

## IMMUNOGENICITY OF RETRANSPLANTED RAT KIDNEY ALLOGRAFTS

Effect of Inducing Chimerism in the First Recipient and Quantitative  
Studies on Immunosuppression of the Second Recipient

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It has already been shown (1, 2) that long-surviving, passively enhanced (AS  $\times$  AUG) $F_1$  rat kidneys originally transplanted to AS strain recipients are not rejected acutely if retransferred to a normal second AS recipients. This contrasts with the fate of normal (AS  $\times$  AUG) $F_1$  primary kidney allografts transplanted to AS recipients that are always rejected within 12 d, and suggests that in this strain combination the retransplanted kidneys have greatly reduced alloimmunogenicity as compared with a primary allograft. Previous work in this laboratory (3) has provided evidence that the reduction in immunogenicity is due to a loss of donor strain bone marrow-derived passenger cells from the kidney during its residence in the first recipient. These experiments also suggested that within the passenger cell component of a kidney graft a specialized subpopulation, the dendritic cells, play a pivotal role in conferring the strong primary alloimmunogenicity associated with primary kidney allografts. As few as  $5 \times 10^4$  donor strain dendritic cells injected into the secondary recipient at the time of receiving a retransplanted kidney allograft led to acute graft destruction, whereas  $5 \times 10^5$  splenic adherent cells or  $5 \times 10^6$  T or B lymphocytes had no effect on graft acceptance. If this passenger cell hypothesis is correct, changing the genotype of the passenger cells in the long-surviving enhanced allograft back to that of the donor strain should restore its immunogenicity. Results are presented here of kidney retransplantation after the induction of donor strain bone marrow chimerism in the intermediate recipient.

In the previous paper, we proposed the hypothesis that class II alloantigen can immunize via two routes (3); the significance of the class II MHS-incompatible dendritic cell lies in its capacity to activate directly helper T lymphocytes ( $T_H$ )<sup>1</sup> of the recipient (route 1). Alloantigen, including class II MHS incompatibility, present on all cells other than dendritic cells (and other specialized APC), is treated like any conventional protein antigen and requires to be picked up, processed, and presented to  $T_H$  by the recipient's own APC (route 2). Route 2 is likely to be a much less efficient mechanism of alloimmunization because the density of the processed alloantigen in association with class II MHS molecules of the recipient's APC is likely to be so much lower than the class II alloantigen molecules expressed on the allogeneic APC involved

<sup>1</sup> *Abbreviations used in this paper:* APC, antigen-presenting cells; MHS, major histocompatibility system;  $T_H$ , T helper cells.

in route 1 immunization. The hypothesis is based on the assumption that the trigger that activates  $T_H$  is altered self class II MHS molecules on APC and that allogeneic class II MHS molecules are "seen" by the  $T_H$  as altered self.

According to our hypothesis, the retransplanted kidney, depleted of all bone marrow-derived cells, is confined to immunizing via route 2, and this explains its reduced immunogenicity. However, there is some strain dependent variation in the survival of retransplanted rat kidney allografts, and in certain donor recipient strain combinations destruction of the retransferred kidney does occur (4) (possible reasons for this variation will be mentioned in the discussion). We predicted that the rejection response to such a kidney, immunizing exclusively via route 2, would be much more easily suppressed than the rejection of a primary graft between the same strains immunizing via routes 1 and 2. Experiments are described here using a strain combination in which the retransplanted kidney allograft is rejected; comparison is then made between the dosage of pharmacological immunosuppression required to maintain prolonged survival and function of a primary graft and that needed to produce the same effect in the retransplanted kidney depleted of highly immunogenic allogeneic passenger cells.

### Materials and Methods

*Animals.* We used the following inbred strains or hybrids derived from them: AS (RTIA<sup>1</sup>) and AUG (RTIA<sup>c</sup>). The parental strains were bred at the National Institute for Medical Research, Mill Hill, and hybrids from them were bred in the experimental animal unit at this institution. All rats were maintained here.

*Kidney Transplants and Retransplants.* These were performed by conventional microsurgical technique, as previously described (1). Animals were inspected at regular intervals for survival and bled for serum urea estimations on days 7, 10, 14, 21, and 28. Enhancement of (AS × AUG)F<sub>1</sub> kidneys transplanted into AS recipients was induced by means of a combined active and passive regime by injecting transplant recipients intravenously with  $5 \times 10^7$  donor strain spleen cells 11 d before kidney transplantation, 1 ml of AS anti-AUG strain antiserum 1 d later, and a further 1 ml of the same antiserum at the time of transplantation.

