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

I. SYNERGY IN GRAFT-VERSUS-HOST REACTIONS PRODUCED BY
CELLS FROM NZB/Bl MICE

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When lymphoid cells from adult animals are injected into immature allogeneic or F₁ hybrid recipients, they produce graft-vs.-host (GVH) reactions and a wasting disease (1, 2). In the mouse this reaction is characterized by loss of body weight, splenomegaly, hepatomegaly, lymph node atrophy, and death (3). Splenomegaly, an early and constant finding, forms the basis of the spleen weight assay developed by Simonsen to measure the vigor of the GVH reaction in recipient mice (4).

Spleen cells from older NZB/Bl mice show an impaired ability to induce GVH reactions (5). After 4 months of age, these mice spontaneously develop Coombs' positive hemolytic anemia and other autoantibodies similar to those seen in certain human autoimmune diseases (6). Impairment of GVH activity (5) and the development of lymphoid neoplasms (7) have been shown to occur after the development of anemia and autoantibodies.

The reduced capacity of spleen cells obtained from older NZB mice to initiate GVH reactions may represent a decrease in the numbers of reactive cells present in the spleen. Alternatively, the accumulation of hematopoietic cells in the spleens of these older animals may result in a dilutional effect. In the present study, the ability of spleen cells from 3- and 12-month old NZB mice (without and with overt disease, respectively) to effect GVH reactions was quantitatively compared in C57BL/6N recipients, using the spleen weight assay. The impairment in GVH activity of cells from older mice could not be wholly accounted for by the dilutional effects of hematopoietic cells. Moreover, the activity of these "older" spleen cells could be fully restored by the addition of small numbers of spleen cells from young NZB mice, suggesting that the

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spleens of the older NZB animals are deficient in a cell population necessary for effecting GVH reactions.

Materials and Methods

Animals.—Donor 3-month old and 12-month old NZB/B1 mice (H-2^d) and litters of recipient C57BL/6N (H-2^b) and NZB/B1 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 was performed (8). Spleens from 3- and 12-month old NZB mice were removed and a cell suspension was prepared in Hanks' balanced salt solution. Doses of cells varying from 2.5×10^6 to 20×10^6 were injected intraperitoneally in a constant volume of 0.05 ml into newborn C57BL/6N mice; uninjected littermates served as controls. 9 days after grafting of cells, litters were sacrificed and a spleen weight to body weight ratio was determined for inoculated recipients and control littermates. An index of spleen enlargement was computed by dividing the spleen weight to body weight ratio of the injected animals by that of their untreated littermates. The mean spleen index for each cell dose was computed by averaging the mean indices obtained from each litter inoculated with that dose. As previously noted (1, 8), there was a linear relationship between the spleen index and the logarithm of the number of grafted cells. Standard reactivity curves were obtained in this manner for spleen cells from "young" (3-month old) and "old" (12-month old) NZB donors. Regression lines for each donor population were calculated by the method of least squares. Spleen indices greater than 1.3 were considered to represent significant GVH activity (4).

The difference in GVH reactivity between the old and young spleen cell populations was quantitated by dividing the number of cells from old mice necessary to produce a given spleen index by the number of cells from young mice needed to produce the same index. Since the regression lines were parallel, the ratio was constant for all cell doses on the linear portion of the curve. A similar formulation has been given by Simonsen (1). This is a standard biometric procedure (9) and has been used previously to measure both enhanced (10) and reduced (8) GVH activity.

Combined Cell Inocula.—Suspensions of old and young cells were combined in vitro in fixed proportions to give mixtures of known ratios. Combined cell populations were injected into newborn C57BL/6N mice in the same volume and route as described for the uncombined cell suspensions. In one experiment, old and young spleen cells were injected separately into the same recipients, 24 hr apart; these recipient mice were assayed 9 days after the first inoculation of cells.

Frozen and Thawed Cells.—Suspensions of old and young spleen cells in Hanks' balanced salt solution were frozen in an alcohol-dry ice bath and thawed. This procedure was repeated 3 times. Frozen and thawed old cells were then added to untreated young cells and inoculated into C57BL/6N recipients. Young cells that had been similarly frozen and thawed were also added to viable populations of old cells prior to inoculation into recipient mice.

