, and 7 occasionally showed a slight increase in class I MHC antigen expression on renal tubular cells, but this was much less intense and more variable than that seen in rejecting and enhanced allografts.

Quantitative Absorption Analysis. The kinetics of donor class I MHC antigen induction in rejecting and enhanced renal allografts were assessed by quantitative absorption analysis of kidney homogenates using the mAb MN4-91-6. This analysis confirmed the immunohistologic findings and showed that in both rejecting and enhanced allografts there was a rapid and substantial increase in class I MHC antigen expression, which by day 5 was severalfold greater than that found in normal kidneys. As shown in Fig. 5, the induction of class I MHC antigens occurred more rapidly in enhanced than in rejecting grafts and even by day 1 enhanced grafts showed a pronounced increase. By day 3 the levels were similar to those detected only by day 5 in rejecting grafts.

Class II MHC Antigen Expression

Immunohistology. Class II MHC expression was detected by immunoperoxidase staining with F17-23-2, which labels DA but not PVG class II MHC antigens, therefore allowing a precise determination of donor class II expression, and also by MRC OX-6 which labels a monomorphic class II MHC antigen determinant (five kidneys from each group for each time point were examined). The distribution of class II MHC antigen in normal DA kidney (indicated by labeling with F17-23-2) is shown in Fig. 4D. Class II antigen expression was most obvious on the interstitial dendritic cells scattered throughout the kidney. In addition, there was weak class II antigen expression by some renal tubules (mostly by the basal half of cells in the proximal tubules), but there was no evidence of class II expression by vascular endothelium nor by glomeruli and the surrounding Bowman's capsule.

By day 1 after transplantation allografts from both unmodified and transfused recipients showed a slight diminution in the frequency of class II positive interstitial dendritic cells. Apart from this feature day 1 rejecting allografts were indistinguishable from normal kidneys, but day 1 enhanced allografts showed a slight focal increase in donor class II antigen expression on proximal tubules. By day 3, both rejecting (Fig. 4E) and enhanced (Fig. 4F) grafts showed foci of increased class II expression and this was most marked in enhanced grafts. In addition, day 3 enhanced, but not rejecting grafts, showed occasional induction of donor class II antigen on arteriolar endothelium and on Bowman's capsules as well as a striking reduction in the number of interstitial dendritic cells. By day 5, both rejecting (Fig. 4G) and enhanced (Fig. 4H) grafts stained strongly for donor class II antigen on all tubular cells, arteriolar vascular endothelium, and Bowman's capsule although the glomeruli remained negative. In addition, donor-type (F17-23-2⁺) interstitial dendritic cells had virtually disappeared and did not appear to have been replaced by recipient-type dendritic cells (i.e., MRC OX-6⁺ cells). These changes persisted in day 7 enhanced grafts. By day 100, the donor class II antigen expression on renal tubules had reverted almost to normal and

