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MEDIATION OF ACUTE BUT NOT CHRONIC REJECTION OF MHC-INCOMPATIBLE RAT KIDNEY GRAFTS BY ALLOREACTIVE CD4 T CELLS ACTIVATED BY THE DIRECT PATHWAY OF SENSITIZATION[1]

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It has been previously postulated that there are two pathways of sensitization of MHC-incompatible kidney allografts: a direct pathway in which the host responder T cells are activated by MHC-incompatible passenger dendritic cells of the graft, and an indirect pathway, in which graft alloantigens are processed like "nominal" T cell antigens by host accessory cells, and presented as self-MHC restricted moieties. We show here that a rat AS anti-August alloreactive CD4$^+$ T cell line, and a presumptive clone, activated through the direct pathway are capable in an adoptive transfer model of initiating rejection of normal August kidney grafts. However, neither the T cell line nor the presumptive clone initiates rejection of passenger cell–depleted August kidneys. The results support the hypothesis that direct pathway–sensitized T cells play a dominant role in early acute rejection, but not in chronic rejection.

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Although the roles of different cells in allograft rejection, especially T lymphocyte subsets, have attracted considerable

experimental investigation, the relevance of the experimental models used in these studies to clinical organ transplantation is normally assumed to be self-evident. However, there are some significant differences between the natural history of the rejection process of vascularized organ grafts in human subjects under immunosuppressive therapy and that seen in experimental animals not receiving immunosuppression. The foremost of these is that, in cases of successful clinical renal transplantation, rejection episodes become less frequent and less acutely destructive with the passage of time after transplantation. Because of this phenomenon, immunosuppressive therapy can safely be tapered to levels that still prevent rejection and also avoid most, but not all, toxic side effects of the therapy. The explanation in cellular and molecular terms of this changing pattern has yet to be defined. We report here an experimental model in rats that allows this problem to be investigated, and demonstrate that $CD4^+$ T lymphocytes that are capable of mediating acute rejection of normal rat renal allografts fail to cause rejection of long-surviving transplants depleted of the allogeneic passenger cell population. The results are consistent with the hypothesis that the T cell populations capable of mediating late graft rejection, after loss of allogeneic "passenger" cells, are different from those mediating early acute rejection.

MATERIALS AND METHODS

Animals. Male AS ($RT1.A^l.B^l$) and August ($RT1.A^c.B^c$) rats were supplied by the National Institute for Medical Research (Mill Hill, UK). Male Brown Norway ($RT1.A^n.B^n$) and WAG ($RT1.A^u.B^u$) were purchased from Olac Harlan Ltd. (Oxon, UK).

Isolation of antigen-presenting cells. August spleen cells were adjusted to $10–20\times10^6$ cells/ml in RPMI 1640 with 5% FCS. Cells were incubated for 60 min at 37°C in plastic Petri dishes (3003, Falcon, Oxford, UK). To remove the nonadherent cells, the plates were then gently washed 5 times with 5 ml of prewarmed medium, care being taken to avoid pipetting directly onto the adherent cells. The plates then received 10 ml of PBS with 5% FCS and were placed for 30 min at 4°C before adherent cells were harvested by vigorous pipetting. After irradiation from a ^{137}Cs source (30 Gy), cells were used as allogeneic APC in mixed lymphocyte cultures.

Production of the AS anti-August T cell line. Thirty million AS responder splenic T cells, enriched by filtration through nylon-wool, were cultured with 5×10^6 irradiated August APC in 10 ml of RPMI supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 28 mM $NaHCO_3$, 5×10^{-5} M 2-ME, and 10% FCS. After 5–6 days at 37°C, blasts were harvested from the culture by layering the cell suspension ($<30\times10^6$ cells/ml) on 24% metrizamide solution (Nycomed, Oslo, Norway) and centrifuging for 10 min at 1200 ×g at 20°C. Cells collected from the interface were >95% viable. One and a half million blasts were then restimulated with 0.5×10^6 irradiated August APC in each well of 6-well plates (Costar, Bucks, UK) in 5 ml of medium. Cultures were then restimulated every three days.