RESULTS

Reactivity of Young Spleen Cells from 3-month Old NZB Mice.—Spleen cells from 3-month old NZB mice were injected in doses of 20×10^6 , 10×10^6 , 5×10^6 , and 2.5×10^6 into newborn C57BL/6N recipient animals. The resulting spleen indices were plotted against the logarithm of the number of inoculated cells (Fig. 1). The data is based upon inoculations of 30 litters of recipient mice (approximately 120 mice). No early runting was noted with inocula of 20×10^6 cells or less.

Reactivity of Old Spleen Cells from 12-Month Old NZB Mice with Enlarged Spleens.—NZB mice 1 yr or older had markedly enlarged spleens which weighed approximately 600 ± 200 mg as compared to a mean spleen weight of 150 ± 35 mg for 3-month old NZB animals. The average cell yield was 350×10^6 from each old spleen compared to 120×10^6 from the young spleens. When cells from old NZB mice were grafted to newborn C57 recipients, significant spleen indices were obtained with doses of 10×10^6 and 20×10^6 cells (see Fig. 1), but not with doses of 5×10^6 or less. The slope of the curve (spleen index

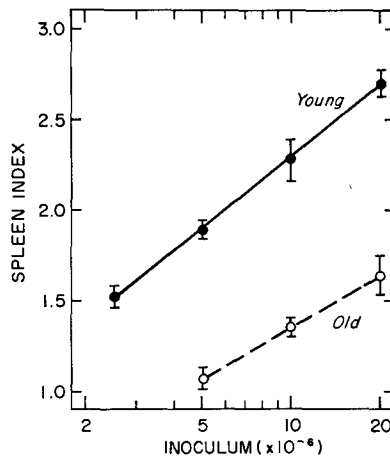


FIG. 1. Reactivity of young and old spleen cells. Mean spleen indices in litters of C57BL/6N newborn recipients after doses of 2.5×10^6 to 20×10^6 young spleen cells from 3-month old NZB donors (\bullet) are compared with indices produced by doses of old spleen cells obtained from 12-month old NZB donors (\circ). Each point represents the mean spleen index of 6–10 litters of C57BL/6N recipients; vertical bars represent the limits of one standard error. Spleen indices are plotted against the logarithm of the number of cells injected. Slopes of the two regression lines as calculated by the method of least squares were not significantly different. Since the reactivity lines are parallel, it can be seen that at all doses the young cells are approximately five times more reactive than the old cells.

against the logarithm of the cell dose) was approximately parallel to that obtained using young cells ($P > 0.10$). Thus, it would require between five and six times as many old NZB cells as young to produce a given spleen index.

Reactivity of Old Spleen Cells from 12-month Old NZB Mice with Unenlarged Spleens.—Occasional old NZB mice were found to have unenlarged spleens. Four unenlarged spleens were pooled and cells from this pool were grafted to newborn C57BL/6N recipients. Cells from these animals gave significant spleen indices at doses of 2.5×10^6 cells or greater (Fig. 2). Using the parallel line assay, it was calculated that these cells were approximately one-half as reactive as young cells, but 3.5 times more potent than cells from 12-month old mice with enlarged spleens.

In one experiment, old cells from one unenlarged spleen were pooled with old cells from four enlarged spleens prior to grafting. It was calculated that cells from the unenlarged spleen comprised 10% of the resulting suspension. To our surprise, the spleen indices obtained with this mixture in doses of 5×10^6 cells were almost identical to those seen when 5×10^6 unenlarged old spleen cells were used alone (Fig. 3).

Combined Inocula of Old and Young Spleen Cells.—This finding suggested that the activity of the weakly reactive old cells might be enhanced by mixture with a potent population of young cells. To test this possibility, equal numbers

