

# TRANSFER OF SPECIFIC UNRESPONSIVENESS TO ORGAN ALLOGRAFTS BY THYMOCYTES

## Specific Unresponsiveness by Thymocyte Transfer\*

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Increased survival of vascularized organ allografts in the rat has been achieved by active host immunization with donor antigen, passive immunization with alloantisera, and a combination of both (1-5). The original concept, as suggested by Kaliss, that immunological enhancement is mediated by host humoral factors, is still widely held, although the exact mechanisms involved in its initiation and maintenance remain unresolved (6). However, passive transfer of serum from enhanced animals rarely increases survival of test allografts, despite detection of humoral blocking factors in some enhancement models (7). The contributions to allograft enhancement by the cellular-immune responses have not been rigorously assessed, although evidence for *in vivo* activity of cells capable of causing specific unresponsiveness has been found in other animal systems: for example, host responses against tumors may be influenced by cells or cellular products (8-10), which also act in delayed hypersensitivity (11-13) and graft-versus-host reactions (14-16). In studies of transplantation tolerance, induced neonatally, suppressor cells have been demonstrated primarily to be recirculating T cells which show specificity *in vivo* to donor strain tissues (17, 18).

We have shown previously that spleen cells and infiltrating leukocytes isolated from well-functioning cardiac allografts in enhanced adult rats suppress spontaneous blastogenesis of normal syngeneic cells *in vitro* (19). Additionally, preliminary observations *in vivo* have suggested that lymphoid cells from enhanced animals bearing a well-functioning organ allograft are able to transfer a state of specific unresponsiveness to unmodified recipients (20). The present studies demonstrate the precise activity of thymocytes in the early phase of immunological enhancement, confirm the specificity of this effect, and describe the necessity of an intact thymus for continued survival of the grafted organ in an enhanced host. Our observations suggest that the phenomenon of enhancement of organ allografts induced by particular immunizing protocols, involves cellular as well as humoral mechanisms.

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*Abbreviations used in this paper:* CDC, complement-dependent cytotoxicity; FCS, fetal calf serum; LMC, lymphocyte-mediated cytotoxicity; MST, mean survival time  $\pm$  SD; SIg<sup>+</sup>, surface-bound immunoglobulin.

### Materials and Methods

**Animals.** Inbred male rats weighing 150–200 g were used in all experiments (Microbiological Associates, Walkerville, Md.). Lewis rats (Lew, Ag-B<sup>1</sup>, RT1<sup>1</sup>) acted as organ recipients; (Lew × BN)F1 hybrids were used as heart donors. Brown Norway rats (BN, Ag-B<sup>4</sup>, RT1<sup>n</sup>) were employed as an antigen source for in vitro assays and for production of alloantiserum in Lew rats. Wistar Furth (WF, Ag-B<sup>4</sup>, RT1<sup>Y</sup>) spleen cells were used as antigen, and (Lew × BN)F1 hybrids as organ donors for specificity experiments.

**Heart Grafting.** Heterotopic cardiac grafts were transplanted to recipient abdominal vessels by using standard microvascular techniques. Ventricular activity was assessed by daily palpation through the flank. Organ rejection was taken as complete cessation of myocardial contractions.

**Thymectomy.** Under ether anesthesia, a midline cervico-thoracic incision was made and the sternum split in its upper one-third. The thymus was exposed, gently teased away from surrounding tissues, and removed intact. Parathymic lymph nodes were always excluded from thymic tissue.

**Cardiac Implants.** Through a midline incision both kidneys were exposed and the renal vasculature clamped, avoiding damage to the ureters. Small areas of kidney capsule were excised and pieces (≈0.5 g) of myocardium placed bilaterally in the defects. The clamps were then removed and hemostasis secured.

**Alloantiserum.** Hyperimmune Lew anti-BN serum was produced in a group of 50 Lew recipients of single orthotopic BN skin grafts, followed by eight biweekly intraperitoneal injections of 10<sup>8</sup> pooled BN spleen, thymus and lymph node, and bone marrow cells. 1 wk after the final injection, the animals were bled and the serum pooled and stored at –70°C. The antiserum was cytotoxic (50% lysis) against BN lymphocytes at 1:256 dilution.

Hyperimmune Lew anti-WF serum was obtained by skin grafting 10 Lew rats with WF skin followed by six biweekly intraperitoneal injections of 10<sup>7</sup>–10<sup>8</sup> pooled WF spleen, thymus, lymph node, and bone marrow cells. 2 wk after the final injection, the animals were bled, the serum pooled, and stored at –70°C. The antiserum was cytotoxic against WF lymphocytes at a 1:512 dilution.

