# Studies on Synergistic Thymus–Bone Marrow Cell Interactions in Immunological Responses[1] (34427)

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Cells from bone marrow and thymus have been found to interact synergistically in the immunological response to sheep red blood cells (SRBC) (1). Miller (2) showed that the thymus cells need to be exposed to the SRBC antigen before such interactions occur. We initiated studies to see if similar conditions were required for cell-mediated immunological responses, specifically the homograft and graft-versus-host (GVH) reactions. The results show that interaction between thymus and marrow cells occurred only under one set of conditions in respect to the homograft reaction, but that this interaction was not specific for the isoantigens used. No synergistic effect was observed for the GVH reaction. A preliminary report on the latter finding has been published (3), and was confirmed recently by Stuttman and Good (4).

*Methods. Mice.* The (C57L × A/He) $F_1$ ($LAF_1$) hybrid mice used in this study are routinely bred at NRDL. The (C3H/He× C57Bl/6)$F_1$ ($C3B/6F_1$) infant (3–10 days old) hybrids used in the Simonsen (5) assay were bred here from parents obtained from the Jackson Laboratory. The skin and isoantigen donors were also from the Jackson Laboratory. The H-2 histocompatibility designations for the various mouse strains used are as follows: $LAF_1$, H-2ab; C3H/HeJ, H-2k; C57Bl/6J, H-2b; $C3B/6F_1$, H-2kb DBA/2J, H-2d; A/He, H-2a. Isoantigenic differences between mouse strains having the same H-2 locus are considered weak while differences across H-2 loci are considered strong.

*Test systems.* For several of the experiments, a system similar to one described by Miller (2) was used. His system and the modifications required for testing the cell-mediated responses are detailed in Tab!e I. The other more simplified test systems are described in the text. The techniques for assay of plaque-forming cells (PFC), (6), for skin homografting (7), and for the Simonsen GVH assay (5) have been described.

*Cell preparation.* All cell suspensions were in Difco medium TC 199 containing 10% fetal calf serum. Bone marrow cells were removed by aspiration of the femurs and tibias. Spleen cells were suspended by gentle homogenization in a very loose fitting glass homogenizer. Lymph node cell suspensions were obtained from the mesenteric and other nodes, which were repeatedly snipped and passed in and out of a pipette. Both the spleen and node cell preparations were filtered through gauze (4 ply) and washed once by centrifugation at 100*g*. Cell populations were combined with or without antigen in v:v proportions just prior to injection. For controls, aliquots of the cells were exposed *in vitro* to 2500 R of X-rays to kill the cells.

*Cell injections.* The number of cells injected was based on viability counts obtained by the eosin exclusion technique. Ten million cells from each tissue were injected intravenously into each recipient except in those cases noted where only $10^6$ lymph node cells were given. In the spleen cell transfers, cells from an entire primary host spleen were transferred intravenously into each secondary or test host. In the Simonson assay the cells from ½ spleen of the secondary host

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TABLE I. Test Systems.

| Primary host | Secondary host | Tests on secondary hosts |
|---|---|---|
| 900 R + antigen (ag) $\xrightarrow[\text{spleen at 1 week}]{\text{Transfer}}$ | Irradiated + syngeneic marrow cells ($10^7$) + antigen (ag) | |
| "Miller system" ag = sheep erythrocytes | 900 R + ag | PFC in spleen |
| Homograft ag = irradiated (2500 R) allogeneic cells | 500 R + ag (thymectomized 3–4 weeks prior) | Skin homograft survival |
| GVH ag = irradiated (2500 R) allogeneic cells | 900 R + ag | GVH reactive cells in spleen (Simonsen assay) |

were injected into each infant intraperitoneally. At least 2 individuals in 3 or more litters were injected. The spleen and body weights were taken 10 days after injection and the data were pooled.

