

## INDUCED IMMUNE DESTRUCTION OF LONG-SURVIVING, H-2 INCOMPATIBLE KIDNEY TRANSPLANTS IN MICE\*

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PLATE

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We have reported in an accompanying paper that kidney transplants to mice regularly produce an immune alteration toward donor antigens without any other treatment (1). This immune alteration takes place over the 1st mo or so after the recipient has undergone kidney transplantation and bilateral nephrectomy. In several strain combinations in which incompatibility determined by genes of the *H-2* system is involved, manifestations of rejection activity directed toward the transplant tend to diminish during the early weeks after transplantation and loss of transplanted kidneys becomes much less common.

The mechanism by which this equilibrium between transplant and recipient is maintained remains to be established in full. In our previous paper we presented evidence that the lymphoid cells of kidney transplant recipients which have survived 1 mo or more are able to respond to donor antigens both in vivo and in vitro, but that they do so to a lesser degree, as a population, than do normal cells. This relative reduction in ability to respond is associated with the presence in the recipients' sera of a substance which interferes with the full development or expression of an immune response, especially the development of killer T cells.

The present experiments made use of mice with long-term, well functioning kidney transplants from *H-2* incompatible donors. These animals offer a study system in which various methods of inducing the immune destruction of the surviving organ can be tested. Each recipient bears a large mass of foreign tissue which presents well-characterized antigenic differences to its host. The function of the allogeneic organ can be evaluated on a continuing basis by measuring the blood urea nitrogen level of its recipient. The experiments described here include treatments in which heightened reactivity to donor tissue antigens was conferred by passive immunization and also maneuvers which awakened the dormant responses of the host so that the recipients responded more strongly to donor antigens resulting in the acute destruction of the transplant.

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## Materials and Methods

*Animals.* Inbred mice were used throughout. As described previously (1) strain combinations in which incompatibilities were restricted to a portion of the *H-2* region were employed. The principal test system involved B10.D2 mice as donors and (C57BL/6 × A/J)<sub>F</sub><sub>1</sub>, abbreviated B6AF<sub>1</sub>, mice as recipients. This strain combination involves an incompatibility determined at the *K* end of the *H-2* complex (H-2.31) as well as an incompatibility in the *Ia* region. Cells from A.SW mice were also used in some experiments as they present different *H-2* incompatibilities (H-2.7, 12, 19, 51) to B6AF<sub>1</sub> recipients from those offered by B10.D2 cells.

Members of the B10.BR strain were also used in one experiment as a source of both kidneys and lymphoid cells for transplantation to B6AF<sub>1</sub> recipients. This donor strain is incompatible with the recipient strain at the *D* end of the *H-2* complex (*H-2D* plus *I-C*), the principal antigenic difference being H-2.32. Members of this strain are less incompatible with B6AF<sub>1</sub> recipients than are B10.D2 mice as skin grafts from them have a median survival time of  $16.5 \pm 1.1$  days as against  $13.5 \pm 1.1$  days for B10.D2 grafts.

A third donor strain, the black mutant B6.C-H(z1), abbreviated H(z1), was also used in grafting to B6AF<sub>1</sub> recipients. Cells from this mutant strain, originally described by Bailey et al. (2), are known to be capable of provoking proliferative lymphocyte responses *in vitro* in mixed cultures with cells from recipients of the genotype employed in our experiments. Skin grafts are rejected promptly. Nevertheless, no evidence of a serologically measurable immune response in this combination has so far been detected (3). These findings have been confirmed in our laboratory by using several techniques for measuring serological immunity by P. Sugarbaker and H. Winn, personal communication, so that it is clear that the response to the alloantigens involved in this strain combination, by comparison with most other strain combinations in which *H-2* incompatibility exists, is weighted heavily toward cellular immunity.

### Operative Procedures

**KIDNEY TRANSPLANTATION.** Kidney transplantation was accomplished by direct vascular union as described previously (1). The recipients' kidneys were removed at the time of transplantation.

The survival of kidney transplants was evaluated by the survival of their recipients, by microscopic examination of sections prepared from them at the time of their removal on selected days (see below), and most sensitively by serial determination of blood urea nitrogen (BUN)<sup>1</sup> levels by using a standard urease micromethod. The latter determination was of particular value in the present experiments because it offered a means whereby transient effects could be detected and measured quantitatively. The normal range is considered to be from 15 to 30 mg/100 ml.

As described previously (1) about 30% of B6AF<sub>1</sub> recipients of B10.D2 kidneys die during the first 30 days. This occurs because of the effects of early rejection activity combined, in some instances, with the late consequences of technical imperfections such as some degree of ureteral obstruction. After about 1 mo the animals enter a relatively steady state and continuing attrition of recipients is low with only 20% more being lost by 100 days. A similar pattern of survival results when donor kidneys are obtained from the B10.BR and the H(z1) strains. Accordingly, the subjects for study in the present experiments were confined to recipients which had survived kidney transplantation for 4 wk or more and which had BUN levels in the normal range.

**SKIN GRAFTING.** Full thickness grafts of flank or ventral surface skin were placed on recipient beds on the lateral thoracic wall as described in detail by Billingham and Medawar (4).

**LYMPHOID CELL SUSPENSIONS.** "Sensitized" cells consisted of lymph node and spleen cells obtained from B6AF<sub>1</sub> animals which had rejected B10.D2 skin grafts and which had received an intraperitoneal injection of  $10^8$  B10.D2 lymph node and spleen cells from 4 to 7 days after graft rejection. The immunized cells were collected 7-14 days thereafter.

Spleen and lymph nodes were dissociated to form cell suspensions by gently passing them through a nylon sieve. The cells were collected in medium L-15 (Microbiological Associates, Walkersville, Md.), incubated with 0.14M NH<sub>4</sub>Cl Tris buffer for 5 min at 37°C to lyse erythrocytes, and resuspended in the medium after two washings. The viability of cells in those suspensions employed was in excess of 80% as determined by trypan blue dye exclusion.

<sup>1</sup> Abbreviations used in this paper: BCG, Bacille of Calmette and Guérin; BUN, blood urea nitrogen; C, complement; CMC, cell-mediated cytotoxicity; CY, cyclophosphamide.

**HISTOLOGICAL PREPARATIONS.** Tissue specimens were fixed in 10% neutral formalin in 50% ethanol, cleared in butanol, embedded in paraffin, and sectioned at 4  $\mu$ m before staining with hematoxylin and eosin.

