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

II. Synergy in Graft-Versus-Host Reactions Produced by Balb/c Lymphoid Cells of Differing Anatomic Origin

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(Received for publication 28 August 1969)

The preceding paper (1) describes an example of synergistic interaction between two cell populations that mediate a cellular immune reaction, the graft-vs.-host (GVH) reaction. Mixtures of spleen cells obtained from NZB/Bl mice at two different stages of an autoimmune disease were shown to produce GVH reactions far greater than could be accounted for by summation of the separate activities of either population (1). The demonstration that the GVH activity of a weakly reactive lymphoid cell population could be enhanced by addition of appropriate numbers of cells obtained from lymphoid tissue comparatively rich in GVH activity suggested that at least two cell types were required to effect a GVH reaction. This hypothesis would be strengthened considerably if synergy could be demonstrated using cells from mice without disease. To this end, the GVH activity of different combinations of lymphoid cells from Balb/c mice was examined.

Previous studies from this laboratory have shown that cells from different lymphoid tissues of mice possess substantially different GVH activity (2). Further, it is known that the GVH activity of lymphoid cells obtained from mice soon after birth (3, 4) or from adult mice that have undergone neonatal thymectomy (5) is markedly reduced or absent. In the present study, appropriate proportions of adult spleen and femoral lymph node cells are shown to act synergistically with thymus cells and with spleen cells from neonatally thymectomized animals. In one case, two populations of lymphoid cells that produced no detectable reactions when injected separately in large numbers were able to produce significant GVH reactions when combined.

Materials and Methods

Animals.—1-wk old and adult (10–20-wk old) male Balb/cAnN (H-2^d) mice and litters of 1–5-day old F_1 hybrid Balb/cAnN by C57BL/6N (H-2^d/H-2^b) mice were obtained from the Rodent and Rabbit Production Section, National Institutes of Health, Bethesda, Md.

Graft.-vs.-Host Assay.—A modification of the spleen assay described by Simonsen (6) was performed. Suspensions of spleen, thymus, and femoral lymph node cells were obtained from adult Balb/c mice as previously described (2). 1–5-day old F_1 hybrid recipients were injected with different doses of cells 9 days before the spleen weight assay was performed. Descriptions of the assay and methods for calculation of GVH reactivity curves are given in the preceding paper (1).

Thymectomy.—Thymectomy was performed at 3 days of age through a mid-sternal incision as described previously (7). Half of each litter underwent thoracotomy but not thymectomy (sham-thymectomy); these served as controls. When thymectomized animals were killed, the mediastinum was examined macroscopically, and in some cases microscopically, to determine whether thymic remnants were present. A small number of mice were found with such remnants and discarded.

RESULTS

Standard Curves of GVH Reactivity for Spleen, Thymus, and Femoral Lymph Node Cells.—Spleen cells from 12-wk old Balb/c mice were injected intraperitoneally into litters of Balb/c × C57BL/6 F_1 hybrid recipients in doses of 2.5, 5, and 10×10^6 cells. Spleen indices were determined in recipient mice 9 days after inoculation and plotted against the logarithm of the number of cells inoculated (Fig. 1). Recipient spleen indices were also calculated in litters that had received from 0.5×10^6 to 2.0×10^6 femoral lymph node cells and 5×10^6 to 20×10^6 thymus cells. Cells obtained from thymus tissue were found to contain substantially less GVH reactivity than either spleen or femoral lymph node cells; at any cell dose, thymus cells contained approximately 20% of the reactivity of spleen cells, and 5% of the reactivity of femoral lymph node cells.