Cloning from the AS anti-August T cell line. One cell per well was seeded into 96-well microculture plates with medium supplemented with 5 units/ml of human recombinant IL-2 (Boehringer, Lewes, UK) and containing irradiated August APC at 1×10^3 cells per well. Every 3 days 100 μl of supernantant medium was removed from each well and replaced with fresh medium containing IL-2 and APC. When confluent, colonies in the wells were transferred into 24-well plates and restimulated every 3 days in 2 ml of medium containing 0.3×10^3 irradiated August APC and IL-2. The series of presumptive T cell clones generated by this protocol is currently being investigated for the Vβ families of the T cell antigen-specific receptor that they express. The cDNA derived from one of them, L12.4, has been amplified by the anchored-polymerase chain reaction using rat Cβ2 3′ primer. Nucleotide sequencing showed that L12.4 expressed a $V\beta8.2^+$ TcR (Alam S, et al., manuscript in preparation).

Flow cytometry. The phenotype(s) of the T cell line and L12.4 were determined by fluorescence-activated cell sorter analysis. Cells were reacted first with one of the following murine MAbs: R7.3, specific for an epitope common to all rat α,β TcRs (*1*); W3/25 (antirat CD4) (*2*); MRC-OX8 (antirat CD8) (*3*); and 3AIE-12H7 (antihuman CD7). After washing, the cells were then incubated with fluoresceinated sheep antimouse Ig (Amersham, Aylesbury, UK). After fixation in 1% paraformaldehyde PBS, cells were analyzed with an Epics Profile (Coulter Electronics, Luton, UK).

The proliferation assay. T cell blasts (1×10^5) purified by centrifugation on 24% metrizamide and varied numbers of irradiated APC were placed in 96-well, round-bottomed microculture plates in 0.2 ml of medium per well. No IL-2 was added to the medium. After 60 hr incubation at 37°C, the cultures were pulsed with 1 μCi of [^{3}H]-thymidine per well (Amersham, Aylesbury, UK) and incubated for a further 12-hr period. TdR incorporation was then measured with a LKB 1219 beta counter (Pharmacia, Milton Keynes, UK).

In experiments utilizing the rat monoclonal antibodies YR5/12 (anti-$RT1.A^c$) and YR5/24 (anti-$RT1.B^c$) (a generous gift from Dr. Butcher, Cambridge, UK) as specific blocking agents, 1, 5, or 25 μl/well of antibody ascites was added to the cultures at the start of the incubation.

The experimental system to investigate kidney allograft rejection. Adult, male AS strain rats were irradiated from a ^{60}Co source (5 Gy) to minimize responses from their own immune systems. One day later, they were allografted with an August kidney (left, orthotopic position), and injected with T cells within 4 hr of completion of the transplant. Rats of a control group received no T cells; other rats received T cells purified from normal, nonsensitized AS males. Rats in the experimental groups were injected with cells of the AS anti-August T cell line, or the presumptive T cell clone, L12.4. The recipients' own right kidneys were removed 4 or 7 days later. Graft rejection was assessed from recipient survival and serial estimations of blood urea nitrogen.

In our experiments, two types of August kidneys were transplanted, either those derived from normal August male donors or August kidneys depleted of native "passenger" leukocytes. This depletion was achieved by "parking" the graft for >50 days in a primary AS strain recipient. Protection of the graft from rejection during its temporary residence in the intermediate AS host was obtained by a short course of cyclosporine (10 mg/kg once per day, by gavage, for 10 days) (*4*). Only "passenger" cell–depleted grafts with satisfactory renal function (BUN <13 mmol/L) were used in the experiments involving retransplantation.

Immunohistology. The rat-antirat YR5/24 MAb (anti-$RT1.B^c$) was purified by affinity chromatography with protein G (*5*). The MAb (2 mg per ml) was then biotinylated with biotin (linker)-N-hydrosuccinimide ester (Pierce, Cambridge, UK; 400 mg/ml) for 2 hr in 0.1 M sodium bicarbonate, pH 8.4. The conjugate was separated from the unbound ester by dialyzing against PBS for 24 hr twice at 4°C. The preparation was stored at −20°C.

