THE ROLE OF SUBREGIONS OF THE RAT MAJOR HISTOCOMPATIBILITY COMPLEX IN THE REJECTION AND PASSIVE ENHANCEMENT OF RENAL ALLOGRAFTS*

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The rejection of rat renal allografts is provoked principally by antigens of the major histocompatibility complex (MHC),¹ *H-1* (1–3). While incompatibility at *H-1* usually leads to acute rejection of the allograft, incompatibilities for minor histocompatibility antigens do not. (There are some exceptions to both generalizations (see reference 4, for a compilation of results obtained in several different strain combinations).

Injection of antibodies directed at MHC antigens of the donor into the recipient can cause prolonged survival of the transplanted allograft (passive enhancement) (5). Furthermore it has been shown that alloantibodies not absorbable by donor-type platelets or erythrocytes (RBC) can enhance rat kidney allografts (6) as had been demonstrated previously for heterotopic rat heart grafts (7). These antibodies appear to be directed at the rat equivalent of the mouse Ia antigens, and it has been suggested that only antibodies of this specificity can mediate enhancement and that they do so by blocking lymphocyte-activating determinants present in the graft (6, 8).

Two subregions of the rat MHC have been defined by recombination (9, 10). The *H-1A* region resembles an isolated mouse *H-2K* or *H-2D* region or possibly both; the *H-1B* region is the probable homologue of the mouse *H-2I* region. By making use of the *H-1^{ac1}* recombinant haplotype (9), it has been possible to study the influence of subregions of the MHC on kidney graft rejection and enhancement. In the strain combinations employed, only kidneys histoincompatible at both the *H-1A* and *H-1B* regions were rejected. In addition, alloantibodies directed at either of the two subregions alone enhanced kidney allografts presenting full *H-1^a* haplotype (*H-1A^a* + *H-1B^a*) differences.

Materials and Methods

Animals. PVG (*H-1^c*) and DA (*H-1^a*) rats were maintained in the SPF colony of the MRC Cellular Immunology Unit, Oxford. The MHC recombinant 1R has been described elsewhere

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¹ Abbreviations used in this paper: BUN, blood urea nitrogen; DAB/FCS/NAN₃, Dulbecco's A and B salt solution containing 10% fetal calf serum and 0.01 M sodium azide; FACS, fluorescence-activated cell sorter; MHC, major histocompatibility complex; NRS, normal rat serum; PBS, phosphate-buffered saline; RBC, erythrocytes; TDL, thoracic duct lymphocytes.

(9). This recombinant defines two subregions of the rat MHC named *H-1A* and *H-1B* (11). The recombinant haplotype *H-1^{ac1}* (which inherits its *H-1A* region from *H-1^a* (DA) and its *H-1B* region from *H-1^c* [PVG]) and the *H-1^a* (DA) haplotype are being back-crossed on to the PVG background in the establishment of a series of congenic strains at the ARC Institute of Animal Physiology, Babraham, Cambridge. Surplus animals from the PVG-*H-1^a* and PVG-*H-1^{ac1}* congenic programs were used in these experiments: *H-1^a/H-1^c* heterozygotes and their *H-1^c/H-1^c* littermates came from the N6 and N7 generations; *H-1^{ac1}/H-1^c* heterozygotes from the N6, N7, and N8 generations. *H-1^{ac1}/H-1^{ac1}* homozygotes came from either the original 1R (old) homozygous line N3F>2 or from the N6F1 generation. (AO × PVG)F₁ rats (*H-1^w/H-1^c*) were also bred at Babraham.

Antisera. Antisera were obtained from animals immunized at three-weekly intervals by subcutaneous and intraperitoneal injection of pooled spleen and lymph node cells, and bled 7–8 days later. Some animals had been primed with a graft of belly skin. Serum donors: (AO × PVG)F₁ anti-PVG-H-1^a (anti-*H-1^a*) was from six ♂ rats, (AO × PVG)F₁ anti-PVG-H-1^{ac1} (1R) (anti-*H-1A^a*) from two ♂ rats and (1 R × PVG)F₁ anti-PVG-H-1^a (anti-*H-1B^a*) from six ♂ rats.