GVH Reactivity of Spleen-Thymus Mixtures and Femoral Lymph node-Thymus Mixtures.—0.5×10^6 femoral lymph node cells, able to initiate barely significant GVH reactions when injected alone, were combined with 4.5×10^6 thymus cells, which were inactive at this dose. Reactions equivalent to those produced with inoculations of 1.3×10^6 femoral node cells or 25×10^6 thymus cells were achieved with this mixture (Fig. 2). When 1×10^6 femoral lymph node cells were added to 4×10^6 thymus cells, reactions were produced that were quantitatively similar to those seen with 3.0×10^6 femoral lymph node cells. Moreover, inocula of spleen cells that were too small to initiate significant GVH reactions were shown to confer reactivity on otherwise unreactive amounts of thymus cells (Fig. 3). Thus, 1×10^6 spleen cells combined with 4×10^6 thymus cells produced spleen indices in recipient F_1 hybrid mice that were usually seen after 2×10^6 spleen cells had been injected.

In order to test a population of lymphoid cells that possessed no inherent GVH reactivity, spleen and thymus cells were obtained from 1-wk old Balb/c mice. No significant reactivity was apparent when these "immature" cells were injected into F_1 hybrid litters in numbers as great as 35×10^6 cells (Table I). Although neither cell population could produce significant reactions separately, even when exceedingly large doses of cells were used, when 10×10^6 cells

from each of these inactive populations were combined, recipients consistently evidenced significant GVH activity. Table I summarizes these data, as well as those obtained using mixtures composed of different proportions of immature spleen and thymus cells.

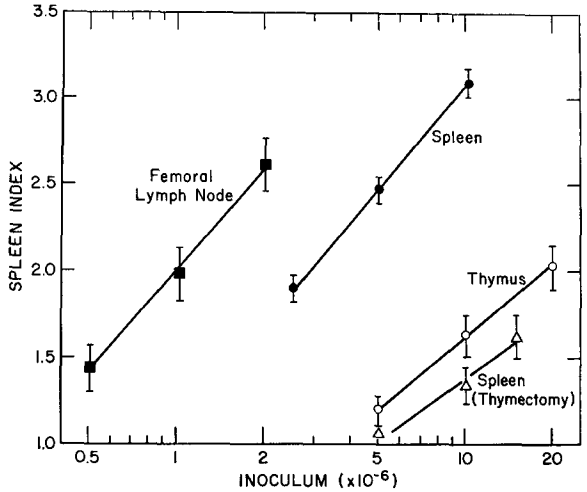

FIG. 1. Graft-vs.-host reactivity of femoral lymph node, spleen, thymus and spleen cells from thymectomized animals. Graft-vs.-host reactions that resulted from the injection of adult Balb/cAnN lymphoid cells of differing anatomic origins into 1–5-day old Balb/c by C57BL/6 F_1 hybrid recipients. The spleen index (ordinate) is a measure of the splenomegaly induced by the grafted cells; it was obtained by dividing the spleen-to-body weight ratio of the injected animals by the spleen-to-body weight ratio of the uninjected littermates. Spleen indices greater than 1.3 are regarded as indicative of significant splenomegaly ($P < 0.01$). Reactivity curves of femoral lymph node (■), spleen (●), and thymus (○) cells are shown; each point represents the mean value obtained from 6–14 recipient litters, with vertical bars denoting the limits of one standard error. Spleen cells from 10-wk old Balb/c donors that had undergone thymectomy 3 days after birth showed markedly reduced activity (△); each point on this curve represents mean values from 3 recipient litters. No signs of overt runting were noted in any of the recipient litters.

GVH Reactivity of Spleen Cells from Neonatally Thymectomized Mice.— Spleen cells from 10-wk old Balb/c mice that had been thymectomized 3 days after birth were injected into F_1 recipient mice. These cells were approximately eight times less reactive than spleen cells from control, sham-thymectomized mice (Fig. 1). Spleen cells from mice that had undergone sham-thymectomy (thoracotomy) were exactly as reactive as those from normal animals (Fig. 1). When 4×10^6 spleen cells from thymectomized animals were combined with 1×10^6 control spleen cells (see Fig. 4), the mixture produced reactions equivalent to those that would have been obtained had the inoculum contained almost