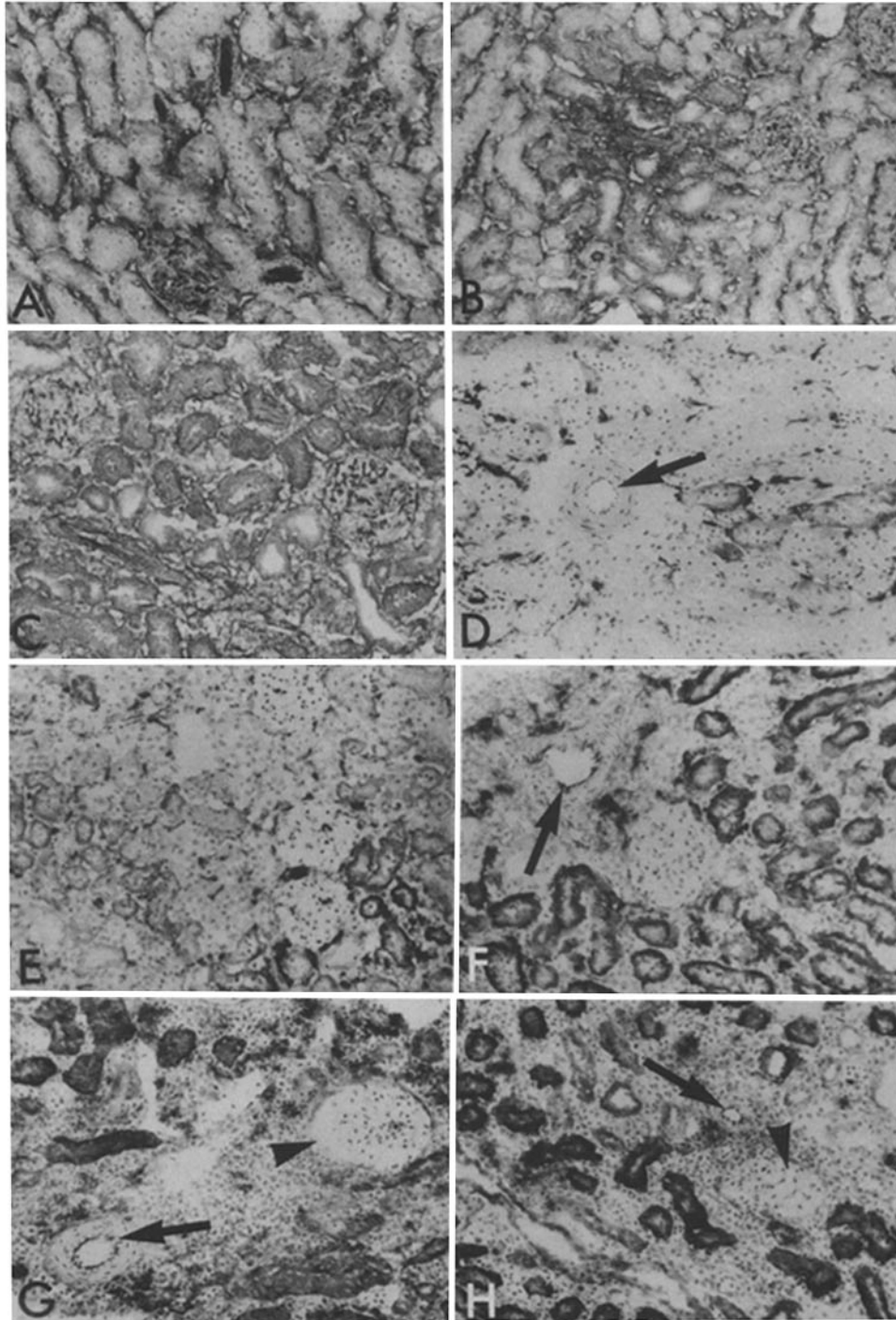


FIGURE 4. Immunohistologic studies of class I and class II MHC antigens in normal DA kidney and in rejecting and actively enhanced DA renal allografts from PVG recipients. Cryostat sections were labeled with mAbs MN4-91-6 against donor class I MHC antigens (A-