These antisera had titers of 1/3,000, 1/3,000, and 1/1,000, respectively. These titers, defined here as the dilution at which the antiserum kills half the number of cells killed at the cytotoxic plateau, were measured in a lymphocytotoxicity test in which serial dilutions of antiserum were incubated for 1 h at 37°C in a final vol of 75 µl containing 50,000 target lymph node cells and 1:5 once-thawed guinea pig serum. Cytotoxicity was measured by trypan blue exclusion. Antisera were stored at –20°C. (AO × PVG)F₁ and (1R × PVG)F₁ normal sera were used as controls.

Renal Transplantation. Left orthotopic renal transplantation was performed as described elsewhere (12). Technical success of the vascular and ureteric anastomoses was checked on the 3rd d after transplantation. Delayed nephrectomy was performed on day 5, before and after which all animals produced urine. Blood urea nitrogen (BUN) levels were determined every day up to day 14, every other day up to day 20, and weekly thereafter. The normal BUN was 30 ± 9 mg %. At the time of transplantation all animals received 1 ml i.v. of one of three antisera or normal rat serum (NRS), or a smaller dose of antiserum diluted to 1 ml with NRS. This treatment was repeated 24 h later.

Cell Preparation. Thoracic duct lymphocytes (TDL) were prepared essentially as described by Ford and Hunt (13). Suspensions of spleen lymphocytes were prepared by pressing the tissue through a wire screen followed by treatment with a Tris-buffered ammonium chloride solution (14) to lyse RBC. Lymphocytes were washed in Dulbecco's A and B solution containing 10% fetal calf serum and 0.01 M NaN₃ (DAB/FCS/NaN₃).

RBC were obtained from rats which had received 1,000 rads ¹³⁷Cs whole body irradiation (120 rads/min) 4–6 days earlier. They were collected into anti-coagulant, washed in phosphate-buffered saline (PBS), and centrifuged 15 min at 460 g through a Ficoll-Isopaque solution (sp gr = 1.094). The resultant RBC suspensions had fewer than 1 leukocyte/10⁶ RBC.

Analysis of Antisera

BINDING ASSAY. A radioimmunoassay described in detail elsewhere (15) was used. 10⁶ target cells (DA RBC, spleen lymphocytes or TDL, prepared as described above) were incubated with 20 µl of antiserum (diluted with DAB/FCS/NaN₃) for 1 h at 4°C. The cells were then washed and incubated with 20 µl of immunoabsorbent purified ¹²⁵I-labeled rabbit-anti-rat FcγG_{2a} and 2b (2.1 µg/ml) (for TDL targets), or rabbit-anti-rat F(ab')₂ (2.8 µg/ml) (for RBC targets). The preparation of these reagents is described elsewhere (15). Cells were washed before gamma counting.

For analysis on the fluorescence-activated cell sorter (FACS) 5 × 10⁶ targets, 50 µl of diluted antiserum, and 50 µl of fluoresceinated second-stage antibody (the same reagents as used in the binding assays) were used in a similar protocol.

ABSORPTION ASSAYS. For the serum absorption studies, 200 or 300-µl samples of diluted antiserum were absorbed once with fourfold increasing numbers of cells for 2 h at 4°C with frequent agitation. After absorption and centrifugation the supernates were tested for residual binding activity to DA RBC or TDL in the binding assay described above. Control absorptions with PVG cells were always included.

FIG. 1. Clinical course of five PVG recipients of $H-1^a/H-1^c$ kidneys ($\blacktriangle$) and two PVG recipients of $H-1^c/H-1^c$ kidneys ($\circ$) presented as the mean $\pm$ SD of the BUN level.

