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

IV. Synergy in the GVH Reaction Quantitated by a Mortality Assay in Sublethally Irradiated Recipients

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It has been reported recently that one can demonstrate synergy among populations of parental lymphoid cells in the elicitation of graft-*versus*-host (GVH)[1] reactions in normal neonatal F_1 recipient mice (1–3). Using the quantitative spleen weight assay (4) as a measure of GVH activity, it was found that mixtures of either peripheral blood leukocytes (PBL) or femoral lymph node cells and thymocytes gave reactions in excess of those expected by summing the reactivities of these populations measured separately.

It is well established that much of the splenomegaly seen in animals undergoing a GVH reaction is due to immunologically nonspecific proliferation of host cells (5–8). Barchilon and Gershon (9) and Hilgard (10) have reported examples in irradiated recipients in which immunologically unreactive cells, such as syngeneic bone marrow, produce increased splenomegaly when combined with allogeneic cells. It was unlikely that such a mechanism was responsible for the synergy observed in the present model employing normal F_1 recipients, since synergy was never observed when either the allogeneic PBL or thymocytes were replaced either by allogeneic bone marrow or by appropriate cells syngeneic with the host (1–3).

Nevertheless, it seemed desirable to attempt to demonstrate this synergy utilizing a GVH assay system which should not depend on those nonspecific factors which contribute to splenomegaly (11). A mortality assay was chosen for two reasons. First, from the studies of Owens and Santos (12) it appeared that one could readily quantitate differences in the lethality of various cell populations and thereby facilitate a demonstration of synergy, if indeed it occurred. Secondly, the studies of Billingham and Silvers (13) and Cantor et al. (14) suggested that the capacity of some tissues to cause lethal runting seemed out of proportion to their capacity to cause splenomegaly.

The results presented in this paper indicate (*a*) that synergy between PBL and thymocytes can be demonstrated using a mortality assay as a measure of the GVH reaction, and (*b*) that the kinetics of cumulative mortality induced by appropriate mixtures of PBL and thymocytes are indistinguishable from

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those induced by large numbers of thymocytes alone and are different from those produced by PBL. Analysis of the relative capacities of different cell populations to cause lethal runting on the one hand and splenomegaly on the other suggests that different cells may in fact be responsible for initiating these different effector activities.

Materials and Methods

Animals.—Adult (8–16 wk old) male BALB/cAnN (H-2^d) mice and 1–5 day old litters of F_1 hybrid C57BL/6N by BALB/cAnN (H-2^b/H-2^d) mice were obtained from the Rodent and Rabbit Production Section, Division of Research Services, National Institutes of Health, Bethesda, Md.

Irradiation.—Recipient F_1 mice placed in a Lucite box were exposed to 400 R total body irradiation 1–3 hr before inoculating them with cells. A Westinghouse Quadrocondex 250 kvp machine was used under conditions of 200 kvp, 15 ma, half-value layer 0.9 mm of Cu, at a distance of 54 cm, and an administered dose rate of 139 R/min.

Cell Suspensions.—Groups of animals were killed by exsanguination from the axillary vessels under ether anesthesia. Suspensions of femoral lymph node, spleen, and thymus cells were prepared by gently teasing the tissue apart and washing the cells once in Hanks' balanced salt solution (HBSS, Grand Island Biological Co., Grand Island, N. Y.). Care was taken when removing the thymus to avoid both contamination with blood and accidental inclusion of parathymic lymph nodes. Peripheral blood was collected by bleeding from the axillary vessels into pipettes rinsed with heparin (1 mg/ml); PBL were obtained as described by Wilson (15). Cells were resuspended in HBSS to the desired concentration of nucleated cells; when combinations of cells were to be used, appropriate concentrations were mixed together immediately before injection.

