

## KIDNEY-SPECIFIC ALLOANTIGEN SYSTEM IN THE RAT

### Characterization and Role in Transplantation\*

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Extensive studies have established that the major histocompatibility complex (MHC)<sup>1</sup> is the main barrier to the transplantation of skin and bone marrow. The analyses with organ allografts have been less detailed, although, at least with kidney grafts, the available evidence definitely supports a major role for the MHC in the primary rejection response. Thus, in the rat, primary kidney graft rejection is weak or nondetectable where donor and recipient strains are matched for the MHC (1), and three studies using a segregating backcross population as kidney donors showed that acute rejection occurred only in MHC-mismatched combinations (2, 3). In other species, including the dog (4), the rhesus monkey (5), and man (6), the rejection response to kidney grafts from MHC-identical siblings is relatively weak.

Kidney-specific antigens have been invoked frequently as potentially important in graft rejection (7-13), but the evidence has never been strong. Such responses could conceivably be directed at kidney-specific alloantigens coded for either within or outside the MHC or at kidney-specific autoantigens. There is evidence for skin-specific alloantigens coded for by loci outside the MHC in both the mouse (14, 15) and the rat (16). However most of our understanding of transplantation antigens is based on serological analyses using lymphocyte or erythrocyte (RBC) targets. Tissue-specific antibodies have thereby been largely excluded from analysis, and it is perhaps for this reason that most polymorphisms of cell membrane molecules have been described on lymphocytes and RBC (17). Therefore we developed an <sup>125</sup>I-anti-Ig binding assay using kidney homogenate as the target tissue (18). By using, as the target in serological analysis, the same tissue used in the immunization of the host, the full spectrum of antibodies can be studied, and the presence or absence of tissue-specific antibodies can be established unequivocally.

In a previous study (18), we showed that ~80% of the antibodies produced by immunizing LEW rats with DA kidney homogenate are directed at kidney-specific antigens: about one-half at a kidney-specific autoantigen, and one-half at a kidney-specific alloantigen (18). In this paper, we have characterized the alloantigen, and studied its production after kidney transplantation and its role in graft rejection.

\* Supported by grants from the National Kidney Research Fund, The Medical Research Council of the United Kingdom, and the Wellcome Trust.

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<sup>1</sup> *Abbreviations used in this paper:* BSA, bovine serum albumin; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; RAR, immunoadsorbent-purified rabbit F(ab')<sub>2</sub> and anti-rat F(ab')<sub>2</sub>; RBC, erythrocyte(s).

### Materials and Methods

*Animals.* Inbred LEW (RT1<sup>b</sup>) and DA (RT1<sup>a</sup>) rats were bred and maintained at the animal house of the Radcliffe Infirmary, Oxford, England. Inbred Fischer (RT1<sup>b</sup>), AGUS (RT1<sup>b</sup>), PVG/c (RT1<sup>b</sup>), and WAG (RT1<sup>b</sup>) were obtained from Bantin and Kingman Ltd., Grimston, Aldborough, Near Hull, Yorkshire, England. Inbred AS (RT1<sup>b</sup>) and August (RT1<sup>b</sup>) rats were from the McIndoe Research Unit, Sussex, England, and inbred BN (RT1<sup>b</sup>) rats were from St. Mary's Hospital, London, England. Outbred Sprague-Dawley and Wistar rats, rabbits and a Dunkin Hartley guinea pig were from the Radcliffe Infirmary animal house. All rats were young adults from 10 to 30 wk of age.

*Kidney Transplantation.* Left orthotopic grafts were performed essentially as previously described (19) using microsurgical techniques for end-to-end anastomosis of the renal vessels and ureter. All ischemia times were <30 min. Right nephrectomy was performed at day 7, and graft function was assessed by serial blood ureas and by animal survival.

*Homogenates.* Kidneys, hearts, livers, and spleens were removed from freshly exsanguinated animals and homogenized either immediately, or after storing at -40°C. All procedures were at 4°C or on ice. Homogenates were prepared by mincing in phosphate-buffered saline (PBS) (Dulbecco's A and B, Oxoid Ltd., London, England) using mechanically driven blades, followed by mechanical homogenization using a Teflon pestle and then by manual ground-glass homogenization. Large particles were removed by centrifuging at 40 g for 1 min. The homogenate was then washed twice in PBS by centrifuging at 35,000 g for 20 min. The second pellet was resuspended in an equal volume of PBS by manual ground-glass homogenization, aliquoted, and stored at -40°C. The protein content of the homogenates was estimated by a modification of the method of Lowry et al. (20), using bovine serum albumin (BSA) as a standard, and was generally in the range of 20-40 mg/ml.

*Cell Suspensions.* All cell suspensions were in 0.5% BSA (Sigma Chemical Co., London, England) in PBS. Single cell suspensions were prepared from thymuses by pressing them through a metal sieve. Contaminating RBC were removed by incubation with Tris-buffered ammonium chloride (21). Viability was assessed by trypan blue exclusion and was almost always >80%. RBC were prepared by two sequential centrifugations with removal of buffy coat and upper RBC layer each each time, followed by centrifugation over Triosil (Nyegaard, Oslo, Norway) -Ficoll (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) of specific gravity 1.090 to remove residual leukocytes. All cell counts were performed on a Coulter Counter, model DN (Coulter Electronics, Harpenden, Herts, England).

#### *Antisera*

**LEW ANTI-DA KIDNEY GRAFT SERUM.** A LEW rat was given a left orthotopic DA kidney graft as described above, except that the recipient's right kidney was not removed. At 4 wk, the rejected graft was removed and replaced with a fresh DA graft, to obtain a second-set response. The rat was bled on days 7, 9, 11, 13, and 15 and exsanguinated on day 17 after the second kidney graft. Sera from all bleeds were pooled, aliquoted, and stored at -40°C.

**LEW ANTI-DA KIDNEY HOMOGENATE SERUM.** This was prepared using DA kidney homogenate in the following schedule. Five LEW male rats were immunized with 1 mg of DA kidney homogenate protein (prepared from perfused male kidneys) emulsified in an equal volume (0.1 ml) of complete Freund's adjuvant and injected subcutaneously into the hind footpads. Boosts as above but in incomplete Freund's adjuvant were given on weeks 4, 7, and 11. The animals were bled by cardiac puncture three times during week 8 and twice during week 12, and were exsanguinated at week 13. All sera were pooled, heat inactivated, aliquoted, and stored at -40°C.