TABLE I
Survival of $H-1^{ac1}/H-1^{ac1}$ and $H-1^{ac1}/H-1^c$ Renal Allografts in PVG Recipients
(an $H-1A^a$ Incompatibility)

Recipients	Genetic status of donors	Gene dose of $H-1A^a$ incompatibility	Survivors* (>35 days)
3	$H-1^{ac1}/H-1^c$ (N6-N8)	1	3/3
5	$H-1^{ac1}/H-1^{ac1}$ (N3F>2)	2	4/5‡
2	$H-1^{ac1}/H-1^{ac1}$ (N6F1)	2	2/2

All rats received 2 ml NRS administered as described in Materials and Methods.

* Only in the single case of rejection (see‡) was any significant rise in BUN level seen.

‡ One rat died on day 10 with no evidence of technical failure, i.e., a presumed rejection.

Results

Rejection. Rejection through the full $H-1^a$ haplotype: since the $H-1^a \rightarrow H-1^c$ combination had not previously been used in experimental kidney transplantation, an initial group of transplants was performed for comparison with published data on other strains. Five PVG rats received (PVG $\times$ DA) F_1 kidneys and 2 ml NRS. They rejected the grafts vigorously and died by day 11 with markedly elevated BUN levels (data not shown). A second group of five PVGs received partially congenic $H-1^a/H-1^c$ heterozygous kidneys (for genetic status of congenic animals used see Materials and Methods) and 2 ml NRS. These rats also rejected their grafts vigorously, dying by day 10 (Fig. 1). Finally two other PVG rats received $H-1^c/H-1^c$ homozygous kidneys (from littermates of the $H-1^a/H-1^c$ donors used above) and 2 ml NRS: they survived long term (Fig. 1). These results demonstrated, as expected, that an $H-1^a$ difference alone was sufficient for rejection irrespective of allotypic differences at loci not linked to the MHC.

REJECTION THROUGH $H-1A^a$. 10 PVG rats received kidneys presenting $H-1A^a$ as a homozygous or a heterozygous incompatibility. Only one recipient rejected its graft (Table I), while no other rat showed a significant rise in BUN level in the post-operative period.

REJECTION THROUGH $H-1B^a$. Four $H-1^{ac1}/H-1^c$ heterozygotes received $H-1^a/H-1^c$

FIG. 2. Clinical course of four $H-1^{ac1}/H-1^c$ recipients of $H-1^a/H-1^c$ kidneys.TABLE II
The Passive Enhancement of $H-1^a/H-1^c$ Renal Allografts in PVG Recipients

Graft type	Treatment	Recipients	No. surviving* longer than controls	No. surviving >35 days
(PVG × DA) F_1	2 ml Anti- $H-1^a$	5	4	4 († d.11)
$H-1^a/H-1^c$	"	5	5	2 († d.12, † d.14, † d.18)
$H-1^a/H-1^c$	2 ml Anti- $H-1B^a$	6	5	5 († d.10)
$H-1^a/H-1^c$	0.2 ml Anti- $H-1B^a$ + 1.8 ml NRS	5	5	3 († d.12, † d.18)
$H-1^a/H-1^c$	2 ml Anti- $H-1A^a$	5	5	3 († d.12, † d.16)

* Control rejections (described in previous section) (PVG × DA) F_1 → PVG 5/5 by day 11. $H-1^a/H-1^c$ → PVG 5/5 by day 10.

heterozygous kidneys, providing an $H-1B^a$ incompatibility in a single gene dose. Although there was considerable elevation of the early BUN levels, all four rats survived (Fig. 2). It appeared, therefore, that incompatibility at both $H-1A$ and $H-1B$ was necessary for acute rejection.

Passive Enhancement. Table II summarizes the results of passive enhancement in the $H-1^a/H-1^c$ → $H-1^c$ combination. Preliminary studies had indicated that this combination was difficult to enhance and that 2 ml of anti- $H-1^a$ serum was approximately the optimum dose. As shown, serum directed against the whole $H-1^a$ haplotype, or against the $H-1A^a$ or $H-1B^a$ subregions in isolation, were enhancing.

