SYNERGY AMONG LYMPHOID CELLS MEDIATING THE GRAFT-*VERSUS*-HOST RESPONSE

V. Derivation by Migration in Lethally Irradiated Recipients of Two Interacting Subpopulations of Thymus-Derived Cells from Normal Spleen*

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Several recent studies have detailed the phenomenon of synergy among certain populations of parental strain lymphoid cells in the elicitation of graft-*versus*-host (GVH)[1] reactions in neonatal F_1 mice (1–5). When either a spleen weight assay in normal recipients or a mortality assay in sublethally irradiated recipients to quantitate the intensity of the GVH reaction was used, mixtures of either peripheral blood leukocytes (PBL) or femoral lymph node cells (LN) and either thymocytes or spleen cells from donors pretreated with a moderate dose of heterologous antithymocyte serum (ATS) regularly gave reactions significantly greater than expected by summing the reactivities of the components of the mixture measured separately.

These results suggested that optimal expression of GVH reactions may require the participation of at least two subpopulations of donor lymphoid cells. Studies utilizing either cells from neonatally thymectomized donors (3) or cells incubated in vitro with anti-θ serum and complement (5) indicated that both of these subpopulations were thymus-derived cells, thereby clearly distinguishing this synergistic reaction from those seen among certain thymus-derived and bone marrow-derived cells in the production of humoral antibodies to a variety of antigens (6–9). In addition, both cell types had to have the capacity to recognize foreign antigens in the F_1 recipients; no synergy was observed when appropriate populations of syngeneic F_1 cells were substituted for either of the populations of parental strain lymphoid cells (1–4), again distinguishing this particular synergy from that described by others in GVH reactions in irradiated recipients (10–12).

A simple two-cell model predicts that the intrinsic GVH reactivity of any

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given lymphoid tissue is the result, at least in part, of an interaction among different cell types. The present experiments were undertaken to see whether the existence of subpopulations within a single lymphoid tissue could be demonstrated more explicitly. The distribution of synergizing populations among different lymphoid "compartments" and their relative susceptibilities to heterologous ATS in vivo (3) both suggested that the relevant cell types might have sufficiently different migratory patterns and/or rates of recirculation to be of use in trying to achieve their separation. The results reported in this paper indicate that within a normal spleen there exist at least two subpopulations of thymus-derived cells with different migratory patterns in lethally irradiated syngeneic recipients. Both are necessary for the recovery of the GVH reactivity characteristic of this tissue.

Materials and Methods

Animals.—7-9-wk old male BALB/cAnN, C3H/HeN, AKR/N, and C57BL/6N × BALB/cAnN F_1 mice and neonatal (1-3-day old) litters of C57BL/6N × BALB/cAnN F_1 were mice obtained from the Rodent and Rabbit Production Section, Division of Research Services, National Institutes of Health, Bethesda, Md.

Irradiation.—2 h before receiving the inoculation of syngeneic spleen cells, mice were exposed to 800 R whole body irradiation. A Westinghouse (Westinghouse Electric Corporation, Baltimore, Md.) Quadrocondex 250 kVp machine was used under conditions of 200 kVp, 15 mA, half-value layer 0.9 mm Cu, at a distance of 54 cm, and an administered dose rate of 139 R/min.

Cell Suspensions.—Groups of animals were killed by cervical dislocation. Suspensions of spleen cells and lymph node cells (pools of inguinal, axillary, cervical, and submandibular nodes only; mesenteric lymph nodes were excluded) were prepared by mincing with scalpels and repeated gentle pipetting through disposable Pasteur pipettes, followed by filtration through gauze to remove debris and cell clumps. All suspensions were prepared and washed once in Hanks' balanced salt solution (HBSS; Grand Island Biological Co., Grand Island, N. Y.) containing 5% heat-inactivated fetal calf serum (FCS, immunoprecipitin tested; Grand Island Biological Co.). Suspensions were adjusted to the desired concentrations of total nucleated cells; when mixtures of cells were used, appropriate concentrations of each were mixed together immediately before inoculation.

In Vitro Treatment with Anti-θ Ascitic Fluid and Complement.—Anti-θ antibodies were raised by a modification of the technique described by Reif and Allen (13). AKR/N mice received six weekly intraperitoneal injections of 10^7 C3H/HeN thymocytes. 4 days after the sixth injection, the mice were given 10^7 Ehrlich ascites tumor cells (generously provided by Dr. Kenneth Sell, National Naval Medical Center, Bethesda, Md.) intraperitoneally and 10^7 C3H/HeN thymocytes subcutaneously. 7 days later the ascitic fluid (anti-θ) was collected, centrifuged to remove cells, heat inactivated at 56°C for 30 min, and frozen at −20°C until use.

