KIDNEY TRANSPLANTS IN MICE

An Analysis of the Immune Status of Mice Bearing Long-Term, H-2 Incompatible Transplants*

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Transplants of living organs among mammals of the same species which differ genetically at the major histocompatibility complex are usually rejected quite promptly. There are, however, some exceptions to this which offer interesting opportunities for further study. The survival of liver transplants among certain pigs which will regularly reject skin grafts or kidney transplants from the same donors (1) is probably such an exception, but the lack of definition of histocompatibility relationships in this species limits detailed studies of the immunological mechanisms involved. Kidney transplants survive longer than skin grafts in some combinations of inbred rats, especially when their survival has been enhanced by the infusion of an antiserum specifically reactive with donor antigens (2–4). In the mouse, however, characterization of the immunogenetics of histocompatibility has been carried further than in other species so that special opportunities are offered for experiments in this species.

This report describes in detail the experimental system of kidney transplantation in the mouse. In preliminary communications we have reported that kidneys transplanted among mice involving varying degrees of histoincompatibility will regularly survive longer than skin grafts among the same strains with survival in good functioning order extending to many months in several strain combinations (5, 6). Transplant survival does not depend upon exogenous immune alterations of any kind. Prompt rejection of kidney transplants in mice can occur, however, if a sufficient immunogenetic disparity between donor and recipient is present. This is the case, for example, when C57BL/6 kidneys are transplanted to (C3H × DBA/2)F_1 recipients in which multiple incompatibilities determined by *H-2* and non-*H-2* complex genes exist. This paper will also describe the current status of our efforts, especially in one particular strain combination, to elucidate the immune relationship between long-term functioning kidney transplants and their apparently healthy, *H-2* incompatible, recipients.

Materials and Methods

Animals. Except where specified otherwise, male mice were used throughout as both donors and recipients. This selection was made because restoration of urinary drainage with our

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technique is simpler in males. In most experiments, recipients were (C57BL/6J × A/J)F_1 hybrids, hereafter referred to as B6AF_1, while donors were of the B10.D2N strain, referred to as B10.D2. Hybrid recipients were selected for greater size and vigor. The B10.D2 donor and B6AF_1 recipient combination constitutes a situation in which the genetic differences are specified at the *K* end of the *H-2* region.

In some experiments A.SW strain mice were employed as cell or tissue donors. These animals present several antigens to B6AF_1 recipients, including at least two private *H-2* specificities (H-2.12,19), which are not present on B10.D2 cells so that they are a suitable source of cells for ascertaining the responsiveness of B6AF_1 mice to foreign histocompatibility antigens other than those presented by B10.D2 donors. B10.BR mice were also used in some specificity control experiments. Cells from this strain present weaker antigenic differences to the recipient strain which are determined at the *D* end of the *H-2* region, including H-2.32. Finally, some donors of the C56BL/6 mutant strain B6.C-H(z1), abbreviated as H(z1) (7), were employed in kidney transplantation to B6AF_1 recipients. This donor strain was of interest as it has been reported by others (8) and confirmed in our laboratory (P. Sugarbaker and H. J. Winn, personal communication) that prompt rejection of skin grafts and active stimulation of recipient strain lymph node cells by donor cells in mixed lymphocyte culture (MLC) occurs with the incompatibilities represented in this combination. Nevertheless, no evidence of detectable humoral antibody production to donor cells, even after repeated stimulation in vivo, has been demonstrated.

Operative Procedures on Mice

SKIN GRAFTING. Full thickness skin grafts were performed according to the method of Billingham and Medawar (9). Median survival times (MST)[1] of skin grafts were calculated, where feasible, by the method of Litchfield (10) and statistical comparisons were made by Student's *t* test.

KIDNEY TRANSPLANTATION. Donor and recipient mice weighing from 25 to 30 g were anesthetized with chloral hydrate (0.1 ml of a 3.6% solution per 10 g of body weight). The left kidney of the donor was mobilized with the ureter and bladder (Fig. 1).

In the recipient the left renal artery and vein were ligated together, and the kidney was removed. After ligation of appropriate lumbar branches, ties were placed around the aorta and vena cava together caudad and individually cephalad to the intended site for the vascular anastomoses. After occlusion of the aorta and vena cava by tightening the loops, longitudinal openings were made in each.

The donor was infused systematically with 0.5 ml of a chilled balanced salt solution containing 7.5% heparin sulfate (7,500 U.S.P. U in 100 ml lactated Ringer's solution) into the vena cava. Aortic arterial inflow to the kidney was occluded and the donor tissue mass was removed including segments of aorta, vena cava, and the ureter with the dome of the bladder. End to side vascular anastomoses were constructed employing a continuous 10-0 nylon suture (Ethicon, Inc., Somerville, N.J.). The previously placed ties were removed allowing blood flow to the transplant. These maneuvers were facilitated by use of a dissecting microscope at varying magnifications for different portions of the procedure. Renal ischemia time was held to less than 35 min.

The donor ureter was next passed under the left vas deferens to reduce the chance of its kinking and the domes of the donor and recipient bladders were tailored to fit. The anastomosis of the two opened bladders was accomplished by using 9-0 nylon suture (Ethicon) in running fashion. The right kidney was then removed. Although the operation could be completed in 98% of recipients, 20–30% of animals failed to survive through the 3-day period immediately thereafter. These deaths were ascribed to the effects of operative trauma and animals were admitted to the various experiments only if they survived for 3 days after transplantation.

The status of the transplanted kidney was evaluated by several means: the continuing survival of the recipient, serial blood urea nitrogen levels (BUN) by using a standard colorimetric urease micromethod which requires about 0.02 ml of serum (blood samples being drawn from the retro-orbital plexus), and by microscopic examination of sections of selected transplants. Technical restrictions did not permit serial biopsy of an individual kidney transplant.

KIDNEY SLICE IMPLANTATION. In some experiments multiple slices of renal tissue, each no

[1] *Abbreviations used in this paper:* BUN, blood urea nitrogen; Con A, concanavalin A; KTxS, kidney recipient serum; MST, median survival time; SRBC, sheep erythrocytes.

