Effect of Donor Immunization with Somatic Polysaccharides on the Graft-*vs*-Host Reactivity of Transferred Donor Splenocytes. (32249)

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Competition of antigens is a relatively innocuous and physiological means of inhibiting with one antigen the sensitization of animals to another(1). A similar inhibition may also be observed when one antigen is given during the inductive phase of immune paralysis towards another ostensibly unrelated antigen(2,3). In this case, the inhibition can be complete and can affect both immediate and delayed hypersensitivity(4). Transplantation immunity, as evidenced by homograft or graft-*vs*-host (GVH) reactions is also depressed by paralysis-inducing treatments with diverse unrelated antigens(5,6).

This period of non-specific inhibition occurring during the inductive phase of immune paralysis is of short duration; persisting antigenic stimulations (antigen mixed with Freund's adjuvant, or skin homografts) are only temporarily inhibited. Sensitization with soluble antigens, however, and GVH reactions can be permanently inhibited(4,5,6). It seems probable, therefore, that transplantation tolerance and donor skin-graft acceptance, could be induced in adult animals if donor lymphoid cells were to colonize irradiated recipients without causing GVH mortality. Treatment of donors with protein antigens has been shown to result in an inhibition or depression of the GVH reactivity of their lymphoid cells. This donor treatment can afford consequent protection of irradiated recipients and, provided the histocompatibility barrier is weak, transplantation tolerance to the donor tissues can be induced (7).

In the case of stronger histocompatibility differences, a significant inhibition of the GVH reaction also occurs; this is demonstrable only with low doses of transferred donor spleen cells, in amounts which thus far have been insufficient to induce transplantation tolerance in recipient animals(7,8).

Previous work indicates that competition of antigens involves quantitative as well as qualitative considerations(9,10). This suggests that a given quantity of a strong competing antigen could better inhibit the GVH reaction against a strong antigen (such as the H_2 locus transplantation antigens) than an equal or larger quantity of a weak competing antigen. Since the somatic antigens of Gram-negative bacteria are considered to be among the most potent of known antigens, it was of interest to assess their capacity to inhibit the GVH reaction for a donor-recipient combination differing at the H_2 locus.

Materials and methods. Animals. Adult male $C_{57}Bl/6N$ mice (H_2b locus) were used as donors and adult male C_3H/HeN mice (H_2k locus) were employed as recipients. These mice were furnished by the animal production branch of the National Institutes of Health. They were housed in air conditioned quarters at 24°C and given Purina Laboratory chow and water *ad libitum*.

Irradiation. Recipient mice were irradiated with 500 r from a Westinghouse Quadrocondex constant potential 200 kilovolt and 15 milliampere source with a half value layer of 0.825 mm copper. Treatment distance was 54 cm and the dose rate was 122 r per minute. On the average, this 500 r irradiation dosage produced 20-40 per cent mortality in C_3H/HeN mice. This mortality could be forestalled by immediate lymphoid restoration with 5-10 $\times$ 10^6 syngeneic or allogeneic spleen cells.

Preparation of donor spleen cells. Spleens were minced and passed through fine nylon mesh into Hanks' solution with added peni-

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cillin. The lymphoid cell suspensions were centrifuged at 1400 rpm for 10 minutes at 5°C.

The cell pellet was resuspended in a small volume of Hanks' solution by aspiration, first with a Pasteur pipette and then with a 27 gauge needle. The resuspended cells were enumerated in a hemocytometer chamber and their exclusion of 0.5% trypan blue dye was ascertained. Measured numbers of cells were then transferred to the recipients by intravenous injection. As estimated by trypan blue exclusion, cell viability regularly ranged from 80-90 per cent of the total nucleated spleen cell count. Varying dosages of viable donor cells were transferred intravenously to groups of irradiated recipients within 4 to 6 hours following irradiation.

Antigens. Two bacterial antigens were employed in this comparative study:

1. Somatic polysaccharide of *"Salmonella enteritidis"* (SEP) with a full range of biologic and toxic properties was prepared from viable bacilli by the aqueous-ether procedure(11), by Edgar Ribi, Rocky Mountain Laboratory, NIAID, Hamilton, Montana.