Graft-Versus-Host Assay.—Within 3 hr of irradiation, F_1 recipients, 1–5 days old, were injected intraperitoneally with a constant volume of 0.05 ml of the desired suspension of parental and/or syngeneic cells; injections were made via a 30-gauge hypodermic needle introduced subcutaneously along the medial aspect of the thigh in order to avoid leakage. Mice were counted daily for the first 35 days and then twice weekly for an additional month. Since the majority of deaths among uninjected irradiated mice occurred within the 1st week of observation and since very few recipients of allogeneic cells died after 35 days (see Results), analysis of mortality between days 7 and 35 was used to measure GVH activity. Cumulative mortality was calculated as the percentage of mice dead relative to the number alive on day 7.

RESULTS

Comparison of Normal and Irradiated Recipients.—An initial experiment was designed to compare the mortality of normal and sublethally irradiated (400 R) F_1 recipients of BALB/c spleen cells (Fig. 1). 10 or 25 $\times$ 10^6 spleen cells injected into normal F_1 recipients produced comparable and rather low levels of mortality. When the F_1 recipients were pretreated with 400 R, 5 and 10 $\times$ 10^6 spleen cells produced 35 day cumulative mortalities of 55 and 90%, respectively. Thus, irradiation resulted in at least a threefold increase in the sensitivity of F_1 recipients to lethal GVH reactions. All subsequent experiments utilized irradiated recipients.

The observation period for this experiment was about 60 days. It was found in this and all other experiments reported here that the 60 day mortality did

not exceed the 35 day mortality by more than 10%; therefore, for convenience the data shown extend only through 39 days.

The validity of discounting any deaths occurring within the 1st week after inoculation of cells was established throughout the course of this study. Deaths among irradiated control mice which received no cells usually occurred within this 1st week of observation; of the 255 such control animals, 29 (11.4%) died at some time during the 60 day observation period, and 20 (8%) died within the 1st week. The majority of these early deaths occurred in a small number of
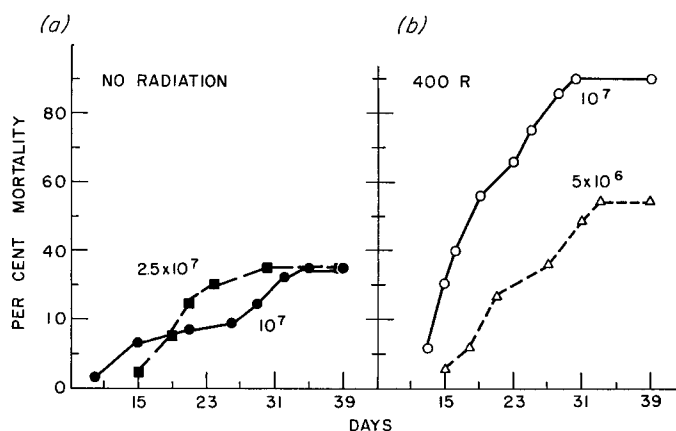

FIG. 1. The effect of sublethal irradiation of F_1 recipients of parental strain cells on observed cumulative mortality. (a) Groups of 30 normal C57BL/6 × BALB/c F_1 neonates, 1–5 days old, were inoculated with either 10^7 (●) or 2.5 × 10^7 (■) adult BALB/c spleen cells. Recipients were observed for 2 months; for convenience the data shown extend only from day 7 through day 39 (see text). Percentage mortality was calculated as the cumulative percentage of mice dead relative to the number alive on day 7. (b) F_1 recipients of 5.0 × 10^6 (△) or 10^7 (○) BALB/c spleen cells were exposed to 400 R whole body irradiation 1–3 hr before inoculation of cells. Mortality among uninjected irradiated mice during the observation period was approximately 11% (see text) and does not differ significantly from the mortality observed among uninjected normal mice of this strain.

litters and were directly attributable either to cannibalism by the mother or to her failure to nurse the litter. In all the experiments reported in this paper (nearly 1000 recipients), the early (1–7 day) mortality of irradiated recipients of parental cells was also 8%, and again the majority of deaths occurred in very few litters, for the same reasons as in controls, and could not be correlated with the size or origin of the cell inoculum.