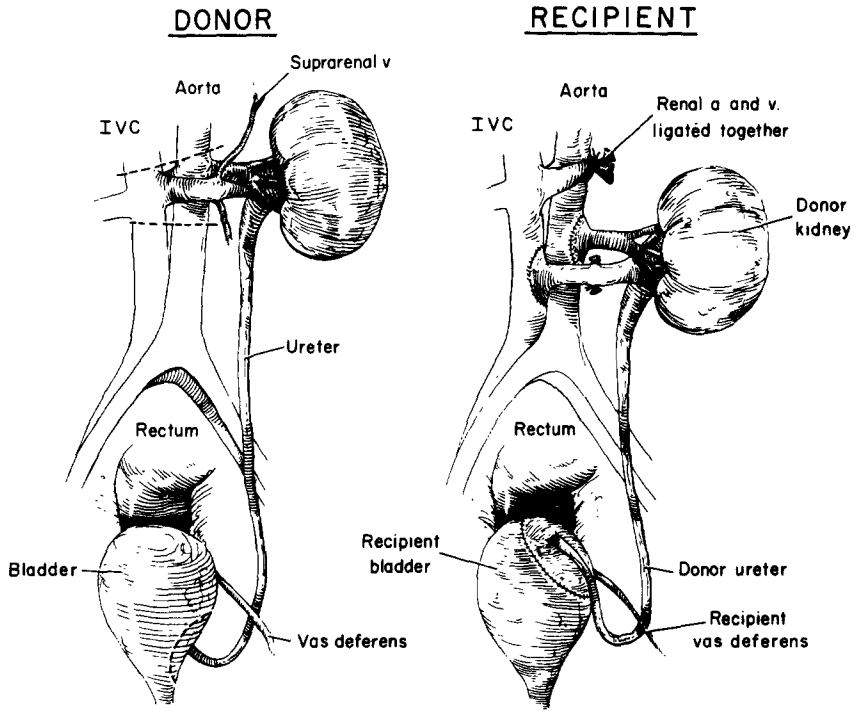

FIG. 1. Diagram of the technique used for kidney transplantation in the mouse. Note that generous portions of donor aorta and vena cava are removed as part of the donor tissue specimen. A similar approach is taken to the restoration of urinary drainage with a large patch of donor bladder. In male recipients the ureter is passed behind the vas deferens (as shown) to avoid kinking (see text).

thicker than about 0.5 mm, were implanted as free grafts into clefts prepared in the hamstring muscles of recipients. In previous experiments with endocrine tissues we have found this to be a satisfactory recipient site for tissue implants of this thickness (11).

SPLENECTOMY. Splenectomy was performed through a subcostal incision with chloral hydrate anesthesia, as described above, and standard surgical technique.

Graft Versus Host Assay. As one method of assessing the immunological capacity of recipient lymphoid cells, the cells to be tested were injected intravenously into neonatal B10.D2 mice within 12 h of birth. The weights of these animals were recorded every other day thereafter and compared to those of uninjected controls reserved in each litter. We have found this "weight gain assay" to be a useful system for demonstrating the immunological competence of murine cells in vivo by their capacity to mount a graft versus host response which is manifested by growth retardation and death of injected mice (12).

Tumor Neutralization Tests. As a further test of the immunological capacity of recipient cells to respond to donor antigens in vivo their ability to inhibit the growth of a tumor of donor *H-2* genotype was evaluated. For this purpose the DBA/2 mastocytoma P815-X2 was employed. Lymph node and spleen cells from various sources were mixed in appropriate ratios with one another and then with tumor cells before injection subcutaneously into $B6AF_1$ female recipients. The dimensions of the resultant tumors were recorded daily thereafter and were interpreted according to the original description by Winn of the "tumor neutralization test" (13). Ongoing estimates of tumor size were expressed as the average of the greatest and least diameters of a tumor.

In Vitro Studies of Lymphoid Cell Responses. Several in vitro assays of the immune responses of lymphoid cells were employed.

MIXED LYMPHOID CELL CULTURES (MLC). Mouse lymphoid cell suspensions were cultured in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N.Y.) containing 5 mM Hepes

buffer (Aldrich Chemical Co., Inc., Milwaukee, Wis.), 6% human serum, 100 U of penicillin G, 150 μg of L-arginine, 2 mM L-glutamine, and 75 μg of kanamycin sulfate/ml for 5 days. Cell suspensions were transferred to 12 × 75-mm disposable glass tubes and incubated at 37°C in a humidified atmosphere containing 5% CO_2. "One-way" cell proliferation was achieved by use of irradiation to block cell division in one of the cell populations. These cells were submitted to 1,000 rads from a ^{137}Cs source at a rate of 950 rads/min. Approximately 18–19 h before the cell cultures were harvested, a pulse of 0.5 μCi of [^{3}H]thymidine/ml was added (sp act 6.0 Ci/mmol; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.). The harvesting procedure has been reported previously (14).

^{51}Cr RELEASE ASSAY. The P815-X2 tumor was utilized as the ^{51}Cr-labeled target ($Na_2{}^{51}CrO_4$; sp act, 1.0 Ci/ml; New England Nuclear Corp., Boston, Mass.) to determine the presence of killer cells directed specifically against H-2K^d antigenic determinants as is appropriate in the combination of strains mainly employed in these experiments (B10.D2 to B6AF$_1$). Concanavalin A (Con A) activated lymph node cells from A.SW mice served as targets to evaluate the specificity of killer cell induction. Blast cells recovered after a 48–72-hour incubation with 5 μg of Con A/ml were labeled in 0.5 ml phosphate-buffered saline with 100 μCi ^{51}Cr for 1 h at 37°C. The cells were then washed three times before use.

The assay as described by Brunner et al., was modified slightly (14). 1 ml each of ^{51}Cr-labeled target cells (2–5 × 10^4/ml) and of lymphoid cells were mixed and centrifuged at 225 *g* for 5 min. At the end of the culture period the cells were briefly resuspended and centrifuged at 900 *g* for 10 min. The percentage of ^{51}Cr released into the respective supernatant solutions was determined by dividing the specific experimental value of radioactive counts by the total amount of radioactivity detected in both the supernatant fluid and pellet of ^{51}Cr-labeled target cells cultured alone.

ASSESSMENT OF THE GENERATION OF KILLER CELLS IN VITRO. 1 ml each of allogeneic irradiated and nonirradiated cells, 2.0 × 10^6 cells/ml, were mixed and incubated together for 5 days. At least 10 tubes of each combination were cultured to obtain a sufficient cell yield so that various dilutions of viable recovered cells could be mixed with ^{51}Cr-labeled P815-X2 target cells in the chromium release assay (see above).

ASSESSMENT OF SERUM-MEDIATED INHIBITION OF CELLULAR RESPONSES IN VITRO. Recipient strain lymph node cells were incubated in a 1:4 dilution of either normal mouse serum or kidney recipient serum (KTxS). The cells were suspended at a concentration of 15 × 10^6/ml of the diluted serum. After a 30-min incubation in a 37°C H_2O bath, the cells were counted, resuspended, and cultured at a concentration of 2 × 10^6/ml with an equal number of irradiated donor strain cells. After 5 days in culture, the cytolytic activity of the cells was determined in a ^{51}Cr release assay. In control experiments an antiserum against H-2K^d antigens was employed. This was collected from normal B6AF$_1$ mice which had been repeatedly injected with B10.D2 spleen and lymph node cells. It had a complement-dependent cytotoxic titer of 1:512.

Assessment of Humoral Response to Sheep Erythrocytes (SRBC). Mice received intravenous injections of 0.1 ml of a 2% suspension of SRBC in saline on days 0 and 7. Blood was drawn repeatedly from individual animals to determine hemagglutination and hemolysin levels in the plasma. The techniques used in these assays were those described by Silver et al. (15).