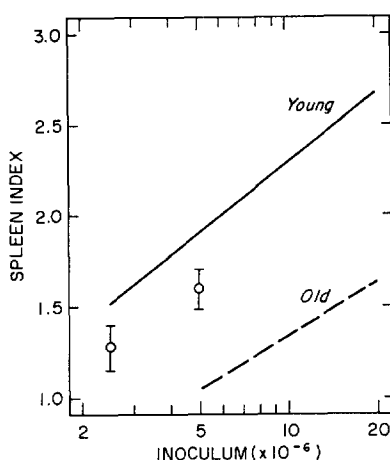


FIG. 2. Reactivity of old spleen cells from NZB mice with unenlarged spleens. Cells obtained from unenlarged spleens from 12-month old NZB mice were injected in doses of 2.5 and 5×10^6 cells into six litters of recipient C57BL/6N newborn mice. The mean spleen index for each dose is plotted against the logarithm of the number of cells injected (O). Standard curves of reactivity are shown for spleen cells obtained from young (3-month old) and old (12-month old) mice. The vertical bars represent the limits of one standard error.

of spleen cells from young and old NZB animals were combined at different dosages and injected into recipient C57BL/6N mice. When 1.25×10^6 , 2.5×10^6 , 5×10^6 , and 10×10^6 old cells were combined with equal numbers of young cells, the mixtures produced spleen indices that were approximately the same as those that would have been obtained had the inocula been composed entirely of young, active cells (Fig. 4). The numbers of old cells added to the inocula possessed minimal or no activity when injected alone (Fig. 1). Thus, when a total dose of 2.5×10^6 cells was used, neither the young nor the old cells in the mixture would have possessed demonstrable activity if injected separately. However, when injected together, reactivity equivalent to 2.5×10^6 young cells was achieved (see Fig. 4).

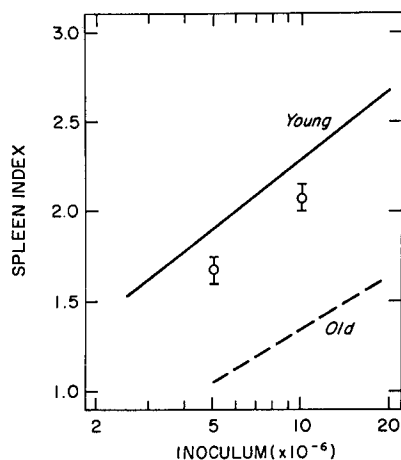


FIG. 3. Reactivity of cells from one unenlarged old spleen pooled with cells from four old enlarged spleens. Cells from the unenlarged old spleen comprised 10% of the mixture. Cell doses of 5×10^6 and 10×10^6 resulted in the mean spleen indices shown (O); vertical bars indicate the limits of one standard error. Each point represents data from four recipient litters. Young (—) and old (-----) spleen cell reactivity curves are included for comparison. Indices obtained with 5×10^6 cells from this pool were approximately equal to those obtained with cell suspensions composed wholly of cells from unenlarged spleens.

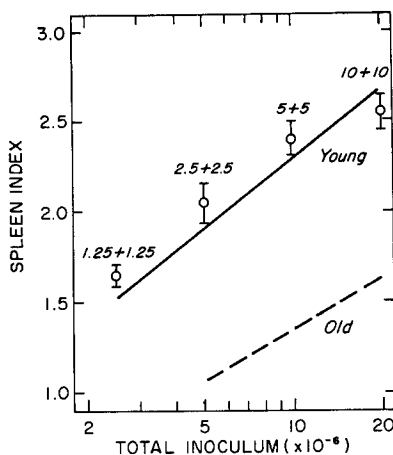


FIG. 4. Reactivity of mixtures of old and young spleen cells in equal numbers. Equal numbers of old and young cells were combined and injected into litters of newborn C57BL/6N recipients. Mean spleen indices (\pm one standard error) that were produced when 1.25, 2.5, 5.0, and 10×10^6 young cells were combined with equal numbers of old cells are shown. Each point represents data from 4-11 recipient litters. Standard curves of reactivity for young (—) and old (-----) spleen cells are shown. The mean spleen indices obtained with this mixture of cells are comparable to those produced had the inocula been composed wholly of young cells.

In additional experiments, the proportion of young cells in the combined inocula was progressively decreased, while the total cell number remained constant. In this way, the importance of changing the young to old cell ratio upon the degree of enhanced activity could be studied. Using a total inoculum of 5×10^6 cells, the number of young cells was progressively decreased from 3.5×10^6 to 1×10^6 (see Fig. 5). The inocula were made up to 5×10^6 cells by the addition of appropriate numbers of old cells. The numbers of old cells included in these inocula were insufficient to produce significant spleen indices when injected alone (Fig. 1). The inclusion of 2.5×10^5 to 1×10^6 young cells.