**SEROLOGY.** Complement-dependent cytotoxic antibody titers were determined in a two stage test as described previously (5). Trypan blue dye exclusion was employed to determine cell viability. A pooled antiserum against B10.D2 strain antigens was prepared from repeated bleedings of B6AF<sub>1</sub> female mice after at least four weekly injections of B10.D2 lymphoid cells. This pool had a cytotoxic titer of 1:512. Rabbit serum which had been exhaustively absorbed against mouse tissue was used as the source of complement (C) for infusion into mice.

**<sup>51</sup>Cr RELEASE ASSAY FOR CELL-MEDIATED CYTOTOXICITY (CMC).** A modified version of the Brunner CMC assay, described previously (6), was used to detect the presence of killer cells in the spleens of B6AF<sub>1</sub> which had received B10.D2 kidney transplants. P815-X2 tumor cells were used as <sup>51</sup>Cr-labeled targets since they share H-2K<sup>d</sup> antigenic determinants with the kidney donor. A mixture of lymphoid test cells and tumor target cells, at the appropriate ratios, was centrifuged and allowed to incubate for 16-18 h at 37°C. The cells were then resuspended and centrifuged and the amount of <sup>51</sup>Cr label released into the supernates was determined.

**BCG AND CYCLOPHOSPHAMIDE.** Bacille of Calmette and Guérin (BCG, Tice strain) was obtained from the University of Illinois Medical Center in vials containing 2-8  $\times 10^8$  viable organisms. The BCG was reconstituted to 1 ml with sterile water, and an individual dose of 0.1 ml (approximately 2  $\times 10^7$  organisms) was given intravenously. Cyclophosphamide (CY) from Mead Johnson Laboratories, Evansville, Ind. was dissolved in sterile water (20 mg/ml) and given i.p. in a dose of 4 mg/animal (160 mg/kg).

## Results

*Treatment with Sensitized, Recipient Strain Lymphoid Cells.* Five B6AF<sub>1</sub> recipients of B10.D2 kidneys were used in this experiment. The transplants had been in place from 45 to 307 days and all were functioning normally. Two recipients received an intraperitoneal injection of 10<sup>8</sup> spleen and lymph node cells from B6AF<sub>1</sub> mice previously immunized to B10.D2 antigens. Two additional animals received a second injection of the same number of cells on the succeeding day, and a fifth animal received three daily injections of 1.5  $\times 10^8$  cells from immunized donors. Fig. 1 shows the effects of these injections on the BUN levels of the recipients. At the first two dosage levels evidence of kidney damage was apparent by the 8<sup>th</sup>-15<sup>th</sup> day. The transplanted kidneys continued to support life in their recipients, however, and there was evidence of improvement in kidney function by about 3 wk after the injections. The transplant in the mouse which received the largest number of cells from immunized donors showed little response. At the doses of cells employed, it is apparent that definite damage to the transplant can result but that it did not progress and there was evidence of some late repair of function. With increasing doses of cells there was no marked increase in the degree of damage to the transplants.

*Treatment with Alloantibody to Donor Antigens with and without Added Complement.* Seven B6AF<sub>1</sub> recipients of B10.D2 kidneys were used in this experiment. Recipients received rabbit serum alone (0.5 ml) as a source of C, B6AF<sub>1</sub> anti-B10.D2 serum alone (0.5 ml), or the antiserum followed 6 h later by C. All injections were delivered intravenously. As inspection of Fig. 2 will reveal, neither antiserum nor C alone appeared to have any effect on kidney function under the conditions of these experiments except in one case (see middle panel) in which a sharp BUN elevation occurred by 5 h after C injection. This kidney went on to complete destruction. The recipient in this instance was known to have had an unusually high cytotoxic antibody titer to B10.D2 cells

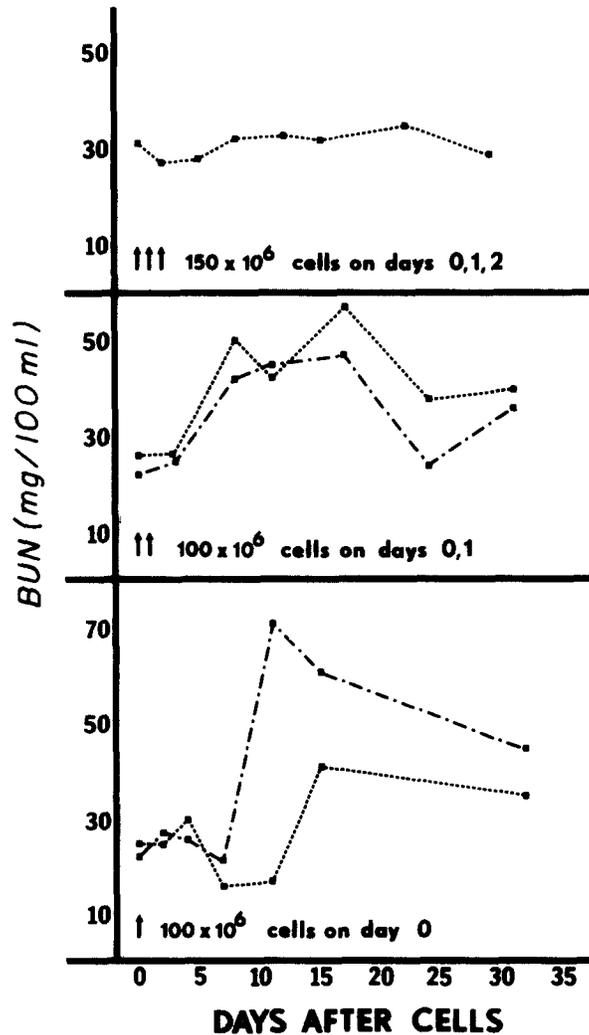


FIG. 1. The effect on transplant function of infusions of lymph node and spleen cells sensitized to donor (B10.D2) antigens. B6AF<sub>1</sub> recipients bearing normally functioning kidney transplants for 45-307 days received ascending doses, as indicated, of lymphoid cells intraperitoneally. Prompt evidence of kidney damage, as reflected in elevations of BUN levels, is apparent. Considerable improvement of renal function follows. With increasing cell dosage (within the limits tested) there is no evidence of increased kidney damage. Indeed, the least damage was seen at the highest cell dose.

(1:256). In five recipients it was found that antibody infusion, followed within a few hours by C, would cause very prompt kidney damage with striking elevation of BUN levels shortly after the C infusion. Thereafter kidney function returned to normal.