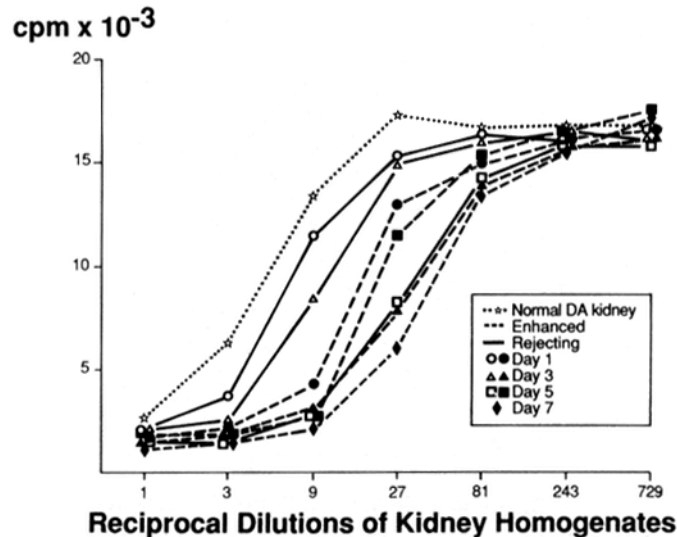


FIGURE 5. Kinetics of donor class I MHC antigen induction in rejecting and actively enhanced DA kidneys from PVG recipients. Quantitative absorption analysis was performed with the MN4-91-6 antibody, which was absorbed with tripling dilutions of kidney homogenate. Residual antibody was assayed by incubating with DA RBC and detecting binding with ¹²⁵I-labeled sheep anti-mouse.

only occasional expression of donor class II MHC antigen on arteriolar vascular endothelium was apparent. The day 100 enhanced allografts contained a normal complement of interstitial dendritic cells that expressed only recipient-type class II antigen (i.e., were F17-23-2⁻ but MRC OX-6⁺). None of the DA isografts examined at day 3, 5, or 7 showed any increase in class II expression after transplantation.

Quantitative absorption analysis. The kinetics of donor class II MHC antigen induction in rejecting and enhanced allografts were assessed by quantitative absorption analysis using polyspecific PVG anti-DA spleen serum as the source of antibody, and microtiter plates coated with purified DA class II antigen as the target. This analysis (Fig. 6) confirmed the immunohistologic findings and showed that donor class II MHC induction occurred more rapidly in the enhanced than in the rejecting renal allografts. By day 1 after transplantation

(C) and F17-23-2 against donor class II MHC antigens (D-H) using the immunoperoxidase technique. All sections $\times 160$. (A) Normal DA kidney labeled with MN4-91-6 antibody. Note strong staining of interstitial cells and vascular endothelium, moderate staining of glomerulus and weak staining of tubules. (B) Rejecting and (C) enhanced renal allografts 3 d after transplantation labeled with MN4-91-6. Note increased staining of tubules and a decrease in C of positively stained interstitial cells. (D) Normal DA kidney labeled with F17-23-2. Note strong staining of interstitial dendritic cells, weak staining of some proximal tubules, and lack of staining of vascular endothelium (arrow). (E) Rejecting and (F) enhanced renal allografts 3 d after transplantation labeled with F17-23-2 antibody. Note increased staining of tubules (more marked in enhanced graft) and staining of arteriolar endothelium (arrow) in enhanced graft. (G) Rejecting and (H) enhanced allografts 5 d after transplantation labeled with F17-23-2 antibody. Note heavy staining of all tubular cells in the absence of staining of the large number of host infiltrating cells. Arteriolar endothelium is positively stained (arrows) but glomeruli (excepting Bowman's capsule) remain negative (arrowheads) and there are very few positively stained interstitial cells. In all experiments, labeling with MRC OX-21 (mouse anti-human C3b inactivator) was completely negative.

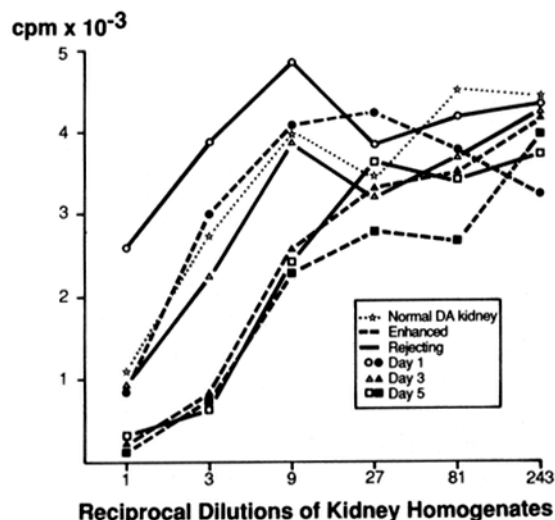


FIGURE 6. Kinetics of donor class II MHC antigen induction in rejecting and actively enhanced DA kidneys from PVG recipients. PVG anti-DA serum was absorbed with tripling dilutions of the kidney homogenates. Residual antibody was assayed by incubating the absorbed serum on PVC microtitre plates to which pure class II MHC antigens had been bound. Binding of the alloantiserum was detected with ¹²⁵I-labeled rabbit anti-mouse.