**DA ANTI-LEW KIDNEY HOMOGENATE SERUM.** This was from pooled bleeds of DA rats after immunization with LEW kidney homogenate in adjuvant on weeks 0, 4, and 7, as described above.

**IMMUNOADSORBENT-PURIFIED RABBIT F (AB')<sub>2</sub> AND ANTI-RAT F(AB')<sub>2</sub> (RAR).** This was prepared, iodinated, and fluorescein labeled as described previously (18).

*Binding Assay.* This was performed essentially as described by Morris and Williams (22), except that homogenate and not a single cell suspension was used on the target. All procedures were at 4°C or on ice. Kidney homogenate to be used as target was thawed, diluted ~10-fold

in PBS, spun at 1,500 *g* for 15 min, and the pellet was resuspended to 2 mg homogenate protein/ml in 0.5% BSA/PBS. Virtually all of the homogenate protein was recovered in this pellet.

Duplicate 25- $\mu$ l samples of sera to be assayed were transferred to LP3 tubes (Luckham Ltd, Burgess Hill, Sussex, England). If the sera had been used in absorption analysis, they were prespun at 6,000 *g* for 5 min immediately before assay. 25  $\mu$ l of target homogenate suspension was added to each tube, and this was incubated for 1 h on ice. The target homogenate was then washed twice by spinning at 1,500 *g* for 15 min and 100  $\mu$ l of RAR at 25  $\mu$ g/ml in 0.5% BSA/PBS with a trace of  $^{125}\text{I}$ -labeled RAR ( $\sim$ 400,000 cpm per assay tube) was added to the pellet of the second wash. After resuspension, the homogenate was incubated for an additional 1 h on ice and washed twice as above. The pellet of the second wash was resuspended in 0.5 ml PBS, transferred to fresh LP3 tubes, and the homogenate-bound radioactivity measured on a Packard gamma counter (Packard Instrument Co., Downers Grove, Ill.) The results are expressed as nanograms of RAR bound per assay, which can be calculated, assuming that labeled and unlabeled RAR bind equally well. In one analysis,  $^{125}\text{I}$ -RAR was used alone, without unlabeled RAR, to increase sensitivity (22) and in this case, the results are expressed as counts per minute bound per assay.

*Absorption Analysis.* Initial titrations of the sera were performed to choose a dilution which represented conditions of target antigen excess in the assay system. Absorptions were then performed at this serum dilution, by incubating the serum with equal volumes (80  $\mu$ l) of tripling dilutions of homogenates or cell suspensions in 0.5% BSA/PBS. Again, all procedures were performed at 4°C or on ice. Absorbing tissue was removed by centrifugation and the absorbed serum stored at -40°C until all the absorptions for a particular analysis had been completed.

For the absorption, homogenates were used at starting concentrations as prepared above, i.e.,  $\sim$ 50% solid tissue. The alignment of the absorption curves for homogenates was done on the basis of protein content. On this basis, the starting concentration of the homogenates was approximately equivalent to lymphocytes at  $10^9$ /ml (23).

Exhaustive absorptions were performed by absorbing with the pellet after centrifugation (to avoid antiserum dilution) of an equal volume of homogenate or cell suspension at the starting concentration. After spinning down the homogenate or cells, the preabsorbed serum was absorbed quantitatively as described above.

*Lymphocytotoxicity Assay.* This was a two-stage  $^{51}\text{Cr}$ -release assay using blood lymphocyte targets and guinea pig serum as the source of complement. 2  $\mu$ l of doubling dilutions of serum were mixed with 2  $\mu$ l of  $^{51}\text{Cr}$ -labeled lymphocytes at  $2 \times 10^6$ /ml and incubated at 20°C for 30 min in humidified Terasaki plates (humidified microtest plates; Falcon Labware, Div. of Becton Dickinson & Co., Oxnard, Calif.). 5  $\mu$ l of guinea pig serum was then added, and the plates were incubated for a further 60 min at 20°C. At the end of this incubation, the cells were spun down, and the radioactivity of the supernate was measured in a Packard gamma counter.

*Backcross Analysis.* 20 LEW  $\times$  (DA  $\times$  LEW) $F_1$  backcross rats underwent right nephrectomy. Each kidney was immediately cooled in ice-cold PBS, and individually homogenized as described above. The kidneys were used in absorption analyses to detect three antigens: (a) RT1-A<sup>a</sup> (i.e., DA SD-type antigens) using LEW anti-DA spleen serum assayed on DA RBC targets; (b) RT1-B<sup>a</sup> (i.e., DA Ia-type antigens) using exhaustively DA-RBC-absorbed LEW anti-DA spleen sera assayed on thymocytes; and (c) the DA allele of the kidney-specific antigen system using LEW-kidney- and DA-spleen-absorbed LEW anti-DA kidney homogenate serum assayed on DA kidney homogenate. The specificity of the assays for the RT1-A<sup>a</sup> and RT1-B<sup>a</sup> antigens has been carefully established previously (24), and the same sera were used in this study.

*Immunofluorescence.* Cryostat-cut sections of frozen kidney were transferred to gelatinized slides, dried for 15 min at 4°C, and washed once with PBS. All subsequent procedures were carried out at room temperature. The sections were incubated for 30 min with unlabeled RAR at 100  $\mu$ g/ml to block any immunoglobulins present, and washed three times by incubating for 10 min with PBS. Test sera were added to the sections for 30 min, and the sections were washed three times. Fluorescein-labeled RAR at 50  $\mu$ g/ml was added for 30 min, and the sections again washed three times. They were then mounted in 90% vol/vol glycerine in PBS, and examined with a Leitz Ortholux II microscope (E. Leitz, Inc., Rockleigh, N. J.).

