The Effects of Antigen Overloading on Survival of Renal Allografts.* (31640)

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Pretreatment of adult recipients with antigenic materials from the donor has been shown in some situations to induce partial or complete specific allograft tolerance(1,2). Viable cells(3), as well as homogenates and cell fractions(4,5) have been successfully employed to this effect. Multiple injections by the intravenous route have been found to be most effective, but even these regimens often produce transient sensitization prior to induction of tolerance(6). In some studies effective antigenicity was found to be increased by extraction, purification and solubilization of the antigen from cells,(5,7,8). This may, however, be accomplished at the expense of the permanence of the tolerance induced, since chimerization is prevented and with it antigenic persistence.

Administration of antimetabolites concurrently with the antigen has also been used in attempts to increase dose effectiveness. Tolerance has thus been produced both to protein antigens(9) and to allogeneic donors(10,11, 12). However others, using donor cell and 6-MP administration, could produce no prolonged homograft survivals(13).

In the larger, non-inbred animals in whom large doses of antigen can be expected to be required for the induction of tolerance, the practically unlimited strain-specific antigen source available in inbred mice does not exist. Individual-specific antigen from the donor, expendable amounts of which are limited, has to be employed for pretreatment. In an attempt to induce tolerance in the face of such limited amounts of available (expendable) donor antigen, in the current studies a new approach was added to antigen extraction and antimetabolite potentiation. Using an hyperbaric organ culture technique the actual amount of usable donor antigenic material was increased(14,15).

Almost all studies of antigen-induced tolerance have been performed with skin serving as the test graft. The latter may well be an unduly severe test for the evaluation of tolerance, since parenchymatous organs have been found to be more easily protected from rejection than have skin allografts(16). Significant differences in the behavior of skin and kidney allografts in antigen-pretreated dogs have also been described(17). Because of these two considerations kidneys were used as the test grafts throughout these studies.

Methods. Animals: Cross matched, A-compatible mongrel dogs weighing 14-18 kg were used. The animals were quarantined for 3 weeks and de-wormed prior to use. They were housed in individual cages in air-conditioned quarters. All operations were performed aseptically, under pentobarbital anesthesia.

Preparation of antigens: After surgical exposure of the splenic pedicle the main arteries supplying the spleen were ligated proximally and injected with 1 mg of epinephrine in 10 cc of saline. The spleen, now contracted and emptied of pooled blood, was rapidly removed and immersed into a glucose-containing balanced salt solution (BSS) (provided by Travenol Laboratories as Tis-U-Sol) at 4°C. All further procedures were carried out at this temperature. The spleen was allowed to cool for 5 minutes during which time further contraction occurred and more blood was expelled. The spleen then was weighed. When primary antigen extraction was to be performed the spleen was incised along one end and its cellular contents removed by gently stripping them out through the incised end with a scalpel handle. A thick cell suspension was thus obtained, leaving capsule and fibrous stroma behind. The suspension was put

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through a 40-mesh screen into 100 cc of BSS (approximately 50 cc/10 g spleen). After centrifugation at 1,500 × *g* for 10 minutes the supernatant was discarded.

Saline method:(5) The sediment was broken up by sieving and resuspended in a mixture of 50 cc BSS and 50 cc distilled water, in order to lyse remaining erythrocytes. After centrifugation for 10 minutes at 4,500 × *g* the sediment was once again sieved, and resuspended in 100 cc distilled water. Recentrifugation yielded a faintly pink gelatinous mass as the sediment. This was made up to 100 cc with distilled water and homogenized in a mechanical blender first at 6,000 rpm for 5 minutes, then at 12,000 rpm for 3 minutes. The ionic strength was raised to 0.15 M by addition of 2 M NaCl, and the brei homogenized at 6,000 rpm for one more minute. It was then centrifuged at 4,500 × *g* for 10 minutes. The sediment (consisting of subcellular debris and no intact cells) was discarded. The supernatant ("crude semi-soluble antigen" of Medawar) was slowly titrated at room temperature to pH 7.6 with 0.1 M NaOH during vigorous mechanical stirring.