Anti-θ absorbed with brain tissue served as a control for specificity. BALB/cAnN or AKR/N brain was homogenized in a TenBroeck homogenizer and then washed several times with large volumes of HBSS. Anti-θ was then absorbed with packed brain tissue (3:1 vol/vol) for 30 min at room temperature, with gentle shaking every 5 min to resuspend the tissue, centrifuged, and stored at −20°C until use.

The anti-θ used in this study killed 50% of a suspension of 10^6 BALB/c thymocytes at a dilution of 1:250 (dye exclusion method). Anti-θ absorbed with BALB/c brain was not cyto-

toxic at a dilution of 1:5. Anti-θ absorbed with AKR brain killed 50% of BALB/c thymocytes at a dilution of 1:220.

Cells to be treated were resuspended in RPMI 1640 (Grand Island Biological Co.) containing 1% bovine serum albumin (BSA, Path-o-cyte 5; Miles Laboratories, Elkhart, Ind.) to a concentration of 100×10^6/ml. Tubes containing 0.5 ml of this suspension and 0.3 ml of either unabsorbed or absorbed anti-θ were incubated at 4°C for 30 min. The cells were then washed once in 10 ml RPMI 1640 and resuspended to 0.5 ml. To this was added 1.0 ml of a 1:3 dilution of guinea pig complement (BBL Division, Becton-Dickinson & Co., Cockeysville, Md.) that had previously been absorbed for 1 h at 4°C with BALB/c spleen cells (1 spleen eq/ml diluted complement). After incubation at 37°C for 45 min, the suspensions were washed twice in HBSS + 5% FCS and resuspended in the same diluent to the desired concentration of nucleated cells.

GVH Assay.—The Simonsen quantitative spleen weight assay (14) was performed as described by Mandel and Asofsky (15). Briefly, litters of 1-3-day old C57BL/6N $\times$ BALB/cAnN F_1 mice were inoculated intraperitoneally with 0.05 ml of cell suspension. Two or three mice in each litter served as uninjected controls. Whenever combinations of cells were injected, other litters were also inoculated with the relevant populations alone as internal controls. 9 days later the litters were sacrificed, and the resultant splenomegaly was quantitated as follows: spleen index = (spleen weight to body weight ratio of experimental mouse) ÷ (mean spleen weight to body weight ratio of uninjected littermate controls). Regression lines of spleen index as a function of the logarithm of the cell inocula were calculated by the method of least squares. The slopes of these reactivity lines and individual data points were compared by means of a t test.

Experimental Design.—The basic model employed throughout these experiments is illustrated in Fig. 1. 100 million normal adult spleen cells (either BALB/c or C57BL/6 $\times$ BALB/c F_1 donors) were injected intravenously into syngeneic adult recipients that had received 800 R whole body irradiation 2 h previously. These mice were sacrificed 24 h later, and single cell
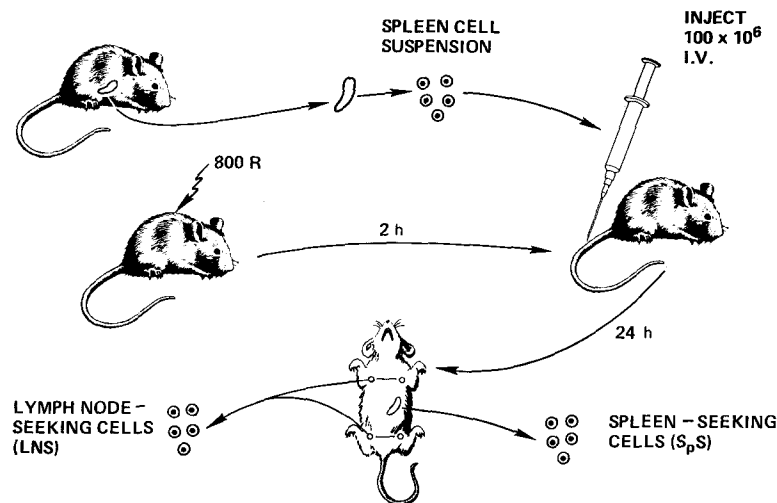

Fig. 1. Experimental design. Spleen cells from normal adult mice were always injected into adult syngeneic irradiated recipients (either BALB/c or C57BL/6 $\times$ BALB/c F_1). Mesenteric lymph nodes were excluded from the pools of LNS.[2] Various numbers of LNS and/or SpS were then injected into neonatal C57BL/6 $\times$ BALB/c F_1 litters and the GVH splenomegaly quantitated 9 days later.

suspensions were made of either the pooled spleens or the lymph nodes (excluding mesenteric nodes).[2] These populations have been operationally defined as either "spleen-seeking spleen cells" (SpS) or "lymph node-seeking spleen cells" (LNS). Various numbers of these cells either alone or in combination were then injected into neonatal F_1 recipients as described above.