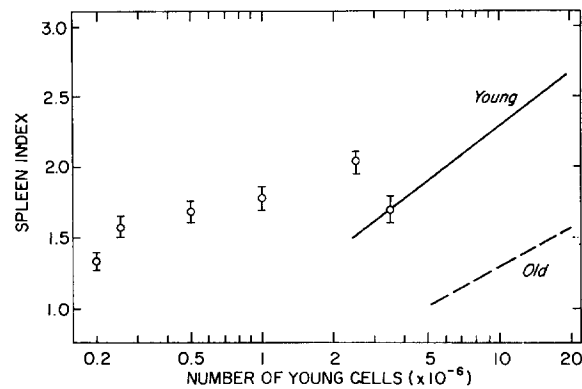


FIG. 5. Reactivity of mixtures of old and young spleen cells in various ratios in a total inoculum of 5×10^6 cells. Doses of young cells varying from 0.2×10^6 to 3.5×10^6 were made up to a total of 5×10^6 cells by the addition of appropriate numbers of old cells. The spleen indices obtained with these mixtures are plotted against the logarithm of the number of young cells included in each mixture (\circ). Each point represents the mean spleen index produced in four recipient C57BL/6N litters; vertical bars represent the limits of one standard error.

which also were inactive when injected separately, resulted in significant activity approaching that obtained with 5×10^6 young, active cells. Significant spleen indices were obtained when as few as 2×10^5 young cells were added to 4.8×10^6 old cells. However, when 10^5 young cells were added to 4.9×10^6 old cells, no significant activity was noted. When the ratio of young cells was increased to 70% of the total inoculum, indices fell to those normally seen with this number of young cells alone.

“Efficiency” of Synergy.—Reference to the standard curves of reactivity for the young and old cells (Fig. 1) permits calculation of the degree of enhanced activity obtained with combinations of the two cell populations at different ratios. These calculations are presented in Table I. Enhancement is described in terms of young rather than old cell activity, since this method avoids extrapolation of the standard curves. Since the curves for the two cell popula-

tions are parallel, the use of either method results in quantitatively identical "synergy factors". The amounts of young (y) and old (o) cells in each inoculum are indicated. The number of old cells is converted to the equivalent number of young cells (y_o) using the standard curves of reactivity. The total young cell strength in the inoculum (y_t) is obtained from the sum of y and y_o . The number of young cells normally required to produce the spleen indices obtained

TABLE I
The "Efficiency" of Synergy

Total dose $\times 10^{-6}$	y Number of young cells in inoculum $\times 10^{-6}$	o Number of old cells in inoculum $\times 10^{-6}$	y_o Young cell activity contained in old cells $\times 10^{-6}$	y_t Total young cell activity $\times 10^{-6}$	SI Observed mean spleen index	y_n Young cells normally required for this index $\times 10^{-6}$	y_n/y_t Efficiency of enhancement
2.5	1.25	1.25	0.25	1.50	1.62	3.0	2.00
	0.50	2.0	0.40	0.90	1.64	3.0	3.33
5.0	3.5	1.5	0.3	3.8	1.70	3.5	0.92
	2.5	2.5	0.5	3.0	2.05	6.2	2.06
	1.0	4.0	0.8	1.8	1.82	4.2	2.33
	0.5	4.5	0.9	1.4	1.66	3.1	2.21
	0.25	4.75	0.9	1.35	1.57	2.7	2.00
	0.20	4.80	0.9	1.10	1.30	2.0	1.81
10.0	0.10	4.90	0.9	1.00	1.07	—	No reaction
	5.0	5.0	1.0	6.0	2.39	11.5	1.91
	3.0	7.0	1.4	4.4	2.19	8.2	1.86
	1.0	9.0	1.8	2.8	1.79	4.0	1.42
	0.5	9.5	1.9	2.4	1.22	—	No reaction
20.0	10.0	10.0	2.0	12.0	2.57	16	1.33

This table shows the amount of enhancement above expected activity given by mixtures of young and old spleen cells. For convenience, all spleen cell numbers are converted to equivalent numbers of young cells by reference to the standard curves (see text).