*Antigen.* Allogeneic spleen, lymph node, and marrow cells which were suspended in TC 199 and exposed to 2500 R of X-rays *in vitro* were used as isoantigen. These killed cells (2.6–3.6 $\times$ $10^7$/recipient) were mixed with the viable cells under test just before intravenous injection. Washed sheep erythrocytes ($10^8$/recipient) were used as antigen when the PFC response was measured.

The X-irradiation and animal maintenance have been described previously (8). The 95% confidence intervals were calculated using the *t* distribution.

*Results and Discussion.* Intially we repeated the work of Miller (2) to verify that synergistic interactions between thymus and marrow cells occurred in the PFC response to SRBC antigen. Thymus cells and SRBC antigen were injected into the irradiated primary host; the spleen cells of that host were transferred 1 week later to a secondary irradiated host together with bone marrow cells and additional SRBC. The spleen of the secondary host (Table II, group 1) contained significantly greater numbers of PFC than those in the control situations (group 2–7) except where lymph node cells were used (group 6). Here, as expected, high levels of PFC were found. These results confirm those of Miller (2).

*The homograft response.* The effect of thymus and/or bone marrow cell injections on the restoration of homograft reactivity in sublethally irradiated mice was then evaluated. The $LAF_1$ mice exposed to 500 rad of X

TABLE II. Synergistic Interaction between Thymus Cells Previously Exposed to Sheep Erythrocytes and Marrow Cells in the Production of Plaque-Forming Cells (PFC).

| No. | Primary hosts (900 R) | Secondary hosts (900 R) | PFC/Secondary spleen ± SD (at 6 days) |
|---|---|---|---|
| 1 | Thymus[a] + SRBC | 1° spleen[b] + marrow[a] + SRBC | 870 ± 346[c] |
| 2 | Thymus only | 1° spleen + marrow + SRBC | 82 ± 57[c] |
| 3 | Irradiated thymus + SRBC | 1° spleen + marrow + SRBC | 46 ± 33 |
| 4 | Thymus + SRBC | 1° spleen + irradiated marrow + SRBC | 4 ± 4 |
| 5 | Thymus + SRBC | 1° spleen + marrow | 11 ± 7 |
| 6 | Lymph node[a] + SRBC | 1° spleen + SRBC | 1477 ± 606 |
| 7 | Marrow + SRBC | 1° spleen + marrow + SRBC | 9 ± 4 |

[a] $10^7$ syngeneic ($LAF_1$) thymus marrow or lymph node cells injected.

[b] 1° = primary.

[c] $0.025 < p < 0.05$.

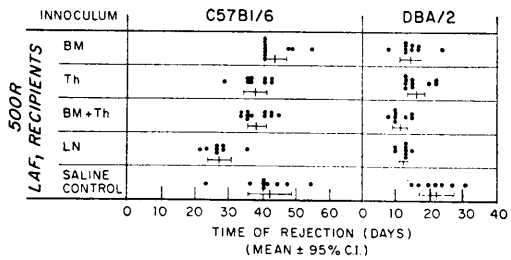


FIG. 1. Fate of skin allografts on sublethally X-irradiated $LAF_1$ recipients injected with syngeneic bone marrow (BM), thymus (Th), lymph node (LN) or a combination of these cells ($10^7$ each).

rays were injected with syngeneic thymus, bone marrow, or a combination of these cells ($10^7$ each) and then were grafted 1 day later with skin from allogeneic donors of varying antigenic disparity. The results are shown in Fig. 1. The C57Bl/6 allografts (H-2 similar) were rejected in about 40 days in each case, a time similar to that of the saline-injected controls. No synergism as evidenced by an accelerated response was noted. The mice injected with $10^7$ lymph node cells as a positive control showed an accelerated time for graft rejection. The rejection time of the DBA/2 (H-2 different) allografts followed a different pattern. In this case, the groups of mice injected with marrow, thymus, or node cells showed a decreased mean survival time (mst) relative to the controls (15 days versus 23 days). This indicates that the bone marrow and thymus cell populations by themselves have the ability to restore competence towards the more antigenically disparate allografts in a manner similar to the adoptive restoration afforded by lymph node cells. Again, however, no synergism between thymus and marrow cells was evident.