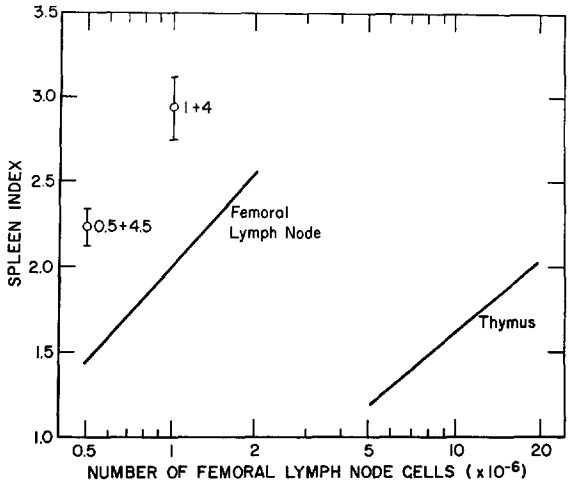

FIG. 2. Graft-vs.-host reactivity of mixtures of femoral lymph node and thymus cells. Standard curves of reactivity for femoral lymph node cells and thymus cells are shown. When 4.5 × 10^6 thymus cells were added to 0.5 × 10^6 femoral lymph node cells, spleen indices equivalent to those produced by 1.3 × 10^6 femoral lymph node cells alone were obtained. When 4 × 10^6 thymus cells were added to 1 × 10^6 femoral lymph node cells, reactions quantitatively similar to those seen with 3 × 10^6 femoral lymph node cells were produced. Reference to the standard curve for thymus cells shows that 5 × 10^6 thymus cells were insufficient in number to produce significant reactions. The two points shown above represent data obtained from 14 recipient litters; vertical bars denote the limits of one standard error.

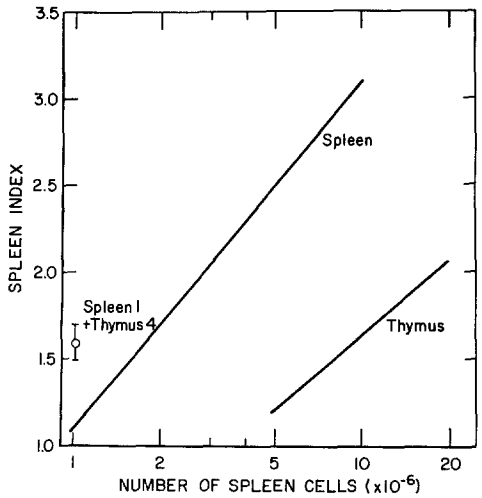

FIG. 3. Graft-vs.-host reactivity of mixtures of spleen and thymus cells. The mean spleen index produced by inocula composed of 1 × 10^6 spleen cells and 4 × 10^6 thymus cells is shown; vertical bar indicates the limits of one standard error. Reference to the two standard curves shows that neither component of the mixture can produce significant reactions separately. The reactivity of this mixture is equivalent to that obtained with almost 2 × 10^6 spleen cells.

3×10^6 control spleen cells or 22×10^6 spleen cells from the thymectomized mice. Reference to Fig. 1 shows that neither dose of these cell populations separately was capable of producing significant reactions. When the propor-