Acetone-fixed frozen kidney sections were preincubated for 20 min in TBS (0.5 M NaCl; 0.015 M Tris; pH 7.6) containing 1% rat serum. The sections were labeled for 30 min with biotinylated YR5/24 MAb (1/100), washed three times in TBS, then incubated with FITC-avidin conjugate (1/200; Extravidin; Sigma Chemicals, Poole, UK) for 30 min.

RESULTS

The objective of our initial experiments was to characterize in vitro the properties of the AS anti-August T cell line and the presumptive clone L12.4. Both the polyclonal T cell line and L12.4 proliferated specifically when cocultured with irradiated August stimulators (Fig. 1). There was a weak proliferative response detected in the AS anti-August line against a third-party stimulator cell (WAG strain, $RT1^u$), but no response of L12.4 against the third-party stimulator, BN APC

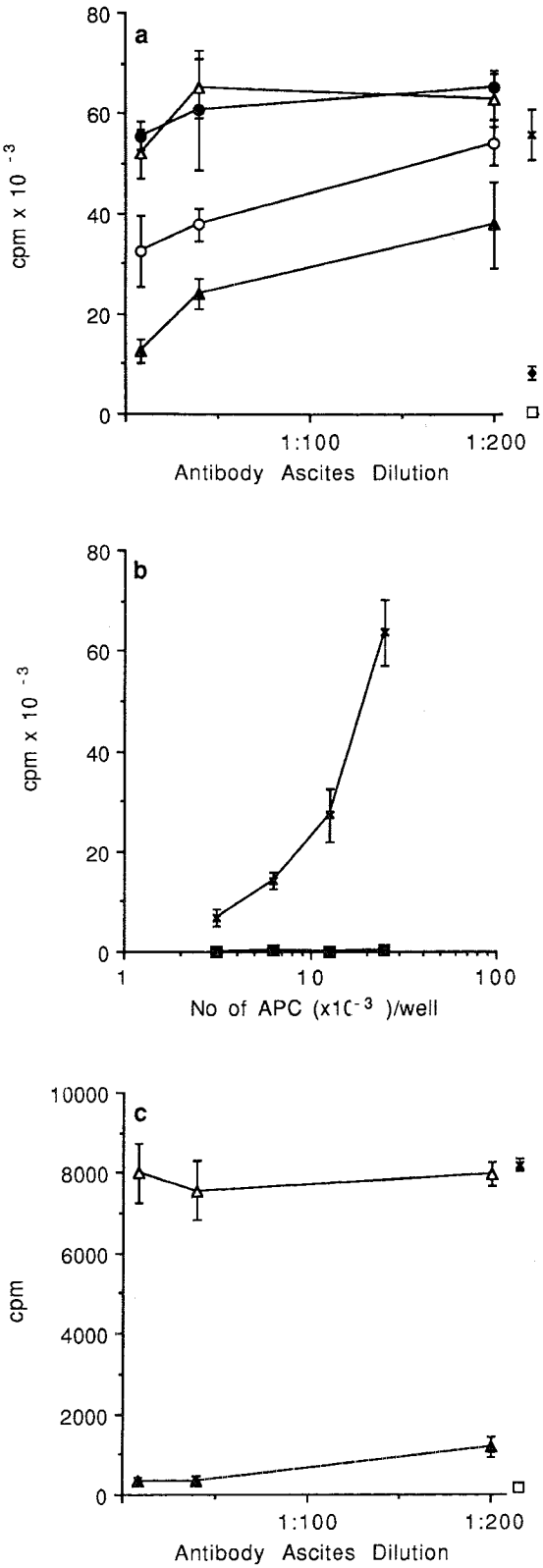

FIGURE 1. Specific proliferative responses of the AS anti-August cell line and L12.4. AS anti-August line (a) or L12.4 (b, c) cells (100×10^3 cells/well) were cultured alone (□) or with irradiated August (x), WAG (♦), or Brown Norway (■) APCs. The proliferative responses against irradiated August APCs (12.5×10^3 cells per well) were tested in the presence of YR5/12 (△), YR5/24 (▲), W3/25 (○), or MRC-OX8 (●) MAbs (a, c). Plotted values are the mean of quintuplicates ± SD.