These enhancement studies demonstrated that a renal allograft presenting a full $H-1^a$ haplotype difference to the recipient can be enhanced by antisera directed at two distinct subregions of that haplotype. These antisera, anti- $H-1A^a$ and anti- $H-1B^a$, have been shown to detect K/D-like and Ia-like antigens of the rat, respectively (9, 16). Since antibodies against rat Ia-like antigens are thought to be active components of enhancing alloantisera (6, 8) the results with the anti- $H-1A^a$ serum were a surprise. An analysis of this antiserum was undertaken to discover whether it contained any antibodies against Ia-like antigens. This was possible if the cross-over which had produced the $H-1^{ac1}$ haplotype had occurred within (rather than outside) the rat equivalent of the mouse I region, so that the recombinant inherited an I region locus

FIG. 3. Exhaustive absorption of the anti- $H-1^a$ serum with DA RBC leaves a plateau of binding to DA TDL. ■, ●: binding to DA TDL after absorption with DA RBC and spleen cells, respectively. △: binding to DA RBC after absorption with DA RBC. ▲, □: binding to DA RBC and DA TDL, respectively, after nonspecific absorption with PVG RBC. ▽, ▼: NRS binding to TDL and RBC, respectively.

as well as the K/D -like locus(i) of $H-1^a$ origin. Exhaustive absorption of the anti- $H-1^a$, anti- $H-1A^a$, and anti- $H-1B^a$ sera with leukocyte-depleted preparations of RBC was performed to determine whether detectable quantities of anti-Ia-like antibodies were present in the anti- $H-1A^a$ serum. The three antisera were diluted (anti- $H-1^a$ and anti- $H-1A^a$ to 1/100, and anti- $H-1B^a$ to 1/50) and 300- μ l samples were absorbed with serial fourfold doses of DA RBC, DA spleen cells, or PVG RBC (for cell preparation and absorption methods see Materials and Methods). The absorbed sera were assayed for binding to DA RBC and DA TDL, using unabsorbed serum and NRS as positive and negative controls, respectively.

DA spleen cell absorptions reduced the binding activity on DA RBC and TDL targets to the NRS level in all cases, while absorptions with PVG RBC had no effect at all on the binding of the three sera. The effect of absorption with DA RBC was different in each of the three cases:

(a) absorption of the anti- $H-1^a$ serum (1/100) with DA RBC successfully removed binding activity for DA RBC targets, but only reduced binding on DA TDL to a plateau which was well above the NRS control (Fig. 3). Hence substantial binding activity directed at non-RBC antigens was present in this serum. The unabsorbed and the DA RBC-absorbed anti- $H-1^a$ sera were also analyzed in the FACS for binding to TDL using the second stage fluoresceinated anti-rat Fc reagent. Whereas the unabsorbed serum produced bright labeling of 98.7% of TDL, the DA RBC-absorbed serum did so for only 33.9%, and simultaneous labeling with anti-rat $F(ab')_2$ demonstrated that these were the surface Ig-positive cells (33.4% labeled with both reagents together). This analysis of the anti- $H-1^a$ serum showed that antibodies against Ia-like antigens could readily be detected in a complex antiserum by this method.

FIG. 4. Exhaustive absorption of the anti-*H-1B^a* serum with DA RBC does not reduce the level of binding to DA TDL. Absorption with DA spleen cells (▲) reduces the binding to DA TDL to the NRS base line (■). The specific DA RBC (●) and the nonspecific PVG RBC (Δ) absorptions remove no binding activity.

(b) Absorption of the anti-*H-1B^a* serum (1/50) with $>10^{10}$ DA RBC/300 μ l had no effect on its binding to DA TDL (Fig. 4). FACS analysis showed that both before and after absorption of this antiserum with DA RBC, 33.5% of a TDL population were labeled, and these were shown to be the Ig-positive cells. This demonstrated that the contamination of the DA RBC preparation with cells bearing Ia-like antigens was very low and unlikely to affect the analysis of the anti-*H-1A^a* serum within fairly wide limits.

(c) Absorption of the anti-*H-1A^a* serum (1/100) with DA RBC reduced binding to both DA RBC and TDL to the same level as the NRS control (Fig. 5): there was no plateau of residual binding to TDL. Similarly, FACS analysis of the DA RBC absorbed anti-*H-1A^a* serum with a fluoresceinated anti-rat Fc second stage reagent showed no cell-labeling greater than NRS, while all cells were labeled by the unabsorbed antiserum. Binding activity against Ia-like antigens was therefore not detectable in the anti-*H-1A^a* serum.