Histology. Tissue specimens were fixed by immersion in 10% neutral formalin in 50% ethanol, cleared in butanol, cut at 4 μm after embedding in paraffin, and stained with hematoxylin and eosin.

Cytotoxic Antibody to Cells. Cytotoxic antibody levels were determined in vitro against donor strain spleen or lymph node cells in a two stage test. Rabbit serum was used as the source of complement and trypan blue dye exclusion as the criterion of cell viability as described previously (16).

Results

Renal Isotransplants. Bilateral nephrectomy and renal isotransplantation from B6AF$_1$ donors was performed in 10 B6AF$_1$ mice. Three animals died on the 2nd day and were therefore not considered further in the study. One of the remaining seven recipients still survives at 876 days (Fig. 2). One animal died on day 51 and five were sacrificed on days 48, 499, 512, 516, and 656 days. The

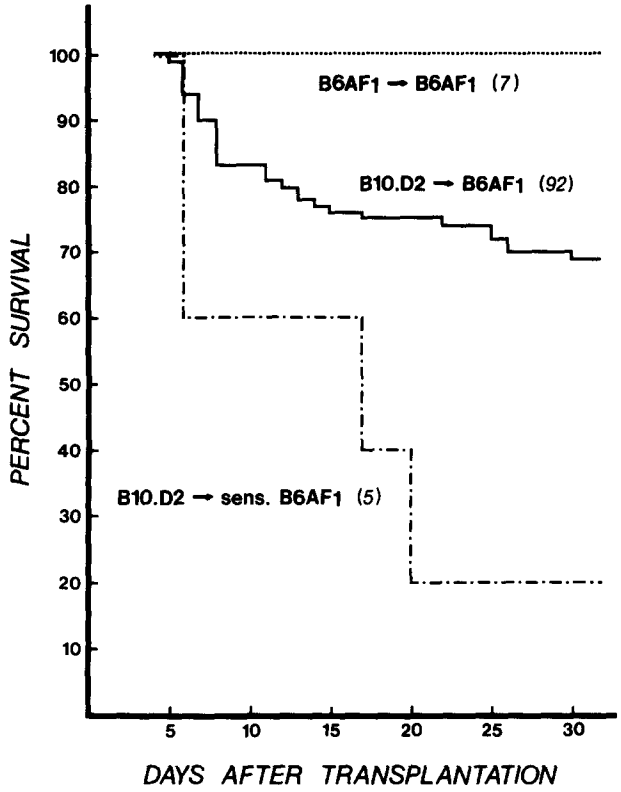

FIG. 2. The survival of renal isotransplants ($B6AF_1$ to $B6AF_1$) is compared to that of allotransplants from B10.D2 donors to normal $B6AF_1$ recipients or to $B6AF_1$ recipients which have been previously immunized to B10.D2 cells (see text). The number of animals in each group is indicated. Only those recipients which survived for at least 3 days are included in these data. About 30% of the transplants to normal recipients fail during the 1st mo after transplantation. Very few are lost thereafter. A much higher percentage of transplants to preimmunized recipients is destroyed early.

BUN levels of the seven survivors, measured between 30 and 45 days after transplantation, were 20, 22, 23, 24, 26, 27, and 40 mg/100 ml (average 26.0 mg/100 ml). On examination of sections of fixed tissue from these transplants little evidence of inflammation was seen and signs of significant ureteral obstruction were generally minimal.

In other control experiments it was found that eight animals undergoing unilateral nephrectomy survived indefinitely, maintaining BUN levels in the normal range, i.e., 15–30 mg/100 ml with an average of 22.4 mg/100 ml.

Bilaterally nephrectomized $B6AF_1$ mice succumbed to uremia within 36 h with BUN levels from 80 to 200 mg/100 ml.

Survival of Allogeneic Kidney Tissue Transferred to Normal Recipients

KIDNEY TRANSPLANTS TO NORMAL RECIPIENTS. B10.D2 kidneys were transplanted to normal $B6AF_1$ male mice and the course of the animals was followed. The pattern of survival of these animals is depicted graphically in Fig. 2. Since

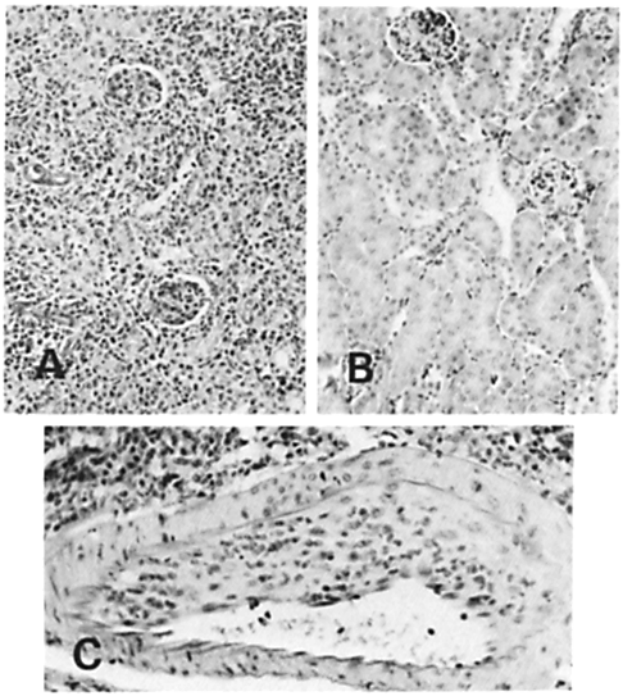

FIG. 3. Histological changes observed in B10.D2 kidneys transplanted to $B6AF_1$ recipients. (A) Allotransplant removed at 9 days after transplantation. An intense mononuclear infiltrate is present and is associated with tubular damage and margination along arterial endothelium. This and other sections are stained with hematoxylin and eosin (no. 338, × 190). (B) Allotransplant removed at 56 days after transplantation. No infiltrate is present and the tubules appear normal. The top glomerulus has a segmental scar (no. 697, × 190). (C) Allotransplant removed at 71 days after transplantation. This large artery shows marked intimal proliferation and infiltration by mononuclear cells. Only a scant interstitial infiltrate is present in the cortex and the smaller arteries are normal (no. 687, × 300).

early survival of kidney isotransplants exceeds that of allotransplants, the loss of about 30% of the allotransplants in the 1st mo must be attributable mainly to rejection. By following the course of 92 recipients of allografts, it was apparent that deaths became much less common after the 1st mo. Late kidney failure may, of course, be due to the cumulative consequences of technical factors as well as rejection activity. During the 70-day period from 30 to 100 days postoperatively, the additional loss of animals with gradually advancing uremia was about 20%. Several recipients have survived for as long as 1 yr with normal renal function. Mice that survive longer than this tend to have a gradual rise in their BUN levels.