(RT1^n). Specific proliferation of both the AS anti-August line and L12.4 was inhibited by MAb against the RT1.B^c allele (YR5/24) but not by MAb against the RT1.A^c allele (YR5/12) (Fig. 1). Similarly, the antirat CD4 molecule MAb, W3/25, inhibited proliferation of the T cell line, but MRC-OX8, antirat CD8, had no effect. FACS analysis demonstrated the T cell line to be 86–95% CD4$^+$ depending upon the passage sample tested. Greater than 97% of L12.4 reacted with W3/25 (Fig. 2). A weak cytolytic activity against ^{51}Cr-labeled August T blast cells was detected in the T cell line, but none in L12.4 (data not shown). Finally, L12.4 was found to produce IL-2 (assayed on the IL-2-dependent line CTLL-2) when stimulated by irradiated August APCs (data not shown). The above data indicated that the AS anti-August T cell line was composed of a majority population of noncytotoxic, CD4$^+$ cells with specificity for RT1.B^c APC, and the presumptive clone L12.4 was derived from the majority population.

The effector function of these cells in kidney allograft rejection was then examined, using as target grafts kidneys harvested from normal August rats, and August kidneys that had been "parked" for >50 days in AS recipients, thus depleting them of August strain passenger cells. All recipients were male AS strain rats that had been irradiated (5 Gy) one day before transplantation. At completion of the transplant, the rats were injected intravenously with T cells of the AS anti-August line, L12.4 cells, T cells harvested from normal AS rats, or no cells. The survival times of the different groups of rats are depicted in Figure 3.

Figure 3a shows that the irradiated AS recipients not given any T cells accepted the kidneys from normal August donors for more than 50 days. There was a weak early rejection episode in 5 of 8 transplanted animals that resolved spontaneously, with blood urea nitrogen levels falling to normal levels by 2–3 weeks (BUN at day 21 = 11.39±1.75 mmol/L). Two animals did not show any elevation of their BUN during the posttransplantation period. One exceptional rat developed chronic rejection (with persistent high BUN: 17.1–57.2 mmol/L) and died

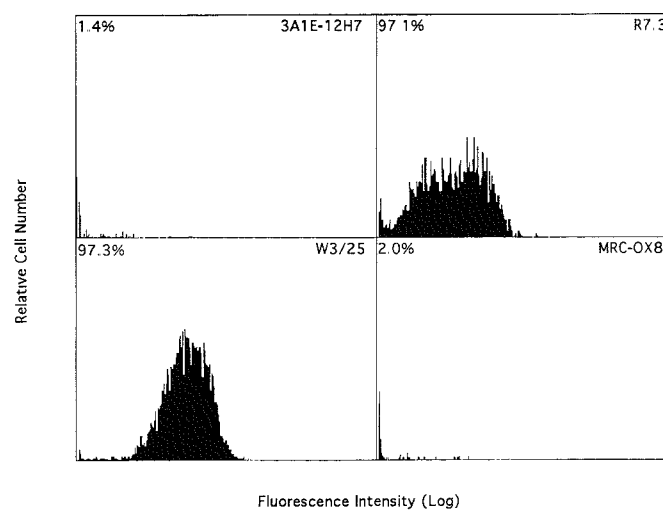

FIGURE 2. Phenotype expressed by clone L12.4 after three months of continuous culture. L12.4 cell blasts were isolated and stained with R7.3 (antirat α,β TCR), W3/25 (antirat CD4), MRC-OX8 (antirat CD8), or 3A1E-12H7 (antihuman CD7) MAbs and fluoresceinated sheep antimouse Ig. Histograms shown were gated to exclude dead cells.