This analysis of the anti-*H-1A^a* serum suggested that antibodies against K/D-like antigens of the rat were indeed able to enhance renal allografts. It was clear, however, that better evidence in this matter would have to come from different approaches to the problem; special arguments involving very small amounts of highly potent enhancing antibodies, or enhancing antibodies restricted to Ig class(es) not detected by the second stage binding reagent, could not be discounted by these experiments. Monoclonal antibodies against *H-1A^a* region K/D-like determinants, which had recently been produced (17, 18) provided a much more stringent test of whether or not enhancement of renal allografts could be achieved through determinants of K/D-like antigens.

One such monoclonal antibody, R3/13, derived from an AO (*H-1^w*) anti-DA immunization (17, 18) was used. R3/13 culture supernate was salt precipitated and

FIG. 5. Exhaustive absorption of the anti- $H-1A^a$ serum with DA RBC reduces the level of binding to DA, RBC, and TDL to the level of NRS binding. ■, ●: binding to DA TDL after absorption with DA RBC and spleen cells, respectively. Δ: binding to DA RBC after absorption with DA RBC. ▲, □: binding to DA RBC and DA TDL, respectively, after nonspecific absorption with PVG RBC. ▽, ▴: NRS binding to TDL and RBC, respectively.

FIG. 6. The enhancement of 2/3 $H-1^a/H-1^c$ kidneys in PVG recipients treated with 2 ml R3/13 clone product (●; ○; □) compared with the rejection of similar transplants by hosts treated with culture medium alone (▲; △; ■).

concentrated ninefold, and 2 ml was given at the time of transplantation to each of three PVG recipients of $H-1^a/H-1^c$ kidneys. As a control, a sample of virgin culture medium was treated in the same way and injected into three more such recipients. In this experiment all rats received immediate contralateral nephrectomy. All control rats and one of the experimentals rejected their kidneys and died by day 6. The other two recipients of R3/13 antibody survived long term, however, one after initial BUN elevation (Fig. 6). This trial demonstrated that antibodies against K/D-like antigens

could enhance rat renal allografts, and that enhancement could be achieved through a single K/D-like determinant.

Discussion

The experiments described in this paper have used a recombinant of the rat MHC for the genetic analysis of the rejection and passive enhancement of renal allografts. Only a full *H-1^a* haplotype difference led to rejection: neither an *H-1A^a* nor an *H-1B^a* difference was sufficient to cause rejection (comparing single haplotype disparities only). Thus it appeared that the combination of both *H-1A* and *H-1B* region antigens was required for rejection.

Transplants in the *H-1^a/H-1^c → H-1^c* combination were difficult to enhance and complete success was not achieved. Nevertheless, antisera against antigens of the entire *H-1^a* haplotype or against either of the two subregions, *H-1A* or *H-1B*, were able to enhance such grafts. Furthermore a monoclonal antibody, R3/13, directed at an *H-1A^a* antigenic determinant, was also able to enhance in a full *H-1^a* difference.

Rejection. The requirement for both *H-1A^a* and *H-1B^a* disparities to induce rejection in these experiments does not necessarily imply synergistic interaction between subsets of recipient lymphocytes responding to the two sets of antigens. There is no clear evidence whether an additive or an interactive effect is at work. If additive, it may simply reflect a need for multiple incompatibilities to achieve rejection, a requirement clearly seen in the rejection of mouse kidney grafts where multiple *H-2* and non-*H-2* differences led to late rejection, while kidneys presenting *H-2K* end (including I-A and I-B) or *H-2D* end (including I-C) differences were accepted indefinitely (19). The consistent early elevation of BUN seen in recipients of *H-1B^a* incompatible grafts (Fig. 2) suggested that this region does indeed include a histocompatibility locus for kidney, but that the response was just too weak to reject the allograft before other immune mechanisms benign to the graft came into play. Thus *H-1B* (Ia-like) antigens probably play more than just an amplifying role in renal allograft rejection, although the nonspecific increase in the strength of a rejection episode caused by allogeneic MLR-stimulating determinants is not denied. Such an allogeneic effect has been clearly demonstrated for thyroid allografts by Sollinger and Bach (20).