## Results

*Analysis of Alloantibodies After Removal of Autoantibody Component.* In our previous analysis of the LEW anti-DA kidney homogenate serum, >50% of the antibodies were found to be directed at autoantigens (18). To begin with we therefore examined the LEW anti-DA kidney graft serum for autoantibodies, by absorbing the serum with LEW kidney and assaying on DA kidney homogenate. The serum did contain autoantibodies but the amount was, as expected, less than in the anti-homogenate serum prepared with adjuvants.

Both the anti-graft and anti-homogenate serum were absorbed exhaustively with LEW kidney to remove autoantibodies and then reanalyzed. The results are given in Fig. 1A and B. It can be seen that the removal of autoantibodies was complete because in both sera, LEW kidney failed to absorb any further antibody. Absorption with DA spleen homogenate clearly subdivided the alloantibodies into two components, one present on both kidney and spleen (almost certainly the DA MHC antigens) and another present on kidney but absent from spleen. From the point of view of transplantation, it is of interest that this component was produced after kidney allografting, as well as after immunization with homogenate.

The specificity absent from spleen was studied by exhaustively preabsorbing the LEW and anti-DA kidney homogenate serum with LEW kidney and DA spleen before further quantitative absorptions. The results are given in Fig. 2 and show that the alloantigen absent from spleen is also absent from heart and liver, and we have therefore designated it as kidney specific. Because the kidney-specific antigen was more easily characterized by the stronger anti-homogenate serum, this serum was used in most subsequent studies.

*Genetic Linkage of the Gene Coding for the Kidney-specific Antigen.* Whether or not the gene coding for the kidney-specific alloantigen was linked to the rat MHC was tested by typing the kidneys of 20 LEW  $\times$  (DA  $\times$  LEW) $F_1$  backcross animals as outlined in Materials and Methods. Both the SD and Ia antigens of the MHC were typed in addition to the kidney-specific alloantigen, and the results are given in Fig. 3. It can be seen from Fig. 3A that the kidneys could be divided into two distinct groups; 5 of the 20 kidneys were able to absorb out the kidney-specific alloantibodies, and therefore they were typed positive for the antigen. It is clear that only one kidney-specific locus was being studied ( $\chi^2$  for two loci was 24.1,  $P < 0.001$ ). Indeed, the number of positive kidneys was sufficiently small to raise the possibility that more than one gene was necessary for the expression of the antigen ( $\chi^2 = 4.1$ ,  $P \approx 0.05$ ). Fig. 3B and C shows that 8 of the 20 kidneys typed positively for the DA MHC antigens, and as expected from the low recombination frequency between the SD and Ia loci, the 8 animals that were positive for the SD antigens were the same that were positive for the Ia antigens. Of the kidneys which typed positively for the DA kidney alloantigen (closed symbols), four were not associated with the DA MHC, and one was. It is clear, therefore, that there is no genetic linkage between the gene(s) coding for the kidney-specific alloantigen and the MHC.

*Strain and Species Distribution of the DA Kidney-specific Alloantigen* Kidneys of all the strains depicted in Table I were homogenized and typed by absorption as described in the backcross studies. The results show that most of the strains carried the DA allele of the kidney-specific alloantigen system, the exceptions were the AS and LEW strains. The species cross-reactivity of the specificity was tested by absorption with

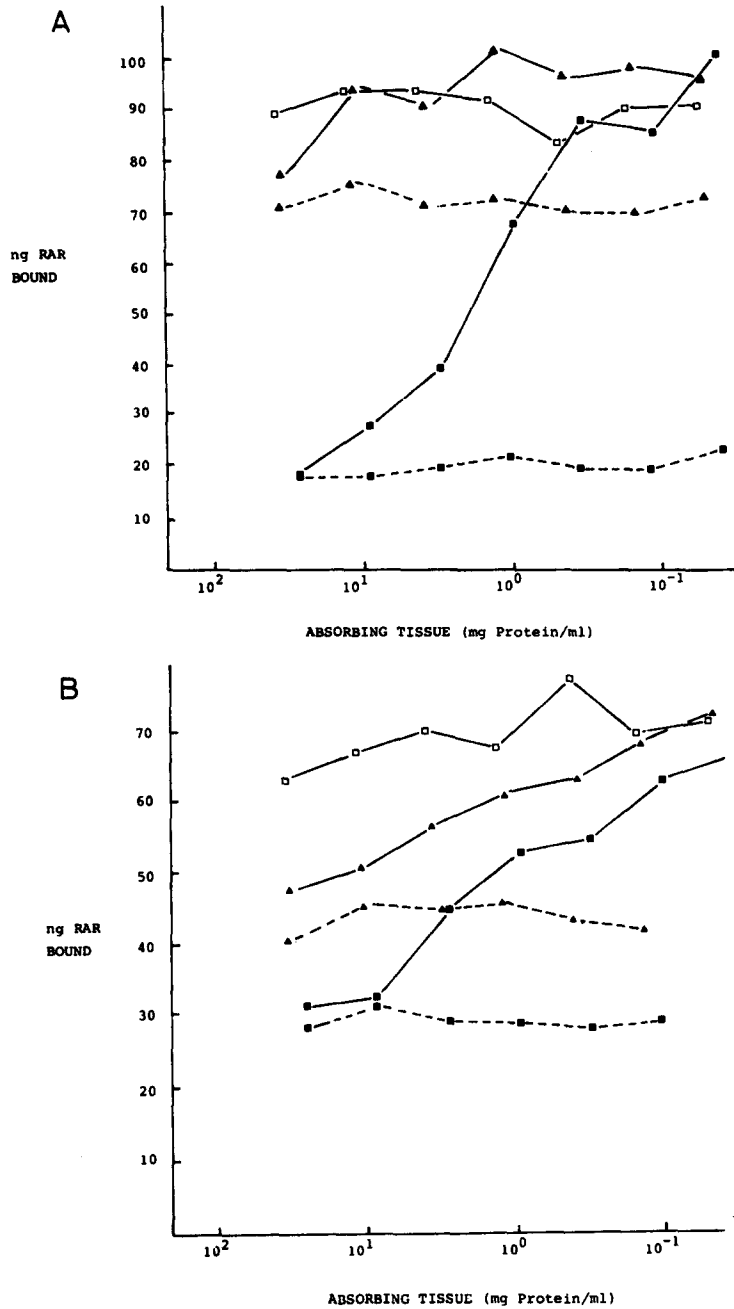


FIG. 1. Allospecificities recognized by LEW anti-DA kidney sera. LEW anti-DA kidney homogenate serum at a 1/10 dilution (A) and LEW anti-DA kidney graft serum at a 1/4 dilution (B), both preabsorbed with LEW kidney homogenate to remove autoantibodies, were absorbed with LEW kidney (□); DA kidney (■-■); DA kidney × 2 (■-■-■); DA spleen (▲) and DA spleen × 2 (△). DA kidney homogenate was used as the target tissue. Designation "× 2" indicates that the serum had been preabsorbed with the pellet after centrifugation (to avoid serum dilution) of an equal volume of the tissue indicated before the quantitative absorption shown.