Early rejection is characterized by intense mononuclear infiltration. Mononuclear cells invade tubules, accumulate along the arterial endothelium, and may mediate tissue damage at these sites (Fig. 3A). An adaptive phase occurs subsequently as time progresses. Samples taken during this period showed a remarkable regression of the interstitial infiltrate and little or no evidence of residual tubular damage (Fig. 3B). A late phase follows in which some tissue

damage, especially to arterial vessels and glomeruli, gradually appears. The changes consist of some mononuclear cell infiltration and intimal proliferation beginning in larger renal arteries (Fig. 3C). Similar lesions are well known features of human renal allografts (17). Glomerular damage seems to be slowly progressive over the duration of survival of the allografts. The mechanisms for these late changes have not yet been established.

Seven B10.D2 kidneys from female donors were transplanted into female $B6AF_1$ recipients to explore the possibility of a difference in responsiveness of female recipients to transplanted kidneys in this strain combination. Five of these survived in excellent condition for over 4 wk, thus demonstrating no detectable difference attributable to the sex of the recipient.

12 H(z1) kidneys transplanted to $B6AF_1$ recipients were studied. Of these, 9 survived beyond 3 days in excellent condition with BUN levels ranging from 19 to 36 mg/100 ml for at least 5 wk. Accordingly, their survival was quite similar to that seen for B10.D2 kidneys to $B6AF_1$ recipients.

KIDNEY IMPLANTS. Two to four B10.D2 renal slices comprising, in aggregate, at least one third of a kidney were implanted bilaterally into the hamstring muscles of each of 12 adult male $B6AF_1$ recipients. Donor kidneys were perfused exactly as for transplantation before preparing the tissue slices to eliminate any variation in number of "passenger leukocytes." In selected cases blocks of muscle tissue containing the implants were excised 11–14 days after grafting for histological examination. By this time the normal renal architecture of the implants was scarcely recognizable as it was largely effaced by a dense, mononuclear cell infiltrate which was considered to be entirely characteristic of allograft rejection. Donor strain skin grafts were applied later to some of these recipients (see below).

Kidney Transplants to Preimmunized Recipients. Five male $B6AF_1$ mice, which had received and rejected B10.D2 skin grafts, underwent bilateral nephrectomy and transplantation of B10.D2 kidneys from 1 to 14 days after skin graft rejection was complete. Their survival is shown in Fig. 2. Early loss of transplants was much greater in this group. Indeed, over half were unable to support the life of their recipients for more than 5 days. Nevertheless, it is of interest that one animal survived for a prolonged period (122 days) before being sacrificed.

Kidney Transplants to Splenectomized Recipients. B10.D2 kidneys were transplanted to nine recipients which had undergone splenectomy from 12 to 27 days previously. Several reports have appeared (18–20) that splenectomy of a recipient impairs active immunological enhancement of transplants, although this is not agreed to by all authors (21). Accordingly, it was noteworthy that five of these kidney transplants survived in excellent condition for many weeks, thus demonstrating no influence of prior splenectomy on renal transplant survival in this group of animals.

Survival of Test Skin Grafts to Kidney Recipients. 15 $B6AF_1$ recipients were selected which had born B10.D2 kidney transplants for 40–60 days. Skin grafts from B10.D2 donors to these recipients survived as recorded in Fig. 4. The presence of a surviving kidney transplant greatly extended the life of a *later* skin graft from the donor strain with over 65% of grafts surviving for more than 1 mo. Beyond this period many grafts gradually lose their hair, shrink in area,

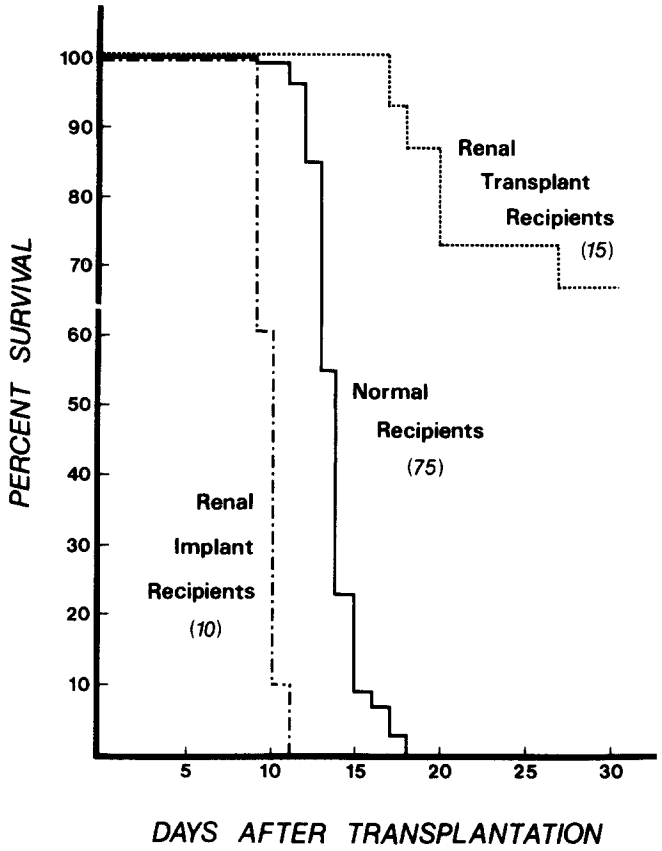

FIG. 4. The survival times of donor strain (B10.D2) skin grafts to normal $B6AF_1$ recipients or to recipients of previous grafts of B10.D2 kidney tissue. Kidney tissue was transferred either by transplantation or in free implants of renal slices to the leg muscles. The number of animals in each group is indicated. Transplant recipients, which are healthy and maintaining BUN levels in the normal range, support the prolonged survival of donor strain skin grafts whereas recipients of kidney tissue implants reject grafts in accelerated fashion. This alteration is specific for the donor strain (see text).

and are slowly rejected in succeeding weeks. In several instances, however, the grafts remained in excellent condition for well over 1 yr. In no case was there any evidence that the presence of the skin graft influenced the function of the kidney transplant. This behavior of skin grafts differed markedly from that observed with normal $B6AF_1$ recipients (MST 13.5 ± 1.1 days).

That this alteration in recipient responsiveness was particularly evident toward the donor strain is shown by the fact that skin grafts from A.SW donors to nine $B6AF_1$ kidney transplant recipients during the same period after transplantation were rejected with an MST of 10.2 ± 1.03 days (MST of A.SW skin grafts to normal $B6AF_1$ recipients is 10.5 ± 1.1 days). Another test of the specificity of immunological reactivity of kidney transplant recipients consisted of measuring their responses to SRBC. Four $B6AF_1$ recipients of B10.D2 kidney transplants which were in good condition from 13 to 166 days after transplantation were tested. Their responses to SRBC immunization, as compared to the

responses of eight normal controls, were unimpaired. For example, at 21 days after SRBC, the transplant recipients showed hemagglutinin titers ranging from 160 to 640 which is the same range as that found in normal animals. This is further evidence that transplant recipients are capable of mounting normal immune responses to antigens not present in the donor tissue.