*Treatment with Donor Strain Cells with or without BCG and CY.* BCG and CY were employed according to a protocol originally described by Mackness

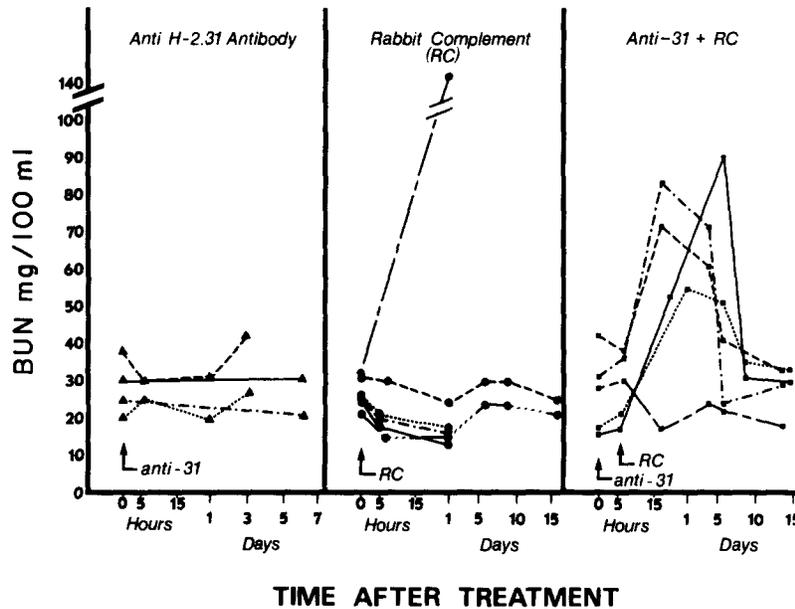


FIG. 2. The effect of intravenous infusions of antibody and C on the function of long-surviving B10.D2 kidneys transplanted to B6AF<sub>1</sub> recipients. In the left panel the BUN records of four transplant recipients are shown. At time 0 all received 0.5 ml of an antibody directed to donor antigens (see text). This did not influence the function of the transplanted kidneys. In the middle panel six recipients received injections of C (0.5 ml of rabbit serum absorbed with mouse cells). No effect was seen except in the case of one recipient known to have an unusually high titer of antibody to donor antigens. In the right panel, antibody infusion to five recipients was followed in 6 h by C. Impairment of kidney function was apparent by elevation of BUN levels in four of the five animals within 24 h. This damage was transient and kidney function after this treatment returned to normal in all animals.

and Lagrange which they devised to alter the state of specific unresponsiveness to sheep erythrocytes which can be induced in mice by large doses of this antigen (7).

**CONTROL. TREATMENT WITH RECIPIENT CELLS ALONE.** This control was performed to rule out a possible nonspecific effect resulting from the injection of large numbers of spleen and lymph node cells which might lodge in the transplanted kidney or cause damage to the kidney in some other nonspecific fashion. Accordingly, three B6AF<sub>1</sub> mice, which had received B10.D2 kidney transplants at least 4 wk previously, were injected with  $1.5 \times 10^8$  normal B6AF<sub>1</sub> lymph node and spleen cells intraperitoneally on 2 successive days. No effect on BUN levels was observed during a subsequent month of observation.

**TREATMENT WITH DONOR STRAIN CELLS ALONE.** Four B6AF<sub>1</sub> kidney recipients received  $1.5 \times 10^8$  pooled B10.D2 lymph node and spleen cells intraperitoneally on 2 successive days. An increase in BUN levels was observed in all mice by the 6<sup>th</sup> day after the initial cell injection. The damage induced in the kidney transplants was transient, however, as BUN values steadily returned to near normal levels within 4 wk (see Fig. 3). Two of these four recipients underwent

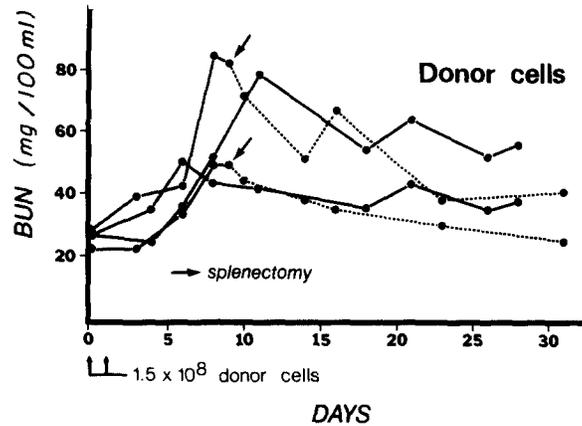


FIG. 3. The effect of donor cells on transplant function. Intraperitoneal injections of  $1.5 \times 10^8$  donor strain lymph node and spleen cells on 2 succeeding days resulted in elevations of BUN levels in all four B6AF<sub>1</sub> mice which were long-term recipients of B10.D2 kidney transplants. The damage provoked by these cells tended to be repaired gradually. Injection of the same number of recipient strain cells to three other animals (not shown) failed to induce any evidence of transplant damage.

splenectomy at the height of the induced response so that their spleens could be assessed for the presence of killer cells as measured by <sup>51</sup>Cr release from P815-X2 target cells (see Fig. 4). Evidence of only a sluggish immune response was revealed by this test especially as compared to the level of cell-mediated cytotoxicity found in the spleens of animals which had recently received and rejected skin grafts. It was also of some interest that removal of the spleen at this point did not alter the course of the response as compared to the other animals tested.

**TREATMENT WITH BCG AND CY.** BCG ( $2 \times 10^7$  organisms) was given intravenously to two B6AF<sub>1</sub> mice with normally functioning B10.D2 kidney transplants at 8–15 wk. The BUN levels remained stable thereafter and were not influenced adversely when an injection of CY (4 mg) was given 20 days later (see Fig. 5).

**TREATMENT WITH CY AND DONOR CELLS.** Five B6AF<sub>1</sub> recipients of B10.D2 kidneys received injections of 4 mg of CY followed 2 days later with the first of two daily intraperitoneal injections of  $1.5 \times 10^8$  B10.D2 spleen and lymph node cells. By the 10<sup>th</sup>–12<sup>th</sup> days after the initial cell injection four out of five of the treated mice showed marked BUN elevations and three of these animals were sacrificed, two with BUN levels of over 200 mg/100 ml. The fourth animal recovered considerably from the damage incurred and survived for several months. The remaining transplant showed only a minor degree of damage during the observation period (see Fig. 6).