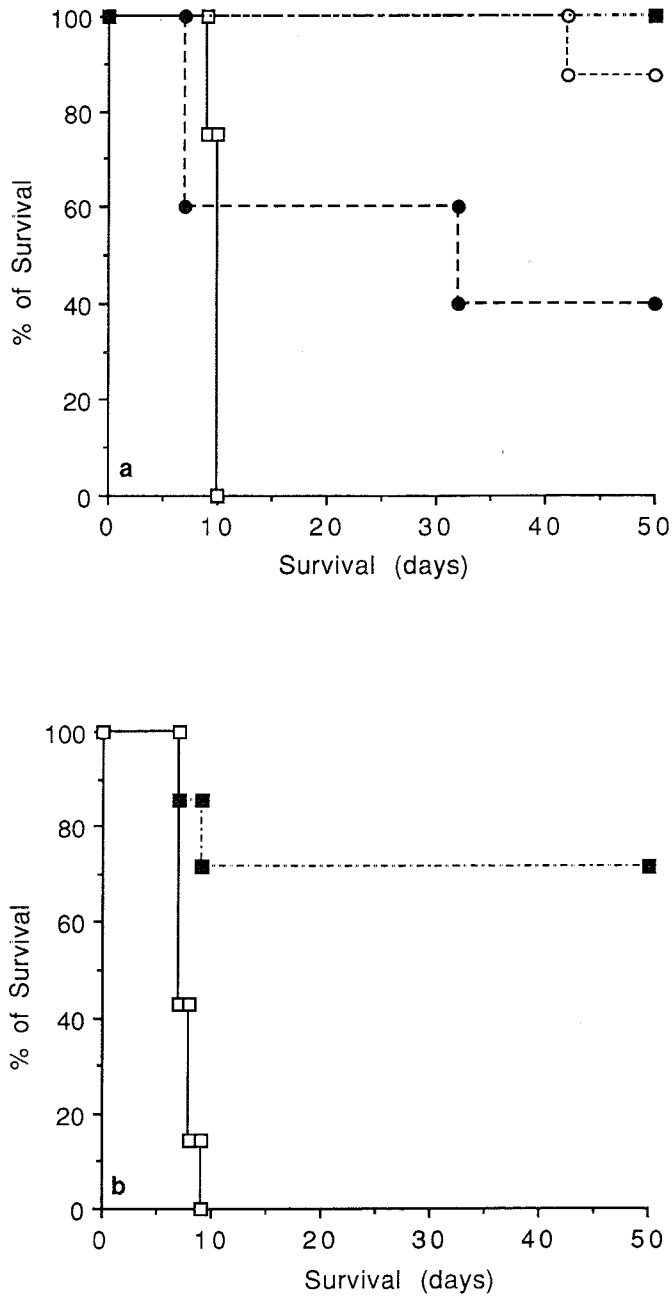

FIGURE 3. Survival of August kidney grafts in sublethally irradiated AS recipients reconstituted with different T cell populations. (a) AS recipients were transplanted with normal August kidneys and perioperatively reconstituted with no cells (n=8) (---------), 55–83×10^6 nylon wool-purified AS splenic T cells (n=5) (- -●- -), or 10×10^6 AS anti-August alloreactive line cells (n=4) (—□—)—or with "passenger" cell-depleted August kidneys—and reconstituted with 10×10^6 AS anti-August alloreactive line cells (n=4) (- - -■- - -). (b) AS recipients were grafted with normal August kidneys (n=7) (—□—), or with "passenger" cell-depleted August kidneys (n=7), (- - -■- - -) and perioperatively reconstituted with 7–9×10^6 L12.4 cells.

at day 42. The rats that were transplanted with kidneys from normal August donors but received 55–83×10^6 normal AS spleen T cells suffered early acute or subacute graft rejection in 3 of 5 recipients, but the remaining 2 in the group lived beyond 50 days (BUN at day 50 = 10.95±1.01 mmol/L). All the rats transplanted with normal August kidneys that also received 10×10^6 cells of the AS anti-August line rejected their grafts acutely. In contrast to the results of the previous group, rats receiving "passenger" cell-depleted kidneys and 10×10^6 cells of the AS anti-August T cell line failed to suffer severe acute rejection, resulting in graft failure. All four animals in the group survived beyond 50 days. A weak rejection episode was detectable at 10–14 days, but this was followed by spontaneous recovery (BUN at day 21 = 10.28±2.57 mmol/L).