**Cell Suspensions.** Single-cell suspensions were prepared from spleen, lymph nodes, or thymus by disrupting the organs in RPMI-1640 medium (Associated Biomedic Systems, Inc., Buffalo, N.Y.), filtering through cotton wool, and washing twice. Mononuclear leukocytes were harvested from heparinized peripheral blood after dilution three times in saline, and layering on Ficoll-Hypaque (sp gr 1.090, Pharmacia, Upsala, Sweden). Erythrocyte-free splenic leukocytes were obtained after brief treatment with hypotonic ammonium chloride and washing; the final preparation contained < 0.1% erythrocytes (21). Pure preparations of erythrocytes were obtained by layering heparinized whole blood over Ficoll-Hypaque and centrifuging at 500 g for 10 min. The erythrocyte pellet was washed twice in Eagle's minimal essential medium (Associated Biomedic Systems, Inc.). This preparation contained <0.5% leukocytes. All transferred cells were injected intravenously into recipient animals.

**Harvesting of Cellular Infiltrates.** Cardiac allografts were transplanted in groups of 8–10 animals. The procedures used in the collection of infiltrating cells are modifications of techniques previously described (22). At serial intervals after grafting, the hearts were removed and cleaned of surrounding tissue and clot. Each myocardium was then diced in 5 ml of tissue culture medium (RPMI-1640) containing 0.005 M Hepes (N 1 hydroxyethylpiperazine N-2 ethane sulfonic) acid buffer (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) and 4% (vol/vol) heat-inactivated fetal calf serum (FCS) (Associated Biomedic Systems) with 0.1 ml 0.2% EDTA (sodium ethylene diaminetetracetic acid, Sigma Chemical Co., St. Louis, Mo.). The tissue was then expressed through 60-gauge stainless steel mesh and filtered through cotton wool. The suspension of infiltrating cells was centrifuged twice at 200 g for 15 min, washed, and incubated for 20 min at 25°C, then 20 min at 37°C in 10 ml buffered RPMI-1640 containing 0.4 mg/ml deoxyribonuclease (DNAse, Pentex Biochemical, Kankakee, Ill.). The cell suspension was then washed, resuspended, and layered over Ficoll-Hypaque. After centrifugation at 350 g for 15 min, the cells were collected from the interface layer, washed in medium, and counted.

**Lymphocyte-Mediated Cytotoxicity (LMC)<sup>1</sup>.** LMC was determined by a modification of the

technique of Brunner et al. (23), as previously described (22). Infiltrating cells from cardiac allografts, and leukocytes from peripheral blood, lymph nodes, and spleen were used as the attacking cell populations; donor strain (BN) thymocytes labeled with  $^{51}\text{Cr}$  acted as target cells ( $\text{Na}_2^{51}\text{CrO}_4$ , New England Nuclear, Boston, Mass). Attacking to killer cells were assayed in a ratio of 100:1.  $^{51}\text{Cr}$ -labeled Lew thymocytes were used as controls. All LMC assays were incubated in microtiter plates (3040 Micro Test II, Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) for 4 h at 37°C. The percentage of specific cell lysis was calculated:

$$\text{percent} = \frac{\text{experimental cpm} - \text{control cpm}}{\text{freeze thaw cpm} - \text{control cpm}}$$

**Complement-Dependent Cytotoxicity (CDC).** Serial twofold dilutions of sera (25  $\mu\text{l}$ ) were incubated with  $^{51}\text{Cr}$ -labeled BN lymph node cells ( $1 \times 10^4$  in 25  $\mu\text{l}$ ) for 60 min. The cells were washed twice, 50  $\mu\text{l}$  of rabbit complement (1:10 dilution) (Pel-Freeze Biologicals, Inc., Rogers, Ark.) was added to the target cells and serum and incubated at 22°C for 90 min. After this incubation 150  $\mu\text{l}$  of medium was added and the microtiter plates were centrifuged at 200 g. The supernates were then counted for released  $^{51}\text{Cr}$ .

**Surface-Bound Immunoglobulin (SIg+) Lymphoid Cells.** Lymphocyte suspensions were incubated with fluorescein isothiocyanate conjugated rabbit anti-rat IgG (heavy and light chain) (N. L. Cappel Laboratories, Inc., Cochranville, Pa.). Cells with surface immunoglobulin were easily detected by fluorescence microscopy (24). By using this technique, thymocyte suspensions used for transfer were found to contain <1% SIg+ cells.

**Identification of T Lymphocytes.** Rabbit anti-rat brain antiserum was produced and rendered specific for T lymphocytes by the method of Golub (25). The heat-inactivated serum was absorbed with (Lew  $\times$  BN)F1 erythrocytes and lyophilized liver powder six times, ultracentrifuged and diluted 1:10 before indirect immunofluorescence cell staining. Antiserum (25  $\mu\text{l}$ ) was mixed with  $10^6$  rat lymphoid cells in 50  $\mu\text{l}$  medium and incubated for 30 min at 4°C. After washing the cells thrice in medium, the suspension was centrifuged for 10 min at 200 g. FITC conjugated goat anti-rabbit IgG (50  $\mu\text{l}$ ) (N. L. Cappel Laboratories, Inc.) was then mixed with the pellet, incubated for 30 min at 4°C and washed thrice with Hanks' medium containing 0.1% sodium azide. The cell suspension was then examined for immunofluorescence membrane cell staining.