rejecting allografts showed a reduction in class II MHC antigen expression whereas enhanced allografts showed levels similar to those in normal kidney. This may be explained by a reduction in the number of interstitial dendritic cells in both rejecting and enhanced allografts (inasmuch as these cells make up a large proportion of class II positive structures in the normal kidney), which in the case of day 1 enhanced grafts is compensated for by the rapid induction of class II antigen on renal tubular cells. By day 3 the expression of class II in rejecting allografts had increased to levels slightly greater than those in normal kidney. By contrast, day 3 enhanced allografts showed a marked increase in class II induction, with levels similar to those subsequently seen in day 5 rejecting grafts. There was no further increase in class II expression by day 7 in either enhanced or rejecting grafts (data not shown).

Discussion

The prolonged survival of MHC incompatible organ grafts, achieved by prior administration of donor antigen, depends on several factors that include the strain combination, the type of organ transplanted, and the nature, dose and timing of the antigen pretreatment (2, 32, 33). We have shown in this report that, although a single preoperative transfusion of donor strain blood markedly prolongs rat renal allograft survival, there is an accelerated immunological response to the graft which is characterized by early loss of graft dendritic cells, rapid and substantial leukocyte infiltration, alloantigen-specific and -nonspecific cytotoxic activity, and unexpectedly, an accelerated and striking increase of donor strain class I and class II MHC antigen expression within the graft. These findings are of relevance to both the process of renal allograft rejection and to an understanding of the induction phase of active enhancement.

Bone marrow-derived interstitial cells (passenger leukocytes) are highly immunogenic components of organ allografts and are generally considered to be important initiators of the rejection response (7, 34, 35). The disappearance of donor interstitial dendritic cells from rejecting and enhanced renal allografts may have been due to either destruction *in situ* or migration from the graft. The more rapid disappearance of dendritic cells from enhanced grafts corresponded with, and possibly initiated, the accelerated leukocyte infiltrate, the composition of which was similar to that seen in rejecting grafts, with the notable exception that enhanced grafts contained a reduced number of MRC OX-8⁺ cells and MRC OX-39⁺ cells. Whether this reflected important differences in the proportions of functionally diverse MRC OX-8⁺ cells in rejecting and enhanced grafts is not clear from the present study but the increased number of IL-2 receptor-positive cells suggests a greater degree of cellular activation in rejecting grafts.

The striking induction of class I and class II MHC antigens in rejecting allografts is widely reported (12, 25, 36, 37). However, the accelerated and substantial increase in not only class I but also class II MHC antigens in enhanced allografts was surprising. It is of particular interest because increased expression of class I and class II MHC antigens may, in principle, contribute to rejection both by activating the rejection responses and by making target cells more susceptible to them. In particular, specific cytotoxic T cell lysis directed preferentially at class I MHC targets (38) may be augmented by increased expression of class I antigens whereas DTH responses and the action of Th cells, directed primarily at class II targets, may be augmented by increased expression of class II MHC antigens (39, 40). Because the induction of class II MHC antigens is almost certainly mediated by IFN- γ (13, 14) and other lymphokines released from activated graft infiltrating cells, it might be expected that an effective immunosuppressive regimen would prevent class II induction. This suggestion is supported by the recent report that nonrejecting rat renal allografts in cyclosporin-treated recipients showed diminished induction of class I and no induction of class II MHC antigens (41). The findings in the present study therefore suggest that there are, within the enhanced grafts, activated T cells, which have differentiated to the stage of secreting lymphokines such as IFN- γ but which are unable to mediate effective graft damage. If the accelerated MHC induction in enhanced grafts does, as seems likely, increase their vulnerability to rejection, it is clearly insufficient to overcome the protective mechanisms that are initiated during enhancement. Moreover, the accelerated MHC induction may be a necessary component of the early phase of enhancement which, after prior transfusion, provides the appropriate signal for induction of suppressor mechanisms.