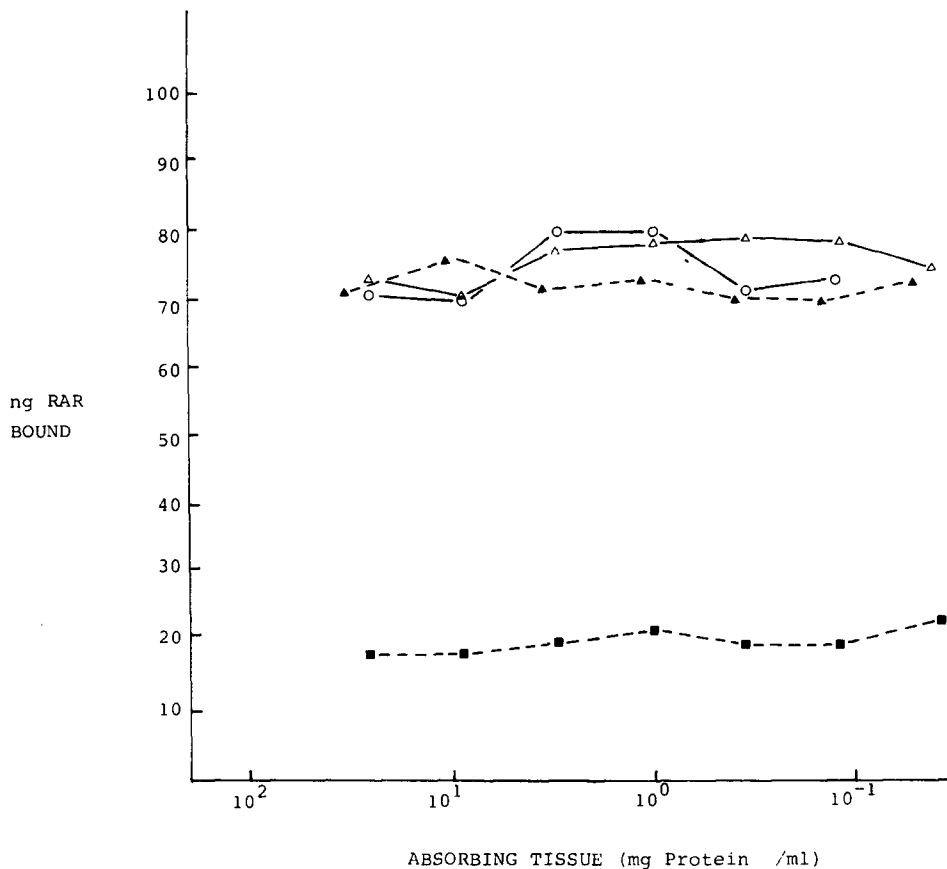


FIG. 2. Demonstration of kidney-specific alloantigen. LEW anti-DA kidney homogenate serum at a 1/10 dilution, preabsorbed with LEW kidney and DA spleen, absorbed with DA spleen (▲); DA heart (○); DA liver (Δ); and DA kidney  $\times 2$  (■). DA kidney homogenate was used as the target tissue. Designation " $\times 2$ " as in Fig. 1.

kidney homogenates from guinea pig, rabbit, dog, and man, and the results are given in Fig. 4. Rabbit, dog, and human kidneys all showed substantial cross-reactivity, although the absorptive capacities of these kidneys was only 10–30% that of DA kidney. Exhaustive absorptions were not performed, but dog kidney appeared to absorb to a plateau, suggesting that there was a dog-rat cross-reactive component of the antibodies, and another reacting with rat but not dog. The guinea pig kidney tested showed no absorption.

*Localization of the Kidney-specific Alloantigen.* This was done by incubating the LEW-kidney- and DA-spleen-absorbed LEW anti-DA kidney homogenate serum with freshly frozen sections of DA kidney, followed by an incubation with fluorescein-labeled RAR, as outlined in Materials and Methods. LEW kidney was used as a control, and showed no staining other than faint, granular staining of some glomeruli. With DA kidney there was a striking linear staining of tubular basement membranes as shown in Fig. 5A. Not all tubular basement membranes were stained, and it seemed that it was only the proximal convoluted tubules that were positive. Glomeruli were almost always negative, though occasionally a part of Bowman's capsule was