TABLE I

Graft-vs.-Host Reactivity of Thymus and Spleen Cells from 1-Wk Old Balb/c Mice

Number of immature spleen cells	Number of immature thymus cells	Individual spleen indices	Mean spleen index
(*a*)	35×10^6	1.29, 1.27, 1.17, 1.20, 1.02	1.19 ± .05
	25×10^6	1.08, 1.04, 1.11, 1.10, 1.18, 1.15, 1.20, 1.06, 1.08	1.11 ± .02
	20×10^6	1.10, 1.21, 1.47, 1.18, 1.18, 1.76, 1.23, 0.87, 1.21, 1.13, 1.11, 1.14, 1.13, 1.18, 1.19, 1.12, 1.16, 1.32	1.21 ± .04
	10×10^6	1.06, 1.04, 1.04, 1.10, 1.01, 1.16 1.23, 1.03, 1.07	1.07 ± .03
(*b*) 30×10^6		0.96, 1.06, 1.14, 1.06, 1.08, 0.99, 0.98	1.04 ± .03
20×10^6		1.17, 1.34, 1.21, 1.20, 1.08, 0.93	1.16 ± .06
10×10^6		1.02, 1.05, 0.89, 1.23, 0.97, 1.07, 1.69	1.13 ± .10
(*c*) 10×10^6	10×10^6	1.77, 1.67, 1.52, 1.58, 1.47, 1.34, 1.74, 1.87, 1.54, 1.34, 1.45, 1.42, 1.37, 1.36, 1.29, 1.39, 1.46, 1.63, 1.41, 1.48, 1.59, 1.32, 1.89, 1.04, 1.35, 1.76, 1.19, 1.50, 1.67, 1.19, 1.50, 1.28, 1.44, 1.63, 1.52, 1.33, 1.48, 1.24, 1.85, 1.47, 1.18, 1.29, 1.12, 1.50, 1.38, 1.55	1.47 ± .03
10×10^6	5×10^6	0.94, 1.15, 1.17, 1.19, 1.15, 1.14, 0.91, 0.99	1.08 ± .04
5×10^6	15×10^6	1.22, 1.67, 1.49, 1.93, 1.91, 1.38	1.59 ± .10
5×10^6	5×10^6	1.27, 1.11, 1.11	1.16 ± .05

Spleen indices produced in individual F_1 hybrid recipients are shown for (*a*) inocula of immature thymus cells, (*b*) inocula of immature spleen cells, and (*c*) inocula composed of mixtures of immature thymus and spleen cells in different proportions. Spleen indices greater than 1.30 indicate significant GVH reactions.

tions of spleen cells from thymectomized and sham-thymectomized mice in the mixture were reversed, no synergy was noted. Indices obtained with this mixture (1×10^6 spleen cells from thymectomized mice + 4×10^6 spleen cells from sham-thymectomized mice) were identical to those usually produced using 4×10^6 spleen cells from sham-thymectomized mice (Fig. 4). When only

1×10^6 spleen cells from sham-thymectomized mice were added to 1×10^6 cells from thymectomized animals, reactions were produced equivalent to those obtained with 2×10^6 control spleen cells from sham-thymectomized mice.

In another experiment, thymus cells from adult Balb/c mice were combined with spleen cells from neonatally thymectomized mice. When 1×10^6 thymus cells were combined with 4×10^6 spleen cells from thymectomized mice,

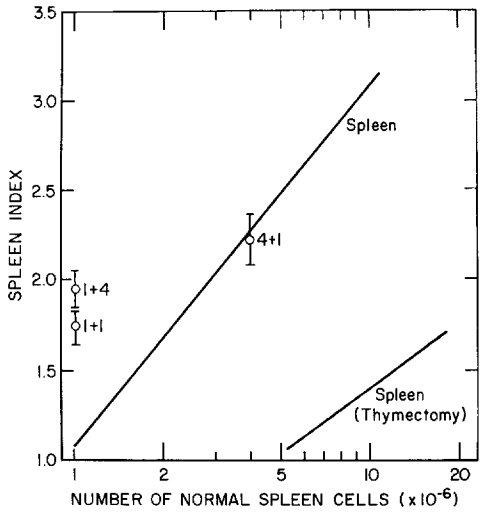

FIG. 4. Graft-vs.-host reactivity of mixtures of spleen cells from thymectomized and normal mice. Spleen cells obtained from Balb/c mice that had been thymectomized 3 days after birth possessed impaired GVH activity when compared with those from sham-thymectomized control littermates. Mixtures composed of small numbers of control spleen cells (1×10^6) combined with equal numbers of spleen cells from thymectomized mice (1 + 1) or in a 1:4 ratio (1 + 4) showed markedly enhanced reactivity. However when the latter ratio was altered so that the mixture was composed of 4×10^6 control spleen cells and 1×10^6 thymectomized spleen cells from thymectomized mice (4 + 1), spleen indices were produced that were normally seen with 4×10^6 control spleen cells alone. The mean spleen index for each mixture represents 3–4 recipient litters; vertical bars denote the limits of one standard error.