Sucrose method:(8) The spleen cell suspension was sieved into 100 cc 0.25 M sucrose solution containing 0.025 M KCl, and 0.005 M $MgCl_2$ and buffered to a pH of 7.5 with tromethamine. The suspension was homogenized in the fashion described under the saline method. The resulting brei was centrifuged for 10 minutes at 1,500 × *g* and the supernatant ("cytoplasmic fraction" of Monaco *et al*) used for injection.

When cultured cells were used for preparation of antigenic fractions the above techniques were applied to the harvested cells. At the end of the culture period the spleen slices were put through a wire mesh and similarly extracted. Antigenic extracts were stored at −20°C until use.

Culture methods: After removal, cooling and weighing the spleens were cut transversely into 3 mm slices. These were placed on stainless steel screens and incubated on the surface of Eagle's medium‡ (10 cc media/g of tissue) containing glutamine, penicillin, streptomycin and 15% fetal calf serum. One-half cc of phytohemagglutinin P was added to each 100 cc. The culture dishes were pressurized in cast aluminum chambers to 45 p.s.i.g. with 98% oxygen and 2% CO_2 at 34 to 36°C. The cell yield was harvested every 48 hours from the surface of the slices, the media and the bottoms of the culture dishes. The slices were turned and the media changed at each harvesting. Cultures were carried for 10 to 12 days.

Cell counts were performed using a Coulter counter after trypsinizing of spleen cell suspensions and culture yields for 5 minutes. Cell viability studies utilized Hanks' eosin exclusion method(18). Differential counts were performed using Wright's stain.

Kidney transplants were performed after flushing the kidney with 50 cc cold heparinized BSS. The renal artery was anastomosed end-to-end to the external iliac artery, the renal vein end-to-side to the common iliac vein. The ureter was implanted into the bladder through a submucosal tunnel. Both of the recipient's kidneys were removed at the same operation. Postoperatively urine specimens were collected daily; determinations of blood urea nitrogen and of endogenous creatinine clearance were performed twice a week. The length of survival of the recipient animal was accepted as the most accurately measurable criterion of the length of functioning survival of the renal allograft. All animals dying after transplantation were autopsied. Histologic studies were performed on all transplanted kidneys, and the recipient's spleen and representative lymph nodes.

Results. Controls: Eleven dogs bearing control allografts without pretreatment survived 8.4 ± 1.5 days (range 6 to 11). The kidneys of these dogs were congested and hemorrhagic, showing predominantly cortical perivascular round cell infiltration and necrosis.

Donor-spleen pretreatment: Fifteen atropinized dogs were injected over a 90-minute period with 2.4 to 5.3 × 10^9 living donor spleen cells suspended in BSS. Two dogs died. Six dogs received kidney allografts from the donor of the spleen one week after infusion.

‡ Provided by Hyland Laboratories, Glendale, Calif.

TABLE I. Effects of Donor Spleen Cell Injection on Survival of Subsequent Renal Allografts.

	# Dogs	MST*	S.D.	Range (days)
Control	11	8.4	1.5	6 to 11
Fresh spleen cells 2.4-5.3 $\times$ 10^9 I.V.				
KT† on day 7	6	6.5	1.5	5 to 9
KT on day 21-24	7	9.1	1.8	6 to 12
Cultured spleen cells 0.7-6.5 $\times$ 10^{11} over 10 days				
KT on day 6-8	9	8.2	2.2	6 to 12
KT on day 27-30	8	10.1	2.0	5 to 13
6-MP and cultured spleen cells 0.9-7.9 $\times$ 10^{11} over 10 days				
KT on day 9-12	6	8.3	2.4	7 to 12
KT on day 22-26	7	12.2	1.2	10 to 16

* Mean survival time (of recipient) in days.
† Kidney transplant.