**TREATMENT WITH BCG AND DONOR CELLS.** Four B6AF<sub>1</sub> recipients of B10.D2 kidney transplants received injections of  $2 \times 10^7$  BCG organisms from 42 to 144 days after transplantation. On the 22<sup>nd</sup> and 23<sup>rd</sup> days after BCG treatment, they received intraperitoneal injections of  $1.5 \times 10^8$  B10.D2 lymphoid cells. All of these animals showed a sharp rise in BUN by the 7<sup>th</sup>–8<sup>th</sup> day after the initial cell

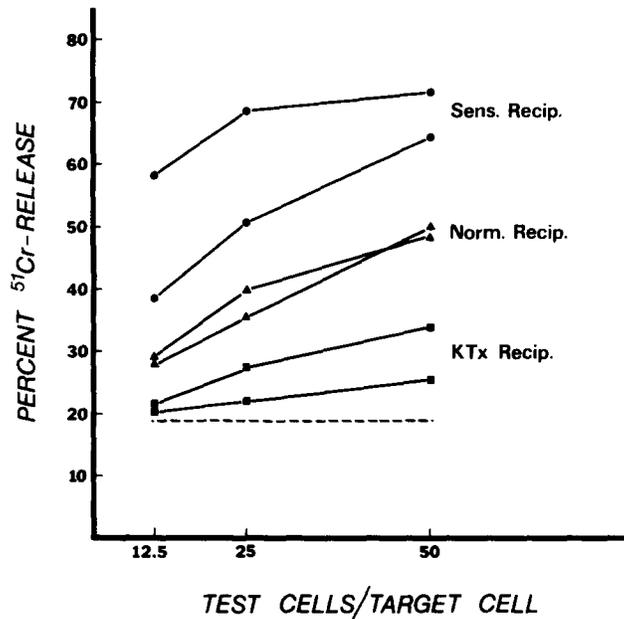


FIG. 4. Normal B6AF<sub>1</sub> animals, B6AF<sub>1</sub> animals which had recently rejected B10.D2 skin grafts, and B6AF<sub>1</sub> recipients of B10.D2 kidneys were injected i.p. with  $1.5 \times 10^8$  B10.D2 lymphoid cells 8 and 9 days before removal of their spleens. Increasing numbers of spleen cells from each animal were mixed with <sup>51</sup>Cr-labeled P815-X2 cells and incubated for 16 h before the supernates were harvested. Spontaneous release (---) = 19.8%. The capacity of kidney transplant recipients to produce cytotoxic cells in vivo under these conditions was less than that of normal mice and markedly less than that specifically immunized animals.

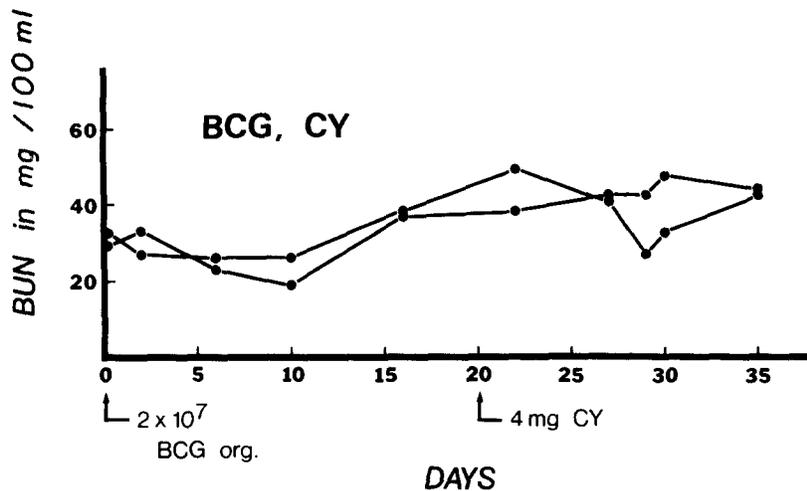


FIG. 5. The administration of BCG and CY in the dosages indicated failed to produce any evidence of renal damage in two control B6AF<sub>1</sub> mice which were long-term recipients of B10.D2 kidney transplants.

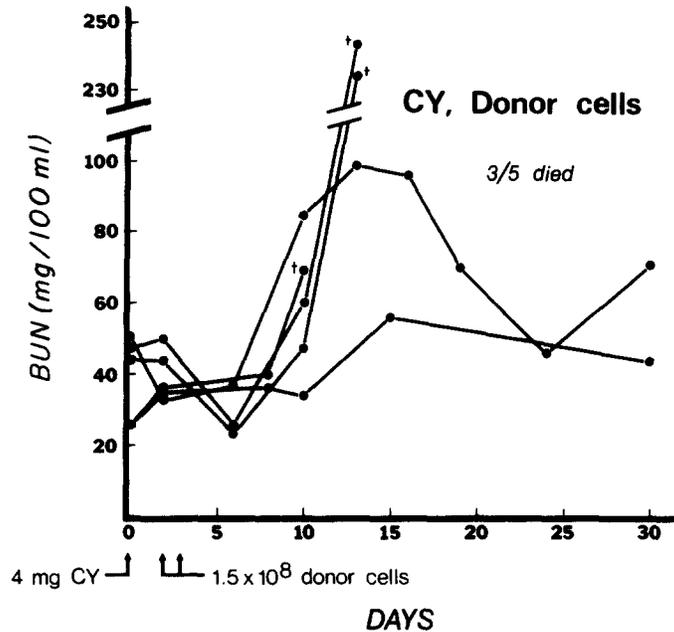


FIG. 6. Treatment of five B6AF<sub>1</sub> recipients of B10.D2 kidney transplants with CY followed by two daily intraperitoneal injections of donor strain lymphoid cells rapidly provoked marked evidence of renal damage in all but one animal the BUN of which showed only moderate elevation. Three of the five animals became moribund and were sacrificed, whereas two recovered.

injection. Three went on to severe uremia and were sacrificed when moribund. The fourth animal survived with a BUN at a slightly elevated level (see Fig. 7).

**TREATMENT WITH BCG, CY, AND DONOR CELLS.** As further controls for this particular experiment, two unilaterally nephrectomized B6AF<sub>1</sub> mice received injections of BCG and of CY 20 days later in the standard doses described. 2 and 3 days after this they received intraperitoneal injections of  $1.5 \times 10^8$  B10.D2 lymphoid cells. Their BUN levels remained normal throughout this period and for at least 30 days thereafter.

Six B6AF<sub>1</sub> recipients of B10.D2 kidney transplants received the "triple treatment" of BCG, CY, and donor strain lymphoid cells according to the schedule described above. By the 6<sup>th</sup> day after the first injection of B10.D2 cells, the BUN levels of all animals had begun to rise sharply. All transplants went on to complete cessation of function and their recipients were sacrificed as they became moribund (see Fig. 8).

At sacrifice the spleens of three of these mice were removed and were tested *in vitro* for cell-mediated cytotoxicity to donor antigens by evaluating their ability to cause release of label from <sup>51</sup>Cr-tagged P815-X2 mastocytoma cells. As can be seen in Fig. 9, active killing of target cells was demonstrable in all cases even at low lymphocyte to target cell ratios. Such killing is similar to that produced by cells from mice which have recently rejected skin grafts, but has never been observed with cells from long-term kidney transplant survivors (1).