<div align="center">RESULTS</div>

Comparison of Normal and Irradiated Unreconstituted Tissues.—Preliminary studies were designed to examine the cell yields from the spleens and lymph nodes of adult mice 24 h after lethal irradiation and to test the specific GVH reactivity of such cells. In three separate experiments the average yield of nucleated cells from irradiated BALB/c mice was about 8×10^6 cells/spleen and approximately 1.5×10^6 cells/lymph node pool. These numbers represent about 15% of the cells recoverable from normal untreated donors.[3]

The reactivities of normal BALB/c spleen cells and lymph node cells (individual data points not shown) are illustrated in Fig. 2.[2] As few as 4×10^5 LN or 8.5×10^5 spleen cells were sufficient to produce significant splenomegaly (in our hands, a spleen index greater than 1.3; see also reference 14) in F_1 recipients. Injection of as many as 1.6×10^6 LN or 8×10^6 spleen cells from donors given 800 R 24 h previously in no case resulted in spleen indices greater than 1.3. Since large numbers of these cells were inactive, any contribution of residual host cells to the directly measured GVH reactivity of tissues from irradiated reconstituted mice would be negligible.

Reactivities of SpS and LNS Alone.—The spleens of lethally irradiated BALB/c mice 24 h after injection of 100×10^6 normal BALB/c spleen cells contained approximately 22×10^6 nucleated cells/spleen, i.e., about three times the number recovered from unreconstituted animals (see above). The directly measured GVH reactivity of various numbers of these SpS is illustrated in Fig. 2. Each point represents the mean spleen index (± 1 SEM) of 5–15 litters (total number of litters = 45).

It is clear that SpS were less active than normal spleen cells by two criteria. More SpS than normal spleen cells were required to produce a minimal significant spleen index of 1.3, approximately 3.0×10^6 SpS vs. 8.5×10^5 normal spleen cells. Secondly, the regression line for SpS was significantly flatter than that for normal spleen cells, that is, many more SpS than normal spleen cells were required to produce a given increment in the observed splenomegaly. The calculated slopes of the regression lines were 1.75 for normal spleen cells and 0.90 for SpS ($P < 0.001$).

Suspensions of lymph node cells from irradiated mice injected 24 h previously with 100×10^6 normal adult spleen cells contained approximately 4×10^6

[2] Tigelaar, R., and R. Asofsky. 1972. Synergy among lymphoid cells mediating the graft-*versus*-host response. VI. Comparison of the amplifying activities of spleen, inguinal lymph node, and mesenteric lymph node cells on the GVH reactivity of thymocytes. Manuscript in preparation.

[3] Tigelaar, R. Unpublished observations.

nucleated cells/animal, again about three times as many as recovered from the lymph nodes of irradiated unreconstituted donors. The directly measured GVH reactivity of these LNS is shown in Fig. 3; each point represents the mean spleen index (±1 SEM) of three to eight recipient litters (total number of litters = 39). In a comparison with the population from which they were derived, normal spleen cells, it is clear that LNS, like SpS, show a strikingly flat dose-response line. The slope of the LNS regression line was 0.55 and
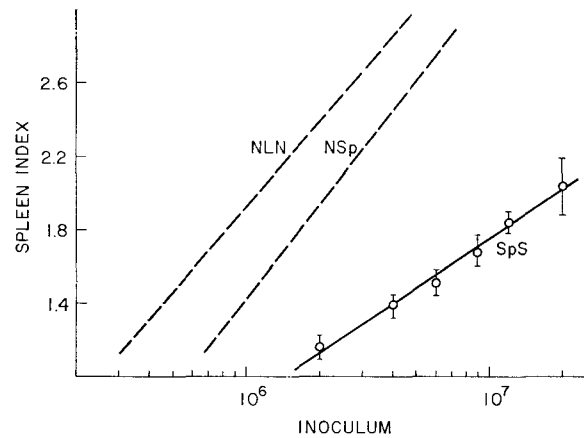