In the next series of experiments we studied the effect of *in vivo* exposure of thymus cells to isoantigen prior to injection with bone marrow cells. A test system similar to Miller's (2) was used (cf. Table I). Syngeneic $LAF_1$ thymus cells were injected together with DBA/2 (H-2 different) isoantigen into an irradiated primary host. The spleen from the primary host (containing a portion of the injected thymus cells) was removed after 1 week and the cells injected with $10^7$ marrow cells and more isoantigen into a secondary thymectomized test host which had been sublethally irradiated (500 R.) The test mice were skin grafted the next day and results are shown in Fig. 2. The mst values for the DBA/2 allografts are similar in pattern to those in Fig. 1 except there was an acceleration of the rejection time. This acceleration

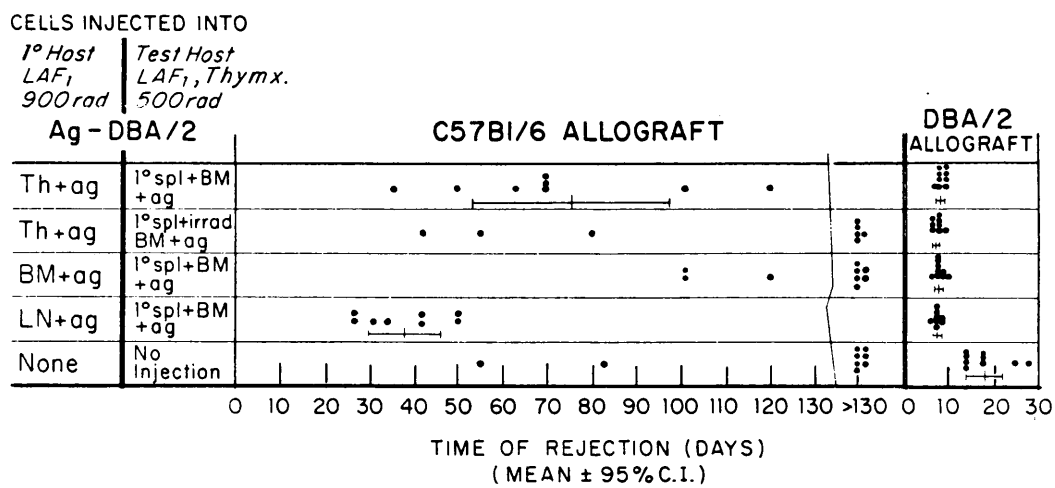


FIG. 2. Fate of skin allografts on thymectomized sublethally X-irradiated $LAF_1$ mice injected with syngeneic bone marrow (BM) cells, DBA/2 isoantigen (ag) and spleen cells from primary irradiated recipients. The primary recipients were injected 1 week earlier with $10^7$ syngeneic thymus (Th), bone marrow or lymph node (LN) cells and DBA/2 isoantigen. The test hosts were grafted 1 day after injection.

is presumably due to sensitization by the DBA/2 isoantigens. These test hosts rejected the DBA/2 allografts with a mst of 7 to 8 days, no matter if the primary host had previously been injected with thymus, marrow, or lymph node cells. Noninjected controls had an mst of 17 days. Other controls not presented in Fig. 2 showed that when the primary hosts were injected with (i) thymus cells only; (ii) irradiated thymus cells only; (iii) irradiated thymus cells plus antigen, the test hosts responded to the DBA/2 allografts in a manner similar to the noninjected controls (mst ranged from 14 to 18 days). In addition, the presence of DBA/2 isoantigen in the *test host* (in contrast to its presence in the primary host) was not necessary for the accelerated response, nor was the injection of viable bone marrow cells (line 2). Therefore, it appears that thymus, bone marrow and, as expected, lymph node cell populations were each able to adoptively restore the irradiated test host under these conditions. No synergism between marrow and thymus cells was evident, although it could have been masked by the immunocompetence conferred by the individual cell populations.