Comparison of Various Doses of Thymocytes, PBL, and Femoral Lymph Node Cells.—The results of injecting different numbers of parental thymocytes or PBL into irradiated F_1 neonates are shown in Fig. 2. The mortalities shown are the means of at least three separate experiments at each particular inoculum. Increasing the number of inoculated cells increased the observed 35 day mor-

tality. 1 $\times$ 10^6 PBL caused a 35 day mortality of approximately 80%; comparable levels of mortality were seen with 25 $\times$ 10^6 thymocytes. In addition to showing the obvious quantitative differences between the lethality of PBL and thymocytes, Fig. 2 also reveals an interesting qualitative difference between these tissues. If one arbitrarily chooses a day midway in the assay, such as day 21, one can see that well over half of the recipients of any given inoculum of PBL which will ultimately succumb are already dead by this time. On the other hand, relatively few of the recipients of thymocytes which ultimately died had done so by day 21. For any given level of mortality, recipients of thymocytes survived approximately a week longer than recipients of PBL.
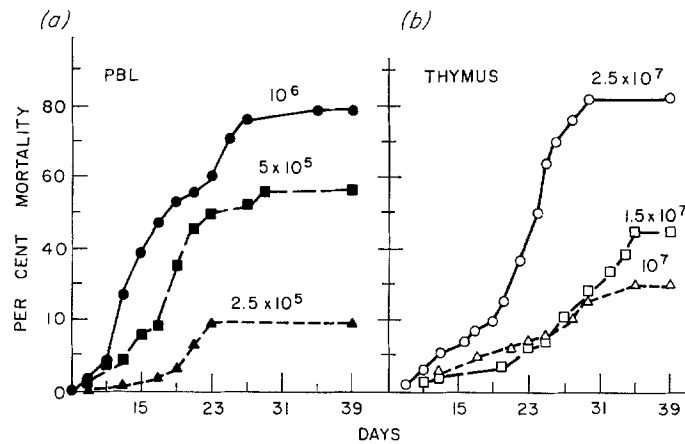

FIG. 2. Comparison of effects on observed cumulative mortality among irradiated F$_1$ recipients of various numbers of adult BALB/c peripheral blood leukocytes (PBL) or thymocytes. (a) Irradiated recipients were inoculated with 2.5 $\times$ 10^5 (▲), 5.0 $\times$ 10^5 (■), or 1.0 $\times$ 10^6 (●) PBL. (b) Irradiated recipients were inoculated with 1.0 $\times$ 10^7 (△), 1.5 $\times$ 10^7 (□), or 2.5 $\times$ 10^7 (○) thymocytes. Mortalities shown are the means of at least three separate experiments (60–100 recipients) at each inoculum.

Thus, in addition to being quantitatively more effective than thymocytes, PBL also tended to cause significantly earlier mortality than did equivalently lethal numbers of thymocytes.

Similar experiments were carried out employing different numbers of BALB/c femoral lymph node cells. The 35 day mortality induced by 1.5 $\times$ 10^6, 1.0 $\times$ 10^6, and 5.0 $\times$ 10^5 lymph node cells was 84, 67, and 30%, respectively. In addition, the mortality produced by these cells occurred relatively early, as was the case with PBL, i.e., animals began dying in significant numbers late in the 2nd week of observation.

In order to quantitate the differences in lethality of cells obtained from these various tissues, the percentages of 35 day mortalities were plotted on a probit

scale as a function of numbers of cells injected as plotted on a logarithmic scale. These results are shown in Fig. 3. A linear relationship between the probit of the per cent of mortality and the logarithm of the number of inoculated cells was observed for each of the tissues tested. In addition it can be seen that the reactivity curves for each tissue form parallel lines with each other, thus permitting a quantitative comparison of the reactivities of the cell populations. The regression lines shown in Fig. 3 were estimated visually. However, the slopes of the regression lines for different cell populations calculated by the method of least squares were not significantly different from each other, i.e., $P > 0.5$. PBL, which were the most lethal cell population, were about 30 times
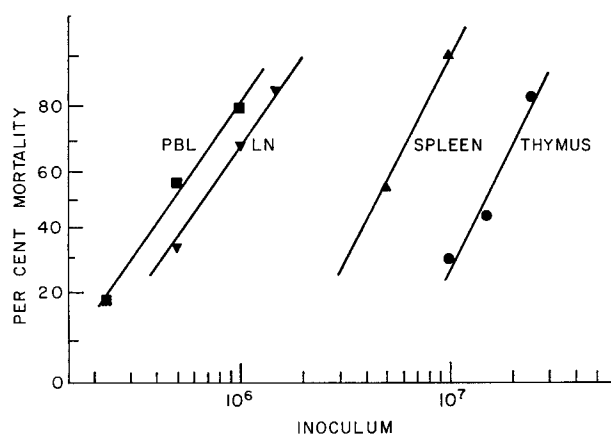