Fig. 2. GVH reactivity of BALB/c SpS alone. Spleen indices are plotted against the logarithm of the number of cells injected. Each point (O) represents the mean spleen index (±1 SEM) of 5–15 litters. Regression lines calculated by the method of least squares. Shown for reference (dotted lines) are the regression lines (data points not shown) for normal BALB/c ymph node (NLN) and normal BALB/c spleen NSp) cells.[2]

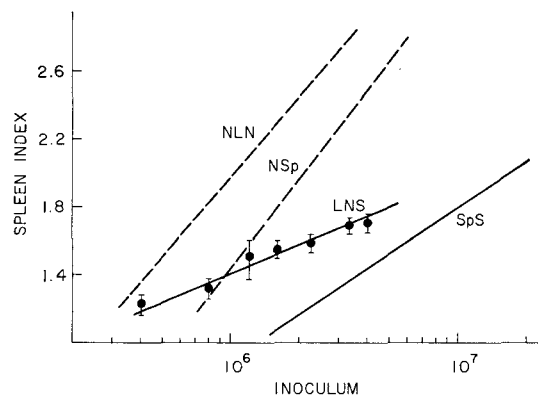

Fig. 3. GVH reactivity of BALB/c LNS alone. Each point (●) represents the mean spleen index (±1 SEM) of 3–8 recipient litters. Regression line calculated by the method of least squares.

differed significantly from both that of normal spleen cells ($P < 0.001$) and that of SpS ($P < 0.005$). Thus the in vivo passage of normal spleen cells into irradiated syngeneic recipients resulted in the recovery of two populations each of which had a significantly impaired capacity to initiate GVH reactions in neonatal F_1 recipients.

It was considered that the extreme flatness of the lines of the LNS and SpS populations might be due to some sort of nonspecific suppressive activity exerted or released by dying or residual radioresistant host cells. Three different experiments failed to provide any evidence for such a suppressor activity. (a) Normal spleen cells were incubated in vitro for 2 h at room temperature with an equal number of spleen cells from donors irradiated 24 h previously. The dose-response curve of this mixture was identical with that obtained with the same numbers of normal spleen cells alone. (b) Before injection into F_1 recipients, a suspension of SpS was layered on top of a two-step discontinuous BSA gradient (10% top layer, 35% cushion). Tubes were centrifuged in the cold at 10,000 rpm for 30 min. As Bianco and Nussenzweig (16) also noted, the pellet (below 35% BSA) contained the majority of the erythrocytes and nonviable leukocytes, while the layer above the 35% BSA was enriched in viable nucleated cells. The dose-response line for these viable cells was as shallow as that seen with unfractionated SpS (Fig. 2). (c) Normal spleen cells were injected into mice irradiated 24 h (instead of the usual 2 h) previously, and 24 h later the GVH activity of the SpS population was tested. The dose-response line of these cells was again identical with that illustrated in Fig. 2.

Reactivity of Mixtures of LNS and SpS.—Having established the regression lines for LNS and SpS tested separately, we then performed experiments in which these two subpopulations of normal spleen cells were recombined in a constant but arbitrary ratio of 1:5 immediately before injection into F_1 recipients. The results of these experiments are summarized in Fig. 4. The data points refer to the mean spleen indices (± 1 SEM) seen in recipients inoculated with 2.0×10^6, 4.0×10^6, or 6.0×10^6 SpS each combined with one-fifth that number of LNS (i.e., 4.0×10^5, 8.0×10^5, and 1.2×10^6, respectively). For convenience the points have been plotted according to the number of SpS in the inoculum. Each point represents data pooled from four to nine separate experiments and is the mean of 8–25 recipient litters (total number of litters = 44).

