FAILURE OF LONG SURVIVING, PASSIVELY ENHANCED KIDNEY ALLOGRAFTS TO PROVOKE T-DEPENDENT ALLOIMMUNITY

I. Retransplantation of (AS × AUG)F1 Kidneys into Secondary AS Recipients*

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One of the features of long surviving, passively enhanced kidney allografts is their failure to induce repeated and sustained rejection episodes. Theoretically, this could be attributed to the induction of immunological unresponsiveness in the recipient, and/or to antigenic changes in the kidney graft. There is ample evidence showing that the immune response of the long-surviving recipient is specifically suppressed in some, but not all, respects (1–4), whereas the graft appears to have a normal and full complement of major histocompatibility antigens as measured by antibody absorption (5) or radioactively labeled antibody uptake (6). However, the presence of major histocompatibility complex (MHC)1 gene products on a tissue does not necessarily confer alloimmunogenicity. Platelets, erythrocytes, partially purified lymphocyte or liver membranes, and liposomes bearing Ag-B (rat H-1) antigens are examples of natural or artificial cell membranes which fail to elicit T-cell dependent, primary alloimmune responses despite injection of one to three orders of magnitude more MHC specificity than that present on an immunogenic dose of intact allogeneic lymphocytes (7, 8, and our unpublished results). As rat Ia-like alloantigens were present on both the lymphocyte membranes and liposomes, failure to induce T-cell dependent, primary alloimmune responses cannot be attributed to absence of these antigens, and it is evident that additional factors are involved.

It is therefore possible that although long surviving, passively enhanced kidneys carry MHC antigen, they may nonetheless be poorly or nonimmunogenic. This possibility is supported by two brief previous studies in which the retransplantation of immunologically enhanced kidneys was accompanied by the delayed onset of rejection (9, 10). The experiments in this present study were undertaken to test directly the immunogenicity of long surviving, passively enhanced kidneys. They show that in the (AS × AUG)F1 donor to AS recipient strain combination, such kidneys when retransplanted into nonimmune secondary AS recipients do not induce normal T-cell dependent immune responses; instead the grafts survive for prolonged

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1 Abbreviations used in this paper: CFT, complement fixation test; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; 1°, primary; 2°, secondary.
periods, and induce a state of nonresponsiveness. If the secondary AS recipients were previously sensitized against AUG tissue, strong immune responses occur and the kidneys are acutely rejected.

Materials and Methods

Animals. We used the following inbred strains, or hybrids derived from them: AS (Ag-B1 or H-1'), AUG (Ag-B5 or H-It); and WF (Ag-B2 or H-lW). Rats were bred and maintained at this laboratory.

Kidney Transplants. These were performed by conventional microsurgical technique with end to end anastamoses of the renal vessels, and the ureter. Grafts were transplanted into the left orthotopic position; recipients own remaining right kidneys were removed 7 d later. Animals were inspected at 1- to 2-d intervals for survival. Dead animals were autopsied and the kidneys were examined histologically unless advanced postmortem changes were present. Passive enhancement of (AS × AUG)F1, kidneys transplanted into AS recipients was induced as previously described (11), by injecting 0.5 ml of AS anti-AUG strain antisera i.v. into transplant recipients at the completion of the vascular anastamoses. Some rats also received an additional similar dose of enhancing serum i.p. 24 h later.

Lymphocytotoxicity Tests. Cells (10 μl of a 2 × 10^6/ml peripheral lymphocyte suspension in complement fixation test (CFT) buffer) were added to a 10 μl of antibody dilution in the wells of U-form microtiter test plates. 10 μl of guinea pig or rat complement (see below) was added immediately and incubation continued for 60 min at 20°C. After incubation, plates were held inverted and excess fluid removed by a short sharp downward motion of the arm. 10 μl of a 2% trypan blue (in 1.5 strength phosphate-buffered saline [PBS]) was then added to each well and 4 μl of each mixture transferred to a well of a Terasaki plate for reading. This method is based on the trypan blue test of Gorer and O'Gorman (12).