Of interest was the additional finding that B10.D2 skin grafts to 10 $B6AF_1$ mice about 4 wk after receiving B10.D2 kidney implants were rejected in accelerated fashion (MST 9.1 ± 1.02 days, Fig. 4). Accordingly, a striking difference in immune responsiveness to donor antigens occurred depending upon whether the donor kidney tissue was implanted as a free graft or was transplanted with primary vascular union.

Analysis of Immune Responsiveness of Kidney Transplant Recipient Lymphoid Cells. Several tests of the immune responsiveness of lymph node and spleen cells from $B6AF_1$ recipients bearing B10.D2 kidney transplants for 1 mo or more were made, both in vivo and in vitro.

In Vivo Tests

CELL TRANSFER TO NORMAL ADULT MICE. Lymph node cells from 11 long-term $B6AF_1$ recipients of B10.D2 kidney transplants were pooled and transferred i.p. to normal $B6AF_1$ mice in a dosage of 2×10^8 cells per mouse. This cell dosage was selected as it had been shown by Kilshaw et al. to demonstrate marked suppressor cell activity in mice in which specific unresponsiveness to allogeneic skin grafts had been induced by treatment with donor strain lymphoid cells, anti-lymphocyte serum, and *Bordetella pertussis* (22). Three animals were treated in this particular experiment with the prescribed number of cells. 2 days after receipt of the cells, B10.D2 skin grafts were placed on these animals. These grafts were rejected in 12, 14, and 15 days (MST 13.0 ± 1.03 days). The MST of B10.D2 skin grafts to 4 $B6AF_1$ mice which had received the same number of cells from mice which had recently received and rejected B10.D2 skin grafts was 10.0 ± 1.02 days. These figures were compared also with the MST of B10.D2 skin grafts to normal $B6AF_1$ recipients of 13.5 ± 1.1 days. All these skin grafts were performed at the same time as we have found that slight differences in skin graft survival can be observed from time to time even under seemingly standardized conditions. The survival of skin grafts to animals which had received cells from kidney transplant recipients did not differ statistically from the normal MST ($P > 0.05$) whereas significant acceleration of rejection was demonstrated after transfer of immunized cells ($P < 0.01$). In other experiments, done at several different times, pooled lymph node and spleen cells from a total of 17 kidney transplant recipients were transferred to normal $B6AF_1$ mice at the dosage mentioned above. No evidence of suppressor cell activity was apparent by observing skin grafts to 10 $B6AF_1$ recipients treated in this way.

Larger numbers of cells were not employed as this experimental design required an uneconomical use of cells from the necessarily small number of kidney transplant recipients available. Further tests for the presence of suppressor cells accordingly made use of other types of cell transfer systems and of in vitro tests in which fewer cells could be employed (see below).

INJECTION OF LYMPHOID CELLS INTO NEWBORN MICE. The potential for cells

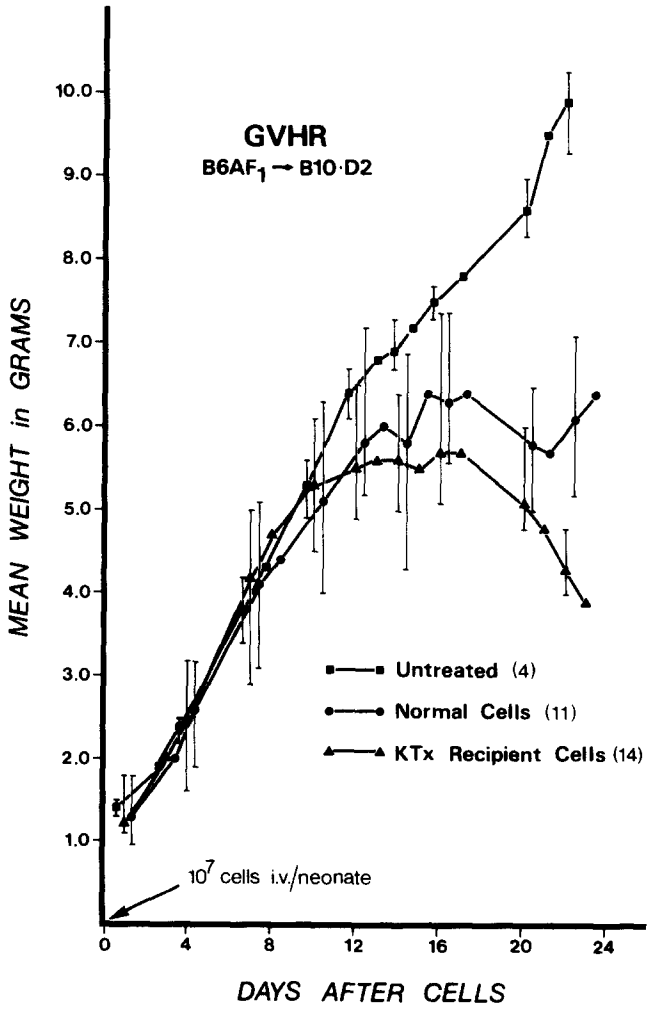

FIG. 5. The capacity of pooled spleen and lymph node cells to produce a graft versus host reaction (GVHR) (runt disease) was determined by following the gain in body weight of neonatal B10.D2 mice injected intravenously with the cells to be tested. Each of several litters was divided into three groups, some receiving 10^7 viable spleen and lymph node cells from normal adult $B6AF_1$ donors, some the same number of cells from $B6AF_1$ recipients of B10.D2 kidneys, and some receiving no treatment. The mean weights of each group are plotted. At the cell dosage tested, no difference is detected by this test between the responsiveness of cells from normal and kidney transplant recipient animals. The number of animals entering each group is indicated, and the range of recorded weights is plotted at representative points.

from long-term kidney transplant recipients to generate a specific immune response to donor strain antigens in vivo was tested by delivering 1×10^7 pooled lymph node and spleen cells intravenously to newborn B10.D2 mice and observing their gain in body weight thereafter. The results from four separate litters were combined. In each litter some neonatal mice received normal $B6AF_1$ cells, some received kidney transplant recipient cells and some were left uninjected. As Fig. 5 shows, these cells were fully capable of mounting a graft-versus-host

response in neonatal hosts as they produced a retardation in growth which was indistinguishable from that after the injection of the same number of cells from normal adult B6AF$_1$ mice. Littermate controls which were uninjected developed at the normal rate.

CELL RESPONSIVENESS AS EVALUATED BY THE "TUMOR NEUTRALIZATION TEST." For these experiments the target employed was the P815-X2 mastocytoma. 5,000,000 viable tumor cells injected subcutaneously into each B6AF$_1$ recipient produced a tumor mass which reached a size of about 7–10 mm in greatest diameter by 6–8 days before the tumor cells were gradually rejected by the active immune responses of the host. When this number of tumor cells was mixed with an equal number of pooled lymph node and spleen cells from preimmunized B6AF$_1$ mice, the combined inoculum of tumor cells and immunized lymphoid cells resulted in a barely palpable growth. Lymph node and spleen cells from kidney transplant recipients mixed in equal numbers with tumor cells had no detectable influence on tumor growth in normal B6AF$_1$ mice.