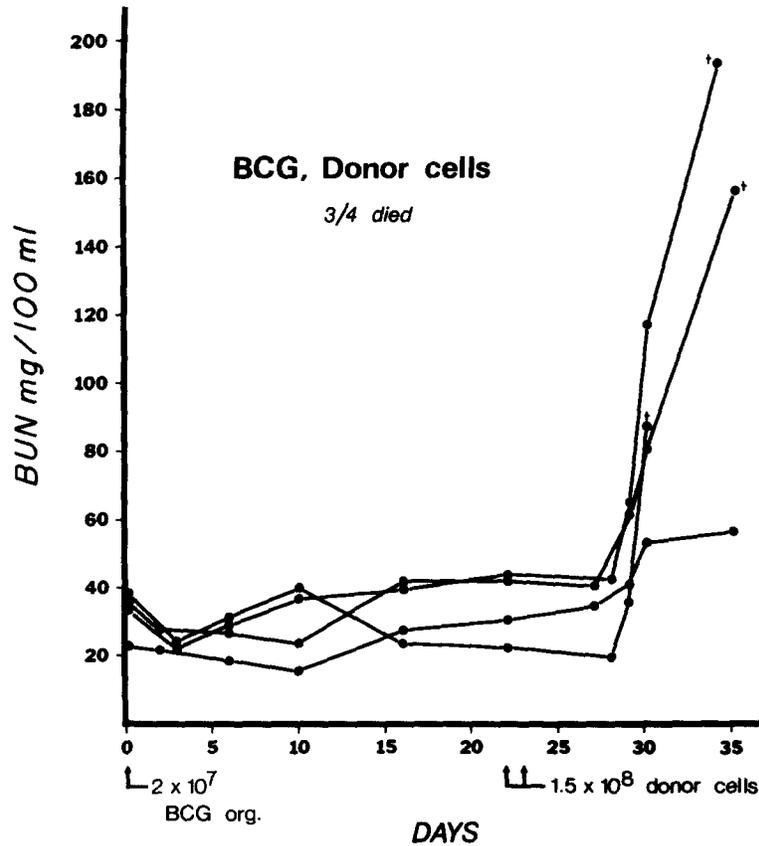


FIG. 7. Treatment of four B6AF<sub>1</sub> recipients of B10.D2 kidneys with BCG followed 22 and 23 days later with two intraperitoneal injections of  $1.5 \times 10^8$  donor strain lymphoid cells. Evidence of transplant damage was apparent by 7 days in all animals. In one of these, the damage failed to go to completion and the animal survived.

No cytotoxic antibody was detected in serum from any of these three animals at the time they were sacrificed.

To determine whether or not the allogeneic cell inoculum must share foreign specificities with the kidney transplant to induce a damaging effect against it, two B6AF<sub>1</sub> mice were employed which had born normally functioning B10.D2 kidney transplants for 69 days or more. Each was treated with BCG and CY as above, but the cell infusions were derived from A.SW donors. Neither animal showed evidence of kidney damage or alteration in its state of apparent well being during a period of several weeks of observation after the completion of the treatment.

The "triple treatment" regimen was also applied to four B6AF<sub>1</sub> recipients of B10.BR kidney transplants. Once again, by the 7<sup>th</sup> day after the first donor strain cell injection, the BUN levels of all animals became distinctly elevated from previously normal levels and all animals became fatally uremic by the 9<sup>th</sup> day (see Fig. 10). A similar result was obtained with three B6AF<sub>1</sub> recipients of

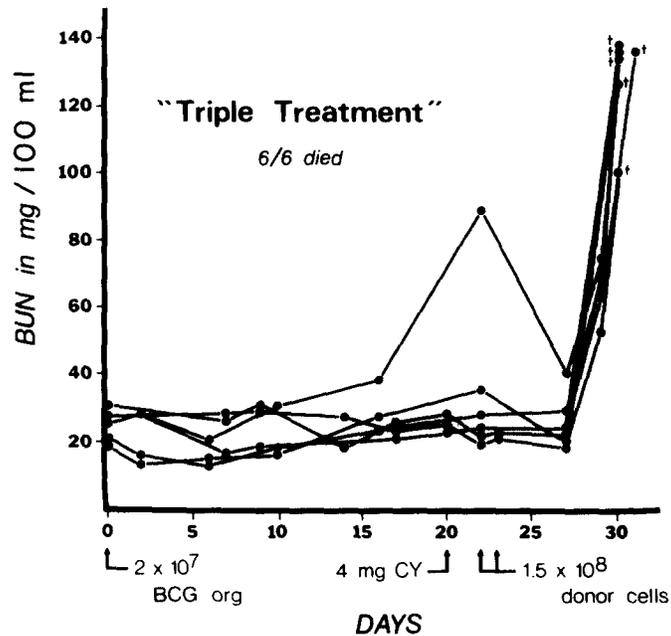


FIG. 8. Six B6AF<sub>1</sub> recipients of B10.D2 kidney transplants received BCG, CY, and donor cells in the "triple treatment" program as indicated. All manifested severe renal damage by the 7<sup>th</sup> or 8<sup>th</sup> day and either died or required sacrifice in a moribund state.

H(z1) kidney transplants. All mice became markedly uremic by the 6<sup>th</sup> day and all were sacrificed in a preterminal state by the 12<sup>th</sup> day after the receipt of donor strain cells.

Sections of fixed renal tissue were examined microscopically in a number of instances and the morphologic findings are summarized in Table I. Transplants from untreated recipients showed minimal focal mononuclear and plasma cell infiltrates that were localized principally around the vessels in about 5% of the cortex. Large arteries tended to show intimal thickening and mononuclear infiltration, and the glomeruli manifested moderately extensive focal and segmental scarring. No mononuclear cell accumulation was found on the endothelial surfaces. Kidney transplants from B10.BR and B10.D2 donors were examined microscopically 6-7 days after donor cells were given in the "triple treatment" regimen. In sharp contrast to the appearance of kidneys from untreated recipients, all of these showed an intense, diffuse infiltrate of mononuclear cells in the interstitium and focally within tubules (Fig. 11). The most striking change was the accumulation ("sticking") of mononuclear cells on arterial endothelial surfaces throughout the arterial system. These cells resembled lymphoblasts with basophilic cytoplasm and occasional nucleoli. Cells with indented nuclei, consistent in appearance with monocytes, were also present as well as occasional neutrophils. Many arterial lumina contained a loose meshwork of pale eosinophilic fibrillar material. The endothelial cells were occasionally basophilic; sometimes no endothelial nuclei were seen in an entire vessel cross section, indicating endothelial loss. However, necrotic endothelial cells or