Evidence of synergism was observed with the C57Bl/6 grafts (Fig. 2). Note that as a result of thymectomy, a "permanent" impairment of the response to this "weakly" antigenic graft was evident and 7 out of 9 test hosts maintained this allograft for more than 130 days in the noninjected controls. However, when thymus cells were exposed to the isoantigen (line 1) all of the test recipients rejected the C57Bl/6 allograft (mst = 75 days) even though the allograft was not of the same strain as the DBA/2 isoantigen used. Hence, it appears that there was a nonspecific stimulation of homograft reactivity in the test mice under these conditions. With the exception of the lymph node group each of the other control groups had from 3 to 6 mice (out of 8 or 9) which remained "permanently" incompetent. These control groups include in addition to those shown in Fig. 2, groups in which the primary hosts were injected with thymus or irradiated thymus cells only, or with irradiated thymus cells plus isoantigen.

The above synergistic interaction was obtained when antigenically strong isoantigen (H-2d vs. H-2ab) was used. In an identical experiment using a weak isoantigenic difference, *i.e.*, C57Bl/6 isoantigen (H-2b vs. H-2 ab) no such synergism was evident. In this situation the response to C57Bl/6 allografts remained permanently suppressed in many test hosts even then thymus cells and C57Bl/6 isoantigen were injected into the primary host. Also, the response to the nonspecific graft (DBA/2) was not accelerated (mst = 17 days). Improved responses did occur in 2 control groups, namely when the primary hosts received lymph node or bone marrow cells in place of thymus cells. The mst values for C57Bl/6 allografts in these cases were 29 days and 61 days, respectively. The responses to DBA/2 allografts were not effected. Hence it appears that sensitization of the marrow cells occurred under these conditions in a fashion analogous to the expected sensitization of the lymph node cells. The important finding here, however is that the weak, non-H-2 isoantigen was unable to stimulate or initiate thymus-marrow cell interaction similar to that found when the more disparate isoantigen was used (Fig. 2).

*The graft-versus-host response.* The effect of thymus–bone marrow cell interaction was then tested using the Simonsen splenomegaly (5) assay for GVH reactions. In initial experiments marrow, thymus, or thymus + marrow cells ($10^7$ each) from parental C3H donors were injected into infant $C3B/6F_1$ hybrid hosts (Fig. 3). The relative spleen weights for the hybrid hosts 10 days after injection was between 400 and 500 mg/100 g of body weight. These values were not different from the controls injected with highly irradiated cells. The positive controls however, which received $10^6$ parental lymph node cells, showed an increased spleen weight, indicative of a GVH reaction. Thus, these data showed no synergism between thymus and bone marrow cells and were similar to the results of Stuttman (4).

It was considered possible that more time might be required for the interaction to occur. Therefore two additional experiments were designed, one in which the thymus and

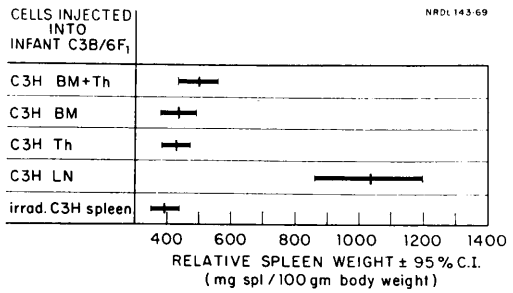


FIG. 3. Relative spleen weight of infant (3–10 day old) C3B/6F$_1$ hybrids injected ip with $10^7$ parental strain (C3H) bone marrow (BM), thymus (Th), lymph node (LN), or a combination of these cells ($10^7$ each). Controls received an equivalent number of X-irradiation (2500 R)-killed spleen cells. Weight determinations were made 10 days after injection.

marrow cells were passaged for 1 week through an intermediate irradiated host, and the second where a system similar to Miller's (2) was used. In the latter case, the spleen of secondary recipients were tested for GVH activity 1 week after injection.