A main objective in using this system was to determine whether cells from kidney transplant recipients would suppress the action of preimmunized cells on the tumor in vivo as compared to normal recipient strain cells. In these experiments inhibition of growth of the tumor caused by an equal number of sensitized cells was reduced after the addition of a like number of either normal or kidney transplant recipient cells, but kidney transplant recipient cells did not have a greater influence in this regard. Indeed, it seems likely that the effect of secondarily added cells from either of these sources was simply to dilute the impact of the sensitized cells on the tumor.

In summary, these experiments yielded no evidence that cells from long-term kidney transplant recipients can suppress the specific immune reactivity of previously immunized lymphoid cells against donor antigens in vivo under the conditions employed.

In Vitro Tests

CELL-MEDIATED CYTOTOXICITY. Spleen cells from 16 kidney transplant recipients were evaluated individually to determine whether immune cytotoxic killer cells could be detected at times ranging from 42 to 372 days after transplantation. The average quantity of ^{51}Cr released from labeled target cells did not differ significantly ($P > 0.05$) from that produced in cultures with cells from normal B6AF$_1$ mice. Assays were performed under various conditions including rocking versus stationary cultures, 4, 6, 9, or 16 h of incubation, and test to target cell ratios of 50:1, 100:1, or 200:1 (detailed data not shown). Mice which had recently rejected B10.D2 skin grafts or had received a series of intraperitoneal injections of B10.D2 lymphoid cells yielded spleens which contained easily detectable killer cell activity.

Experiments in which test cells were mixed in varying proportions with target cells indicated that few if any cytotoxic killer cells are present in the spleens of kidney transplant recipients at 40 days or longer after transplantation. Similar results followed when lymph node cells were tested in this way (Fig. 6).

MIXED LYMPHOCYTE CULTURES. The proliferative responses of lymphoid cells from kidney transplant recipients after stimulation with irradiated donor

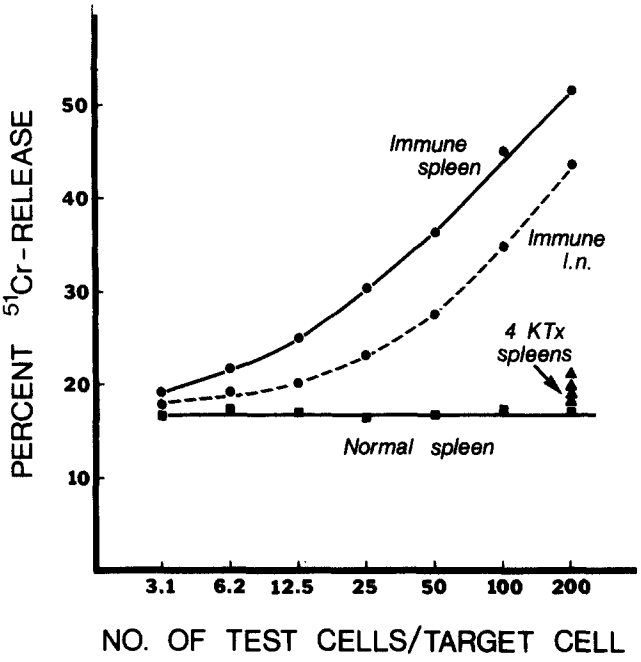

FIG. 6. A comparison of the ability of lymphoid cells from kidney transplanted, immune and normal animals to cause killing in vitro. Specifically immune lymphoid cells were obtained from B6AF$_1$ mice which had been grafted on two consecutive occasions with skin from B10.D2 donors (days 0 and 19), and which were then injected on day 29 with 7×10^7 B10.D2 lymphoid cells. Spleens and lymph nodes were removed 4 days later and titrated for killer cell activity in a ^{51}Cr release assay against P815-X2 tumor cells. The kidney transplant recipients tested had received B10.D2 kidneys 54, 56, 59, and 63 days before removal of their spleens and lymph nodes (l.n.). Lymphoid cells from normal B6AF$_1$ mice served as controls. Supernates from the assays were evaluated for ^{51}Cr-release after an 8 h incubation. Spontaneous ^{51}Cr-release was 19.8%. Active killer cells were not detected in the spleens of kidney transplant recipients as compared to the killing activity of different numbers of specifically immune cells.

strain cells were compared to the levels of response attained by normal recipient strain cells. The responses of three long-term survivors of kidney transplants (118–199 days) were similar to normal levels of response (Table I).

GENERATION OF KILLER CELLS. Kidney transplant recipient spleen cells from these same mice were also incubated with irradiated donor strain cells to determine their ability to generate cytotoxic effector cells. While significant cytolysis was detected in the subsequent CMC assay, the levels of killing attained were substantially lower than the levels of killer cell activity generated by cells from normal B6AF$_1$ mice (Fig. 7). Further studies have confirmed these results in that nucleated splenic cells from five out of six mice bearing foreign kidney transplants for 118 days or longer produced lower levels of killer T cells than cells from normal mice.

In another series of experiments, spleen cells from kidney transplant recipients were sensitized in vitro against irradiated B10.D2 and A.SW cells. While only one out of five recipients yielded detectable levels of killer cells against the

TABLE I

Responses in MLC of KTxR and Normal B6AF$_1$ Cells

Kidney transplant recipients*					Normal B6AF$_1$		
Animal no.	Days post Tx	Cell source	Alone	+B10.D2 (1,000 rads)	Cell source	Alone	+B10.D2 (1,000 rads)
			cpm			*cpm*	
529	118	LN	1,677	53,145	LN	11,744	148,588
		Spleen	22,937	108,145	Spleen	25,195	97,149
507	199	LN	3,028	113,216	LN	729	82,756
535	134	LN	15,488	49,818	LN	2,737	37,606

* B10.D2 into B6AF$_1$ kidney transplants.