FIG. 3. Comparison of the 35 day cumulative per cent of mortality among irradiated F_1 recipients of graded numbers of BALB/c PBL (■), femoral lymph node (▼), spleen (▲), or thymus cells (●). The 35 day per cent of mortalities are plotted on a probit scale as a function of the logarithm of the cell inoculum. The slopes of each of the regression lines calculated by the method of least squares were not significantly different from each other, i.e., $P > 0.5$.

as active as thymocytes, while femoral lymph node cells and spleen cells were slightly more than 20 and 3 times as reactive as thymocytes, respectively.

Activity of Combinations of PBL and Thymocytes.—Experiments were next performed to examine the lethality of combinations of PBL and thymocytes injected together. When 10×10^6 thymocytes were injected together with 2.0×10^5 PBL into a group of 30 F_1 mice, the 35 day mortality was 82% (Fig. 4). By reference to Fig. 3, one can calculate that 2.0×10^5 PBL are the equivalent of 6.0×10^6 thymocytes. If the reactivities of PBL and thymocytes combined were simply additive, i.e. the sum of their reactivities measured separately, one would have expected to see a 35 day mortality equivalent to that of approximately 16×10^6 thymocytes, or about 60%. The observed activity of 82%, however, is equivalent to the reactivity of 25×10^6 thymocytes, or about 1.6 times the expected reactivity.

The mortality as a function of time observed when mice were injected with 10×10^6 thymocytes combined with 1.0×10^5 PBL is also illustrated in Fig. 4 and represents the mean of four separate experiments involving approximately 90 recipient mice. The observed 35 day mortality of nearly 80% is again equivalent to that seen with 25×10^6 thymocytes alone, while the expected reactivity of this combination of cells if the activities were merely additive was that of
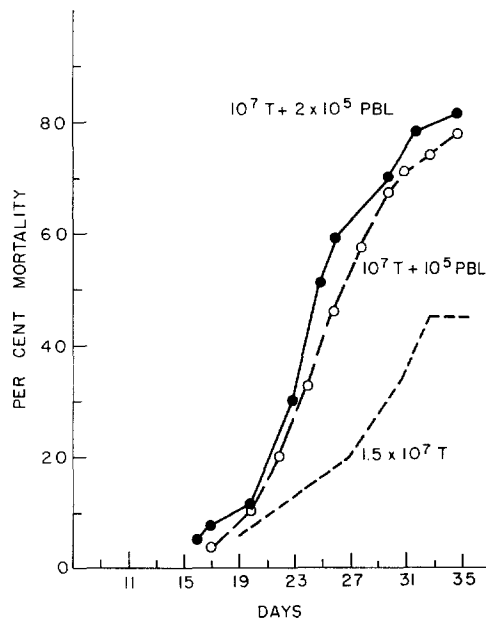

Fig. 4. Cumulative mortality among irradiated F_1 recipients of combinations of BALB/c PBL and thymocytes. A group of 30 recipients was each inoculated with a mixture of 10^7 thymocytes (T) and 2.0×10^5 PBL (●). The mortalities shown for recipients of mixtures of 10^7 thymocytes and 1.0×10^5 PBL (○) represent the means of four separate experiments (90 recipients). The mortality observed with 1.5×10^7 thymocytes alone (Fig. 2) is shown as a reference.

approximately 13×10^6 thymocytes. In this case the observed reactivity was nearly twice the expected reactivity.