The striking return of the calculated regression line to a slope (2.23) similar to that observed with normal spleen cells ($0.1 > P > 0.05$) by combining two subpopulations each with very shallow dose-response lines is obvious. The slope of the regression line of the combination differs significantly ($P < 0.001$) from that seen with either LNS or SpS alone. Had no synergy occurred within the mixture of LNS and SpS, the dose-response line of the mixture would have been expected to be flat like those of the populations from which the combination was derived.
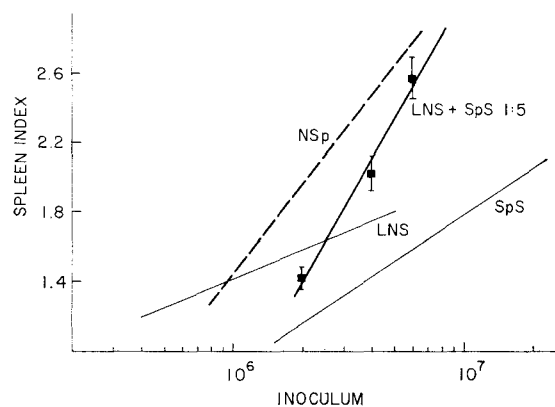
FIG. 4. GVH reactivity of mixtures of BALB/c LNS and SpS. The data refer to the mean spleen index (± 1 SEM) of 2.0, 4.0, or 6.0 $\times$ 10^6 SpS each combined with one-fifth that number of LNS. For convenience the points have been plotted according to the number of SpS in the inoculum. Each point represents data pooled from 4–9 separate experiments (8–25 recipient litters per point). Regression line calculated by the method of least squares.

Since the slopes of the LNS and SpS regression lines are significantly different from each other, it is difficult to calculate accurate expected (i.e. assuming no synergy) spleen indices for the combinations. Nevertheless, a rough estimate of such expected additive reactivity can be made. The combination of 8.0 $\times$ 10^5 LNS + 4.0 $\times$ 10^6 SpS can serve as an example. By referring to the standard reactivity lines for LNS and SpS alone, one can see that the reactivity of 8.0 $\times$ 10^5 LNS alone is equivalent to that seen with about 3.5 $\times$ 10^6 SpS alone. Additive reactivity by the mixture would be expected to be that shown by about 7.5 $\times$ 10^6 SpS alone (3.5 $\times$ 10^6 + 4.0 $\times$ 10^6) or, reading from the SpS line, a spleen index of about 1.70. The observed reactivity of this combination, however, was 2.02 $\pm$ 0.05, a spleen index expected from nearly 20 $\times$ 10^6 SpS tested alone. Thus the observed reactivity of this particular combination was more than twice that expected by addition.

In spite of the observation that both lymph node and spleen cells from donors lethally irradiated but not reconstituted with syngeneic spleen cells were unable to initiate significant splenomegaly in F_1 recipients when tested directly, the possibility remained that the synergistic reactions seen in combinations of LNS and SpS in fact involved these residual, perhaps radioresistant, host cells. Under certain conditions thymus-derived helper cell activity in humoral antibody responses has been remarkably radioresistant (17, 18). In order to test this possibility, 4.0 $\times$ 10^6 SpS were combined immediately before injection into F_1 recipients with 8.0 $\times$ 10^5 lymph node cells from irradiated unreconstituted donors. The reciprocal combination was injected into other F_1 recipients, i.e., 8.0 $\times$ 10^5 LNS + 4.0 $\times$ 10^6 spleen cells from irradiated

unreconstituted donors. In neither case was there any evidence of synergistic reactivity. The reactions were similar to those seen with the reconstituted population alone; the cells from irradiated unreconstituted donors neither enhanced nor depressed the reactivity of the active population in the mixture.

Specificity of the Reaction.—Previous studies (1–4) had shown that synergy was no longer observed if F_1 cells were substituted for either of the populations of parental strain cells in a synergistic combination of thymocytes and PBL, indicating that both populations had to have the capacity to recognize foreign antigens in the F_1 recipient. It was of interest to test if the same were true in the present model.

Irradiated adult F_1 mice were injected with 100×10^6 normal adult F_1 spleen cells, and 24 h later suspensions of lymph nodes and spleens were prepared. At the same time, similar suspensions were prepared from irradiated BALB/c mice injected 24 h before with normal BALB/c spleen cells. Various numbers of mixtures of LNS and SpS in a ratio of 1:5 were again injected into neonatal F_1 recipients, except that now one of the populations in the mixture consisted of cells syngeneic to the F_1 recipient.

The results are illustrated in Fig. 5. Each point represents data pooled from two experiments and is the mean spleen index (± 1 SEM) of a minimum of six
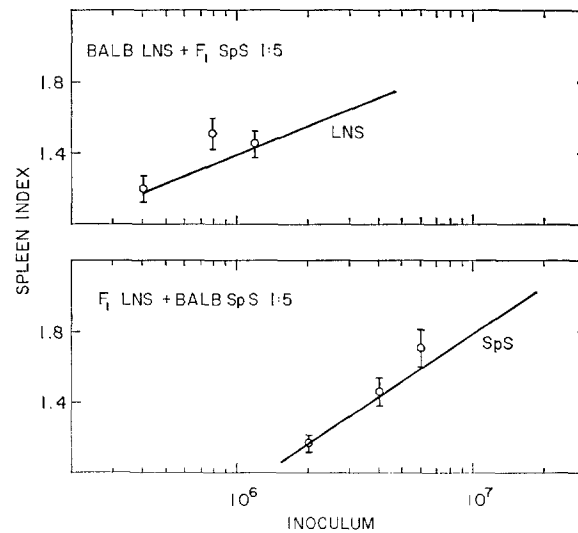