**Identification of Mononuclear Cells Bearing an Fc Receptor.** A 2% solution of ox erythrocytes was incubated with a subagglutinating dilution of rabbit anti-bovine erythrocyte antibody (IgG fraction) (Baltimore Biological, Baltimore, Md.) for 90 min at 37°C (26). 10  $\mu\text{l}$  lymphocytes ( $5 \times 10^6/\text{ml}$ ) was added to 10  $\mu\text{l}$  of the IgG-coated bovine erythrocytes (2%) and 10  $\mu\text{l}$  FCS in a microtiter tray, and centrifuged for 15 min at 250 g at 4°C. After incubation at 4°C for 45 min, the percent of rosette-forming lymphocytes was determined by counting the cells under the fluorescence microscope with acridine orange stained cover slips.

**Trypsinization of Transferred Thymocytes.** Thymocytes were incubated in protein-free medium containing 10  $\mu\text{l}/\text{ml}$  of trypsin (Sigma Chemical Co.) for 30 min at 37°C. The cells were then washed three times with culture medium before transfer into syngeneic recipients of test allografts. Under these gentle conditions the majority of SIg is removed from spleen cells (E. Milford, unpublished observations).

**Statistics.** Statistical significance was determined by use of Student's two-tailed *t* test.

## Results

Previous experiments in this laboratory have established the optimal immunization regimen for enhancement of Lew recipients of (Lew  $\times$  BN)F1 cardiac allografts (5). Best graft survival occurs when Lew animals are pretreated with  $5 \times 10^7$  BN spleen cells and 1 ml of Lew anti-BN alloantiserum 11 and 10 d before transplantation, respectively. This pretreatment regimen increases graft survival to a minimum of 26 d, with 20% of the grafts functioning indefinitely, significant prolongation when compared to grafts in untreated hosts (mean survival time  $\pm$  SD [MST] =  $7 \pm 1$  d,  $P < 0.001$ ). The object of the following series of experiments was to define further previous

observations that thymocytes from enhanced, engrafted animals prolong test graft survival after adoptive transfer into unmodified hosts (20).

*Graft Survival after Cell Transfer.* Transfer of  $10^8$  syngeneic thymocytes from enhanced Lew rats bearing well functioning (Lew  $\times$  BN)F1 cardiac grafts (thymocyte donors), to unmodified animals receiving (Lew  $\times$  BN)F1 cardiac grafts 24 h later (test graft recipients), successfully prolonged the test grafts (MST =  $4 \pm 3$  d,  $P < 0.001$ , Fig. 1). However, transfer of  $10^8$  thymocytes from normal Lew rats or from unmodified Lew rats 6 d after receiving (Lew  $\times$  BN)F1 cardiac allografts did not effect test graft survival (MST =  $6 \pm 1$  d).

The kinetics of the phenomenon of transferred unresponsiveness mounted by thymocytes from enhanced engrafted rats was then examined. Thymocytes harvested 3 or 12 d after transplantation did not prolong test graft survival after adoptive transfer (MST =  $7 \pm 1$  d), whereas cells transferred a 9 d produced moderate prolongation of test graft survival (MST =  $9 \pm 2$  d,  $P < 0.05$ ).

Having established that transfer of thymocytes 6 d after transplantation gave best results, the effect of cell dose was then investigated (Fig. 2). The longest survival of test grafts was obtained upon transfer of  $10^8$  thymocytes (MST =  $14 \pm 3$  d). Correct cell dosage was crucial, as transfer of fewer or greater numbers of cells was ineffectual (MST of test grafts after transfer  $10^6$  thymocytes =  $8 \pm 2$  d,  $10^7$  thymocytes =  $7.5 \pm 1$  d,  $5 \times 10^8$  cells =  $8 \pm 1$  d).

*Effect of the Enhancing Protocol.* The role of each component of the enhancing protocol on the generation of thymic suppressor activity was studied. Either active (spleen cell) or passive (antiserum) enhancement alone, 11 and 10 d before transplantation, increased graft survival to  $9.5 \pm 1$  and  $10 \pm 2$  d, respectively (27). Thymocytes harvested 6 d after cardiac transplantation from these actively or passively pretreated animals and transferred into test graft recipients did not confer prolonged test graft survival ( $7.5 \pm 1.5$  d and  $6.5 \pm 1$  d, respectively). Thus, the entire immunization regimen for the engrafted thymocyte donor, with both antigen and antibody, was necessary for optimal test graft survival.

The type of antigenic stimulus used for active enhancement was then assessed (Fig. 3). Potential recipients were immunized with  $5 \times 10^7$  BN spleen cells, erythrocyte-free splenic leukocytes, or leukocyte-free erythrocytes, 11 d before heart transplantation. In all cases this was followed 1 d later by 1 ml of alloantiserum. 6 d after grafting,  $10^8$  thymocytes were transferred adoptively to syngeneic rats who received a (Lew  $\times$  BN)F1 test heart graft 24 h later. Best survival of test grafts occurred when whole spleen cells, containing both leukocyte and erythrocyte fractions were used. No significant prolongation was observed when leukocytes alone were used (MST =  $7 \pm 1$  d); intermediate increase in survival of test grafts was noted with erythrocytes alone (MST =  $10 \pm 2$  d,  $P < 0.001$ ).