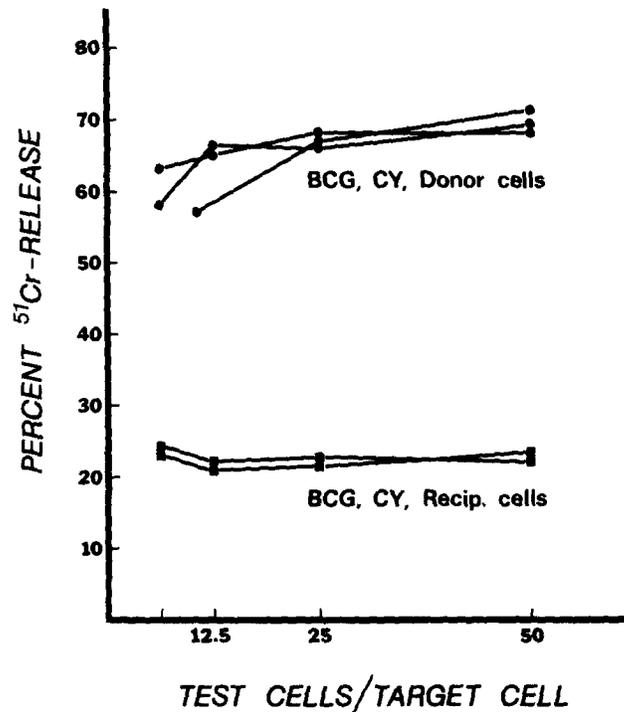


FIG. 9. B6AF<sub>1</sub> recipients of B10.D2 kidneys were treated with BCG, CY, and donor or recipient strain cells as described in the text. Spleens from these animals were removed 8 days after the second cell injection and the splenic cells were mixed in increasing numbers with <sup>51</sup>Cr-labeled P815-X2 cells. The supernates were harvested after a 16-h incubation period. Spontaneous release = 19.5%. Treatment with donor strain cells resulted in the induction of high levels of cell-mediated cytotoxicity which were not seen with recipient strain cells.

frank thrombosis were not identified. Glomeruli showed segmental damage with loss of architecture which was not significantly more severe than that seen in untreated animals. Allografts from animals which had received BCG and cells alone resembled in all respects those that received CY in addition.

#### Discussion

These experiments shed some additional light on the nature of the equilibrium which is arrived at spontaneously between graft and host in the strain combinations studied. The fact that donor antigens remain on display on the surfaces of donor cells is affirmed by their vulnerability to the effects of passively conferred immunity, either humoral or cell mediated. Although we have no information regarding possible changes in the density of such cell surface antigens, they appear to persist widely throughout the parenchyma of the kidney. In particular, the intense and acute reaction which occurs against the endothelium of vessels in transplants after treatment of their recipients with BCG, CY, and donor strain lymphoid cells is evidence against the notion that graft endothelium is replaced by recipient cells with a period of 1 or 2 mo.

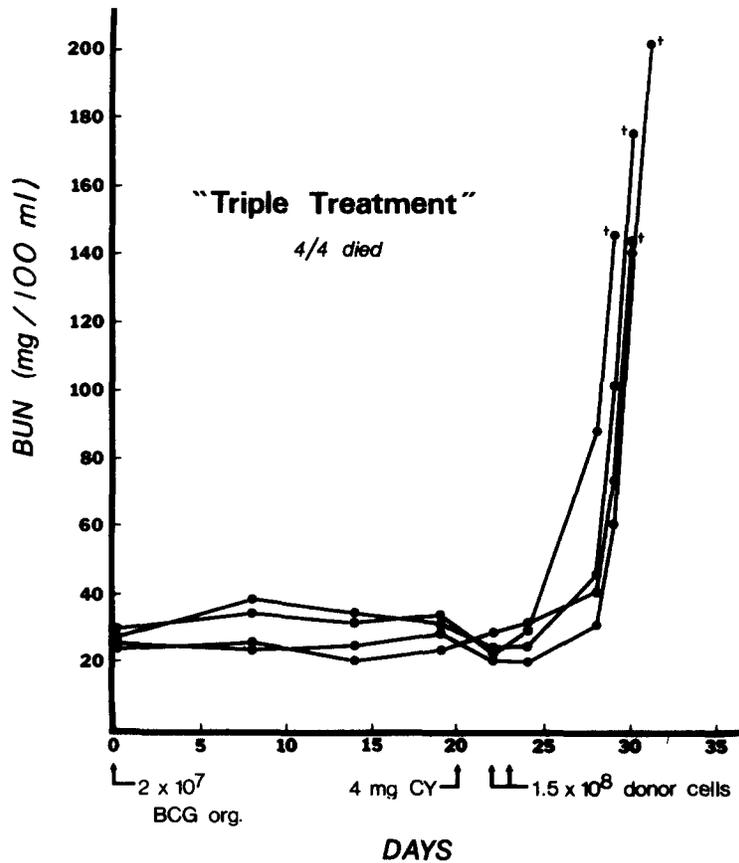


FIG. 10. Long-term B6AF<sub>1</sub> recipients of B10.BR kidneys in which relatively little histoincompatibility is involved underwent the "triple treatment" regimen outlined in the text. Without fail all showed prompt evidence of rejection which rapidly destroyed all the transplants. Primary kidney transplants in this strain combination are rarely rejected (see text and reference 15).

TABLE I  
Morphologic Features of Rejection in Kidney Transplant Recipients after Receiving BCG, CY, and Donor Cells\*

Kidney and cell donor strain	Treatment	Number of animals	Allograft age	Days after donor cells	Infiltrate		Glomerular damage
					Intersti-tial	Endo-thelial	
			<i>days</i>				
B10.D2	None	12	39-181	-	0/+	0	+ / + + +
B10.D2	BCG/CY/Cells	3	132-347	7	+++	+++	++
B10.D2	BCG/Cells	2	76-182	11-12	+++	+++	+++
B10.BR	BCG/CY/Cells	3	61-72	6-7	+++	+++	+++

The findings are described in detail in the text.

\* The extent and intensity of the changes were scored qualitatively from 0 to + + + +.