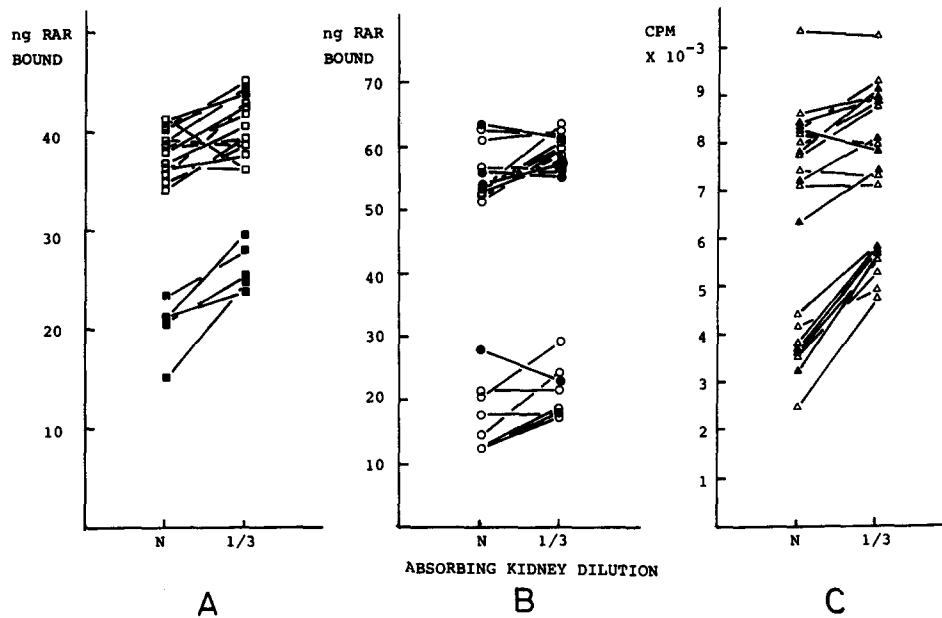


FIG. 3. Typing of 20 LEW  $\times$  (DA  $\times$  LEW)  $F_1$  backcross rats for the DA MHC and the DA allele of the kidney-specific antigen. Right kidneys from each rat were individually homogenized and used for absorption at no dilution and a 1/3 dilution of assays for (A) the DA allele of the kidney antigen; (B) DA SD (RT1-A<sup>s</sup>) antigens; and (C) DA Ia (RT1-B<sup>s</sup>) antigens. For details, see Materials and Methods. Rats typing positively for the DA allele of the kidney antigen are represented with closed symbols.

TABLE I  
*Strain Distribution of the DA Allele of the Kidney-specific Alloantigen System*

Strain	RT1 type	DA allele of kidney-specific system
LEW	l	-
AS	l	-
AGUS	l	+
Fischer	l	+
WAG	$\mu$	+
PVG/C	c	+
August	c	+
BN	n	+
DA	a	+
Wistar (not inbred colony)		+
Sprague-Dawley (not inbred colony)		+

positive, as shown in Fig. 5B. The vasculature was completely negative, as was the kidney medulla; there was a very sharp cutoff of fluorescence at the cortico-medullary junction.

To be certain that the binding assay and the fluorescence studies were detecting the same antigen, biopsies from four kidney-antigen-positive backcrosses and from three kidney-antigen-negative backcrosses were tested for the tubular basement membrane antigen in fluorescence studies as above. All four kidney-antigen-positive

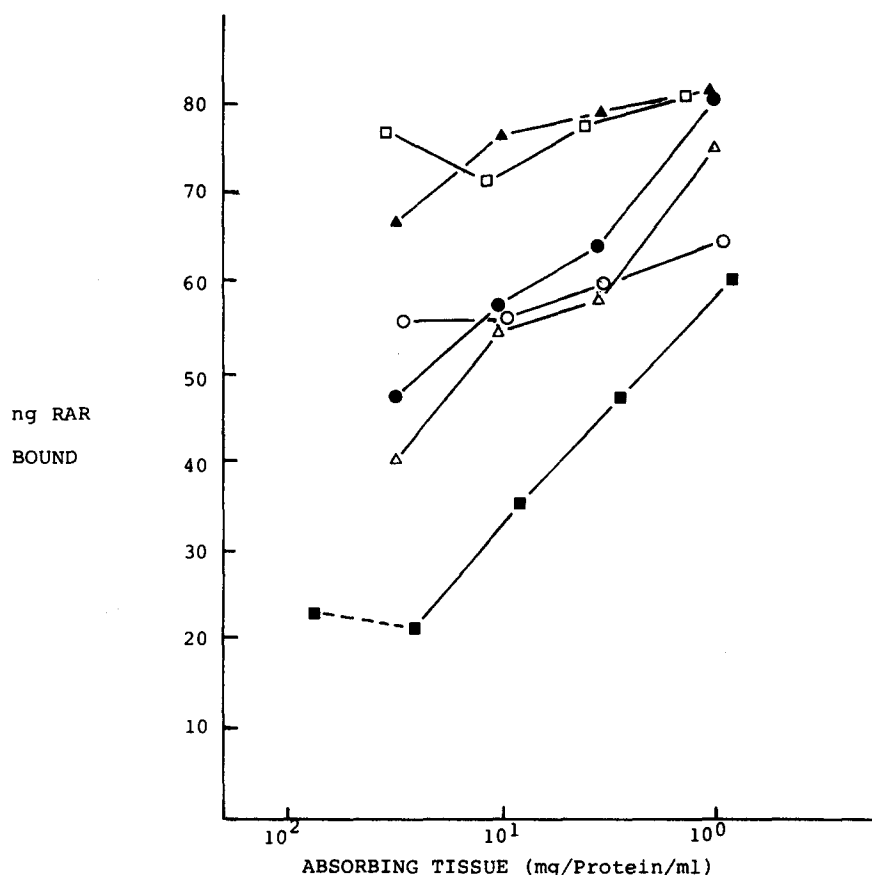


FIG. 4. Species cross-reactivity for the DA allele of the kidney-specific antigen. LEW anti-DA kidney homogenate serum at a 1/10 dilution exhaustively preabsorbed with LEW kidney and DA spleen, was absorbed quantitatively with kidney homogenate of LEW rat (□); DA rat (■); guinea pig (▲); dog (○); rabbit (△); and human (●). DA kidney was used as the target tissue.

backcrosses (so designated by absorption analysis) gave strong staining of the tubular basement membrane, whereas all three kidney-antigen-negative backcrosses had no staining at all.

DA lung tissue did not show basement membrane staining with the LEW and DA kidney alloantigen serum.