To determine the influence of the antigenic stimulus provided by the vascularized heart graft itself on the production of thymocytes with suppressor activity, thymocytes were obtained from enhanced rats who had received either no cardiac allograft or who were given various substitute antigenic stimuli on the day of grafting (Fig. 4). Thymocytes from enhanced Lew animals who had received BN spleen cells and Lew anti-BN serum but no heart grafts, did not prolong survival (MST =  $7 \pm 1$  d). Substituting an intravenous injection of  $5 \times 10^7$ – $5 \times 10^8$  (Lew  $\times$  BN)F1 spleen cells

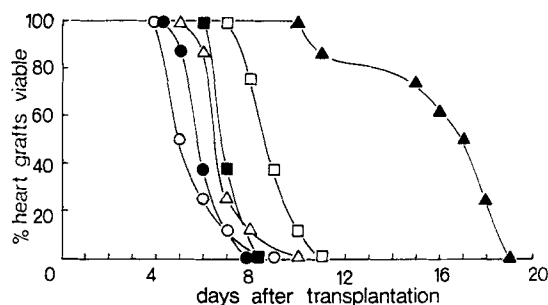


FIG. 1. Survival curves are noted for test (Lew  $\times$  BN)F1 cardiac allografts placed in unmodified recipients 24 h after adoptive transfer of  $10^8$  thymocytes from syngeneic enhanced engrafted animals. The sources of thymocytes are: (○) normal Lew; (●) unmodified Lew bearing acutely rejecting (Lew  $\times$  BN)F1 cardiac allografts at 6 d; and enhanced Lew rats bearing well functioning (Lew  $\times$  BN)F1 cardiac allografts at 3 d ( $\Delta$ ), 6 d ( $\blacktriangle$ ), 9 d ( $\square$ ), and 12 d ( $\blacksquare$ ). There are 8–16 animals in each group. Thymocytes transferred at 6 d prolong test graft survival significantly ( $P < 0.001$ ).

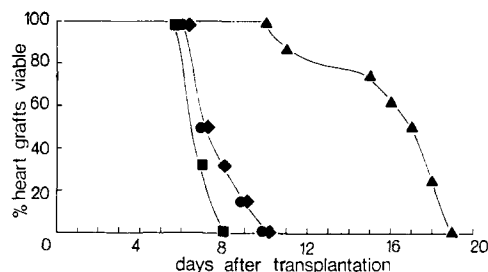


FIG. 2. The effect of dose of transferred thymocytes from enhanced recipients on test graft survival is shown. Cells were adoptively transferred in doses of  $10^6$  ( $\blacklozenge$ ),  $10^7$  ( $\blacksquare$ ),  $10^8$  ( $\blacktriangle$ ), and  $5 \times 10^8$  ( $\bullet$ ). There are 8–12 animals in each group. Transfer of  $10^8$  cells produced optimal results ( $P < 0.001$ ).

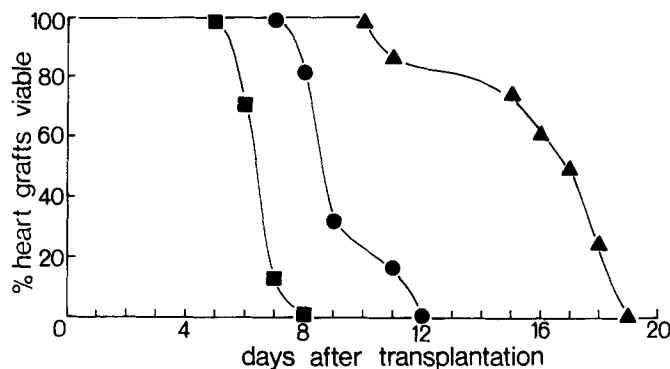


FIG. 3. The effect of the initial active immunizing stimulus of the enhanced recipient upon survival of test allografts after adoptive transfer of  $10^8$  thymocytes is noted. Groups of 8–12 enhanced thymocyte recipients were pretreated with  $5 \times 10^7$  BN whole spleen cells ( $\blacktriangle$ ),  $5 \times 10^7$  leukocyte free BN erythrocytes ( $\bullet$ ), or  $5 \times 10^7$  erythrocyte free BN splenic leukocytes ( $\blacksquare$ ), 11 d before transplantation, followed by 1 ml of hyperimmune serum 1 d later. Although pretreatment with erythrocytes alone increases the effect of transferred thymocytes, optimal activity is achieved by pretreatment with whole spleen cells ( $P < 0.001$ ).

instead of the heart graft, did not prolong test graft survival after transfer of thymocytes. However, when myocardial tissue was implanted into the kidney cortices of enhanced animals, transfer of thymocytes to syngeneic hosts 6 d later resulted in significant prolongation of test grafts (MST =  $10 \pm 2$  d,  $P < 0.001$ ). These