The parallel use of the guinea pig and the rat complements allows convenient detection of IgM cytotoxins, because guinea pig serum complements both IgG and IgM rat alloantibody, whereas rat serum (13) complements rat IgG alloantibody but not IgM.

Blood Ureas. The test-combination Boehringer (Boehringer UK Ltd, Lewes, Sussex, United Kingdom) urea enzymatic kits were used with 3-μl serum samples; other reagent volumes were reduced accordingly.

Cell-mediated Cytotoxicity. Effector cells consisted of peritoneal lymphocytes from which adherent cells had been removed by incubation with glass beads. This assay system was as described previously (14). A centrifugation step (1,000 rpm for ≅ 20 sec) is included at the commencement of effector and target cell incubation, and one-half of the supernates is removed 4 h later, without further centrifugation. The percentage of specific 31Cr release was calculated by the formula:

\[
\text{Experimental release (cpm) - spontaneous release (cpm) × 100}. \\
\text{Total radioactivity incorporated in target cells (cpm)}
\]

Results

Long Surviving, Passively Enhanced Kidney Allografts Fail to Induce Acute Rejection in Naive Secondary Recipients. Passively enhanced (AS × AUG)F1 kidneys which had been in residence for 1-3 mo in AS recipients were retransplanted into naive AS rats (Fig. 1). The survival times of 10 such naive, secondary AS recipients are shown in Table I; prolonged survival of all rats was observed, 4 of the 10 continuing to survive for >100 d. As controls, seven naive AS rats were transplanted with normal (AS × AUG)F1 kidneys. Their survival times were 4 × 10, 2 × 11, and 12 d, results similar to our previous experience (15). Histological examination of the control allografts removed at death showed the presence of advanced rejection. The blood urea levels of the controls and the naive, secondary AS recipients are shown in Fig. 2 and conform to the different patterns of survival.
Long Surviving, Passively Enhanced Kidney Allografts Succumb to Acute Rejection in Sensitized, Secondary Recipients. A similar experiment was performed in which passively enhanced (AS × AUG)F₁ kidneys were retransplanted to secondary AS recipients, but in this case, the recipients had been sensitized against AUG strain lymphocytes 2½-6 mo previously. None of the recipients had detectable lymphocytotoxic antibody at serum
dilutions of 1:2 or greater at the time of transplantation. Six rats received kidney allografts, and five died with acutely rejected grafts, confirmed by histology in four cases (Table I). Three of the five rats, which rejected their grafts acutely, died before blood urea estimations were performed. The blood urea levels of the remaining two rats in this group and the single long survivor (J.533) were all >200 mg/100 ml on days 9–11.

Humoral Antibody Response to Retransplanted Kidney Allografts. As might be expected from the markedly different patterns of survival, humoral responses differed greatly according to whether the secondary recipients were naive or had previously been sensitized against AUG lymphocytes. Naive secondary recipients (Table II) produced no or low levels of lymphocytotoxic IgM antibody. No IgG lymphocytotoxins were found in their sera. In keeping with previous experience (14) the control rats grafted with normal (AS × Aug)F₁ kidneys responded with high titres of lymphocytotoxins against AUG targets; six of seven of the controls had IgM titers exceeding 1/64 at 7 d. At 9–10 d, IgG, as well as IgM, lymphocytotoxins were present in their sera.

The sensitized secondary recipients all produced high titers of IgG lymphocytotoxins against AUG cells within 7 d of being transplanted (Table II). The single rat in this group which survived beyond day 11 initially produced IgG lymphocytotoxins, but by 3 wk, only IgM lymphocytotoxins were present in its serum.