FIG. 5. Failure of mixtures of BALB/c and F_1 LNS and SpS to restore the steep dose-response relationship shown by BALB/c NSp or mixtures of BALB/c LNS + SpS. Upper half: 4.0×10^5, 8.0×10^5, or 1.2×10^6 BALB/c LNS each combined with five times that number of F_1 SpS. Regression line shown is that for BALB/c LNS tested alone (Fig. 3). Lower half: 2.0, 4.0, or 6.0 $\times 10^6$ BALB/c SpS each combined with one-fifth that number of F_1 LNS. Regression line shown is that for BALB/c SpS tested alone (Fig. 2). Each data point is the mean spleen index (± 1 SEM) of six recipient litters.

recipient litters. The upper half of Fig. 5 shows the results obtained when 4.0×10^5, 8.0×10^5, or 1.2×10^6 BALB/c LNS were inoculated together with five times those respective numbers of F_1 SpS. The observed reactions did not differ significantly ($P > 0.3$) from the reactions obtained when BALB/c LNS were injected alone (solid line, Fig. 3). The reciprocal experiments are illustrated in the lower half of Fig. 5, that is, 2.0×10^6, 4.0×10^6, or 6.0×10^6 BALB/c SpS inoculated together with one-fifth those respective numbers of F_1 LNS. Again the observed reactions did not differ significantly ($P > 0.3$) from the reactions obtained with the respective numbers of BALB/c SpS alone (solid line, Fig. 2). Mixtures of F_1 cells after migration and BALB/c cells after migration displayed the reactivity expected from the BALB/c cells alone. There was no indication of such combinations' having the steep dose-response relationship exhibited either by normal BALB/c spleen cells or by mixtures of BALB/c LNS and SpS.

Sensitivity of Mixtures of BALB/c LNS and SpS to Anti-θ Treatment.—Having established that appropriate mixtures of BALB/c LNS and SpS gave a synergistic GVH reaction with dose-response characteristics similar to those of the normal population from which they were derived, we thought it important to test whether the cell types responsible for this synergy were both thymus-derived, as had been found in a previous model (3, 5). Accordingly, suspensions of LNS and SpS were each incubated in vitro with either unabsorbed or brain-absorbed anti-θ and complement. After incubation, various mixtures of treated and untreated cells were injected into litters of F_1 recipients.

The results of these experiments are summarized in Table I. Treatment of either one of the subpopulations with unabsorbed anti-θ completely eliminated the synergistic reaction observed with untreated mixtures. Mixtures of untreated LNS and anti-θ-treated SpS exhibited reactivity not significantly

TABLE I

GVH Reactivity of Synergistic Mixtures of LNS and SpS after In Vitro Incubation with Anti-θ Ascitic Fluid and Complement

Group	LNS	In vitro treatment	SpS	In vitro treatment	Litters	Mean spleen index ± 1 SEM	Significance		
1	8×10^5	None	4×10^6	None	25	2.02 ± 0.05	$P > 0.4$[a]	$P < 0.001$[d]	$P < 0.001$[e]
2	$8 \times 10^{5*}$	Abs. anti-θ + C‡	4×10^6	Abs. anti-θ + C	5	1.95 ± 0.09			
3	8×10^5	None	—	—	8	1.34 ± 0.04	$P > 0.1$[b]		
4	8×10^5	None	4×10^6	Anti-θ + C	8	1.49 ± 0.08			
5	—	—	4×10^6	None	14	1.41 ± 0.03	$P > 0.1$[c]		
6	8×10^5	Anti-θ + C	4×10^6	None	8	1.48 ± 0.04			

* Number of nucleated cells injected into F_1 recipients after in vitro incubation.

‡ Absorbed with homogenate of BALB/c brain.