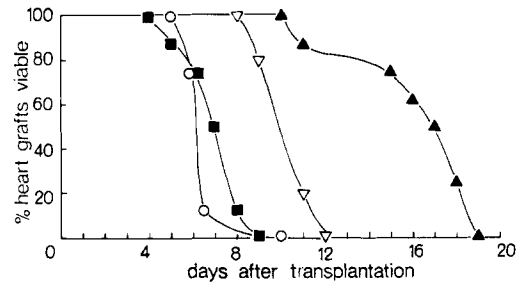


FIG. 4. The effect of the allograft in enhanced recipients upon transferred thymocytes mediating survival of test allografts is shown. Groups of 9–14 Lew animals were immunized actively with  $5 \times 10^7$  BN spleen cells 11 d before and 1 ml Lew anti-BN antiserum 10 d before receiving (●)  $5 \times 10^6$  (Lew × BN)F1 spleen cells, (▽) a (Lew × BN)F1 myocardial implant under the kidney capsule, (▲) a vascularized (Lew × BN)F1 cardiac allograft, or (○) no further antigenic stimulus. Both myocardial implants and a vascularized graft produced prolongation of test graft survival ( $P < 0.001$ ).

experiments show the necessity for active and passive immunization of the enhanced graft recipient and suggest the need for a second antigenic stimulus of sufficient magnitude to marshal a sustained host response against it.

*Transfer of Viable or Nonviable Thymocytes and Serum.* The possibility was tested that passive transfer of cytophilic antibodies or antigen-antibody complexes on the surfaces of transferred thymocytes from enhanced thymocyte donors might themselves increase graft survival (Table I). The ability of viable thymocytes which had been trypsinized, or thymocytes which were allowed to shed overnight at  $37^\circ\text{C}$  in tissue culture medium, was undiminished in prolonging test graft survival. Conversely, transfer of  $10^8$  heat killed thymocytes ( $56^\circ\text{C}$  for 30 min), failed to produce graft prolongation (MST =  $7.5 \pm 1$  d).

The effect of passive transfer of serum from enhanced recipients of well functioning grafts on survival of test grafts was assessed. Enhanced engrafted animals were bled at 6 d after transplantation and their serum pooled and stored at  $-70^\circ\text{C}$ . This serum was then injected into unmodified Lew recipients of (Lew × BN)F1 cardiac grafts, by using the regimen of French and Batchelor (Table I) (3). The serum transfer had no effect on test graft survival (MST =  $7.5 \pm 1$  d).

*Specificity of the Suppressor Effect.* The specificity of the suppressor effect against donor strain and third party strain grafts was examined by using the RT1 disparate WF strain (Fig. 5). Lewis animals were pretreated with  $5 \times 10^7$  WF spleen cells and 1 ml of Lew anti-WF alloantiserum 11 and 10 d before transplantation, respectively. With this enhancement regimen (Lew × WF)F1 cardiac grafts survived  $21 \pm 5$  d; such grafts placed in unmodified animals survived  $7 \pm 1$  d ( $P < 0.001$ ). The survival of (Lew × WF)F1 allografts, after transfer of thymocytes from enhanced Lew recipients of (Lew × WF)F1 heart grafts was prolonged to  $10 \pm 2$  d ( $P < 0.002$ ). However, when thymocytes from enhanced Lew recipients of (Lew × BN)F1 heart grafts were transferred into unmodified Lew recipients of (Lew × WF)F1 test grafts, no prolongation was noted (MST =  $6 \pm 1$  d). Similarly, (Lew × BN)F1 heart grafts in Lew hosts survived only  $6 \pm 1$  d after syngeneic thymocyte transfer from enhanced animals bearing (Lew × WF)F1 cardiac grafts. Hence, specificity of unresponsiveness to the original RT1 haplotype was demonstrated.

*Effect of Host Thymectomy on Enhancement.* Having demonstrated prolongation of test graft survival after thymocyte transfer, the effect of thymectomy on graft survival in

TABLE I

Type of transfer*	No. animals	MST graft survival days
Viable thymocytes ( $10^6$ )		
Trypsinized	5	14 $\pm$ 3‡
Cultured overnight	5	15 $\pm$ 2‡
Heat-killed thymocytes ( $10^6$ )	8	7.5 $\pm$ 1
Serum	8	7.5 $\pm$ 1

\* Cells and serum were taken from enhanced Lew recipients of (Lew  $\times$  BN)F1 cardiac grafts 6 d after transplantation. Cells were then transferred into unmodified Lew test graft recipients transplanted with a (Lew  $\times$  BN)F1 heart 24 h later. The serum was transferred: 1 ml intravenously at time of transplant, 1 ml intraperitoneally at 24 h, and 0.5 ml intraperitoneally at 48, 72, and 96 h, respectively (3).

‡ ( $P < 0.001$ ).