Enhancement. While the enhancement with the antisera against the full *H-1^a* haplotype and the *H-1B^a* region came as no surprise, the success with the anti-*H-1A^a* antiserum and the monoclonal antibody R3/13 was inconsistent with the reported failure to enhance (LEW × BN)_F₁ kidney grafts in LEW recipients with alloantibodies eluted from platelets (21). Enhancement through K/D-like antigens disagrees with the hypotheses of Davies (8) and Soullillou et al. (6) which leave no place for K/D-directed antibodies in passive enhancement, and with McKenzie and Henning (22) who suggested that anti-K/D sera were only graft destructive. The results are consistent, however, with other demonstrations of the enhancing capacity of anti-K/D sera: the findings of Staines et al. (23) for mouse skin (only very slight prolongations, in this case); those of Davis (24) for mouse heart; and those of Duc et al. (25) for mouse sarcoma (Sa 1) and lymphoma (EL 4) allografts.

It would seem that the results obtained in this study are best described in the following way. Both the *H-1A* and *H-1B* regions contain histocompatibility loci for skin allografts (10; Butcher, unpublished observations) which are here presumed to

be relevant to kidney graft rejection. $H-1A^a$ and $H-1B^a$ antigens are insufficient, however, when presented alone, to cause rejection of a kidney. When presented together, however, the kidney is rejected. Treatment of the allograft recipient with alloantibodies specific for antigens of one of the subregions (A or B) of $H-1$ produces a hyporesponsiveness towards the histocompatibility antigen(s) of that region alone (perhaps by opsonisation of antigen reactive cells as suggested by Hutchinson and Zola (26), leaving responsiveness to the other subregion (B or A) unaffected. In the case of the $H-1^a/H-1^c \rightarrow H-1^c$ combination used here, this leads to graft survival since, as demonstrated, a single A^a or B^a subregion incompatibility is insufficient to produce rejection.

Such a description can be made more general: the vigor of a rejection episode will depend on the strength of the response to each of the histocompatibility antigens presented by the graft. In different strain combinations the strengths of the responses to $H-1A$ and $H-1B$ antigens will vary: MHC-linked immune response gene control of the rejection of $H-1A$ disparate skin grafts has recently been demonstrated (Butcher, unpublished observations) and there is no reason to expect kidneys to differ in this respect. In some combinations the $H-1A$ and $H-1B$ disparity alone may be sufficient to provoke rejection. Such considerations may explain the disagreement between the results presented here and those of Catto et al. (21) who failed to enhance (LEW $\times$ BN) F_1 ($H-1^l/H-1^n$) kidneys in LEW ($H-1^l$) recipients with LEW anti BN antibodies eluted from BN platelets. If in this latter combination the $H-1B^n$ incompatibility is alone sufficient to cause rejection (unlike the $H-1B^a$ difference in the present paper) then a hyporesponsiveness to $H-1A^n$ antigens produced by injection of the antibodies eluted from platelets would be irrelevant to rejection through the $H-1B$ region. This emphasizes the importance of using several genetic combinations in the study of alloimmune phenomena.

Summary

The laboratory recombinant haplotype $H-1^{ac1}$ of the Norway rat has been used in studies of the rejection and passive enhancement of kidney allografts. While the full $H-1^a$ haplotype provoked acute rejection, neither of the isolated subregions, $H-1A^a$ and $H-1B^a$, did so. It was also found that alloantisera raised against either the $H-1A^a$ or the $H-1B^a$ antigens would enhance the grafts. It is suggested that both MHC subregions contain a histocompatibility locus (i) for kidney (as they do for skin) and that in the genetic combinations studied only incompatibility for both provokes a response sufficient for rejection. In other combinations, however, single region incompatibilities may be sufficient.

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