When y_n/y_t is greater than 1.0, the reaction obtained with that ratio of young and old cells is greater than that expected from the sum of their separate reactivities.

with these mixtures (y_n) is compared to the young cell equivalents (y_t) actually present in the mixture of old and young cells. The efficiency of synergy (y_n/y_t) was substantially reduced as the total inoculum was increased from 2.5×10^6 cells. For doses of cells less than 10×10^6 , ratios of 20% young cells in the total inoculum produced the most efficient enhancement of activity. When larger inocula were used, the percentage of young cells in the mixture required for optimal enhancement was markedly increased.

Factors Affecting Synergy.—Injections of young and old cells were separated by 24 hr. In one case, 1×10^6 young cells were injected intraperitoneally into

newborn recipient mice, followed by the injection of 4×10^6 old cells 24 hr later. In a second experiment, old cells were injected 24 hr before young cells. These experiments resulted in significant GVH activity, comparable to that

TABLE II
The Failure of Delay Between Injections of Different Cell Populations to Affect Synergy

First injection	Second injection	Delay	Individual spleen indices	Spleen index
1×10^6 young cells	4×10^6 old cells	15 min	1.75, 2.43, 2.11, 1.93 1.81, 1.56, 1.49, 1.18	1.91 ± 0.18
4×10^6 old cells	1×10^6 young cells	15 min	2.26, 2.08, 2.52, 2.21	2.27 ± 0.09
1×10^6 young cells	4×10^6 old cells	24 hr	2.17, 2.02, 1.85, 1.61 1.52, 1.51, 1.45, 1.28	1.68 ± 0.11
4×10^6 old cells	1×10^6 young cells	24 hr	2.09, 2.20, 2.31	2.20 ± 0.06
Expected (1×10^6 young + 4×10^6 old cells injected simultaneously)				1.82 ± 0.07

The expected reactivity (bottom line) is obtained from the data shown in Fig. 5. Neither 1×10^6 young nor 4×10^6 old spleen cells produced detectable reactions when injected separately. Spleen indices measure recipient spleen enlargement, characteristic of the GVH reaction in mice, compared with spleens of uninjected littermates. Indices greater than 1.3 indicate significant GVH reactions.

TABLE III
The Requirement For Viable Spleen Cells

Spleen cell inoculum ($\times 10^{-6}$)			Mean spleen index of individual litters			Mean
Young	Old	Total				
5	5	10	2.40	2.82	2.41	2.54 ± 0.13
5	5 frozen and thawed	10	1.75	2.13	1.53	1.80 ± 0.18
5 frozen and thawed	5	10	1.20	1.15	0.99	1.11 ± 0.06

No enhanced reactivity was obtained if either population was killed by freezing and thawing 3 times. The mean spleen index shown on line 2 (1.80) is the index given by 5×10^6 young cells alone (see Fig. 1).

obtained with this ratio of young and old cells injected simultaneously in the same syringe (Table II).

Suspensions of old and young cells were separately frozen and thawed three times. When 5×10^6 frozen and thawed young cells were mixed with 5×10^6 untreated old cells, no significant GVH activity was obtained (Table III).

In addition, when 5×10^6 frozen and thawed old cells were combined with 5×10^6 untreated young cells, the mixture produced reactions equivalent to that obtained using inocula of 5×10^6 young cells alone.

In order to rule out the possibility that lymphoid cells from the old and young NZB mice were reacting to each other in the recipient animal and somehow producing splenomegaly as a result of "autoimmunity", litters of NZB mice 1-5 days old were used as recipients. Injections of spleen cells from old and young animals in doses as high as 30×10^6 did not cause significant splenomegaly (Table IV).

TABLE IV
Failure of NZB Spleen Cells to Produce GVH Reactions in NZB Recipients

Spleen cell inoculum ($\times 10^6$)		Spleen indices in individual mice	Mean
Young	Old		
—	30	1.19, 0.60, 0.69, 0.84, 0.87, 1.49, 0.90	0.94 ± 0.12
30	—	1.01, 1.13, 0.90, 0.96, 1.20	1.04 ± 0.06
15	15	1.10, 1.16, 1.01, 1.26, 0.14, 0.86	1.06 ± 0.06

Neither old nor young cells inoculated into 6-day old NZB/B6 recipients produced detectable splenomegaly. In addition, a 1:1 mixture of the two populations produced no detectable reaction.