reactions were produced that were similar to those obtained had the inocula been composed of 9×10^6 spleen cells from thymectomized mice (Table II). When the proportions between the two cell populations were inverted, so that 4×10^6 thymus cells were combined with 1×10^6 spleen cells from thymectomized mice, the resulting mixture produced no significant GVH reactions.

Genetic Requirements for Synergy.—In order to test whether genetic incompatibility of both synergizing cell populations was a prerequisite for synergy, one cell population was obtained from donors that were genetically incapable

TABLE II

Graft-vs.-Host Reactivity of Spleen Cells Obtained from Neonatally Thymectomized Mice Alone and in Combination with Adult Thymus Cells

Number of thymectomized spleen cells	Number of thymus cells	Individual spleen indices	Mean spleen index
9×10^6		2.03, 1.52, 1.43, 1.72, 1.91, 1.93	1.76 ± .10
	5×10^6	1.30, 1.25, 1.23, 0.90, 1.21, 1.15, 1.09, 1.50, 1.07, 1.07, 1.00, 1.20, 1.22, 1.46	1.19 ± .05
4×10^6	1×10^6	2.38, 1.70, 1.60, 1.60, 1.57	1.77 ± .15
4.5×10^6	4.5×10^6	1.34, 1.60, 1.39, 1.43, 1.51, 1.65 2.03, 3.19	1.77 ± .22
1×10^6	4×10^6	1.13, 1.50, 1.36, 1.80, 1.13, 1.19 0.70	1.26 ± .13

TABLE III

Failure of Combinations of F_1 and Parental Strain Cells to Interact Synergistically in GVH Reactions

	Balb/c Donor	Balb/c by C57BL/6 donor	Individual spleen indices	Mean spleen index
(*a*)		10×10^6 spleen cells	1.01, 1.10, 0.97, 1.07, 1.20, 1.01, 1.08, 1.06	1.06 ± .03
		10×10^6 thymus cells	1.09, 0.96, 0.90, 1.18, 1.21, 1.11, 1.06, 1.04	1.07 ± .04
	1×10^6 spleen cells		1.10, 1.19, 1.21, 1.06, 1.10	1.13 ± .03
	0.5×10^6 femoral lymph node cells		1.30, 1.20, 1.36, 1.16	1.26 ± .05
(*b*)	1×10^6 spleen cells	4×10^6 thymus cells	1.06, 1.10, 1.16, 1.26, 1.28, 1.31	1.20 ± .04
	0.5×10^6 femoral lymph node cells	4.5×10^6 thymus cells	1.09, 1.24, 1.22, 1.04, 1.06, 1.11, 1.08, 1.18, 1.01	1.11 ± .03
	4.5×10^6 thymus cells	0.5×10^6 femoral lymph node cells	0.86, 1.06, 1.10, 0.90, 1.35, 0.73, 0.94, 0.96, 1.08, 1.02, 1.20, 1.10	1.03 ± .05

(*a*) Graft-vs.-host reactions produced by thymus, spleen, and femoral lymph node cells obtained from adult (12-wk old) F_1 hybrid Balb/c by C57BL/6N donors and Balb/c donors grafted to newborn C57Bl/6 recipients are summarized. (*b*) Reactions produced by mixtures of F_1 hybrid and Balb/c lymphoid cells on C57BL/6N recipients are shown.

of responding to recipient antigens. Substantial numbers of thymus and spleen cells from Balb/cAnN by C57BL/6N F_1 hybrid adult donors (12-wk old) produced no significant GVH reactions in C57BL/6N newborn recipients, as expected (Table III). When 1×10^6 Balb/c spleen cells were combined with 4×10^6 F_1 hybrid thymus cells, no reaction in C57BL/6N newborn recipients was detectable, in contrast to the synergy observed when both these cell populations had been obtained from Balb/c donors. In another experiment, Balb/c peripheral lymph node cells were included with F_1 hybrid thymus cells; these mixtures produced no reactions, in contrast to identical numbers of peripheral node and thymus cells obtained from Balb/c donors. The reciprocal experiment, combining femoral lymph node cells from F_1 hybrids and thymus cells from Balb/c donors, was also negative (see Table III).