Because the AS anti-August T cell line is polyclonal, and the possibility existed that a minority population might have selectively expanded in vivo, leading to unexpected immunoregulatory effects, the presumptive AS anti-August T cell clone L12.4 was also examined in the same system. A similar result was observed (Fig. 3b). All of 7 normal August kidneys were acutely rejected within 9 days following the transfer of 7–9×10^6 L12.4 cells; the transfer of the same number of L12.4 cells failed, in most animals, to cause severe acute rejection of the "passenger" cell-depleted August grafts. Of the 2 rats in the group that died, one had normal blood urea in all blood samples tested and its death was thought to be due to causes other than rejection. The remaining 5 animals survived beyond 50 days, with normal BUN levels after day 14 (12.38±3.89 mmol/L).

Normal AS kidneys, normal August kidneys, and passenger cell-depleted August kidneys were compared for the expression of RT1.B^c products. Frozen tissue sections were immunolabeled with biotinylated YR5-24 MAb and avidin-FITC conjugates. Although AS kidneys were negative, both normal and passenger cell-depleted August kidneys displayed extensive fluorescence staining (Fig. 4). YR5-24 binding was mainly localized on the tubular epithelial cells. Brightly stained cells, identified as dendritic cells, were detected in the glomeruli and intertubular spaces of normal August kidneys. These YR5-24$^{bright+}$ cells were absent from long-term surviving August allografts. Interestingly, endothelium cells were found negative for the expression of YR5-24 ligands in both normal and passenger cell-depleted August kidneys.

DISCUSSION

The results reported in this study show that the vulnerability of an August kidney to acute immunological destruction by anti-class II-specific T cells depends upon whether that graft contains the highly immunogenic population of August "passenger" cells. Once these have been depleted, the August kidney is no longer susceptible to rejection by these cell populations. This does not, of course, mean that the "passenger" cell-free August kidney is invulnerable to rejection by other cell populations of a different lineage, or by CD4$^+$ cells with a different specificity.

One possible explanation for our results could be a lack of expression of target RT1.B molecules by "passenger" cell-depleted August kidneys. To clarify this, immunohistological studies were carried out on normal AS and August strain kidneys, as well as long-surviving August grafts taken from AS recipients that had been treated with cyclosporine. In confirmation of experience with other strain combinations in which long-surviving kidney allografts express RT1 class II molecules of graft donor specificity (*6*), we observed specific staining of tubule epithelial cells of passenger cell-depleted August kidneys incubated first with biotin-labeled YR5/24 Mab, and then with avidin-FITC (Fig. 4).