The kinetics of the mortality curves obtained with combinations of PBL and thymocytes which gave synergistic levels of 35 day mortality (Fig. 4) are indistinguishable from those seen with a large inoculum of thymocytes alone (Fig. 2). Animals did not die in significant numbers until the end of the 3rd week, with the majority of deaths occurring within the next several days.

Requirement for Allogeneic Cells to Observe Synergy.—In the previous studies using the spleen weight assay it was found that no synergy was observed if either the parental strain PBL or thymocytes in the combination were replaced

by F_1 cells, indicating that both populations had to have the capacity to specifically recognize foreign antigens in the F_1 recipients. Similar experiments were designed to determine whether the synergy observed in the mortality assay also required that both populations be allogeneic.

Inoculation of either 1.5×10^6 F_1 PBL or 35×10^6 F_1 thymocytes into irradiated F_1 recipients produced mortality identical to that seen among control animals receiving no cells. Animals inoculated with a combination of 10×10^6 F_1 thymocytes and 1.0×10^5 BALB/c PBL also showed control levels of mortality at 60 days. The reverse combination, that is, 10×10^6 BALB/c thymocytes and 1.0×10^5 F_1 PBL, produced a 35 day mortality of 32%, identical to that observed in recipients of 10×10^6 BALB/c thymocytes alone. Therefore,

TABLE I

Comparison of Relative Reactivities of Cell Populations in the Mortality Assay with Their Reactivities in the Spleen Weight Assay

Cell source	Parallel line method		Limiting dilution method	
	Mortality assay	Spleen weight assay	Mortality assay	Spleen weight assay
Femoral lymph node	22.7	14.6	25.9	15.0
PBL	31.3	25.0	35.0	20.0
Spleen	3.3	7.6	2.9	6.0

The reactivity of thymocytes was assigned an arbitrary value of 1.0; other reactivities were determined relative to thymocytes. Measurements using the parallel line method in the spleen weight assay employed standard procedures for bioassay (16, 17). Measurements using the parallel line method in the mortality assay used the number of cells producing 50% mortality. The minimum number of cells resulting in a spleen index of 1.3 was regarded as the limiting dilution for that population in the spleen weight assay (4). The minimum number of cells causing 15% cumulative mortality was used as the limiting dilution for that population in the mortality assay.

synergy was not observed in this assay when either of the populations of cells allogeneic to the host was replaced by an equivalent population syngeneic with the host.

Comparison of the Relative Reactivities of Cell Populations in the Mortality Assay with their Reactivities in the Spleen Weight Assay.—Parallel line assays have been employed previously (1–3, R. T., unpublished observations) to quantitatively compare the GVH reactivities, as judged by the spleen weight assay, of the same cell populations and the same strain combinations used in the present study. With the establishment of another set of parallel lines in this mortality assay (Fig. 3), it became possible to compare the relative reactivity of a given cell population in the two assays. The results of this comparison are shown in Table I. Thymocytes, the least reactive cell population in both assays, have arbitrarily been assigned an activity value of 1.0 in each assay, and the other tissues a figure corresponding to their reactivities relative to thymocytes.

A value of 2 would indicate that the population was twice as reactive as thymocytes. The limiting dilution (the lowest number of cells necessary to observe a significant effect) for thymocytes was essentially the same in both assays, i.e., approximately 7×10^6 cells. The rank order of reactivity from lowest to highest of thymus, spleen, femoral lymph node, and PBL was the same in the mortality assay as in the spleen weight assay. Quantitatively, however, it can be seen that spleen cells were more than twice as effective in causing splenomegaly (7.6) than they were in causing mortality (3.3). The opposite effect was observed for femoral lymph node cells, which were about 1.5 times as active as judged by the mortality assay as when judged by the spleen weight assay. PBL, the most active cell population, also tended to show a higher reactivity in the mortality assay than in the spleen weight assay. The value of 25 for PBL in the spleen weight assay and of 31.3 in the mortality assay are averages, since these ratios relative to thymocytes varied slightly with the position on the dose-response curve at which the comparison was made. A similar analysis can be done using limiting dilutions, i.e., the smallest number of cells needed to produce a detectable reaction. The results obtained by this method are almost the same as those obtained by the parallel line method. Thus, although the rank order of tissue reactivities was the same in both GVH assays, spleen cells were relatively less active and PBL and femoral lymph node cells relatively more active in leading to death than they were in producing splenomegaly.