Cellular Immune Response to Retransplanted Kidney Allografts. Cytotoxic lymphocytes were harvested from the peritoneal cavities of naive and sensitized secondary AS recipients, control AS rats grafted with normal (AS × AUG)F₁ kidneys, and untransplanted but hyperimmune AS rats given a single challenge of 30–40 million AUG lymphocytes on day 0. Assays for the activity of effector cells from these rats against AUG targets are shown in Fig. 3. It was possible to harvest only sufficient effector cells to test a single effector:target ratio. Therefore, the single ratio of 100:1 has been used throughout. The cellular immune response of the naive, secondary recipients remained at a consistently low level slowly rising to its maximum on day 23. In contrast, control recipients and sensitized secondary recipients developed substantial responses within 7–8 d. The subsequent fall in cellular immunity seen in
TABLE II

Lymphocytotoxic Antibody Titers* (vs. AUG Targets) in Naive and Sensitized Secondary as Recipients

| Rat No.  | 7/8 d | 10/11 d | 14 d | 21 d | 28 d |
|----------|-------|---------|------|------|------|
| J.513 (N)‡ | 0     | 0       | 0    | 3 (IgM) | 0    |
| J.516 (N) | 0     | 0       | 0    | 3 (IgM) | 0    |
| J.517 (N) | 0     | 0       | 0    | 0     | 0    |
| J.518 (N) | 0     | 0       | 0    | 0     | 0    |
| J.523 (N) | 0     | 0       | 0    | 4 (IgM) | 0    |
| J.524 (N) | 0     | 0       | 0    | 5 (IgM) | 0    |
| J.529 (N) | 0     | 0       | 0    | 0     | 0    |
| J.531 (N) | 0     | 0       | 0    | 0     | 0    |
| J.533 (N) | 0     | 0       | 0    | 0     | 0    |
| J.534 (N) | 0     | 8 (IgM) | 0    | 5 (IgM) | 0    |
| J.533 (S)‡ | 6     | 6       | 6    | 6 (IgM) | 5 (IgM) |
| J.535 (S) | 6     | 6       | —    | —     | —    |
| J.544 (S) | 6     | —       | —    | —     | —    |
| J.545 (S) | 6     | —       | —    | —     | —    |
| J.551 (S) | 6     | 6       | —    | —     | —    |
| J.552 (S) | 6     | —       | —    | —     | —    |

S, Sensitized recipient.
See Table I for details.

* Titers, highest dilution expressed as reciprocal of log2; end point, 30% kill of target cells; (indicates that the rat is dead).
‡ N, naive recipient.

FIG. 3. Comparison of cell-mediated immunity in primary and secondary AS recipients (naive and sensitized). Results are the means of the net chromium release produced by peritoneal lymphocytes from three to five rats. No SE of the means was >3% 51Cr release. Effector: target cell ratio = 100:1. Target cells were (AS X AUG)F1 thymocytes.

the control group coincided with the development of severe uraemia, and has been noted previously. Hyperimmune AS rats (without kidney allografts) challenged on day 0 also showed strong cellular immunity which waned during the 4th wk.

Development of Nonresponsiveness in the Naive Secondary Recipients. Approximately 2 mo
after they had been allografted, four of the naive secondary recipients (J.516, 518, 523, and 529) were challenged with $30 \times 10^6$ (AUG × WF)$_1$ lymphocytes. The objective here was to determine whether they had been rendered unresponsive to graft antigens. Two challenges were given separated by 6 wk, the first being given at least 7 wk after the retransplantation. After each challenge blood ureas and lymphocytotoxic antibody titers against both AUG and WF target cells were measured. The rats remained clinically well after the antigenic challenges and showed stable blood ureas (Table III). There was marked suppression of the antibody response against AUG target cells. The titers after the second challenge are shown in Table IV.

Three of four rats produced good responses against Wistar cells (Table IV) but insufficient data is available for us to say that the suppression is completely specific for AUG antigens. It should be noted that antibody produced after the first challenge persisted up to the time of the second challenge in the three control rats. The control rats therefore have cytotoxic antibody at day 0 (Table IV).