Survival of AUG kidneys transplanted into AS recipients was achieved by one of two methods. In some cases, the recipients had previously received an enhanced (AS × AUG)F<sub>1</sub> kidney transplanted at least 1 mo previously; the original transplant was then removed and an AUG kidney put in its place. Alternatively, the recipients were normal AS rats treated with the same enhancing regime as for recipients of (AS × AUG)F<sub>1</sub> kidneys; in addition, these animals were injected with cyclophosphamide, 5 mg/kg per d for 14 d. The first dose was given intravenously at the time of operation, and all subsequent doses were given intraperitoneally. At least 1 mo was allowed to elapse before retransplanting the AUG kidneys.

*Serum Ureas.* The glucose creatinine urea analyser 919 (Instrumentation Laboratories Ltd.) was used, with a urea enzymatic rate reaction method of 40  $\mu$ l samples.

*Cyclophosphamide.* Cyclophosphamide B.P. (W. B. Pharmaceuticals, Bracknell, England) was dissolved immediately before use in sterile distilled water to yield a solution of 20 mg/ml. This was then diluted 1:10 in sterile PBS and injected intravenously or intraperitoneally as appropriate. When the required dosage was 5 mg/kg, the dilution step was 1:20 to reduce error in measuring the volume to be injected.

*Induction of Bone Marrow Chimerism.* Strain AS rats bearing a long-surviving enhanced (AS × AUG)F<sub>1</sub> kidney were given a lethal dose of whole body x irradiation (1,000 rad). The following day, a monodiscrete suspension of  $7 \times 10^7$  (AS × AUG)F<sub>1</sub> nucleated bone marrow cells was injected intravenously into each irradiated animal.

In all rats, chimerism was assessed 2 wk later by differential cytotoxicity using an hyperimmune AS anti-AUG antiserum and comparing the degree of killing of peripheral blood lymphocytes from a normal AS (background), a normal (AS × AUG)F<sub>1</sub> (positive control), and the bone marrow-injected animals. Animals were accepted as "chimeric donors" if the highest

titer at which 50% specific lysis of their cells was observed was no more than one dilution less than observed in the positive control. Retransplantation of the allogeneic kidneys carried by the chimeric AS rats into normal AS secondary recipients was performed shortly after confirmation of the chimerism.

## Results

*Repopulation with Donor Strain Passenger Cells Restores the Immunogenicity of Long-surviving, Passively Enhanced Kidney Allografts.* Whereas previous studies on the role of passenger cells have involved the injection of suspensions of donor strain cells intravenously or intraperitoneally into the recipient of a retransplanted kidney allograft, in this experiment the immunogenicity of passenger cells *in situ* in the graft was tested, mimicking more closely the constitution of a primary allograft. The experimental system was to take an intermediate AS recipient bearing a long-surviving, enhanced (AS × AUG)F<sub>1</sub> kidney and render the recipient chimeric by injection of donor strain (AS × AUG)F<sub>1</sub> bone marrow cells. The (AS × AUG)F<sub>1</sub> kidneys of the chimeric intermediate recipients were then retransplanted into normal AS rats after serological confirmation of chimerism. From Table I, it can be seen that all the animals in this group experienced severe acute graft rejection. All of them had very high levels of serum urea on day 10. Of the five animals, three died from graft destruction within 11 d, a fourth recipient (M2) died on day 15 having had a serum urea of >88 mmol/liter on day 10, and the fifth recipient M55 survived for 42 d, despite having had a serum urea of 66 mmol/liter on day 10. These results provide confirmation for the hypothesis that the reduced immunogenicity of retransplanted kidneys is due to the loss of donor strain passenger cells.

*Retransplanted AUG Kidney Allografts Are Rejected.* The altered immunogenicity of retransplanted allografts is most apparent when the outcome is indefinite graft survival, as occurs in the (AS × AUG)F<sub>1</sub> to AS strain combination. Using certain strain combinations, however, retransplanted kidneys are rejected, usually at a slightly slower tempo and with minimal cytotoxic antibody production (4). Table II shows the results in five AS rats given retransplanted fully allogeneic homozygous AUG kidney allografts. All the rats in the group died within the first 4 wk, the median survival time being 22 d. Three animals had extremely high serum urea values at 10 d (>80 mmol/liter).