The fact that no apparent damage to transplants was inflicted by the infusion of antibody specifically directed against graft antigens without the further addition of rabbit C probably attests to the relative inefficiency of mouse complement in such reactions. A similar result has been reported by Koene et al., who found that skin allotransplants between mice of the same strains as those used in many of our experiments (B10.D2 to B6AF<sub>1</sub>) will be destroyed acutely only if infusions of antibody to the donor strain are supplemented with rabbit complement (8). Another similar result was reported by French who found that acute destruction of rat kidney transplants, surviving as a consequence of infused antibody directed toward donor antigens (immunological enhancement), could be induced by the administration of an effective complement in the form of guinea pig serum (9). It is not clear exactly why the effects which are noted on the function of our transplanted mouse kidneys after antibody and C treatment do not generally result in irreversible changes. The most likely explanation can probably be found, however, in the availability of sufficient quantities of all of the components required for the full expression of an inflammatory reaction rather than any lack of susceptibility of transplanted kidneys to such injury. Previous studies performed in our Unit of the damaging effects of infused antibody on surviving skin grafts established that although the ready availability of polymorphonuclear leukocytes and complement are important to the onward progress of antibody mediated destruction, other factors can participate in the process (10), W. Soper and H. J. Winn, personal communication.

The donation to transplant recipients of large numbers of lymphoid cells from mice highly immunized to the kidney transplant antigens was demonstrated to produce a definite impairment in renal function but only transiently. It was somewhat surprising that no definite increase in the destructive potential of cells transferred from preimmunized donors was seen with increasing doses. This result may be similar to those reported by Batchelor and Welsh and Bowen et al. (11, 12). They found that rats, which can be made to accept transplanted kidneys for long periods by early treatment with antibody against donor strain antigens, will enter a "steady state" during which no further treatment is required for the continuing survival of the transplants. The transfer of large numbers of specifically immunized lymphoid cells to these survivors, or even the parabiosis of such recipients with syngeneic animals immunized to the kidney transplant antigens, failed to bring about rejection of the transplanted tissue. In our system the available evidence suggests that an important point at which interference with the normal evolution of the immune response to transplanted kidneys occurs is in the generation of killer T cells (1). It may be that even in cell populations from preimmunized donors, the number of active killer cells is relatively small and that the recruitment of additional killer cells is inhibited under the conditions prevailing in long-term transplant recipients.

The above considerations make it particularly striking that the cell-mediated immune processes of the host can be called into such vigorous action by treatment with donor antigens reinforced by BCG, CY, or both. These treatments can result in the complete rejection of a transplant which the host is incapable of rejecting either through its own active processes or even after

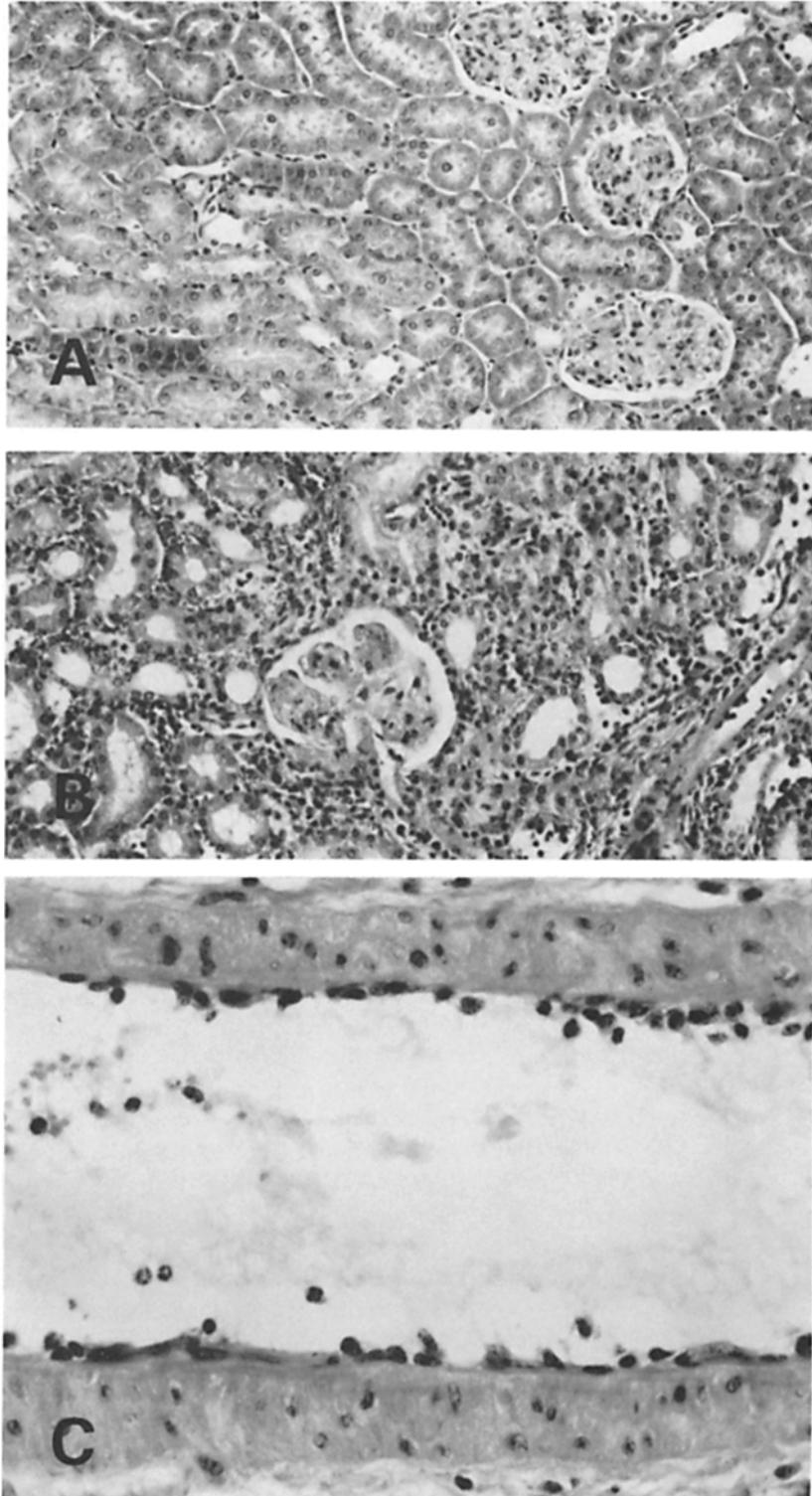


FIG. 11

receiving large numbers of cells from specifically immunized donors. Evidence that these regimens result in the generation of a vigorous cellular response lies both in the rapid appearance of killer cells in recipient spleens and lymph nodes and in the rich cellular infiltrate which is found in the transplanted kidneys as they cease to function. Under the conditions tested so far, an indispensable part of the treatment regimen is the inclusion of a disseminated wave of donor specific antigens. Although this stimulus, on its own, will result in a readily detectable immune response to the transplant, the response does not cause the complete rejection of any transplants and loses its force in a few days. As reported previously (13), the application of a donor strain skin graft to a surviving kidney transplant recipient results in no discernible reaction against the transplanted kidney. Indeed, donor strain skin grafts in this system survive for prolonged periods and their rejection, when it occurs, is very gradual. Nevertheless, the vigor of the acute rejection which can be called forth especially by the "triple treatment" regimen, in which all surviving transplants are completely destroyed in 7-8 days, is similar in tempo to an accelerated reaction which would ordinarily occur only in the presence of pre-existing immunity. Thus, even though kidney transplant recipients have been shown to be quite deficient in "memory cells" by *in vitro* tests, by the time they have traversed the three and one half weeks required for the "triple treatment" program they behave as though preimmunized. Even though the administration of donor cells appears to be essential to the increase in the immune response which we have observed, supplementary treatment with CY and BCG has been required to provoke rejection in all transplants. It is impressive that this treatment is also effective even in the presence of the weaker immunogenetic disparity represented by B10.BR transplants to B6AF<sub>1</sub> recipients.