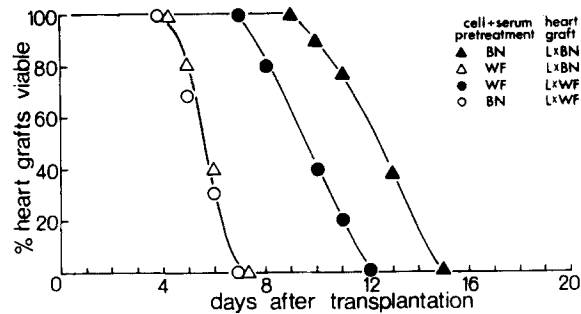


FIG. 5. Prolongation of test cardiac allografts after transfer of thymocytes from enhanced animals is a strain specific phenomenon. The survival of appropriate cardiac allografts is prolonged significantly ( $P < 0.001$ ) in Lew recipients immunized with specific antigen and antiserum. There is no prolongation of specific test grafts after thymocyte transfer from enhanced recipients immunized with inappropriate (third party) antigen and antibody, before grafting.

enhanced rats was examined (Fig. 6). Groups of animals underwent thymectomy at various intervals. Thymectomy at 6 wk followed by enhancement and engraftment 8–10 wk later had no effect upon graft survival (MST =  $32 \pm 4$  d) as compared to nonthymectomized enhanced recipients. Similarly, thymectomy immediately before grafting, or at 3 d after transplantation of enhanced recipients did not diminish graft function (MST =  $31 \pm 3$  d and  $31 \pm 4$  d, respectively). However, thymectomy 6 and 10 d after transplantation reduced significantly graft survival to  $16 \pm 3$  d and  $14 \pm 2$  d, respectively ( $\approx 10$  and 4 d after thymectomy,  $P < 0.001$ ). Thus, removal of the thymus at a critical period after transplantation severely diminished the effect of enhancement on prolonged graft survival.

**Surface Properties of Infiltrating Cells.** Infiltrating cells harvested at 6 d from well-functioning heart grafts and cells from spleens of test graft recipients were identified by their surface characteristics (Table II). These cells were then compared to cells isolated at 6 d from spleens and from acutely rejecting grafts in unmodified animals. Infiltrating cells recovered from cardiac allografts were incubated at  $22^\circ\text{C}$  overnight to permit shedding of cytophilic antibodies, resuspended and washed (28, 29). Spleen weights 6 d after transplant and overall numbers of cells in spleens were comparable

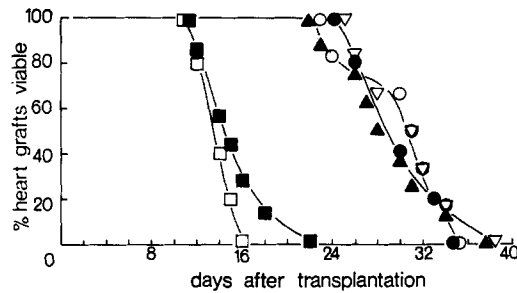


FIG. 6. The effect of thymectomy on survival of cardiac allografts in enhanced hosts is shown. Thymectomy of the enhanced recipients 6-10 d after transplantation causes acute rejection of cardiac allografts. Thymectomy at other intervals has no effect upon long-term survival. Day of thymectomy: ▲, none; ▽, -56; ●, 0; ○, 3; ■, 6; □, 10.

TABLE II  
Lymphocyte Subpopulations\* in Test Graft Recipients Comparison with Acute Rejection‡

	T cells	B cells	Fc+ Receptor cells	LMC	
				6 d	12 d
	%	%	%	%	
Cardiac allografts					
Test grafts	40 ± 9	34 ± 9	30 ± 2	0	0
Acute rejection	70 ± 6	35 ± 13	22 ± 4	37 ± 3	—
Recipient spleens					
Test-grafted animals	36 ± 4	61 ± 11	47 ± 2	0	0
Acutely rejecting animals	65 ± 6	37 ± 7	26 ± 5	14 ± 3	—

\* Mean ± standard deviation.

‡ Each of four experiments include cell pools from grafts of 8-10 animals.

in both groups of animals. The numbers of T lymphocytes in both hearts and spleens of test graft recipients were diminished as compared to animals acutely rejecting their grafts. Conversely, there were increased numbers of SIg+ and Fc+ cells in the spleens of rats from the experimental group, as compared to unmodified recipients.

**Lymphocyte-Mediated Cytotoxicity.** Infiltrating cells from well-functioning heart grafts and leukocytes from peripheral blood, lymph nodes (cervical, axillary, and brachial) and spleens of 34 test graft recipients were tested for direct LMC in four separate experiments, 6 and 12 d after transplantation. Allospecific killing was never observed. This finding contrasts to significant LMC (37% ± 3) mounted by cells isolated from acutely rejecting cardiac allografts at 6 d, and by the spleen cells of the unmodified hosts (14% ± 2) at 6 d, peaking at 8 d after transplantation (40% ± 3, P < 0.001).