TABLE IV

Graft-vs.-Host Reactivity of Mixtures of Adult Balb/c femoral Lymph Node Cells and Adult C57BL/6N Thymus Cells in F_1 Hybrid (Balb/c by C57BL/6N) Newborn Recipients

Numbers of Balb/c femoral lymph node cells	Numbers of C57BL/6N thymus cells	Individual spleen indices	Mean spleen index
0.5×10^6		1.37, 1.03, 1.09, 1.40, 1.27, 1.55, 1.22, 0.98, 2.32, 1.99, 1.57, 1.37, 0.74, 0.90, 1.78	1.44 ± .10
	10×10^6	1.81, 1.08, 1.02, .091	1.21 ± .20
0.5×10^6	4.5×10^6	3.55, 3.24, 2.72, 2.24, 2.11, 2.36, 2.78, 2.75, 2.47, 2.29, 2.11	2.60 ± .14

Finally, mixtures of femoral lymph node cells obtained from Balb/c mice and thymus cells from C57BL/6N donor mice produced reactions in Balb/c by C57BL/6N newborn recipients far in excess of those anticipated from summation of the separate activities of the two cell populations (Table IV). When 0.5×10^6 Balb/c femoral lymph node cells were combined with 4.5×10^6 C57BL/6N thymus cells (which were separately unreactive), reactions were produced equivalent to those seen with inoculations of almost 2.0×10^6 Balb/c femoral lymph node cells.

DISCUSSION

The importance of interaction among lymphoid cells in the development of humoral immune responses is well established. Claman has shown that mixtures of cells from bone marrow and thymus, and of cells from spleen and thymus, interact in lethally irradiated syngeneic hosts to produce a hemolysin response to sheep erythrocytes substantially in excess of the sum of the separate responses of these cell populations (8). Similarly, Miller and Mitchell and colleagues have shown synergy between thoracic duct and bone marrow cells

in the hemolysin response to sheep erythrocytes (9–11). Studies by Davies et al. have suggested that synergistic interaction between thymus and bone marrow may play a role in the development of immunologic memory (12). The present study extends the foregoing to include the graft-vs.-host reaction, a form of cellular immunity.

Synergy was usually demonstrated by combining cells from tissues comparatively rich in GVH activity, such as femoral lymph node and spleen, with cells from tissues with much less activity, such as thymus and spleens from immature or neonatally thymectomized mice. Proper adjustment of the ratio between the cell populations studied was essential for the demonstration of this synergy. Thus, spleen cells combined with thymus cells in a ratio of 1:4, or with spleen cells from thymectomized mice in the same ratio, produced about twice the expected reactivity. Increase in the proportion of spleen cells resulted in a loss of observable synergistic effect. This effect of ratio may explain the failure of synergy between spleen and thymus cells reported in another GVH system (13).

The observations may be explained by postulating that at least two cell types are required to mediate GVH reactions. The ratio between these subpopulations of GVH competent cells in different lymphoid tissues determines the degree of reactivity of that tissue. Weakly reactive tissues such as thymus would contain suboptimal ratios, while inactive tissues such as immature spleen and thymus contain ratios below the critical level for reactivity. Synergy is produced when two populations with complementary deficiencies are combined resulting in a ratio nearer the optimum for activity. The activity obtained by combinations of immature spleen and thymus cells, each inactive in large numbers when injected separately, is a most striking example of this complementary potential. This finding suggests that an important component in the ontogeny of GVH reactivity in such tissues as spleen might be the acquisition of cells derived from the thymus. Dissemination of cells of thymic origin, especially in the neonatal period, has been demonstrated by Weismann (14). Further weight is given to this possibility by the observation that relatively small numbers of thymus cells from adult animals can restore reactivity to hypoactive spleen cells obtained from adult, neonatally thymectomized mice.