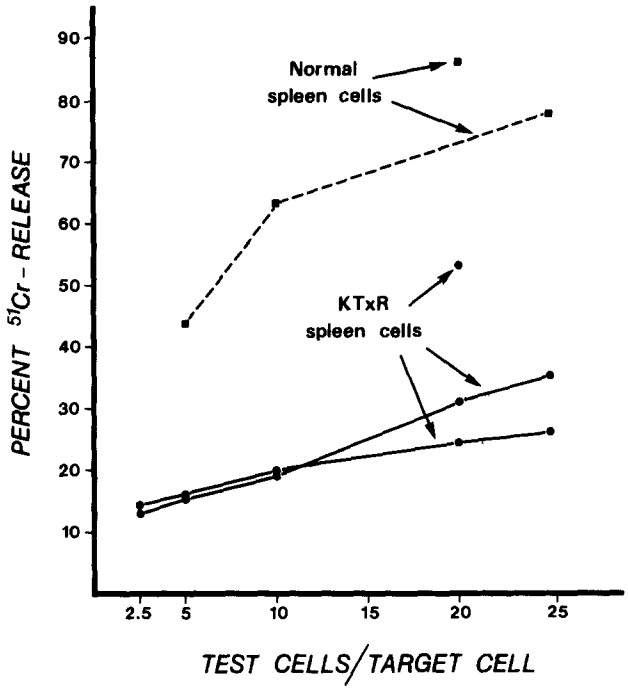

FIG. 7. The ability of B6AF$_1$ lymphoid cells from long-term recipients of B10.D2 kidney transplants to become sensitized in vitro. Nucleated splenic cells from transplant recipients and normal B6AF$_1$ mice were cultured with irradiated donor strain cells (B10.D2) at 2×10^6 each/culture. 5 days later the cultured cells were evaluated in a ^{51}Cr-release assay to measure the level of sensitization that had developed. Spleen cells from kidney transplant recipients show reduced sensitization to donor antigen by this test as compared to cells from normal mice.

donor strain H-2^d targets, five out of six developed significant levels of killer activity directed against the A.SW H-2^s target antigens (data not shown). This is further evidence that transplant recipients have specifically depressed abilities to generate killer cells against the antigens of the donor kidney.

SUPPRESSOR CELL ASSAYS. Two types of tests were employed. One was designed to reveal a suppressive effect on the generation of effector cells from

TABLE II

Effect of Preincubated Kidney Transplant Recipient Cells (KTxR) on Killing Activity of Immune Cells

Preincubated mixtures added to CMC assay‡	% Specific ^{51}Cr-release* Cell donors for CMC assay		
	None	Nonimmune $B6AF_1$	Immune $B6AF_1$
None	–	1.8	55.4
Normal cells alone	0	1.2	56.0
KTxR cells alone§	0	1.2	56.0
Normal cells + P815-X2‖	0	0.3	46.7
KTxR cells + P815-X2	0	0	44.0
Normal cells + B10.D2 (1,000 rads)¶	0	1.6	53.1
KTxR cells + B10.D2 (1,000 rads)	0	1.6	50.9

* Spontaneous release = 33.0% after 17 h CMC assay.

‡ Preincubated at 37°C for 23 h and used in CMC assay at a ratio of 1:1 with nonimmune and immune $B6AF_1$ cells.

§ Pool of spleen cells from two kidney transplant recipients.

‖ Normal and KTxR cells preincubated with P815-X2 at 100:1.

¶ Normal and KTxR cells preincubated with B10.D2 cells at 3.3:1.

normal cells and the other to evaluate a suppressive effect on the expression of immunity by preimmunized cells.

For the first test kidney recipient cells and normal $B6AF_1$ cells were mixed at ratios of 1:6, 1:3, and 1:1 and were sensitized together in culture for 5 days by irradiated donor strain stimulating cells. No suppression of the normal response was detectable at any ratio of kidney recipient cells to normal cells when these cultured cells were tested in a CMC assay.

In the second test for suppression, normal or kidney recipient spleen cells were incubated for 23 h with tumor target and irradiated donor strain cells. The effect of these preincubated cell mixtures on the capacity of immune cells to kill ^{51}Cr-labeled tumor targets was assessed. No suppression of immune effector cell function was evident in any of the combinations (Table II). Similar procedures have been reported to reveal evidence of suppressor cells in NZB mice against syngeneic or autochthonous fibroblasts (23). The lower levels of ^{51}Cr-release in the assays with mixtures preincubated with P815-X2 tumor cells probably reflects the slight inhibition which is to be expected from competition by unlabeled target cells. No killer cells were generated by cells from either kidney transplant recipients or normal $B6AF_1$ animals during the 23 h of incubation with either irradiated B10.D2 cells or P815-X2 tumor cells.

These data indicate that kidney transplant recipients contain approximately normal numbers of lymphoid cells that proliferate in response to H-2 antigenic stimuli (helper T cells) but that long-term recipient mice are deficient in their functional prekiller T-cell subpopulations. Also, despite the presence of the transplant, the cell responses attained in vitro are substantially lower than

would be expected for secondary responses indicating again that these recipients have few or no immune "memory" cells.

Analysis of Serum from Kidney Transplant Recipients. Serum was collected repeatedly from the retro-orbital sinus of 16 $B6AF_1$ recipients of B10.D2 kidney transplants at times ranging from 30 to 300 days after transplantation. The tests to be described were performed with a single pool of serum from these collections. Complement-dependent cytotoxic antibody was not detectable by our technique in this serum pool. Individual serum samples from transplant recipients, however, were sometimes shown to contain cytotoxic antibody (see below).

IN VIVO TESTS. To test the capacity of this serum to enhance the survival of donor strain test skin grafts, a regimen for serum administration was employed which exceeded in dosage (volume) that previously shown in our laboratory to be effective in producing enhancement of skin grafts in this strain combination with several antisera which contained demonstrable cytotoxic activity (24). 10 mice received 0.2 ml of serum i.p. on days 0, 2, and 4 with respect to receiving B10.D2 skin grafts. The MST of these grafts was 14.0 ± 1.1 days. When compared to the MST of such grafts on untreated recipients (13.5 ± 1.1 days) the difference was not significant ($P > .05$).

IN VITRO TESTS. (*a*) Cytotoxic antibody titers were determined repeatedly on 46 $B6AF_1$ recipients of B10.D2 kidneys. The percentage of animals with detectable antibody titers declined from 24% at 4 wk to 7% after 12 wk. Antibody titers also declined from an average of 1:64 at 4 wk to 1:4 after 12 wk.

(*b*) The recipient serum pool was tested to determine whether it could inhibit the cytolytic activity of immune T cells and/or the ability of normal $B6AF_1$ cells to generate effector T cells during sensitization in vitro.

Kidney recipient serum and normal mouse serum, each at a concentration of 1:10, were added to tubes containing decreasing concentrations of sensitized $B6AF_1$ cells. ^{51}Cr-labeled P815-X2 target cells were also added and the mixtures incubated for 4.5 h as outlined in Materials and Methods. No inhibition of killing activity was effected by the kidney recipients' serum (Table III). The inability of this serum to block killing activity of sensitized cells is particularly evident at lower ratios of sensitized to tumor target cells. Antiserum raised in $B6AF_1$ mice against B10.D2 skin and/or lymphoid tissues blocked killing in this assay completely (data not shown).

The same kidney recipient serum pool could, however, suppress the generation of killer cells in cultures in which normal $B6AF_1$ cells were mixed with irradiated B10.D2 donor strain cells. Serum from long-term recipients of B10.BR strain kidneys also depressed the ability of $B6AF_1$ cells to generate killer cells against $H\text{-}2K^d$ antigenic targets (see reference 6 for results with this strain combination). The suppressive effect observed with the kidney recipients' serum, therefore, does not appear to be antigen specific. Its influence, however, is confined to a limited "phase" of the development of the immune response in that the serum does not suppress the activity of immune cells but depresses the ability of nonimmune cells to differentiate into effector cells. The nature of this suppressive substance offers an opportunity for further investigation.