The observation that spleen cells and graft infiltrating cells from both transfused and untreated recipients showed similar levels of cytotoxic activity against NK-sensitive targets is consistent with the consensus that nonspecific cytotoxic effector cells do not play a major role in graft rejection (3, 29). However, the finding that unmodified and transfused graft recipients also showed similar levels of alloantigen-specific cytotoxicity was surprising. Assuming that the *in vitro* cytotoxicity assay is an appropriate indication of cytolytic activity within the graft this suggests that either specific cellular cytotoxicity is not the predominant

mechanism of graft destruction in the DA into PVG strain or that the observed Tc activity is effectively suppressed within the graft. Interestingly, the levels of alloantigen-specific cytotoxicity were lower than those seen in other rat strain combinations (e.g., DA into Lewis) under identical conditions, which may be attributable in part to Ir gene control and the "low responder" status of PVG recipients to this aspect of the alloimmune response (42, 43). Nevertheless, unmodified PVG recipients reject DA renal allografts in times comparable to other recipient strains, raising the possibility that additional mechanisms, notably DTH, play a significant role in the rejection response in this strain combination. Irrespective of the mechanism of graft rejection, however, the present study emphasizes the overwhelming suppressive effect of preoperative donor blood transfusion despite the presence of both large numbers of potential effector cells and increased target antigen expression within the graft.

Summary

Administration of 1 ml of donor whole blood 7 d before renal transplantation produces long-term (>100 d) graft survival in the DA (RT1^a) into PVG (RT1^c) rat strain combination. Using this model, the pattern and phenotype of infiltrating leukocytes were examined in rejecting and enhanced renal allografts, at days 1, 3, 5, and 7 after transplantation, by immunohistologic techniques. Paradoxically, enhanced grafts showed a more rapid and substantial leukocyte infiltrate, the phenotype of which was similar to that in rejecting grafts except for a reduced number of MRC OX-8⁺ cells and MRC OX-39⁺ cells. Graft infiltrating cells and splenocytes from transfused animals showed similar, although modest, levels of both nonspecific cytotoxicity and alloantigen-specific cytotoxicity. Immunohistologic analysis of MHC antigen distribution within the allograft revealed, unexpectedly, that enhanced grafts underwent an accelerated and extensive induction of both donor class I and class II MHC antigens. These findings were confirmed by allospecific quantitative absorption analysis, which showed severalfold increases in class I and class II MHC antigens by day 3 in enhanced grafts but not until day 5 in rejecting grafts. An additional observation was the more rapid disappearance of donor interstitial cells from enhanced grafts. These findings emphasize the overwhelming suppressive effect induced by an organ allograft after preoperative blood transfusion despite the associated induction of large numbers of potential effector cells and increased target antigen density within the graft.

The authors are grateful to Professor J. Fabre, Blond McIndoe Centre for Medical Research, East Grinstead, Sussex for allowing the class II quantitative analysis to be performed in his department and to Dr. C. Milstein, MRC Laboratory of Molecular Biology, Cambridge, for permission to use the Y3 rat myeloma cell line. We would like to thank Professor W. R. Lee, Department of Pathology, Western Infirmary, Glasgow for advice and help with photomicrographs and Yvonne Galbraith for preparation of this manuscript.

Received for publication 6 October 1986 and in revised form 19 November 1986.

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