*Rejection of Retransplanted AUG Kidney Allografts Can Be Suppressed with Low Dosage Cyclophosphamide.* Our prediction was that the rejection response to a passenger cell-

TABLE I  
*Survival and Function of (AS × AUG)F<sub>1</sub> Kidney Allografts Retransplanted from Primary as Recipients Rendered Chimeric with (AS × AUG)F<sub>1</sub> Bone Marrow into Normal Secondary as Strain Recipients*

Rat number	Serum urea (mmol/liter)						Survival <i>d</i>
	Day 0	Day 7	Day 10	Day 14	Day 21	Day 28	
M55	6.9	5.8	66.1	39.1	ND	37.4	42
M57	9.7	6.4	>88				10
M59	16.6	6.0	>88				10
M1	12.1	8.4	>88				11
M2	19.1	13.5	>88	48.4			15

TABLE II  
*Survival and Function of AUG Kidney Allografts Retransplanted into Secondary as Recipients:  
 No Further Treatment*

Rat number	Serum urea (mmol/liter)						Survival
	Day 0	Day 7	Day 10	Day 14	Day 21	Day 28	
							<i>d</i>
A10	9.8	7.7	24.2	31.6	—	—	16
A12	8.7	6.9	>83	—	—	—	11
A17	17.5	11.3	46.3	ND	ND	—	22
A18	13.3	8.3	81.5	35.7	48.4	—	22
A20	21.5	ND	80.9	22.6	21.9	31.6	28

TABLE III  
*Survival and Function of AUG Kidney Allografts Retransplanted into Secondary as Recipients Injected with  
 Cyclophosphamide from Day 0 to Day 13*

Rat number	Serum urea (mmol/liter)						Survival
	Day 0	Day 7	Day 10	Day 14	Day 21	Day 28	
							<i>d</i>
A13*	11.5	12.7	33.1	21.2	18.7	10.7	50
A15*	11.0	5.8	23.3	22.7	10.0	12.4	90
A16*	8.3	8.0	13.3	15.3	13.1	8.3	>100
A21‡	9.6	5.9	16.8	83	—	—	15
A22‡	8.2	7.4	9.8	12.1	8.5	11.2	>100
A23‡	9.7	8.9	16.4	10.3	8.6	7.8	>100
A40‡	7.9	7.4	31.2	30.6	23.6	27.3	35

\* 2.5 mg/kg.

‡ 1.25 mg/kg.

depleted allograft, generated exclusively via route 2 of alloimmunization, would be readily suppressed by small doses of an immunosuppressive drug. Table III shows the behavior of two groups of recipients treated with two different dosages of cyclophosphamide for the first 14 d after receiving a retransplanted AUG kidney. All three rats treated with 2.5 mg/kg per d enjoyed very prolonged survival, two animals experienced a small rise in serum urea at day 10, but by day 28 all the kidneys were functioning well. In the group treated with 1.25 mg/kg, three of four rats survived beyond 1 mo.

*The Dosage of Cyclophosphamide Required to Prevent Rejection of a Primary AUG Kidney Allograft Is 5× the Minimum Dose Effective in Preventing Rejection of a Retransplanted AUG Kidney.* The primary allograft stimulates the recipient's immune response via routes 1 and 2, as predicted from this hypothesis, and the results set out in Table IV show that the minimum effective dose of cyclophosphamide in recipients of primary grafts is substantially higher than that required to prevent acute rejection of the retransplanted kidney. The animals receiving 2.5 or 5.0 mg/kg per d of cyclophosphamide died from acute graft destruction within 12 d, as expected for untreated animals. Only when 7.5 mg/kg was injected was survival prolonged, and even at this dose one of four rats died of uremia at 15 d.

TABLE IV  
*Survival and Function of AUG Kidney Allografts Transplanted into Primary as Recipients Injected with Cyclophosphamide from Day 0 to Day 13*

Rat number	Serum urea (mmol/liter)					Survival
	Day 7	Day 10	Day 14	Day 21	Day 28	
A5*	12.0	>88	—	—	—	<i>d</i> 12
A6*	10.9	>88	—	—	—	12
A7*	7.6	>81	—	—	—	10
A8*	9.0	>82	—	—	—	10
A25‡	6.2	>83	—	—	—	10
A26‡	ND	>83	—	—	—	10
A27‡	8.2	76.1	—	—	—	10
A28§	6.7	26.8	20.4	11.6	11.4	>60
A29§	7.2	23.1	82.9	—	—	15
A37§	6.1	17.5	9.8	14.0	13.0	>43
A38§	9.0	11.2	9.3	8.8	8.6	>44

\* 2.5 mg/kg.