Their survival averaged 6.5 $\pm$ 1.5 days (range 5 to 9), significantly below that of the control group. The kidneys were pale, less swollen, and showed only moderate hemorrhage and cellular infiltration in comparison to the control group. No clear-cut vascular damage was evident. This picture is compatible with the behavior of a second set graft. The other 7 dogs received kidney transplants 21 to 24 days after the splen injection. These animals survived 9.1 $\pm$ 1.8 days (range 6 to 12). Their kidneys were comparable to those of the control group. Thus definite sensitization was seen in dogs which received all the living cells from the donor's spleen in a single I.V. injection. The sensitization appeared to be shortlived; by the end of 3 weeks it was no longer demonstrable (Table I).

Cultured cell pretreatment: The cell yield of the 20 to 25 g spleens stimulated with phytohemagglutinin P and cultured in hyperbaric oxygen averaged 3.7 $\times$ 10^{11} cells (range 7.1 $\times$ 10^{10} to 6.5 $\times$ 10^{11}) over the 10 to 12 day culture periods utilized, with 84% overall viability. Seventy-five to 80% of these cells were of the lymphocytic-mononuclear series. Half of the remainder were other leukocytes (with a few myelocytes and blast forms) and half appeared to be reticuloendothelial cells.

The cultured cells were injected immediately upon harvesting, thus extending the exposure to repeated doses of viable cells over 10 to 12 day periods. Seventeen dogs were pretreated with cultured donor spleen cells in this fashion. All survived pretreatment. Results are shown in Table I. Nine dogs received kidney transplants 6 to 8 days after the last donor cell injection and survived 8.2 $\pm$ 2.2 days (range 6 to 12). Eight dogs transplanted 27 to 30 days after the end of spleen cell injections survived 10.1 $\pm$ 2.0 days (range 5 to 13). These results indicate that a major increase in the number of donor cells administered prevents the early sensitization brought about by the lower doses of fresh cells obtained from single spleens ($p<0.05$).

To rule out qualitative or quantitative changes in the antigenicity of the cultured cells (see also 15) 6 dogs were injected with 4 $\times$ 10^9 cultured cells (a dose comparable to that obtained from a single spleen) and grafted 7 to 8 days later. These animals survived 5.8 $\pm$ 1.5 days (range 4 to 8) showing sensitization comparable to that produced by fresh spleen cells. To evaluate the effect of multiple injections, 6 dogs were injected 5 times (over a 10-day period), with a total of 4 $\times$ 10^9 cultured donor-spleen cells. These animals also received renal allografts 7 to 9 days after the last spleen cell injection. They survived 6.1 $\pm$ 1.5 days range 4 to 8). Thus it would appear that early sensitization consistently results from administration of donor spleen cells in the lower dosage range, be they fresh or cultured cells, and administered in a single injection or over a prolonged period.

Cultured cells and 6-MP. Fifteen dogs received 5 mg/kg of 6-MP (kindly provided by Dr. George Hitchings, Burroughs Wellcome & Co.) daily for a 14-day period. On

days 1, 3, 5, 7, 9 (and 11 in some) cultured donor spleen cells were infused intravenously to an average total dose of 5.1 $\times$ 10^{11} cells (range 9.3 $\times$ 10^{10} to 7.9 $\times$ 10^{11}). Two dogs died of hemorrhagic pneumonitis during pretreatment. Results are shown in Table I. Six animals received a kidney allograft from the spleen donor on day 20 or 21 (*i.e.*, 9 to 12 days after the last spleen cell dose and 6 to 7 days after discontinuing 6-MP). These dogs survived 8.3 $\pm$ 2.4 days (range 7 to 12). Seven animals were transplanted on days 33 to 35 (*i.e.*, 22 to 26 days after the last spleen injection), and had a mean survival of 12.2 $\pm$ 1.2 days (range 10 to 16). Thus incorporation of 6-MP into the pretreatment regimen using high doses of cultured donor spleen cells had no major effect on early transplants. On the other hand, late allograft survival was significantly prolonged over control values ($p<0.001$).