The mechanisms to be considered as possible contributors to this sharp change in the balance between transplant and host could lie in several areas, and each agent in the treatment program could play one or more roles. Among the factors to be considered are: (a) influences on suppressor cells, (b) the restoration of responsive cells to a population made deficient in them through clonal deletion or inactivation, or (c) effects on serum factors inhibitory to the evolution of a fully effective state of cell mediated immunity. Our experimental observations are entirely consistent with those of Mackaness and Lagrange (8) who designed the BCG and CY treatment regimen we have used. To restore delayed hypersensitivity reactions to their animals additional treatment with antigen was required just as we find in our system. The precise requirements for the antigenic stimulus and timing of delivery for the components of the

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FIG. 11. Demonstration of morphological changes in transplanted kidneys after "triple treatment". (A) An allograft from an untreated mouse 134 days after transplantation. Little cellular infiltrate is present (no. 535,  $\times$  190). (B) An allograft 132 days after transplantation. The mouse received BCG, cyclophosphamide, and donor cells. The last dose of cells was given 7 days before sacrifice. The kidney shows an intense, widespread interstitial mononuclear infiltrate and tubular damage. The segmental obliteration of glomerular architecture illustrated was also found in the controls (no. 571,  $\times$  190). (C) Longitudinal cross section of an artery in the allograft in Fig. 11B. Numerous cells, largely mononuclear, have accumulated along the endothelial surface. Focal loss of endothelium has occurred (no. 571,  $\times$  430). All sections are from B10.D2 to B6AF<sub>1</sub> transplants.

treatment have not been established in kidney transplant recipients. Mackaness and Lagrange postulated that the influence of CY in their system was attributable to its selective inactivation of B cells which thus resulted in a reduction in antibody levels and consequently in antibody feedback inhibition of T-cell activity. BCG was believed to interfere with "serum blocking factors".

Only a preliminary consideration of the various mechanisms is possible from the experiments reported in this communication and the accompanying paper (1). We have demonstrated that our animals do not manifest marked T-cell unresponsiveness to donor antigens, i.e., they are not tolerant in the conventional sense, and no direct evidence to support the presence of suppressor cells has been uncovered. Thus, however plausible it might be, we cannot attribute the action of CY to a depletion of suppressor T cells as it is reportedly capable of producing in some systems (14). Humoral antibody with complement-dependent cytotoxic activity for donor cells can be detected in low titers, especially in the early weeks after transplantation, in some B6AF<sub>1</sub> recipients of B10.D2 kidneys, but current evidence makes it unlikely that suppression of T-cell function by antibody directed to H-2 determined histocompatibility antigens could be a full explanation for the long-term survival of these transplants. This evidence includes the fact that kidneys transplanted from H(z1) donors to B6AF<sub>1</sub> recipients survive very well even though no humoral antibody to donor cells in this strain combination can be produced by a variety of methods. Nevertheless, there is evidence for the presence of a substance in the sera of long-term recipients which inhibits the ability of nonimmune cells to differentiate into effector cells (1). We have not yet made efforts to determine whether this substance is eliminated by the "triple treatment" regimen, but it would seem logical to expect that this might be at least one of its results.

Whatever the mechanisms may be for the generation of this marked immunological reaction, they do not appear to result in potentiation of the humoral arm of the response since no evidence of cytotoxic antibody production could be detected after the "triple treatment". These results serve to emphasize the complexity of the immune relationship which can exist between a vascularized mass of cells bearing foreign surface antigens and its host. That this immune balance can be so decisively upset should stand as a point of caution in clinical organ transplantation but perhaps as a source of encouragement to oncologists.

### Summary

Various modes for producing the specific immune destruction of surviving kidney transplants between mice were tested in these experiments. Kidney transplants among mice which were incompatible at the *H-2* locus but which were surviving in excellent condition for several weeks without immunosuppression were utilized as subjects for these experiments. When immune damage to these surviving organs resulted from the treatments being tested it was readily detectable by changes in the blood urea nitrogen levels of their recipients. The treatments included means of heightening immune reactivity to donor antigens passively by the transfer of either specifically activated cells, immune serum, or by the active generation of increased responsiveness.

Infusions of an antiserum specifically reactive with the histocompatibility

antigens of the transplant were ineffective in causing damage to the kidney unless exogenous complement, in the form of rabbit serum absorbed with mouse tissue, was also given. The injection of lymph node and spleen cells from recipient strain mice which had been highly immunized to donor antigens caused definite but transient damage to transplanted kidneys.

Stimulation of recipient responsiveness by additional donor tissue antigens presented as skin grafts was ineffective although intravenous injections of lymphoid cells provoked an evanescent reaction. Combining donor strain lymphoid cell treatment with a prior injection of cyclophosphamide (4 mg, 2 days before cell injection) and/or treatment with Bacille of Calmette and Guérin ( $2 \times 10^7$  organisms, 22 days before cell injection) caused an intense and specific immune response to donor antigens with rapid onset of transplant damage. The maximal effect observed followed the combined use of all three agents in which case every transplant was fully rejected by 7 or 8 days after donor cell injection.

These results demonstrate that an otherwise stable balance between an incompatible transplanted organ and its host can be decisively upset by treatments which provoke a heightened, specific immune response. The conferral of immunity passively by transferring either serum or lymphoid cells from sensitized donors was much less successful in causing damage to transplants. This information must be interpreted in the light of the evidence we have presented in an accompanying paper (1) that the maturation of fully competent killer T cells is retarded in long-term kidney transplant recipients and that no direct evidence of suppressor cell activity can be found in them. The fact that only transient damage to transplants occurred even after the transfer of large numbers of immunized lymphoid cells, however, has made us refrain from dismissing altogether the participation of an active suppressor mechanism.

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