2. Methanol extracted endotoxin of *"Serratia marcescens"* (SMP) modified so as to drastically reduce toxicity(12) was provided by Alois Nowotny, Temple University School of Medicine, Philadelphia, Pa.

Results. GVH mortality of recipient mice following transfer of normal donor spleen cells. Several groups of irradiated adult C_3H/HeN mice were given measured numbers of viable $C_{57}Bl/6N$ spleen cells and the resulting GVH mortality for these recipients differing at the H_2 histocompatibility locus, was evaluated. Fig. 1 is a graphic representation of the observed mortality. Transfer of 10×10^6 or fewer normal donor spleen cells failed to produce GVH mortality, whereas 22.5×10^6 or more normal donor cells invariably produced 100% mortality. Between the extremes of 10 and 22.5×10^6 normal spleen cells, GVH mortality was directly related to the number of cells transferred; within this critical dosage range an essentially straight-line relationship was developed (Fig. 2).

This defined relationship between normal donor cell dosage and ensuing mortality in

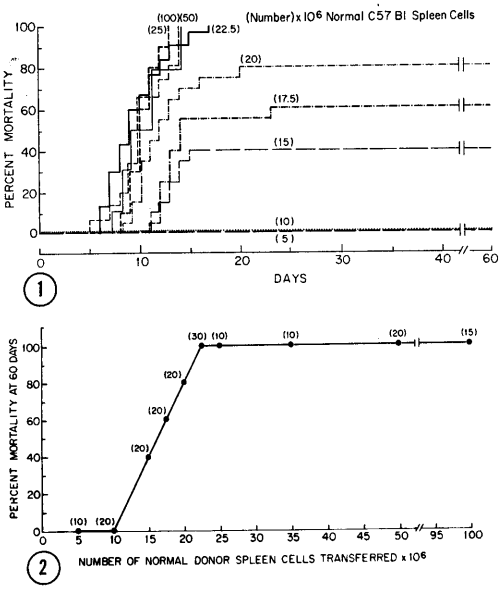

FIG. 1. Mortality in midlethally irradiated (500 r) C_3H/HeN mice receiving measured numbers of normal $C_{57}Bl/6N$ spleen cells.

FIG. 2. Final mortality levels in midlethally irradiated (500r) C_3H/HeN mice receiving measured numbers of normal $C_{57}Bl/6N$ spleen cells. Numbers in parentheses are numbers of animals per experimental group.

recipients, provided a standard dose-response reference plot against which the quantitative effectiveness of spleen cells from antigen treated donors could be matched and compared.

Effect of donor treatment with SEP. Groups of $C_{57}Bl/6N$ donor mice were given 7 daily intraperitoneal injections of 30 μg SEP. Preliminary experiments showed that larger doses of SEP resulted in high mortality of donor mice. At the end of this treatment period, measured numbers of harvested donor spleen cells were injected intravenously into groups of irradiated recipients. The results of this experiment are reported in Table I. It can be seen that in all experimental groups, a significant proportion of the recipients survived at the 15th day as compared with 30%, 0% and 0% survival in the corresponding control groups. Most of the animals in these experimental groups were still alive on the 60th day. Survival at 60 days was considered evidence that the transferred cells were incapable of initiating a fatal form of GVH disease; although survi-

TABLE I. Effect of Pretreatment with SEP or SMP on Competence of Donor Spleen Cells to Produce GVH Mortality.

Donor treatment							
Antigen	Amt/day (μg)	No. of days	No. of recipients	No. of donor cells transferred $\times 10^6$	% Survival at 15 days	% Survival at 30 days	Final survival 60 days
None	—	—	20	20	30	20	20
(controls)	—	—	20	50	0	0	0
	—	—	15	100	0	0	0
SEP	30	7	28	20	57	54	54
			12	50	75	67	50
			8	100	25	12	12
SMP	200	6	8	20	100	100	100
			8	50	100	100	100
			11	100	91	73	64

vors were often maintained up to 6 months, no deaths occurred after 45 days.