[a] Group 1 compared with group 2; [b]group 3 compared with group 4; [c]group 5 compared with group 6; [d]group 1 compared with group 4; [e]group 1 compared with group 6. Student's *t* test used throughout.

higher than that seen with untreated LNS alone ($P > 0.1$). Similarly, mixtures of untreated SpS and anti-θ-treated LNS exhibited reactivity not significantly higher than that seen with untreated SpS alone ($P > 0.1$). Furthermore, absorption of the anti-θ with BALB/c brain tissue, which is known to contain large amounts of the appropriate θ isoantigen (13), completely removed the inhibitory activity from the ascitic fluid, whereas anti-θ absorbed with AKR/N brain (data not shown) produced an effect identical with that of unabsorbed anti-θ. The activity of the anti-θ, therefore, appeared to be due to antibodies reactive with thymus-derived cell antigens, not to antibodies of other specificities that may be present in such a preparation (19–22).

DISCUSSION

It is well established that cell populations from different lymphoid tissues have been heterogeneous, but characteristic, migratory patterns (23–28). Lance and Taub (27, 28) have shown that reproducible percentages of ^{51}Cr-labeled lymph node lymphocytes injected into either normal or irradiated syngeneic recipients can be found 24 h later in both the lymph nodes and spleen. If these populations are then inoculated in a secondary syngeneic recipient, the original lymph node-seeking cells "home" in even greater proportion to lymph nodes, as do the spleen cells to the secondary host's spleen. These migratory patterns most probably do not reflect an absolute tendency for a given cell type to localize in a specific organ, but rather, relative recirculatory rates; that is, at any given time there is a greater probability of recovering cells belonging to the rapidly recirculating pool from lymph nodes than from spleen. As Lance and Taub (27) point out, the mechanism(s) governing the specificity of "homing" is unknown; such characteristics as specific membrane structures and/or affinity for postcapillary venular endothelium in lymph nodes (23) may well be involved.

In spite of our relative ignorance of the specific mechanism involved, the phenomenon of homing in syngeneic recipients provides a useful tool for studying lymphocyte heterogeneity. The most common application has been the direct assessment of the distributional characteristics of different lymphoid populations, i.e., the definition of populations according to their migratory patterns (27, 28). A second and to date less frequently used approach employs migration as a means of fractionating a population of lymphoid cells in order subsequently to test the resultant subpopulations for their relative functional activities.

Lance and Taub (27) reported that lymph node-seeking thymocytes, when compared with the original thymocyte suspension, appeared to have an increased capacity, on a cell for cell basis, to cause graft-*versus*-host disease. Cantor (29) found that the capacity to amplify an antihapten antibody response in adoptive hosts was significantly greater, on a cell for cell basis, for carrier-primed lymph node-seeking spleen cells than for the original carrier-

primed spleen cell population. Stobo and Paul (30) have very recently shown that lymph node-seeking spleen cells differ from normal spleen cells in their relative capacities to be stimulated in vitro by the mitogens phytohemagglutinin and concanavalin A.

Studies of the phenomenon of synergy among certain populations of parental strain lymphoid cells in the production of GVH reactions (1–5) have yielded data consistent with the idea that optimal expression of such reactions may involve some sort of interaction between at least two thymus-derived cells. One cell is present in high concentrations in tissues rich in rapidly recirculating lymphocytes, such as peripheral blood and lymph nodes; such cells demonstrate a high degree of sensitivity to in vivo treatment with heterologous ATS. A second population appears to be relatively more sessile, since it is found in higher concentrations in such tissues as spleen and thymus; these cells appear to be relatively more resistant to the effects of ATS. These differences in distribution and ATS sensitivity are compatible with the idea that the relevant populations might have different migratory patterns in syngeneic recipients.

The migration model employed in these studies offered an opportunity to test the hypothesis, on the basis of a simple two-cell model, that the intrinsic GVH reactivity of a normal lymphoid tissue is the result, at least in part, of an interaction among subpopulations with different migratory patterns. The results reported here are consistent with this hypothesis. The lymph node-seeking and spleen-seeking subpopulations of normal spleen cells when tested alone each displayed impaired GVH reactivities compared with the reactivity of the population from which they were derived; each of the subpopulations had dose-response lines significantly flatter than those seen with normal spleen cells (or with any normal lymphoid tissue previously tested [1–3, 5]).[2] Appropriate mixtures of the two subpopulations (in this case a constant but arbitrary ratio of 1 part LNS to 5 parts SpS), however, completely and dramatically restored the slope of the resultant reactivity line to a slope characteristic of the normal parent population. Clearly the reactivity of the mixtures was not explained by simple addition of the reactivities of the components of the mixture measured separately; such additive reactivity should have resulted in a dose-response line with a slope intermediate between those observed with either LNS or SpS alone.