DISCUSSION

The use of a mortality assay as a means of measuring the efficacy of various cell populations to provoke GVH reactions has been known for many years (4). In general, however, its use has been limited by a relative lack of sensitivity, particularly when normal adult F_1 recipients are used. Relatively larger numbers of cells must be given in order to see significant mortality as compared to the numbers required to give rise to significant splenomegaly (18). Furthermore, rather large increments in numbers of allogeneic lymphoid cells inoculated may produce little or no increase in mortality (reference 13, also see Fig. 1). Pretreatment of adult F_1 recipients with sublethal irradiation (11, 19) or cyclophosphamide (12) dramatically increases the sensitivity of these mice to the lethal effects of inoculation of parental strain cells. The reason(s) for this increased susceptibility to lethal GVH reactions is (are) not fully understood, and the present experiments did not address themselves to that question. However, a similar increase in sensitivity was noted in this study when neonatal F_1 mice were exposed to 400 R whole body irradiation before inoculation of parental strain cells. Many fewer parental spleen cells were required to produce given levels of cumulative mortality in irradiated recipients than in normal recipients. Although irradiation alone did not produce significant mortality, such pretreatment increased the susceptibility of the recipients to parental cells so that essentially 100% 35 day mortality was observed with a sufficient

number of lymphoid cells from any of the sources examined in this study. Furthermore, the limiting dilutions in this mortality assay are of the same order of magnitude as those seen when one uses splenomegaly as the assay of the GVH reaction.

The major purpose of these experiments was to determine whether the inoculation of mixtures of parental thymocytes and PBL would lead to lethal GVH reactions in more animals than would be predicted if the activities of these cell populations when mixed were merely the sum of the reactivities measured separately, i.e., whether synergy could be seen. Such synergy had been observed when GVH reactions were quantitated by a spleen weight assay (1–3). These observations have been explained by postulating a requirement for the presence of at least two cell populations for optimal expression of GVH reactivity. The two cell populations have been shown to have the following characteristics: (a) One population is present in high concentrations in the pool of recirculating lymphocytes; the second is a population of relatively sessile cells found in high concentrations in thymus and spleen (2, 3). (b) Both populations must have the capacity to recognize the host as foreign (1–3). (c) Both of the cell types involved in this interaction are "thymic dependent" (2, 3, 20). (d) The cell type present in excess in thymocyte suspensions appears to act as a precursor of cells which inflict immunologic injury while the cell present in excess in PBL amplifies the activity of the former (2, 3).

The present studies confirm and extend previous observations to another quantitative assay of GVH reactions. Inoculation of 10×10^6 thymocytes with as few as 1.0×10^5 PBL resulted in 35 day cumulative mortality in excess of that expected from the separate reactivities of the constituent cells. The observed reactivity of this combination was equivalent to that seen with 25×10^6 thymocytes alone, or nearly twice the expected reactivity. The limiting number of either population which can be used to give increased reactivity in this assay has not yet been determined, but preliminary experiments employing mixtures of 5.0×10^6 thymocytes and 1.0×10^5 PBL have clearly resulted in levels of mortality which show synergy. As in the spleen weight assay, the substitution in the mixture of thymocytes and PBL of syngeneic cells for either parental strain cell type resulted in cumulative 35 day mortalities similar to those seen with the one parental cell type alone. Therefore, both cell populations must be specifically reactive against the host to produce synergy.