Recipients of spleen cells from SEP treated donors also displayed a significant delay in the time at which they succumbed to GVH disease. In Table II is reported the mean fatality interval (MFI) which is the mean survival time for *only* those mice in which the GVH reaction was fatal. It is apparent that lowering the dose of transferred normal spleen cells from 100 $\times$ 10^6 down to 15 $\times$ 10^6 cells, did not substantially delay the GVH reaction in control groups (10.3 to 12.9 days, respectively, 11.3 days on the average). For groups of recipients given cells from SEP treated donors, the fatality interval was significantly delayed, the average being 19.4 days.

Effect of donor treatment with SMP. Preliminary assays disclosed that methanol extracted *Serratia marcescens* endotoxin (SMP) was far less toxic for mice (LD 50 = greater than 3 mg/mouse) than SEP. It was thus possible to treat donor $C_{57}Bl/6N$ mice with 200 μg of SMP per day for 6 days, before transferring their spleen cells into groups of irradiated C_3H/HeN recipients.

TABLE II. Mean Fatality Intervals (MFI)* of C_3H/HeN Mice Undergoing GVH Reactions After Receiving $C_{57}Bl/6$ N Spleen Cells.

Donor treatment	No. of donor spleen cells transferred $\times 10^6$ 15	17.5	20	22.5	25	50	100
None (controls)	12.9	13.6	11.8	9.5	10.9	10.2	10.3
SEP			16.4		24.9		17.0
SMP			**		**		25.3

* In days, for further explanation, see text.
** No deaths.

The results of this experiment, reported in Table I, show that pretreatment of donor mice with SMP drastically reduced recipient GVH mortality; all of the recipients given either 20 $\times$ 10^6 of 50 $\times$ 10^6 spleen cells survived and only 4 of the 11 mice given 100 $\times$ 10^6 spleen cells died from their GVH reactions. Furthermore the evolution of the GVH reaction in these recipients was greatly delayed, MFI values being more than double those of controls (25.3 *vs* 11.3) (Table II).

The efficiency of SMP in inhibiting donor cell GVH reactivity motivated further study of this antigen involving other modes of donor treatment: a 4-day donor treatment with 30 μg of SMP per day and single immunizing injections of 10 μg SMP either 7 or 4 days prior to spleen cell transfer. The results of these experiments (Table III) indicate that even these modest donor treatments resulted in important survival of recipients in most experimental groups. Inhibition of donor cell GVH activity following these modes of treatment, however, was not as great as that following 6 days of donor treatment with 200 μg daily (Table I). In general, diminution of total antigen dosage appeared to reduce its inhibitory effect on donor cell GVH reactivity.

Discussion. The present work extends our earlier observations(6,7) indicating that intensive immunization or immune paralysis of donors results in a significant alteration in the capacity of their transferred lymphoid cells to produce GVH disease. Since a direct

TABLE III. Effect of Donor Pretreatment with Various Doses of SMP.

Donor treatment	No. of donor cells transferred $\times 10^6$	No. of recipients	No. of survivors at 15 days	30 days	60 days	% Final survival
None (controls)	20	20	5	4	4	20
	50	20	0	0	0	0
	100	15	0	0	0	0
SMP 120 μg (30 μg/day for 4 days)	20	8	8	8	8	100
	50	8	7	7	7	87
	100	8	8	3	2	25
SMP 10 μg (once, 7 days before spleen transfer)	20	6	5	5	5	83
	50	6	2	2	2	33
	100	5	0	0	0	0
SMP 10 μg (once, 4 days before cell transfer)	20	8	7	6	6	75
	50	8	2	2	2	25
	100	8	0	0	0	0

TABLE IV. Comparative Influence of the Antigens Tested on the Capacity of Donor Spleen Cells to Produce GVH Mortality.