Antigenic extract. Attention was next turned to the use of antigenic extracts, using pretreatment with both early and late grafts. Extracts made by both methods outlined were administered to 25 dogs I.V., one-fifth of each lot at a time, without ill effects. Results are shown in Table II. Using both the saline and sucrose extracts, early transplants (9 to 11 days after termination of antigen injections) uniformly showed accelerated rejection, with survivals being 6.2 $\pm$ 1.6 and 6.0 $\pm$ 1.7 days respectively. Late transplants (after 24 to 26-day delays) survived, much as did un-pretreated recipients, 9.2 $\pm$ 2.0 days after saline extracts and 9.8 $\pm$ 1.6 days after sucrose extracts. Thus the pattern of behavior was comparable to that seen after fresh spleen cell injections, containing comparable amounts of antigen.

TABLE II. Effects of Donor Splenic Antigen Injections on Renal Allograft Survival.

	Survival:
Saline extract I.V. on days 1, 3, 5, 7 & 9	
Transplant on days 18-20 (6 dogs)	6.2 $\pm$ 1.6 (range 5-9)
Transplant on days 33-35 (6 dogs)	9.2 $\pm$ 2.0 (range 7-13)
Sucrose extract I.V. on days 1, 3, 5, 7 & 9	
Transplant on days 18-20 (7 dogs)	6.0 $\pm$ 1.7 (range 4-8)
Transplant on days 33-35 (6 dogs)	9.8 $\pm$ 1.6 (range 7-12)

TABLE III. Effects of Donor Antigen—6-MP Pretreatment on Renal Allograft Survival.

	Survival:
Control:	
Non-donor spleen extract I.V. on days 1, 3, 5, 7 & 9; 6-MP on days 1-14	
Transplant on days 33-36:	
Saline extract (6 dogs)	8.3 $\pm$ 1.9
Sucrose extract (5 dogs)	8.0 $\pm$ 1.6
Experimental:	
Saline extract I.V. on days 1, 3, 5, 7 & 9; 6-MP on days 1-14	
Transplant on days 33-35 (7 dogs)	14.1 $\pm$ 2.6 (range 11-20)
Sucrose extract I.V. on days 1, 3, 5, 7 & 9; 6-MP on days 1-14	
Transplant on days 33-36 (6 dogs)	15.0 $\pm$ 2.7 (range 12-19)

Because of the pattern of transient early sensitization only late transplants were studied, when 6-MP pretreatment was combined with the administration of antigenic extracts. The results are shown in Table III. Control studies using pretreatment with non-donor splenic antigen and 6-MP showed the usual first set survivals in the recipients. Using donor spleen extracts and 6-MP after saline extracts survival was 14.1 $\pm$ 2.6 days, after sucrose extracts 15.0 $\pm$ 2.7 days. These differences are statistically significant, $p<0.001$.

In order better to evaluate the findings of prolonged survival in the antigen-6-MP group the permissive effect of post-transplantation steroid administration was utilized. Prednisolone was given in the manner of Zukoski *et al*(19), *i.e.*, 1 mg/lb/day p.o. or s.c., beginning 2 days before transplantation. Results are shown in Table IV. The administration of prednisolone alone led to survivals in allograft bearing dogs of 14.7 $\pm$ 2.4 days. Prednisolone combined with non-donor spleen extract and 6-MP pretreatment produced no different survivals: 16.6 $\pm$ 2.8 days after saline extract, 15.1 $\pm$ 2.7 days after sucrose extract. Turn-

TABLE IV. Effects of Antigen—6-MP—Prednisolone Pretreatment on Renal Allograft Survival.