**Complement-Dependent Cytotoxicity.** Sera from untreated recipients of acutely rejecting grafts, from enhanced rats bearing well-functioning grafts, and from animals with test grafts surviving for prolonged periods after thymocyte transfer, were collected serially after transplantation and tested for complement-dependent cytotoxicity (Fig. 7). Unmodified Lew recipients of (Lew × BN)F1 cardiac allografts showed a rising titer of antibody around the time of acute rejection at 5-7 d, which persisted for 1 wk thereafter. Little CDC activity was demonstrable in enhanced recipients of well-functioning grafts. Test graft recipients which had received syngeneic thymocytes



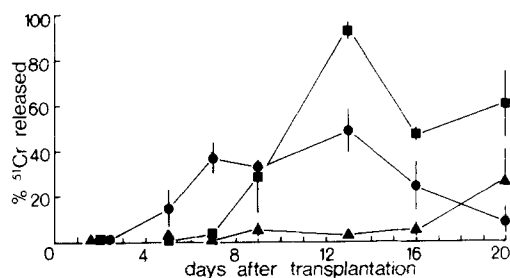


FIG. 7. Serial determinations of CDC is noted. There is an increase in cytotoxicity during the time of acute rejection in unmodified recipients of heart grafts, and marked increase in activity around the time of rejection in animals who had received immunologically unresponsive thymocytes from enhanced, engrafted animals. ●, untreated; ▲, enhanced; ■, suppressed.

from enhanced engrafted thymocyte donors showed no cytotoxicity for 7 d after transplantation. However, cytotoxicity became intense ( $\approx 90\%$  lysis) by day 12, near the time of rejection, and still remained high at day 20. Additionally, CDC was tested against nylon wool separated T and B lymphocyte enriched BN lymph node cells. CDC against separated lymph node cells by using serum at day 12 and 14 from acutely rejecting and suppressed animals revealed comparable killing of T and B cells.

### Discussion

Just as graft rejection appears to be the culmination of many different interrelated cellular and antibody-mediated responses, the induction and maintenance of graft survival in immunologically enhanced animals appears increasingly to be related to a series of connected host events. Although immunological enhancement was first described as a humoral phenomenon, passive transfer of serum from enhanced animals rarely increases survival of test allografts. We have shown previously that cell populations producing immunological unresponsiveness emerge in enhanced, engrafted animals (19, 20). Transferred thymocytes and spleen cells from enhanced recipients of cardiac allografts increase significantly the survival of test grafts in unmodified syngeneic animals. The phenomenon seems to be mediated primarily by T cells, as thymocytes produce optimal prolongation of test grafts, although nylon wool nonadherent spleen cells have somewhat greater activity than the nylon wool adherent fraction. The concept that cell-mediated suppression of the immune responses acts as an active immunoregulatory mechanism has been demonstrated in many systems, including depression of antibody formation (30, 31), delayed hypersensitivity responses (11–13), the mixed lymphocyte reaction (32, 33), graft-versus-host responses (14–16) and immunological tolerance. Indeed, Mitchison in 1960 demonstrated evidence for active suppressor mechanisms which continuously maintain a tolerant state (34).

In the present experiments, the role of thymocytes in enhancement of cardiac allografts has been defined further and the immunological specificity of the phenomenon established. Adoptive transfer of thymocytes from enhanced thymocyte donors to unmodified syngeneic test graft recipients doubled the survival of donor strain test allografts. This activity was highly time dependent, detected only 6–9 d after transplantation, and also markedly dose dependent. That  $5 \times 10^8$  transferred thymocytes gave significantly poorer results than  $10^8$  thymocytes suggests a fine

balance existing between suppressor and some other cell types whose effects become evident above or below the optimal dose of cells with suppressor activity. Such dose dependency has been described in other models (35). Additionally, acute rejection of previously well-functioning allografts in enhanced animals occurs after thymectomy between days 6 and 10 only, the same time as the optimal effect of transferred thymocytes. This confirms the importance of an intact thymus at a critical period after induction of enhancement. The influence of the thymus has also been noted in studies of tolerance, in which thymectomy may prevent induction of neonatal tolerance to skin grafts (36).

Prolonged allograft survival after passive cell transfer in adults has been described predominantly in immunocompromised hosts. Specific tolerance to skin grafts in the rat has been achieved by transferring T-enriched thoracic duct lymphocytes from neonatally tolerized rats to sublethally irradiated syngeneic recipients (18). Transfer of normal thymocytes to slightly irradiated young, syngeneic chickens also effects responsiveness towards allografts, as survival of subsequent skin grafts is prolonged (37). Kilshaw and Brent have induced unresponsiveness to skin allografts in adult mice by pretreatment with donor strain crude liver extract combined with *Bordetella pertussis* vaccine and anti-lymphocyte serum (35). This state of unresponsiveness can be adoptively transferred to partially immunosuppressed recipients by splenocytes harvested from mice with long surviving grafts. Additionally, Stuart et al. have noted that transfer of splenocytes from combined active and passive enhanced recipients of kidney allografts, harvested 80–150 d after transplantation delayed the onset of rejection of test grafts in five of six instances (38). Batchelor et al., however, failed to demonstrate such an effect in renal allografted rats that were passively but not actively enhanced (39).