1 Donor treatment (total dose)	2 No. of donor cells transferred $\times 10^6$	3 % Final survival at 60 days	4 No. of normal cells equivalents transferred $\times 10^6$	5 MCV,* % of normal
None (controls)	20	20	20	
	50	0	50	100
	100	0	100	
SEP (210 μg)	20	54	15.7	
	50	50	16.2	31
	100	12	20.9	
SMP (1,200 μg)	20	100	10 or <	
	50	100	10 or <	20
	100	64	14.5	
SMP (120 μg)	20	100	10 or <	
	50	87	12	25
	100	25	19.5	
SMP (10 μg)†	20	83	12.2	
	50	33	18.2	43§
	100	0	22.5 or <	
SMP (10 μg)‡	20	75	13	
	50	25	19.5	46§
	100	0	22.5 or <	

* MCV = Mean Cumulative Value: for further explanation, see text.
† 7 days prior to cell transfer.
‡ 4 days prior to cell transfer.
§ Computed with only the 20 and 50 $\times 10^6$ cell dosage values.

relationship between normal spleen cell dosage and consequent GVH mortality had been established (Fig. 2) it was possible to relate the quantitative GVH effectiveness of spleen cells from immunized donors in terms of normal spleen cell equivalents. This was accomplished simply by matching observed mortality with the identical mortality caused by equivalent numbers of normal donor splenocytes. Column 4 of Table IV records this relationship for recipients of spleen cells from treated donor mice. It can be seen for example that 100 $\times 10^6$ spleen cells from SEP treated donors were required to produce GVH mortality ordinarily elicited by only 20.9 $\times 10^6$ normal spleen cells.

Comparison of donor spleen cell GVH activity on this basis makes it possible to compute a mean cumulative value (MCV) which relates the effectiveness of donor cells

throughout the entire cell dosage range employed. This MCV (Table IV, column 5) was derived by dividing the sum of the normal cell equivalents for the three donor cell dosages by the total number of donor cells actually transferred. In the case of SEP, for example, 52.8×10^6 ($15.7 + 16.2 + 20.9 \times 10^6$) was divided by 170×10^6 ($20 + 50 + 100 \times 10^6$) to yield 0.31. On conversion to per cent this indicates that cells from SEP treated donors were on the average 31% as capable of producing fatal GVH disease as were equal numbers of normal donor cells. This value indicates that treatment with 30 μg/day of SEP decreased the GVH activity of donor splenocytes to about ⅓ that of controls. Higher donor dosage of this antigen would likely be more effective in inhibiting the GVH reaction. The toxicity of this product, however, precludes its use at higher dosages.

Contrastingly the relatively much lower toxicity of SMP, allowed administration of up to 200 μg/day of this potent antigen resulting in a decrease of the MCV to only 20 per cent of that seen for controls. When these two antigens were used at comparable dosage levels (30 μg/day) SMP maintained an advantage since its MCV was 25% *vs* 31% for SEP. Even a single dose of 10 μg SMP given either 7 days or 4 days prior to cell transfer substantially attenuated the severity of ensuing GVH reactions. (MCV 43% and 46%, respectively). As with the protein antigens tested in the same GVH system (13), larger total dosages of antigen administered to donors, generally resulted in greater inhibition of subsequent GVH reactions. In this respect modified bacterial somatic antigens such as SMP with relatively low toxicity offer special promise since protein antigens given even at saturating dosages (100 mg/day, for 8 days) produced distinctly less inhibition of the GVH reaction. It may even be that treatment of donor mice with even higher dosages of minimally toxic SMP, would permit transfer of larger numbers of spleen cells, which in turn could lead to the establishment of recipient tolerance to donor tissues.

Summary. The effect of immunization of donor mice with bacterial antigens, on the capacity of their spleen cells to product graft-*vs*-host (GVH) disease has been investigated for irradiated adult recipients differing from donors at the H_2 locus. Fatal GVH disease produced by spleen cells from untreated control donors was a function of the number of cells transferred. Antigen pretreatment of donors caused a reduction in the capacity of their spleen cells to produce GVH mortality. Compared with equal numbers of cells from untreated mice, the magnitude of this reduction was proportional to the total quantity of antigen used in pretreatment: 1.4 mg of *Serratia marcescens* polysaccharide (SMP) decreased donor spleen cell reactivity to 1/5 of control levels whereas 0.12 mg produced a decrease of ¼ and 0.01 mg to somewhat less than ½ that of controls. *Salmonella enteritidis* polysaccharide (SEP) also provided reduction of donor cell GVH reactivity however when employed in similar dosages (SMP 120 μg, SEP 210 μg) SMP was substantially the more active in inhibiting subsequent GVH reactions. In addition, the very low toxicity of SMP permitted its use at much higher and more effective dosages.

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