The results of the 1-week passage in intermediate hosts are shown in Fig. 4. No significant synergistic interaction occurred between marrow and thymus cells. Also, the exposure of thymus cells alone to isoantigen and then transfer to the test animal with added marrow cells did not result in synergism (line 3). However, spleen cells from primary hosts given lymph node cells did exhibit GVH reactivity. Earlier experiments have been reported (3) in which similarly passaged parent strain cells were transferred into adult hybrid recipients which had been

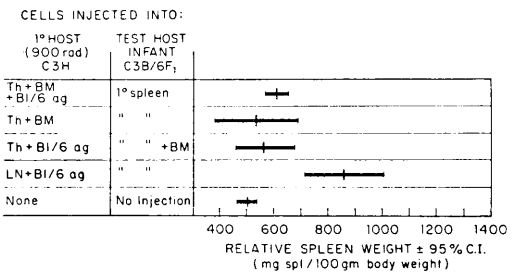


FIG. 4. Relative spleen weights of infant C3B/6F$_1$ hybrid recipients of spleen cells [±C3H bone marrow (BM) cells] from primary irradiated C3H recipients of syngeneic thymus (Th), bone marow (BM), or lymph node (LN) cells ($10^7$ each) and C57Bl/6 isoantigen. The primary hosts were sacrificed 1 week after injection.

sublethally irradiated. These experiments also indicated no synergistic interaction between thymus and marrow cells.

The results of a GVH test employing a system duplicating Miller's (2) are shown in Fig. 5. The isoantigen used in this study was

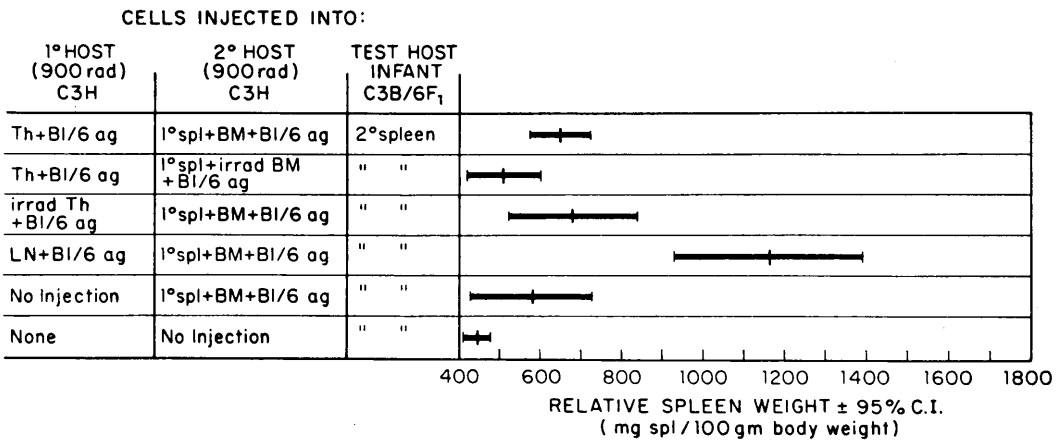


FIG. 5. Relative spleen weights of infant C3B/6F$_1$ hybrids injected with spleen cells from irradiated C3H secondary hosts which had received $10^7$ syngeneic bone marrow (BM) cells, C57Bl/6 isoantigen and spleen cells from primary C3H hosts. The primary hosts were irradiated mice which had received syngeneic thymus (Th) or lymph node (LN) cells ($10^7$ each) and C57Bl/6 isoantigens. The primary and secondary host were sacrificed 1 week after injection.