‡ 5.0 mg/kg.

§ 7.5 mg/kg.

### Discussion

The experiments reported here provide additional support for the passenger cell hypothesis and develop further the implication of the two routes of alloimmunization. Our previous experiments showed that retransplanted kidney allografts devoid of allogeneic passenger cells have reduced immunogenicity. We now show that if the incompatible passenger cells are reintroduced *in situ*, by making the intermediate kidney graft recipient a chimera with donor strain bone marrow cells, then the immunogenicity of the retransplanted kidney is restored to the strength characteristic of a normal primary allograft. Given that it has been shown previously that long surviving enhanced kidneys contain more class I and an equal amount of class II alloantigen compared with a primary allograft (4, 5) and that the vascular endothelium of such kidneys carries abundant class I alloantigen (6), these results provide conclusive evidence that the major mechanism mediating the reduced immunogenicity of the retransplanted kidney allograft is the depletion of allogeneic passenger cells.

The survival and function of a retransferred kidney shows some variation, depending on the donor recipient combination involved, thus, semi-allogeneic donor kidneys fare better than fully allogeneic grafts. For example, as has already been mentioned, (AS × AUG)<sub>F1</sub> kidneys placed in secondary AS recipients function indefinitely, but secondary AS recipients of homozygous AUG kidneys die from graft destruction usually within 3–4 wk. This presumably results from the double dose of alloantigen carried by the homozygous kidney. There is also some strain variation. In contrast to the slight rise in serum urea seen in secondary AS recipients of (AS × AUG)<sub>F1</sub> kidneys, six of eight Lewis recipients of retransferred (DA × LEW)<sub>F1</sub> allografts experienced very high serum ureas at day 10 and two out of eight animals died at 22 and 42 d; the others enjoyed indefinite survival (4). This difference between different strain combinations may represent the effects of Ir gene control and/or different

precursor frequencies of alloreactive cells in the recipients.

Whether or not graft destruction occurs is only a crude measure of immunogenicity. For this reason, a strain combination was chosen in which the retransplanted kidney is rejected, thus allowing a more quantitative measure of immunogenicity. Comparison was made of the dose of cyclophosphamide required to maintain a primary graft, immunizing via routes 1 and 2, and a secondary graft immunizing only via route 2. The results presented here demonstrate a fivefold difference in the minimum dosage of cyclophosphamide that was needed to prevent effective immunization by the two types of graft.

These results have both conceptual and clinical significance. In addition to providing a quantitative comparison of the relative efficiency of the two routes of alloimmunization, they carry the implication that if donor strain APC could be inactivated or removed from a human organ allograft, the necessary dosage of immunosuppressive agents required during the early post-operative phase may be greatly reduced.

### Summary

It has been previously shown that long surviving, enhanced (AS × AUG)F<sub>1</sub> rat kidneys residing in a primary AS recipient are not acutely rejected if transferred into a second AS recipient. The reduced immunogenicity of the retransplanted graft was attributed to a depletion of incompatible passenger cells.

It is shown here that if the primary AS recipient is made chimeric by x irradiation and injection of (AS × AUG)F<sub>1</sub> bone marrow cells, transfer of the long surviving, enhanced graft into a second AS recipient provokes acute graft rejection comparable to that observed when normal (AS × AUG)F<sub>1</sub> kidneys are transplanted into untreated AS recipients.

Transplantation of passenger cell-depleted AUG kidneys into AS recipients leads to graft rejection, with a median survival time of 22 d. Treatment of these recipients with as little as 1.5 mg/kg cyclophosphamide for 14 d induces prolonged graft survival. By contrast, five times as much cyclophosphamide treatment is required to induce prolonged survival of normal AUG kidneys (i.e., containing incompatible passenger cells) transplanted to AS recipients. These results confirm that the major alloimmunogenic stimulus of rat kidney grafts is provided by the incompatible passenger cells.

We thank the Medical Research Council for financial support, Miss B. E. Phillips for excellent technical assistance, and Mrs. N. Fisher for preparing this manuscript. We are also indebted to Dr. B. Roser for helpful advice.

*Received for publication 21 July 1982.*

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