Assays for cytotoxicity of peritoneal effector lymphocytes (Table V) showed that the secondary recipients did develop cellular immunity against AUG targets after the first challenge, but the pattern was similar to that of a primary response, i.e., the peak response did not exceed 20% lysis and occurred during the 2nd wk. After the second challenge, the cellular response against AUG cells was greatly reduced. In contrast, the response against WF targets increased markedly after the second challenge.
Table V
Cell-mediated Immunity after Challenge of Secondary as Recipients with (AUG X WF)F1 Lymphocytes

|                  | Percentage net 51Cr release vs. AUG targets |                  | Percentage Net 51Cr release vs. WF targets |
|------------------|---------------------------------------------|------------------|---------------------------------------------|
|                  | First challenge                              | Second Challenge | Days | 4-5 | 7 | 10-12 | 14 | 4-5 | 7 | 10-12 | 14 | 4-5 | 7 | 10-12 | 14 |
|                  | Days                                         |                  |      |     |   |       |    |     |   |       |    |     |   |       |    |
|                  | 4-5                                          | 7                | 10-12 | 14 | 4-5 | 7 | 10-12 | 14 | 4-5 | 7 | 10-12 | 14 |
| Secondary recipients | 6.59 (2.27)                                  | 14.35 (2.21)     | 18.99 (1.53) | 8.72 (1.56) | 7.77 (1.90) | 4.95 (1.56) | 4.26 (1.80) | 7.77 (2.00) | 4.95 (1.56) | 4.26 (1.80) | 7.77 (2.00) | 4.95 (1.56) | 4.26 (1.80) |
| Normal AS        | 8.67 (2.98)                                  | 12.73 (2.58)     | 14.65 (2.31) | 4.84 (2.20) | 17.26 (2.31) | 9.42 (2.10) | 5.61 (2.00) | 10.13 (2.00) | 5.61 (2.00) | 10.13 (2.00) | 5.61 (2.00) | 10.13 (2.00) | 5.61 (2.00) |

Results are means of triplicates. Standard deviations are only shown if they are over 10% of the net release.

Discussion

The experiments reported here demonstrate the following.

(a) Long-surviving kidney allografts, when retransplanted into naive, second AS recipients, induced only a weak alloimmune response; the grafts showed prolonged survival, alloantibody responses were either absent or consisted only of IgM, and there was a striking reduction in the generation of cytotoxic T cells.

(b) When these second AS recipients were challenged with immunogenic spleen cells they were found to be hyporesponsive; graft rejection was not precipitated, alloantibody reactive with graft antigens was not formed, and a weak cytotoxic T-cell response was induced, but this decreased after a further challenge. The hyporesponsiveness was at least partially specific.

(c) Long-surviving kidney allografts when retransplanted into sensitized second AS recipients are in most cases rapidly rejected; in all cases, strong IgG alloantibody and cytotoxic T-cell responses are induced.

It is worth emphasizing that in the donor/recipient combination used here, normal kidney allografts are rejected within 12 d in both naive and sensitized recipients, and that graft rejection is accompanied by considerable IgM, IgG, and cytotoxic T-cell generation against the graft (14).

Klein et al. (16) have concluded from studies on thymectomized mice that the anti H-2 IgM response appears to be thymus independent, and the IgG response thymus dependent. In the rat, alloantibody responses to Ag-B antigens similarly are markedly thymus dependent (17). For the generation of cytotoxic T lymphocytes, there is evidence that activation of a T-cell subset which recognizes I-region determinants, or their homologues, is necessary for optimum responses to be generated in vitro (18, 19).

Cantor and Boyse (20, 21) have identified the helper cell population which recognizes I-region differences as Lyt-1+ and have shown that it amplifies the maturation of Lyt-
2,3$^+$ cytotoxic T cells. To this extent, the generation of cytotoxic T cells can also be considered as a helper cell influenced phenomenon. Two points are worth noting: (a) it is uncertain whether the helper cell population necessary for the production of IgG alloantibody is the same as that which amplifies the maturation of the Lyt-2,3$^+$ cytotoxic T cell; and (b) maturation of Lyt-2,3$^+$ cytotoxic T cell can proceed without intervention of the Lyt-1$^+$ subset, but the efficiency is very reduced except at high cell concentrations.

The most simple explanation for our present experiments is that long-surviving kidney allografts are unable to induce the T dependent part of the alloimmune response even though they carry MHC antigens. One consequence of the persistent presence of alloantigen in these circumstances is that nonresponsiveness readily develops except in sensitized second recipients. The IgM alloantibody and weak cytotoxic T-cell activity observed in the naive second recipients represents the T-independent part of the alloimmune response which in this donor/recipient combination is not powerful enough to cause rapid rejection. Probably, different donor/recipient combinations will vary in the strength of T-independant responses and this will affect the survival times of retransplanted grafts (9, 10).