We have shown that thymocytes from engrafted rats enhanced by combined active and passive means prolong the survival of test allografts when transferred to untreated syngeneic hosts. In these studies, the full protocol used to initiate enhancement in engrafted thymocyte donors was critical for optimal production of cells mounting specific unresponsiveness. Test graft survival was not prolonged when leukocytes alone were used as the active enhancing agent; erythrocytes alone increased graft function moderately. In previous studies on immunizing regimens using the present model, Baldwin et al. showed that optimal enhancement occurs only when a combination of erythrocytes and leukocytes and serum are used (27). Possibly, erythrocytes and leukocytes work through different mechanisms, i.e., erythrocytes predominantly through cellular host responses and leukocytes predominantly through humoral host responses. Thus, to demonstrate suppressor cell activity in rats bearing enhanced organ allografts, the grafted animals must be both actively and passively immunized. Our results may also offer an explanation for the partial success of Stuart et al. and the inability of Batchelor et al. to prolong test graft survival after adoptive transfer of lymphoid cells (38, 39). The production of immunological unresponsiveness in this model was also dependent upon a further antigenic stimulus after combined active and passive immunization (Fig. 4). Optimal test graft survival after cell transfer was obtained with a vascularized cardiac allograft. A small piece of myocardium placed beneath the kidney capsules gave moderate test graft prolongation, although no effect occurred when allogeneic splenocytes were used as the stimulus. Prolonged exposure to allogeneic tissue or a requirement for organ-specific immunization after active and

passive enhancement may be an absolute requirement for the development of suppressor activity.

The antigenic specificity of transferrable unresponsiveness using BN and WF donor strain animals was striking (Fig. 5). These strains were chosen because they are incompatible for all known major histocompatibility antigens, including genes with the RT1-A class I antigens (containing  $\beta 2$  microglobulin), and the RT1-B region which includes genes for MLR, Ia, and Ir.

Because the cells with the best suppressor activity were derived from the thymus, the effect would appear to be attributed primarily to viable T lymphocytes. As trypsin or shedding overnight in medium did not effect the activity of these cells, transfer of antigen or antigen-antibody complexes is unlikely. Additionally, the inability of serum from enhanced, engrafted animals to prolong test graft survival after passive transfer, would suggest that soluble factors or actively produced alloantibody alone are insufficient to inhibit allograft rejection.

Because passive transfer of thymocytes from enhanced animals is not as fully effective in prolonging graft survival as the complete active and passive enhancement protocol itself, additional host factors may come into play to maintain the integrity of the enhanced state. Cellular and humoral host responses by test graft recipients after transfer of thymocytes from enhanced, engrafted donors are different from those previously demonstrated either in studies of unmodified animals acutely rejecting their grafts or in enhanced rats bearing well functioning grafts (5, 19, 22). The reduction in numbers of T lymphocytes both in grafted hearts and in spleens of test graft recipients is paralleled by the complete absence of direct LMC mounted by the isolated cells. Despite the failure of these animals to produce cell-mediated cytotoxicity, cytotoxic antibody is markedly increased around and subsequent to the time of graft rejection. Thus, the data suggest a preferential effect of suppressor like activity upon cytotoxic T-cell function, with a lesser effect upon antibody-producing cells. In contrast, passive enhancement of recipients of both heart or kidney allografts in the same (Lew  $\times$  BN)F1  $\rightarrow$  Lew rat strain combination produces only minor alterations in generation of cytotoxic T lymphocytes, but ablates the generation of cytotoxic antibody (5, 40). Histologically, this lack of antibody production in passively enhanced recipients of kidney grafts is manifest by abolition of IgG-mediated necrotizing vasculitis and glomerulitis. Furthermore, the combination of active and passive enhancement of recipients of cardiac allografts, does not dampen the extent of infiltration of cytotoxic T lymphocytes into the allografted hearts, a situation quite distinct from that noted in test graft recipients in the present experiments (19). Thus different biological manipulations can modify separate pathways in the complex cellular and humoral host responses towards alloantigens in the organ allografts. These mechanisms must be assessed independently for fuller understanding of the total immunological behavior against such tissues.

### Summary

Prolonged survival of vascularized organ allografts has been produced in unmodified inbred rats by transfer of thymocytes from enhanced, engrafted, syngeneic animals. For these thymocytes to increase significantly the survival of test allografts they must be harvested 6-9 d after transplantation. Thymectomy of the enhanced, engrafted animals during the same critical period causes acute rejection of otherwise

long surviving grafts. For optimal effect, the enhanced thymocyte donor must be actively and passively immunized and receive a cardiac allograft. The necessity for erythrocytes in the initial active immunization regimen is noted. Additionally, the antigenic specificity of the suppressor effect has been established with two histoincompatible donor rat strains.

Cellular and humoral host responses mounted by test graft recipients after thymocyte transfer from enhanced, engrafted donors are different from those mounted either by unmodified animals acutely rejecting their grafts or by enhanced rats bearing well-functioning grafts. Numbers of T lymphocytes are reduced in the grafted hearts and in the spleens of test graft recipients, a finding paralleled by the complete absence of specific direct lymphocyte-mediated cytotoxicity. In contrast, cytotoxic antibody production, although delayed, is increased in magnitude, peaking around the time of graft rejection. These studies provide evidence that different biological manipulations can modify separate pathways in the complex cellular and humoral responses towards organ allografts. They demonstrate that cellular immunity is critically involved in immunological enhancement of vascularized organ allografts, a phenomenon hitherto considered primarily humoral. It seems clear that cells with suppressor activity are present within the thymus during the early phases of immunological enhancement.

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