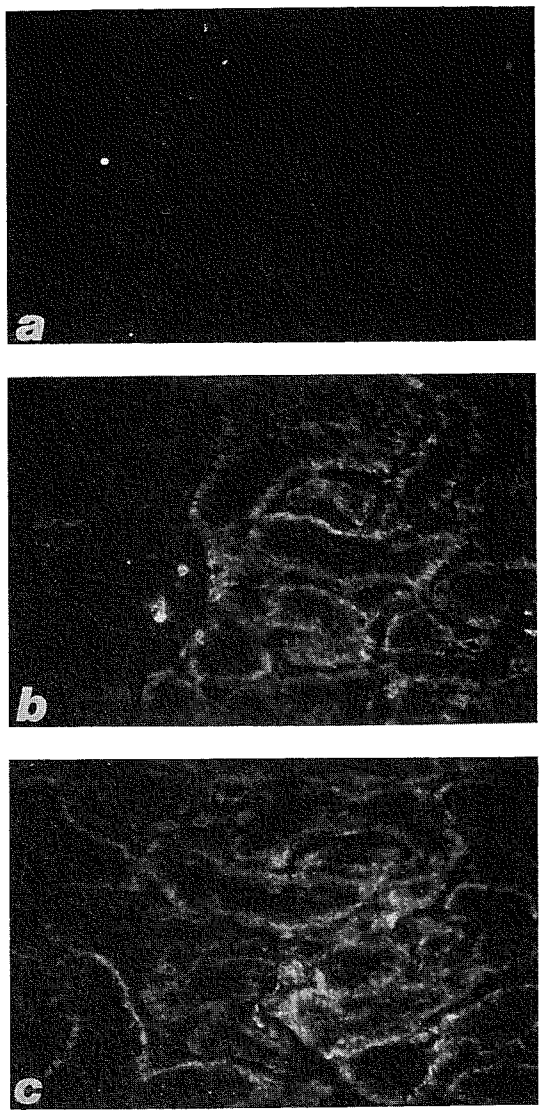

FIGURE 4. FITC-avidin immunolabeling showing RT1.B^c antigen expression in different kidneys. (a) is a section from a normal AS kidney; (b) is a section from a normal August kidney; and (c) is a section from a "passenger" cell–depleted, long-term surviving August kidney (357×).

These results show that adoptive immunization of irradiated AS hosts with defined populations of AS anti-RT1.B^c-specific T cells can initiate acute rejection of normal August kidneys, but not those from which the RT1.B^c genotype "passenger" cells have been depleted. It has been previously demonstrated that the immunogenicity of rat kidney allografts is dependent upon their content of incompatible "passenger" cells—of which the dendritic cell is the most important component (*7*)—and the hypothesis was advanced that there are two pathways of immunization in responses to MHC-incompatible grafts. The direct pathway involves a direct activation of the recipient's CD4$^+$ T cells by the allogeneic dendritic cells within the graft. The second, or indirect, pathway is identical to that followed by nonviable, "nominal" antigens—i.e., macromolecules are internalized by the recipient's APCs, processed into peptides bound by the recipient's MHC class II molecules, and presented at the cell surface of the APC as a self-MHC-restricted allogeneic peptide. Because of the difference in precursor frequencies of T cells responding by the direct and indirect pathways, the former dominates the response against an organ allograft immediately after it has been transplanted.

The AS anti-August T cell line and the presumptive clone, L12.4, used in our experiments are directly activated by RT1.B^c APC suspensions known to contain dendritic cells, and thus belong to the direct pathway. Our results show that they do not contribute to the rejection process once the allograft loses its complement of RT1.B^c dendritic cells. As the long-surviving August kidney graft still expresses class II MHC molecules on its tubules, an explanation for the failure of the transferred T cells to cause rejection is needed. Two possibilities suggest themselves: different peptides might be associated with the MHC class II molecules expressed by renal tubular epithelium and the APCs used to generate the AS anti-August T cell line or L12.4. If the responding T cells are highly specific for the peptide, they may not be activated by the RT1.B^c allele expressed on tubular cells. This may be thought to be improbable in the case of the polyclonal AS anti-August T cell line. The other possibility is that renal tubular cells may not be able to deliver the "second" signal required by T cells for activation. If this is so, do they become anergic instead?

The last issue we will discuss here is the value of the model for investigating graft rejection in which the direct pathway of immunization is no longer the dominant one. Passenger cell–depleted kidney allografts, if transplanted into normal (i.e., nonimmunosuppressed) recipients survive or undergo rejection according to the donor/recipient combination. For example, in AS strain recipients homozygous passenger cell–depleted August kidneys are rejected (median survival time, 22 days [*8*]), but similarly depleted (AS × August)F$_1$ grafts survive indefinitely (*7*). It should therefore be possible to use the former strain combination to identify the T cell population or populations that mediate the rejection of passenger cell–depleted grafts. It seems highly likely that similar populations will be found contributing to chronic graft rejection of human organ allografts. In clinical renal transplantation, successful allografts that have maintained their recipient for one year continue to undergo attrition at approximately 3–5% annually, constituting a substantial loss over 5–10 years (*9*). The specificity and lineage of the T cell populations that mediate these late rejections when incompatible "passenger" cells are presumably no longer present in the allograft have not been defined; this information is needed for monitoring late rejection and designing specific immunotherapy. Whether CD4$^+$ T cells that are specific for allogeneic peptides restricted by self-MHC class II can be detected in chronic rejection remains to be determined, but this is one of the predictions arising from our experiments. Furthermore, the question arises whether such T cells would mediate graft rejection by delayed hypersensitivity or by providing "help" for direct-pathway, cytotoxic, allospecific CD8$^+$ cells.

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