In addition to displaying characteristic quantitative relationships to each other in abilities to cause a given level of 35 day mortality, the peripheral lymphoid tissues also differed noticeably from thymocytes with respect to the average survival time of recipients which eventually died. Recipients of thymocytes survived approximately a week longer than did recipients of equivalently lethal numbers of PBL, lymph node, or spleen cells. The addition of 1.0×10^5 PBL to 10×10^6 thymocytes, which resulted in markedly enhanced levels of 35 day mortality, did not noticeably shorten the average survival time. It is

possible that this assay system is not sensitive enough to detect the potentially subtle alteration in kinetics which could occur with the addition of small numbers of PBL to thymocytes. On the other hand, the fact that the kinetics observed for this combination were similar to those seen with thymocytes alone correlates well with the conclusion drawn from previous experiments (2, 3) that a thymocyte is acting as the precursor of the effector cell while a cell in the PBL suspension is acting to amplify the activity of a cell in the thymocyte suspension. It is also very likely that the cell interactions which produce increased mortality occur among different classes of thymus-derived cells, as is the case in the production of splenomegaly (20).

Since the mortality assay and the spleen weight assay have been shown to yield sets of parallel lines of reactivities for different numbers of cells from various sources, it is possible to compare quantitatively the relative activity of a given cell population in the two separate assay systems. The rank order of reactivity for the various tissues tested was the same in both assays, i.e., in order of decreasing effectiveness PBL, femoral lymph node, spleen, and thymus. When reactivity was expressed relative to the reactivity of thymocytes, femoral lymph node cells and PBL were much more active in the mortality assay than in the spleen weight assay, while spleen cells were nearly twice as effective in causing splenomegaly in F_1 recipients as they were in causing death. These findings are compatible with the observations of Billingham and Silvers (13) and Cantor et al. (14) showing that femoral lymph node cells were particularly effective compared to spleen cells in causing early and subsequently lethal runting in neonatal recipients. This effectiveness was not matched by similar effectiveness in causing splenomegaly in such recipients. It is difficult to ascribe such quantitative differences in relative reactivities in the two assays to a difference in the sensitivities of the assay systems, since it would be expected that such a difference in sensitivity would be reflected equally and and in the same direction for all cell populations tested.

It is now clear that in some cases the optimal expression of GVH reactivity depends on an interaction between at least two thymus-derived cells, one of which is the precursor of the ultimate effector cell, the other of which amplifies the activity of the first. Since this establishes a precedent for the involvement of more than one type of thymus-derived lymphoid cell in a cellular immune reaction, it is possible that different effector functions, in this case the abilities to produce either death or splenomegaly, involve distinct types of precursor cells.

SUMMARY

A mortality assay was used to quantitate graft-*versus*-host (GVH) reactions in sublethally irradiated (400 R) neonatal (C57BL/6 × BALB/c)F_1 recipients of BALB/c lymphoid cells from various tissues. The probit of the 35 day cumulative per cent of mortality was a linear function of the logarithm of the

cell inoculum for any tissue; reactivities of different tissues fell on a series of parallel lines. Peripheral blood leukocytes (PBL), the most active cells, were about 30 times as active as thymocytes, the least active cells studied; femoral lymph node cells and spleen cells were about 23 and 8 times as reactive as thymocytes, respectively. The average survival time of recipients of thymocytes who eventually died was nearly a week longer than that of recipients of comparably lethal numbers of PBL, lymph node, or spleen cells.

Mixtures of PBL and thymocytes gave levels of 35 day mortality significantly greater than those expected if the reactivities of the mixture had been merely the sum of the reactivities of the components measured separately, thereby confirming in any assay independent of host splenomegaly the synergistic interaction of thymocytes and PBL in the GVH reaction. Both populations of cells in the mixture had to be allogeneic to the host in order to observe this synergy.

The kinetics of cumulative mortality observed for mixtures of PBL and thymocytes were indistinguishable from those seen with thymocytes alone, indicating activation of the latter cell type. Finally, comparison of the relative abilities of different cell populations to cause splenomegaly on the one hand and lethal runting on the other has raised the possibility that expression of different effector functions of cell-mediated immune reactions may in fact be initiated by distinct cells.

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