*Role of the Kidney-specific Alloantigen in Kidney Allograft Rejection.* This was tested by transplanting the left kidneys from the LEW × (DA × LEW) backcrosses depicted in Fig. 3, into LEW recipients. There were three groups. First, a group where the donors had the LEW/LEW MHC genotype and were negative for the kidney alloantigen (group 1), this group controlling for the effects of minor histocompatibility locus mismatching. Second, a donor group that had the LEW/LEW MHC genotype but was positive for the kidney alloantigen (group 2), to see if incompatibility for the kidney alloantigen could induce rejection. Finally, a group with the DA/LEW MHC genotype but negative for the kidney antigen were used as donors (group 3) to see if the absence of incompatibility for the kidney antigen influenced acute rejection. The



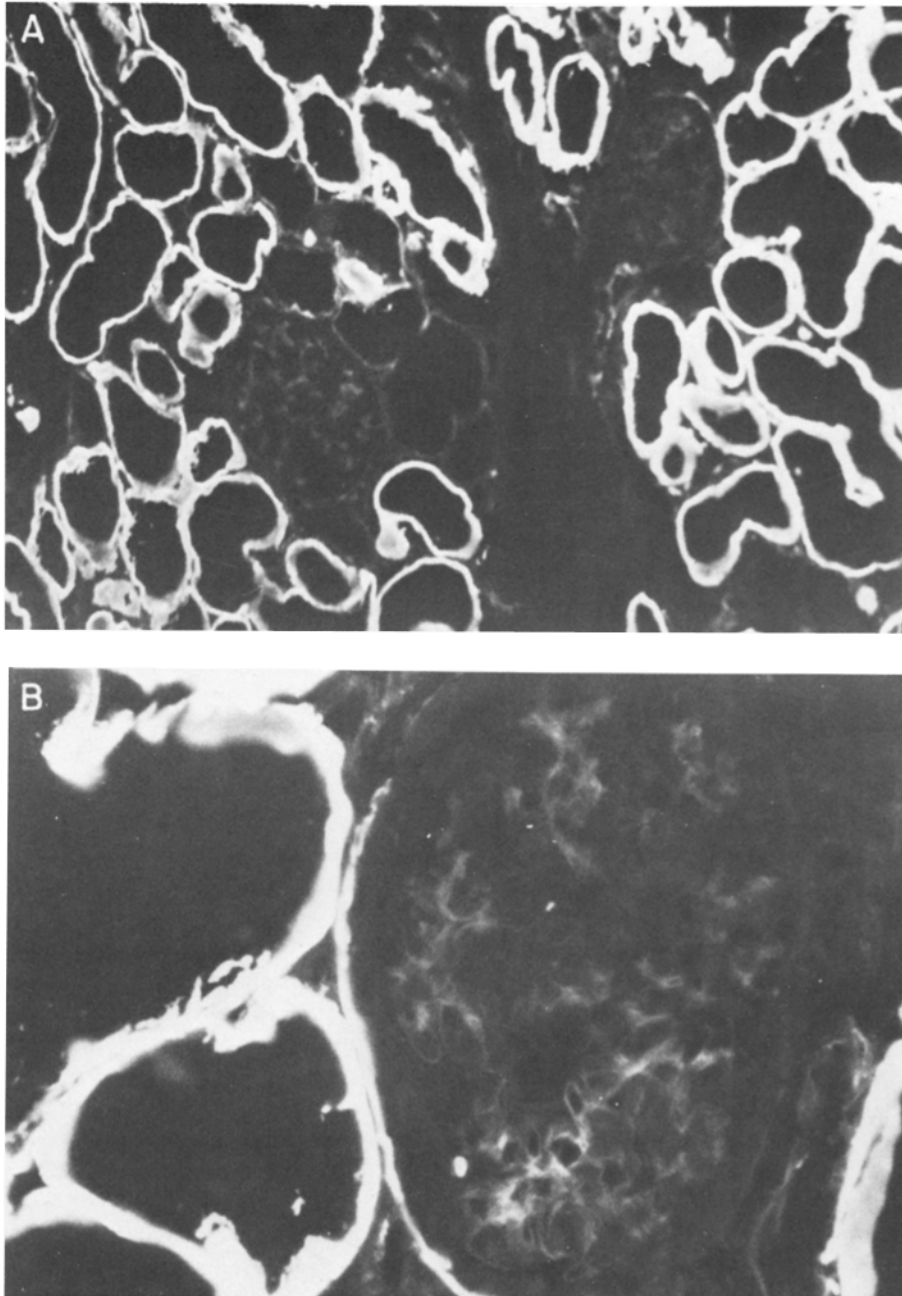


FIG. 5. Localization of kidney-specific alloantigen. Cryostat-cut sections of freshly frozen DA kidney were incubated first with LEW anti-DA kidney homogenate serum at 1/10 (preabsorbed exhaustively with LEW kidney and DA spleen) and then with fluorescein-labeled RAR at 50  $\mu\text{g}/\text{ml}$ . (A)  $\times 125$ . (B)  $\times 500$ . Control sections of LEW kidney had very little background staining, and no staining at all of the basement membranes.

TABLE II  
*Transplantation of LEW × (DA × LEW)F<sub>1</sub> Backcross Kidneys into LEW Recipients*

Donor group	Kidney donor incompatibilities			Mean ± SD of blood urea at week				Survival
	MHC	Kidney alloantigen	Minor histocompatibility antigens	1.5	2	4	12	
				<i>mg/100 ml</i>				<i>d</i>
1	-	-	+	57 ± 19	48 ± 17	51 ± 9	41 ± 5	47*, >100 (four rats)
2	-	+	+	50 ± 12	42 ± 7	55 ± 4	54 ± 8	>100 (four rats)
3	+	-	+	578 ± 78	-	-	-	10, 11, 12, 12, 13

\* This rat died with normal blood urea and normal kidney histology. Cause of death was pneumonia.

results of post-graft blood ureas and animal survival are given in Table II. There are several points to note. First, mismatching for the kidney antigen (group 2) did not influence graft function or survival. Second, the absence of a kidney-antigen incompatibility did not appear to influence the rejection of MHC-mismatched grafts: the rejection times in group 3 are not different from those of (DA × LEW)F<sub>1</sub> kidneys transplanted to LEW rats, where there are incompatibilities for both the MHC and the kidney antigens (25). Third, it is clear that only the MHC influences first-set graft rejection because all nine MHC-matched grafts had no rejection episodes, whereas all five MHC-mismatched grafts had fatal acute rejection.

All of the rats of groups 1 and 2 of Table II underwent graft biopsy at day 7 and graft nephrectomy at ~100 d after grafting. This was done to look for histological differences between the two groups especially as regards tubular changes and interstitial inflammation. However, there were no obvious differences between the groups on routine light microscopy, the only abnormalities were mild mononuclear cell infiltration and mild edema.