"strong" (H-2d vs. H-2k). No evidence of a synergistic thymus–marrow cell interaction was evident in this test. As shown in Fig. 5 (line 1), no splenomegaly occurred under conditions similar to those which did produce an elevated PFC response (Table II) or an accelerated nonspecific homograft reaction (Fig. 2). Since splenomegaly did occur when lymph node cells were injected into the primary hosts (line 4), it is apparent that this system can detect the successive transfer of immunologically reactive cells.

In the last experiment the effect of injections of thymus–bone marrow cell combinations on secondary disease was studied. The injection of combinations of A strain thymus cells and either A or $LAF_1$ marrow cells into irradiated (900 R) $LAF_1$ recipients resulted in the highest percentage of late deaths (66–70%, Table III). However, it cannot be inferred from the data that cellular interactions produced these results. Previous data indicated that allogeneic thymus cell populations alone under certain conditions can initiate homograft (Fig. 1) and GVH responses (3). In addition the combination of $LAF_1$ thymus cells with A marrow cells produced no secondary disease (Table III). Hence, no unequivocal evidence of synergism between thymus and bone marrow cells was seen in the production of secondary disease. Furthermore, no synergism occurred to produce accelerated deaths such as observed when parental strain lymph node cells were injected (Table III).

*Summary and Conclusions.* 1. Marrow cells and thymus cells which had been exposed *in vivo* to SRBC antigen interact synergistically to produce hemolysin plaque-forming cells (Table II) (2).

2. The possibility of similar thymus-marrow cell interaction in producing cell mediated responses was tested in a variety of systems.

3. Synergistic interactions were *not* observed: (a) in the restoration of homograft reactivity of sublethally irradiated mice (Fig. 1); (b) in the production of graft-vs.-host responses as measured by splenomegaly (Figs. 3, 4, and 5) or of secondary disease (Table III) (cf. 4); and (c) in the restoration of the homograft response in sublethally irradiated, thymectomized recipients injected with marrow cells and with spleen cells from irradiated mice which had been injected previously with thymus cells and "weak" isoantigen (non-H-2).

4. A nonspecific stimulation of the homograft response occurred under conditions similar to 3c above but where the isoantigenic disparity was strong.

5. This nonspecific stimulation and the re-

TABLE III. Mortality of Irradiated $LAF_1$ Hybrid Mice Injected with Parental Strain or Isogenic Thymus, Marrow, or Lymph Node Cells.[a]

| Source of cells ($10^7$) injected[b] | | Cumulative mortality (%) by (days): 8 | 25 | 50 | 75 | 100 |
|---|---|---|---|---|---|---|
| A/He | LN[c] | 100 | — | — | — | — |
| A/He | Th | 0 | 100 | — | — | — |
| A/He | BM | 0 | 0 | 10 | 30 | 40 |
| A/He + A/He | Th BM | 0 | 0 | 30 | 70 | 70 |
| A/He + $LAF_1$ | Th BM | 0 | 22 | 33 | 66 | 66 |
| $LAF_1$ + A/He | Th BM | 0 | 0 | 0 | 0 | 0 |
| $LAF_1$ | BM | 0 | 10 | 10 | 10 | 10 |

[a] LN = lymph node; Th = thymus; BM = bone marrow.
[b] 900-R X-irradiated $LAF_1$ recipients.
[c] $10^6$ lymph node cells injected.

sults of Globerson (9) which show a synergism between thymus and marrow cells in the production of *in vitro* GVH responses, offer perhaps the only known evidence that thymus–marrow cell interactions are involved in cell-mediated immunological responses.

6. Specific syngergistic interactions so evident in the initiation of the hemolysin response do not occur when cell-mediated immunological responses are tested under similar conditions. This does not definitely rule out the possibility that thymus–marrow cell interactions are involved in cell-mediated immunological responses.

7. Under certain conditions, thymus and bone marrow cell populations, like lymph node cell populations are each capable of conferring cell-mediated immunological competence. Hence these cell populations may have either one cell type capable of responding to isoantigen, or two or more cell types which interact to produce the response.

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