	Survival:
Controls:	
Prednisolone (1 mg/lb/day) starting 2 days before transplantation (6 dogs)	14.7 ± 2.4 (range 11-18)
Non-donor spleen extract I.V. on days 1, 2, 5, 7 & 9; 6-MP, 5 mg/kg, days 1-14	
Prednisolone (as above):	
Saline extract (5 dogs)	16.6 ± 2.8 (range 13-20)
Sucrose extract (5 dogs)	15.1 ± 2.7 (range 13-19)
Experimental:	
Donor spleen extract I.V. on days 1, 3, 5, 7 & 9; 6-MP days 1-14	
Prednisolone (as above):	
Saline extract (7 dogs)	25.9 ± 8.5 (range 13-38)
Sucrose extract (6 dogs)	31.2 ± 10.5 (range 14-41)
Significance of the difference between control and experimental group:	
Saline extract	$.01 > p > .005$
Sucrose extract	$.01 > p > .005$

ing to the use of donor antigen in this system, saline extracts led to a mean survival of 25.9 ± 8.5 days (range 13 to 38), sucrose extracts to 31.2 ± 10.5 days (range 14 to 41). The differences between control and experimental groups are again significant ($p<0.01$), although the use of prednisolone did not increase the level of significance.

Discussion. Induction of specific allograft tolerance in adult animals of widely different genetic backgrounds (disparate at H-2 loci) has been demonstrated in mice, using skin as a test graft(3,4). Transient sensitization precedes the appearance of tolerance whether single or multiple doses of donor cells or antigen are employed. Whole organ allografts, perhaps more easily tolerated(16), therefore *a priori* more promising, have not been studied in such a system. The ideal sequence of studies would progress from skin allografts, through the exchange of organ allografts between animals with known differences in transplantation antigens, to the use of such grafts in outbred populations. Since technical considerations (size) make the second phase of this progression impractical outbred populations were studied, accepting the limitations and complexities involved.

I.V. administration of single spleen-equivalents of fresh or cultured cells consistently lead to early sensitization. Administration of this dose over a 10-day period did not alter the response. The transient nature of this sensitization is of interest, although perhaps not surprising, in view of the intravenous route of administration(20). Whether the "normal" length of graft survival at 3 weeks repersents low-grade tolerance or simply a reestablished first-set response is unclear, and histologic studies do not help in this differentiation.

Using multiple spleen equivalents of cultured cells early sensitization was abolished. When concurrent 6-MP pretreatment was added, slight but significant prolongation of late graft survival was noted. Finally, using antigenic extracts and 6-MP pretreatment in combination, consistent and highly significant prolongation was produced. This dose-related phenomenon, following pretreatment, potentiated by 6-MP has all the earmarks of partially acquired tolerance. Its specificity is well demonstrated; this feature eliminates all but immunologic enhancement as possible other explanations of the mechanism responsible. Enhancement, unfortunately, cannot be entirely ruled out, because the required serologic studies of transplantation immunity are unreliable and unsuitable in dogs. However, the dose-relatedness, which is the reverse of that seen in enhancement, and the unquestionable potentiation by 6-MP (which affects enhancement adversely) are strong arguments against any role enhancement might play.

The effectiveness of 6-MP pretreatment also gives further support to the contention of Schwartz and Dameshek(9) that not only can sensitization be prevented, but tolerance can be induced by simultaneous administration of antigen and 6-MP. This is contrary to the experience of Goh *et al*(21) using protein antigens, and of Mannick *et al*(13) using donor lymphocytes as pretreatment. The differences between the re-

sults of the latter study and ours are probably explained by the higher doses and the antigen extraction procedures employed in the present series.

Steroids were used in these studies to facilitate the effect of other measures aimed at prolonging allograft survival. This would appear to be a worthwhile method, when minor differences in prolongation need to be expanded for easier evaluation.

The survivals reported by Zukoski *et al* (22) for prednisolone-treated animals bearing kidney allografts are far longer than ours. The reasons for this difference are unclear.

Summary. Intravenous administration of the cells obtained from the future donor's spleen sensitized recipient animals to early but not to late kidney allografts. This early sensitization was prevented by giving increased numbers of spleen cells grown in hyperoxic organ culture. Using antigenic fractions extracted from donor spleen cells for pretreatment a measure of graft protection (prolongation) was produced. The survival was further increased by adding 6-MP to the pretreatment regimen. Because of specificity, direct dose relatedness and potentiation by 6-MP, it is thought that neither antigenic competition nor enhancement are involved, but that partial adult tolerance is the mechanism responsible for the delayed rejections seen in these studies.

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