At the time of graft nephrectomy, the four animals in group 2 were regrafted using backcross rats with the LEW/LEW genotype and that were positive for the kidney antigen as kidney donors. This was done to see if transplantation in the face of an ongoing response to the kidney antigen might give rise to a more vigorous rejection response. However, the post-graft course of these animals as regards graft function, histology of 7-day biopsy, and survival was the same as the first set of grafts. These animals are now at 100 d after their second graft, and have normal blood ureas and urine volumes. More detailed tubular function studies are in progress.

Lymphocytotoxicity studies showed that only the animals mismatched for the MHC produced a significant antibody response to the graft (median titer 1/32, range 1/4 to 1/128). Of the 9 animals grafted with MHC-matched kidneys, 6 had no detectable antibody at all, whereas the other three had titers of 1/1, and 1/1, and 1/2.

*Immunogenicity of the Kidney-specific Alloantigen in Kidney Allografts.* That a kidney-specific response was generated in response to a DA kidney allograft in LEW recipients (Fig. 2B) shows that the kidney-specific alloantigen can induce a measurable antibody response in the transplant situation. However, it is possible that an MHC

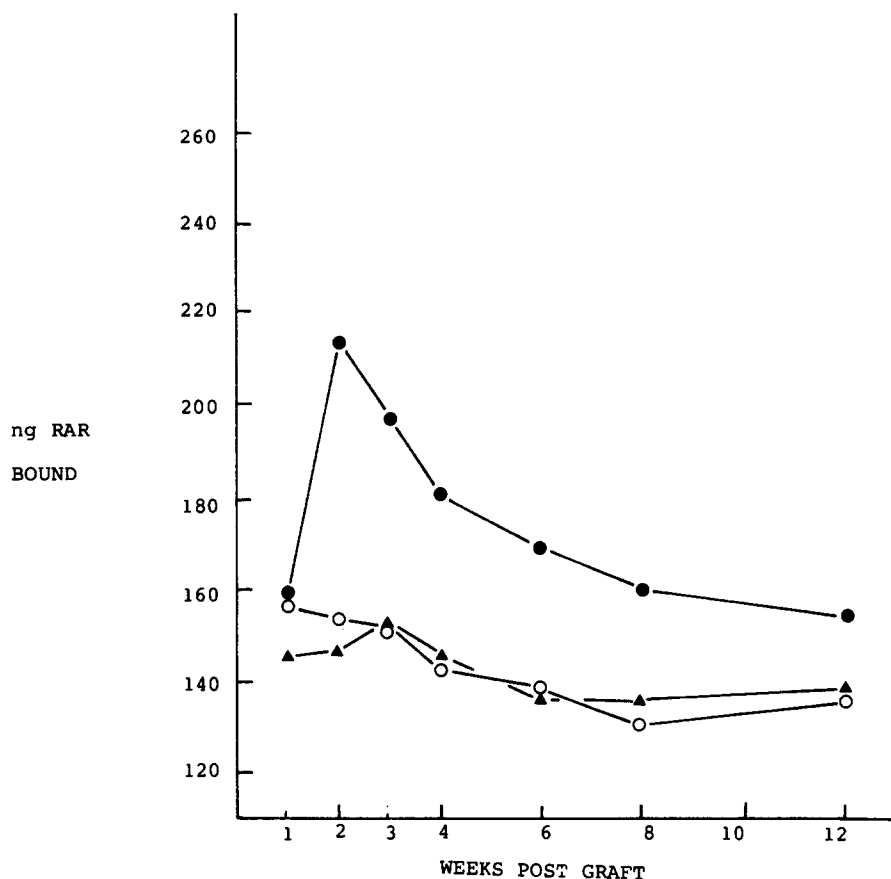


FIG. 6. Production of antibodies to the kidney antigen after grafting. Undiluted sera from LEW recipients of kidney-antigen-positive kidneys (group 2, Table II) and kidney-antigen-negative kidneys (group 1, Table II) were tested for binding to kidney homogenates from LEW/LEW, kidney-antigen-positive targets (group 2, ●) and LEW/LEW, kidney-antigen-negative targets (group 1, ▲; group 2, □). Points represent mean of the four or five rats in each group.

incompatibility is necessary, in addition to the kidney antigen incompatibility, to obtain an antibody response to the kidney antigen. Such adjuvant effects of MHC incompatibilities have been described (26) and if they are important in this case, the animals in group 2 of Table II might not be producing antibodies to the kidney alloantigen, and therefore the conclusion about the lack of importance of kidney alloantigen in graft rejection might be wrong.

This was tested in two ways. First, the post-graft sera of the animals in groups 1 and 2 of Table II were tested for binding to pooled backcross kidney homogenates of (a) kidney-antigen-negative and (b) kidney-antigen-positive animals (both groups were of the LEW/LEW MHC genotype). The results of these binding assays using undiluted sera are given in Fig. 6. The background binding with undiluted serum is high, but nevertheless it can be seen that the rats of group 2 gave higher binding to the kidney-antigen-positive kidney homogenate, and that this was not seen with the rats of group 1. Second, the 2- and 3-wk, post-graft sera of the animals in groups 1 and 2 were tested on frozen sections of DA kidney by immunofluorescence. All the

animals of group 2 but none of the animals of group 1 gave positive staining, and the picture was exactly the same as that seen in Fig. 5, except that the staining was a little less intense.

*Access of Kidney-specific Alloantibody to Target Antigen in Kidney Grafts.* If the antibody is to damage the graft, it is clear that it must have access in vivo to the target antigens. This was tested by taking the kidneys of animals in group 2 of Table II at 100 d post-transplant and by looking for the presence of antibodies bound to the grafts. Frozen sections of the kidneys were made, and these were directly incubated with fluorescein-labeled RAR. There was strong staining of the tubular basement membrane, exactly as seen in Fig. 5, showing that not only were the rats producing the kidney-specific antibody, but that it was binding in vivo to its target antigen. The kidneys of the animals in group 1, also taken at 100 d after grafting, did not show staining of the tubular basement membrane.