Mitchell and Miller have proposed the existence of an "antigen reactive cell" (ARC) which performs the function of specifically identifying antigen (10), and an "antibody-forming cell precursor" which divides, differentiates, and gives rise to antibody-forming progeny after interacting with the ARC and antigen. On the other hand, studies of the "carrier" effect (15, 16) in the secondary response suggest that reactions can be enhanced by the presence of more than one specificity of reactive cell (17, 18). In the case of cellular immune phenomena, the nature of the effector cells—those cells which cause damage to tissue *without* antibody synthesis (19)—is not so precisely known, nor is the

means of effecting the damage. Although the small numbers of cells from tissues rich in GVH activity that are required for synergy suggest that they might be important for the recognition part of the response, the present data do not discriminate between a cooperative action between two cells or a sequential action in reacting to allogeneic tissue.

The failure of mixtures of parental strain and F_1 cells to react synergistically in the other parental strain suggests that active participation of both cell types is a requirement for achieving synergy. This was not a consequence of "allogeneic inhibition" (20) or other such factors which might prevent interaction between parental and F_1 cells, since cells from two parental strains, differing at the H-2 locus, interacted synergistically in F_1 recipients. These observations also make it very unlikely that the synergy described here is the result of provision of a nutritive effect on the reactive cells or the provision of "nonspecific" inflammatory cells (21, 22). The requirement that both cell populations be potentially reactive to achieve synergy is reminiscent of the findings of Green, Paul, and Benacerraf (23), who showed that sensitized lymphocytes from guinea pigs genetically disposed to respond to DNP conjugates of poly-L-lysine could not transfer delayed hypersensitivity to genetic nonresponders. In the present instance, it seems necessary for both cell populations to be geneticaly able to react to the recipient's tissue, a condition that is not fulfilled in the combination of F_1 donor and parental strain recipient.

It appears likely from the data presented here, and from the several examples of synergy in the adoptive transfer of the capacity to form antibody, that synergistic interaction among cells is an important component of immune responses of both cellular and humoral type. Although the mechanism by which synergy is achieved is obscure, the effect is clear—an amplification of reactivity. This amplification mechanism assures that by appropriate adjustments in ratios of reactive cells, optimum reactions can be obtained with minimum increases in the numbers of reactive cells.

SUMMARY

The capacity of cells from different lymphoid tissues obtained from Balb/c mice to produce graft-vs.-host (GVH) reactions was quantitatively determined in C57BL/6N by Balb/c F_1 hybrid recipients. Synergistic responses were observed when small numbers of cells from lymphoid tissues that were rich in GVH activity such as spleen and femoral lymph node were combined with weakly reactive thymus cells. Thymus and spleen cells obtained from 1-wk old mice were separately inactive but produced moderate GVH reactions when combined in equal proportions. GVH activity of spleen cells from mice thymectomized at 3 days of age was partially restored by the addition of small numbers of spleen or thymus cells from adult mice. Changes in ratio between the two cell populations markedly affected the degree of synergy. Synergy was not

observed when Balb/c cells were combined with Balb/c × C57BL/6N F_1 hybrid cells and inoculated into C57BL/6N recipients, but was demonstrated when Balb/c and C57BL/6N cells were combined and inoculated into F_1 recipients, indicating that a genetic disposition to mount GVH reactions in both populations is required to produce synergy. The data indicate that at least two cell types are necessary for GVH reactions, and that synergy between cell populations results from favorable adjustments in the ratio between these two cell types.

We are indebted to Mr. Britton H. Smith for performing thymectomies. We wish to thank Mr. Leslie C. Harne for his excellent assistance.

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