DISCUSSION

Spleen cells from 1-yr old NZB mice have a decreased ability to induce GVH reactions when grafted to newborn C57BL/6N recipients. From purely quantitative considerations, it is unlikely that this reduced reactivity is the result of dilution with hematopoietic or other cell types. Suspensions prepared from old spleens contained only 3 times as many cells as did those from young spleens, while the activity was reduced 5-6-fold. More important, the finding that old cells can be restored to normal or nearly normal activity by addition of young cells suggests that there is a deficiency in the old spleens of a specific population of cells necessary for production of GVH reactions.

The addition of 1×10^6 young cells, which were unable to produce significant GVH reactions when injected alone, to 4×10^6 old unreactive cells, resulted in spleen indices comparable to those obtained with inocula of 5×10^6 young cells. The addition of still smaller numbers of young active cells to an otherwise unreactive population of old spleen cells rendered the mixture immunologically reactive. The most efficient percentage of young cells, using doses of 5×10^6 cells or less, was 20%. Further reduction of the percentage of young cells in the total inoculum resulted in gradual diminution in the degree of enhanced activity, until the minimum number of young cells necessary to confer any measurable activity was reached. These ratio experiments suggest that relatively small

numbers of young active cells are sufficient to confer activity approaching normal upon otherwise unreactive old cells. This cooperative effect was observed when the two populations were injected separately into recipient mice 24 hr apart, and therefore was not dependent upon cell mixing *in vitro*. No synergy was demonstrated if either population of cells was killed by repeated freezing and thawing, indicating that both cell populations actively participate in the reaction.

When the inoculum was 10×10^6 cells or greater, the percentage of young cells in the mixture required for activity was greater than that needed when the total cell dose was less than 10×10^6 cells. If activation of old cells is the result of a cell-to-cell interaction, this concentration effect is puzzling, unless there exists a "space" problem so that only a limited number of donor cells will arrive at strategically significant sites to produce splenomegaly. This decline in the efficiency of synergy seen with increasing numbers of grafted cells is reminiscent of the diminishing effects obtained with increasing numbers of cells in the GVH reaction described by the relationship of spleen index to the logarithm of the number of grafted cells.

It is reasonable to postulate that the population of old spleen cells is deficient in a cell type necessary for initiating the GVH reaction. The addition of this cell type, even in small numbers, to unreactive old cells results in enhanced GVH reactivity of the combined inoculum. These cells present in young spleens, may act in concert with old cells to effect a reaction or may perform a separate specific function necessary for the GVH response, such as the recognition of foreign antigens. By combining populations of spleen cells of differing reactivity, we have favorably altered the ratio of different populations of cells participating in the GVH response and thereby increased the efficiency of the reaction; synergy has been the result.

These experiments have demonstrated synergy using spleen cells from NZB mice at two different stages of an autoimmune disease. This phenomenon is not restricted to cells from diseased animals. The accompanying report (11) describes several examples of synergy in the GVH reaction between different populations of lymphoid cells obtained from Balb/c mice. The relationship of synergy in GVH reactions to other instances of cell-to-cell interaction in immune responses (12-16) will be discussed in that paper.

SUMMARY

The ability of spleen cells from young (3 month) and old (1 yr) NZB mice to induce GVH reactions in newborn C57BL/6N mice was compared quantitatively using the Simonsen spleen assay. Young NZB cells were five times more reactive than cells from older mice. The minimum number of cells producing detectable reactions was 2×10^6 for the young and 10×10^6 for the old. Young and old cells combined and injected together produced GVH reactions quantitatively similar to those obtained with inocula composed of young cells alone.

Mixtures of two cell populations producing no detectable reactions when injected separately into different recipients (1×10^6 young cells and 4×10^6 old cells) produced reactions approximately equal to those obtained with 5×10^6 young cells. As few as 0.25×10^6 young cells were sufficient to effect a reaction when combined with 4.75×10^6 old unreactive cells. Viability of both cell populations was essential for GVH reactivity. This evidence of synergy in GVH reactions indicates that old NZB spleen cells can be rendered immunologically more reactive in the presence of a normally reactive population.

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