This synergistic reactivity was not explained by the contribution, either immunologically specific or nonspecific, of the residual, perhaps radioresistant, host cells known to comprise approximately one-third of the cells in suspensions of either LNS or SpS. Furthermore, the experiments utilizing combinations of LNS and SpS in which either one of the populations consisted of cells syngeneic to the neonatal F_1 recipients indicated that in this model, as in the ones previously described (1–4), both populations in a mixture had to have the capacity to recognize foreign antigens in the F_1 recipient in order for synergy to

be observed. Finally, if either of the LNS or SpS populations in a potentially synergistic mixture was treated in vitro with anti-θ and complement, no evidence of synergy was subsequently observed. This inhibitory capacity of the anti-θ was completely removed by prior absorption with an appropriate mouse brain homogenate. Thus in both of these subpopulations, the relevant cell type(s) that was responsible for the observed synergistic GVH reactions was thymus-derived.

The finding of very flat dose-response lines for either LNS or SpS when tested alone is compatible with a two-cell model in which each of these populations has been relatively depleted of one of the thymus-derived cells necessary for full expression of GVH reactivity. Mixtures of the two populations would then result in a more optimal ratio of the two cell types, with the resultant steepening of the observed dose-response line. Such steepening of the cell dose-response line in the antibody response to sheep erythrocytes has been observed in interactions among thymocytes and bone marrow cells (31) and in interactions among glass-adherent and nonadherent cells (32).

On the other hand, both the marked flatness of the dose-response lines for LNS and SpS tested separately and the finding that these lines were definitely not parallel to each other were not really anticipated. All previous studies testing the separately measured reactivities of normal lymphoid cell populations such as PBL and thymocytes had resulted in parallel and much steeper dose-response lines, even though appropriate mixtures of these suspensions gave reactions consistent with the idea that each of them had a relative excess of a complementary thymus-derived cell subpopulation. The shallow dose-response lines observed with LNS or SpS alone might be at least partially the result of "suppressor" cells derived from the donor inoculum (32–37). No such suppressive activity was demonstrated in cell populations derived from irradiated unreconstituted mice. The possibility that irradiated hosts release suppressive humoral (serum) factors must also be considered. This seems rather unlikely in view of the striking synergy observed when the LNS and SpS subpopulations were recombined.

The relationships between the subpopulations derived in the present model and those utilized in previous studies (1–5) remain to be elucidated. It is, of course, tempting to speculate that LNS are the equivalent of normal PBL or LN and that SpS are the equivalent of normal thymocytes or spleen cells from ATS-treated donors.

Previous studies of synergy in GVH reactions employed the basic design of first establishing parallel dose-response lines for various normal lymphoid tissues and then demonstrating the superadditive reactivity of appropriate mixtures. This could not be explained adequately by a single-cell model in which the difference in reactivities among normal tissues was simply a reflection of differences in the concentrations of this active cell. The results suggested a

more complex model in which the normal tissues were functionally heterogeneous.

The present experiments have provided a more explicit demonstration of such functional heterogeneity within the pool of thymus-derived cells present in a single normal lymphoid tissue. These subpopulations have different migratory patterns and/or rates of recirculation in irradiated syngeneic hosts. Either subpopulation by itself appears markedly deficient in its ability to provoke a GVH reaction, but appropriate mixtures of the two restore the reactivity line to a slope characteristic of the normal population from which the subpopulations were derived. These results offer compelling evidence that the normal expression of GVH reactivity of a given normal lymphoid tissue involves an interaction among distinct subpopulations of thymus-derived cells.

SUMMARY

Spleen cells from normal adult mice were injected into lethally irradiated adult syngeneic recipients. 24 h later, cell suspensions were prepared from the recipients' spleens or peripheral lymph nodes and tested either alone or combined for their capacity to elicit graft-*versus*-host (GVH) reactions in neonatal F_1 recipients, using the Simonsen spleen weight assay.

Either the lymph node-seeking subpopulation or the spleen-seeking subpopulation alone was markedly deficient in its ability to provide a GVH reaction when compared with the normal population from which it was derived. However, an appropriate mixture of the two had a reactivity characteristic of the parent population. Both subpopulations were sensitive to treatment with anti-θ antibody and complement in vitro.

These results provide a convincing demonstration of the functional heterogeneity within the pool of thymus-derived cells present in a single normal lymphoid tissue. They strongly suggest that the normal expression of GVH reactivity of such a tissue involves an interaction among distinct subpopulations of thymus-derived cells.

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