Slight staining of the glomerular basement membrane was seen in the kidneys of both groups 1 and 2 animals. There was a distinct impression that this glomerular staining was stronger in the kidney-antigen-matched (group 1) animals. If this is a valid observation, it could be the result of antigen competition, and the animals with a strong response against the tubular basement membrane might respond less strongly against whatever antigens are involved in the glomerular staining.

*Attempts to Define Alternative Alleles of the Kidney-specific Alloantigen.* DA anti-LEW kidney homogenate serum was directed entirely at autoantigens, as previously published (18), so that an alternative allele could not be demonstrated with this immunization.

### Discussion

This study establishes that in the DA to LEW strain combination (a) there exists a non-MHC-linked locus coding for a kidney-specific alloantigen that, in spite of inducing strong antibody responses, appears not to play any role in graft rejection and (b) the MHC is the only genetic region of importance for primary kidney graft rejection. Although there are a minimum of six non-MHC-linked loci of importance for skin graft rejection in the DA and LEW strains as shown in segregating (DA  $\times$  LEW) $F_2$  populations (27) none of these seems to influence primary kidney graft rejection.

Our assay system was designed to detect all kidney-specific antibodies after immunization with kidney homogenate and after kidney transplantation. Although the kidney-specific antigen detected in the LEW anti-DA immunization seems not to be of importance in graft rejection, it remains possible that other kidney-specific alloantigens were present but did not induce a humoral response. However, if such antigens exist and are important in graft rejection, it is clear that these antigens would have to be coded for by the MHC region. This would seem to be an unlikely possibility.

When immunofluorescence studies were performed to localize the antigen, it was found to be present only on the basement membrane of the proximal convoluted tubules and on a small part of Bowman's capsule of some glomeruli. Antibodies to tubular basement membrane antigens have been detected by immunofluorescence in chronically rejecting kidney grafts in the DA to LEW (28) and (LEW  $\times$  BN) $F_1$  to LEW (29, 30) strain combinations, which is consistent with the strain distribution of the DA allele of the kidney alloantigen (see Table I). Clinically, antibodies to tubular basement membrane have been noted in occasional kidney transplant patients ([31-

36]; and L. C. Paul, L. A. van Es, M. Stuffers-Heiman, G. Brutel de la Riviere, and M. W. Kalf. Antibodies directed against tubular basement membranes in human renal allograft recipients. Manuscript in preparation.) and these would appear to recognize the human homologue of the antigen described in this paper. However, in some of the patients, the antibodies reacted with the tubular basement membrane in the patient's own kidney, showing that they were not directed at allodeterminants on the molecule. Where allospecificity could be shown in the sera ([31, 35]; and L. C. Paul, L. A. van Es, M. Stuffers-Heiman, G. Brutel de la Riviere, and M. W. Kalf. Antibodies directed against tubular basement membranes in human renal allograft recipients. Manuscript in preparation.), 92-98% of random kidneys examined were positive for the specificity, suggesting that in man, as in the rat, one allele seems to predominate.

In the above-mentioned studies in the rat and man, the MHC linkage of the gene responsible for the kidney-specific antigen was not determined, and the role of the antigen in graft rejection could not be evaluated. In addition, the use of immunofluorescence on frozen sections to study the antigen could not give any idea of the strength of the humoral response to the antigen, nor could precise studies of tissue distribution and inter-species cross-reactivity be performed. For example, it is of interest that the amount of antibody formed against the kidney-specific alloantigen was roughly equal to that against the MHC (Fig. 1). The antigen would appear to be highly immunogenic, because, e.g., LEW anti-DA spleen sera are directed virtually entirely at the MHC (24) in spite of there being numerous additional incompatibilities.

Our species cross-reactivity studies show that a homologous kidney antigen exists in the rabbit, in the dog, and in man. The failure of the one guinea pig kidney tested to absorb the antibody (Fig. 4) does not exclude the presence of a homologous antigen in the guinea pig. There is no *a priori* reason to expect cross-reactivity of the serum with homologous antigens of other species. If cross-reactivity with the guinea pig homologue does exist, the particular animal chosen might fortuitously have expressed the less common allele, or different alleles might predominate in the guinea pig.

We had hoped that the reverse immunization of DA rats with LEW kidney would demonstrate an alternative allele of the kidney-specific alloantigen, but no kidney-specific alloantibodies could be detected by this immunization (18). This by no means excludes the possibility that the antigen system is allelic. For example, the LEW kidney is known to contain large amounts of Ia (RT1-B) antigens, but the DA rats also failed to produce any antibodies to these antigens when immunized with LEW kidney (18). Reverse immunizations do not necessarily produce antibodies to alternative alleles, as is well demonstrated in the rat Ly-1 system (37, 38).

Studies in progress have shown that the component of the LEW anti-DA kidney graft serum which is absorbed by DA spleen (Fig. 1 B) is directed at both RT1-A (SD) and RT1-B (Ia) antigens of the MHC. Thus the antibody response mounted by LEW rats to DA kidney grafts consists of four major components directed at (a) a kidney-specific alloantigen; (b) a kidney-specific autoantigen; (c) SD-type antigens of the MHC; and (d) Ia-type antigens of the MHC.

### Summary

The alloantibody response of LEW rats immunized with DA kidney homogenate or given vascularized DA kidney grafts was studied using an  $^{125}\text{I}$ -anti-Ig binding assay

with kidney homogenate as the target tissue. A major component of the sera was found to be directed at a kidney-specific alloantigen. Analysis of LEW × (DA × LEW)F<sub>1</sub> backcross rats showed that the locus coding for this antigen was not linked to the major histocompatibility complex (MHC). The use of these backcross rats, typed for both MHC and the kidney alloantigen, as kidney donors to LEW rats showed that the kidney alloantigen did not induce detectable graft damage and that only the DA MHC was involved in primary graft rejection of DA kidneys by LEW rats. Immunofluorescence studies localized the antigen to the basement membrane of proximal convoluted tubules, and to some Bowman's capsules. Strain distribution studies showed that only 2 of 11 strains were negative for the allele under study; these were the LEW and AS strains. Species cross-reactivity studies showed that rabbit, dog, and human kidneys could absorb this specificity, although more weakly than DA kidney.

*Received for publication 5 November 1979.*

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