It has already been mentioned that the mere presence of MHC antigen is insufficient to elicit a T-dependant alloimmune response. We have shown that the following preparations, although carrying Ag-B specificities in concentrations equivalent to those present on viable allografts, failed to induce primary humoral or cellular alloimmune responses; platelets, erythrocytes (7), lymphocyte membranes, liver membranes, liposomes carrying Ag-B (including IA like) antigens (8), and ultra-violet irradiated lymphocytes (our unpublished observations). For platelets, erythrocytes, and liver membranes, failure to act as a primary alloimmunogen could be attributed to absence or low levels of I-region determinants. However, lymphocyte membranes and liposomes which carry the rat homologue of I-region determinants also failed to elicit significant primary responses. Lafferty and Woolnough (22) and ourselves have concluded that an additional function or signal by the stimulator cells is involved in the generation of optimum primary alloimmune responses. The results of this present study show that long surviving, passively enhanced kidney allografts are unable to provide this additional signal.

Whether I-region differences are essential if allogeneic cells are to be effective, primary (allo-) immunogens is not, at present, clear. In any case, it seems most probable that the long-surviving kidney grafts contain I-region determinants. They have been demonstrated on normal rat kidneys in excess of that which can be accounted for by passenger cells (23). Formal proof of their presence on long-surviving kidneys has not yet been published, but our initial analyses indicate that they are.

In the naive second recipients, the absence of a normal T-dependent response results in the persistent presence of graft antigen and the development of nonresponsiveness. This state is identical to that which develops in conventionally, passively enhanced rats. It strongly suggests that antigen in nonimmunogenic form causes the nonresponsiveness in both experimental systems. A corollary of this view is that normal kidney allografts suffer acute rejection because alloantigen is presented in association with the stimulus causing helper cell activation. The most likely vehicle of this stimulus is the passenger leucocyte, which presumably is absent from long-surviving grafts. Recently, the suggestion has been made that Langerhans cells may
be a particularly effective source of immunogenic stimulation, and the possibility has to be considered that they are the only subpopulation present in a graft which is able to induce primary T-dependent alloimmunity (24, 25).

The requirements for inducing secondary responses are less demanding than those needed for primary responses, either in vitro (26, 27) or in vivo (7, 8). The explanation given for this is that helper cell activation is not necessary for secondary responses. In keeping with this hypothesis, long-surviving kidney allografts when retransplanted to sensitized AS recipients induce strong secondary alloantibody responses and significant cellular immunity.

A number of important implications follow from these experiments. They include the concept that pretreatment of a recipient with MHC antigen need not necessarily jeopardize the survival of any subsequently transplanted graft. Sensitization is only a risk in the naive recipient if the antigen is presented in a form which causes activation of T-helper cells. If presented in a form which does not activate this population, it readily induces hypo or nonresponsiveness. Even when T-independent responses are elicited, they decrease rather than increase on further exposure to antigen. Therefore, MHC antigen in a form which does not activate T-helper cells should now be considered for clinical use to induce specific nonresponsiveness.

Summary

Long survival of (AS × AUG)F₁ rat kidney allografts in AS recipients was induced by passive enhancement with AS anti-AUG antiserum at the time of grafting. After 1–3 mo, the kidney allografts were transferred to second AS recipients, either naive or sensitized against AUG tissue. Naive second recipients did not reject the grafts acutely and failed to mount T-dependent immunity against AUG targets. When later challenged with spleen cells carrying the AUG haplotype, the naive second AS recipients again failed to mount T-dependent immunity, and were nonresponsive.

Sensitized second AS recipients showed strong IgM, IgG, and cytotoxic T-cell responses after grafting, and the kidneys were rapidly destroyed by immune rejection in all but one rat.

It is concluded that long-surviving kidney allografts fail to activate helper T cells and induce in naive second recipients the same state of unresponsiveness observed in the first recipient.

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