TABLE III
Effect of Kidney Transplant Recipient Serum on the Killing Activity of Immune Cells

Type of serum	% ^{51}Cr-release* Sensitized cells/Target cell‡			
	20:1	10:1	5:1	2.5:1
NMS	78.3	70.2	54.2	45.6
KTxR Serum	76.9	71.5	61.6	46.0

* Spontaneous release of ^{51}Cr-labeled P815-X2 = 22.9% after a 4.5-h assay.

‡ B6AF$_1$ cells were sensitized for 5 days in vitro against irradiated B10.D2 cells.

Discussion

These experiments have demonstrated that transplant rejection in several combinations of mouse strains is much less vigorous against kidneys than against skin grafts from the same donor strain. That this difference does not depend upon antigens peculiar to skin was shown by the fact that slices of renal tissue, implanted as free grafts, were rejected at about the same rate as skin grafts. The recipients of implants of renal tissue are specifically immunized after graft rejection. By contrast, however, primarily vascularized renal transplants provoke a much weaker rejection response which tends to diminish with time. This is suggested by the distinct clearing of infiltrating leukocytes from transplanted kidneys after the first 3–4 wk and by the diminished prevalence of complement-dependent cytotoxic antibody in the sera of transplant recipients as time progressed.

In another series of experiments employing primarily vascularized heart transplants between mice, Corry et al., in our laboratory, made some similar observations with B10.BR hearts transplanted to B6AF$_1$ recipients. In this strain combination, the transplanted hearts functioned well for about 3 wk but then entered a period during which the heart beat was greatly reduced before it was restored spontaneously to normal (25). In a later study, Corry found that donor strain skin grafts were rejected in somewhat accelerated fashion in this strain combination if applied soon after heart transplantation but that later skin grafts enjoyed prolonged survival (26). It appears that although heart transplants have the capacity, under the right conditions, to induce an alteration in recipient immune responses, they are less able to do so than are kidney transplants. This is certainly the case in the strain combination we have studied most intensively (B10.D2 donors and B6AF$_1$ recipients) since heart transplants are all promptly rejected in contrast to the long survival of kidneys transplanted between these strains.

The set of circumstances which evolves spontaneously in our animals may be similar to that obtaining with long-surviving kidney or heart transplants in some rats which can be produced by the treatment of recipients with a short course of injections of an antiserum directed against the foreign antigens of the donor. Generally, F$_1$ transplants to a parental strain recipient are required for

long-term survival. Evidence has been advanced, especially by Batchelor and his colleagues (27) that these animals enter a "steady state" which persists long after the originally infused antibody has disappeared. In our untreated recipients it is unclear as to whether the antibody against donor cells which was often detectable contributed to the development of the steady state which developed in them. It is probable that antibody at low levels is present throughout the survival of the recipient. Nevertheless, two facts from our in vivo experiments make it unlikely that antibody is indispensable to the suppression of the early rejection reaction. First, the fact that prior splenectomy, which generally lessens the vigor of active enhancement, did not curtail the survival of transplanted kidneys does not support the concept that conventionally defined enhancement contributes to the onset of the "steady state" in our animals. Secondly, the fact that kidneys from H(z1) donors survived in the absence of any detectable antibody to them is evidence in the same direction.

The immune alteration in recipient responsiveness after the transplantation of a kidney in the strain combinations tested has a considerable degree of specificity. Donor skin grafts (B10.D2) often survived for many weeks whereas skin grafts presenting strong new antigens (A.SW) were rejected normally. The response to sheep erythrocytes was also normal. Nevertheless, there is no justification for classifying this phenomenon as an example of actively acquired immunological tolerance as there is ample evidence that lymphoid cells from kidney transplant recipients can mount vigorous immune responses to donor antigens both in vivo and in vitro. Furthermore, no evidence could be found for the participation of suppressor cell activity by either in vivo or in vitro tests.

Although graft versus host assays established that cell populations from kidney recipients were capable of producing effective immune responses in vivo which were indistinguishable from those generated by cells from normal donors, the cell doses used were large and the discriminatory capacity of the test employed was probably insufficient to reveal reductions in immune responsiveness of the inoculum. In vitro tests offer easy flexibility in varying the numbers of cells in the reaction mixture and these provided evidence for a reduction in cellular responsiveness. It must be remembered that this balanced system probably involves the continual turnover of potentially reactive cells which are constantly being influenced by their surroundings, and single samplings of the components of such a system may not reveal how the balance is maintained. Nevertheless, it was considered significant that long-term recipients, on repeated testing, showed a reduction in functional precursors of killer T cells as well as showing no evidence for the presence of active killer T cells themselves, whereas such cells were readily produced on stimulation by antigens which had not been present in the transplanted kidney. This deficiency of immune cells and in pre-killer cells appears to be specific in that relatively normal levels of killer cells could be induced against A.SW target cells. The fact that almost all transplanted kidneys were rejected promptly when transplanted to recipients which had previously been immunized by donor strain skin grafts shows that such rejection is possible and is consistent with the notion that immune effector cell generation is somehow insufficient to produce decisive rejection by normal recipients.

The nature and significance of the suppressive substance detected in recipient serum by in vitro tests have not been established. Further studies to define the mechanism of this specific deficiency are needed. Meanwhile, an additional dimension of understanding of the immunological balance between the transplanted organ and its surviving host has been gained by various approaches to upsetting this balance as reported in an accompanying paper (28).

Summary

Kidney transplants between strains of mice which are incompatible at either the *K* or the *D* end of the *H-2* complex usually function for prolonged periods supporting the lives of nephrectomized recipients. This occurs with no recipient treatment. With multiple *H-2* and non-*H-2* determined incompatibilities, transplants may be rejected but more slowly than skin grafts. In the strain combination studied most extensively in these experiments (B10.D2 to $B6AF_1$) in which the incompatibility was confined to the *K* end of the *H-2* region, about 70% of recipients survived for many weeks with normal blood urea nitrogen levels. Skin grafts between untreated members of these strains were rejected promptly (mean survival time of 13.5 ± 1.1 days) as were kidney transplants to recipients of prior skin grafts. Donor strain skin grafts to recipients of kidney transplants after kidney transplantation enjoyed greatly prolonged survival whereas skin grafts from a third party (A.SW) were rejected normally. If kidney tissue was transferred in the form of free grafts without primary vascular union, it was rejected promptly leaving its recipient highly immunized.

Cellular and humoral immunity to donor antigens declined over the first few weeks after transplantation, and the spleens of long-term recipients contained no "killer cells." Recipient lymphoid cells could mount active graft versus host reactions to donor strain antigens on transfer to neonatal mice. Nevertheless, they were distinctly less able to respond specifically by the production of killer cells to donor strain antigens after sensitization in vitro. No evidence that this defect was associated with the presence of suppressor cells was forthcoming from several types of in vivo and in vitro tests.

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