Absence of Synergism between Thymus and Bone Marrow in Graft-versus-Host Reactions* (33670)

O. STUTTMAN AND R. A. GOOD[1]

Departments of Laboratory Medicine, Pediatrics and Microbiology, College of Medical Sciences, University of Minnesota, Minneapolis, Minnesota 55455

A synergism between thymus and bone marrow cells in the mouse for the response to sheep red cells was described (1–4). The purpose of the present studies was to determine whether such interaction exists also in cell-mediated thymus-dependent reactions (5, 6). No such synergistic interaction between bone marrow and thymus or spleen cells was detected, in the mouse, using the graft-versus-host reaction as a test system (7).

Materials and Methods. Adult 45-day-old C3H/Bi female mice were used in all experiments. Thymuses were removed under dissecting microscope to avoid contamination with mediastinal lymph nodes. Bone marrow cells were obtained from femurs, tibiae, and humeri by forcing cold lactate-Ringer through with a syringe after cutting off the epiphyses. Spleen and thymus cell suspensions were prepared as previously described (8). All cell suspensions were prepared in cold lactate-Ringer and viability, determined by trypan blue exclusion, ranged from 80 to 98%. Cells were counted in a hemocytometer and the concentrations were adjusted to contain the desired number of cells in 0.2 ml of lactate-Ringer. Mixtures of cells of different origin were obtained by combining the two cell suspensions shortly before injection in a total volume of 0.2 ml. The cell suspensions were mixed by passing several times through a syringe with a 25-gauge needle.

Immunological capacity of these cell suspensions was evaluated by the graft-versus-host assay of Simonsen *et al.* (7). This assay measures the degree of spleen enlargement induced in young F1 hybrid recipients of cells from one of the parental strains. The graft-versus-host reactions has been shown to be one of the cell-mediated, thymus-dependent immunological functions (5, 6).

Suspensions from C3H/Bi tissues were injected intraperitoneally into 8-day-old (C3H $\times$ C57Bl/1)F1 hybrid mice. Two or more litters were used per test. The litters were separated in groups, identified by toe clipping and injected with the following cell types: (a) negative controls receiving 20×10^6 syngeneic spleen cells; (b) positive controls receiving 20×10^6 spleen cells from C3H/Bi origin; and (c) experimental group receiving the cells whose immunologic competence was to be assessed. When cell mixtures were tested, a fourth group was added consisting of animals injected with thymus or spleen cells in same numbers as in the cell mixture. Eight days after intraperitoneal cell administration the recipient animals were sacrificed, their body and spleen weights were determined, and a relative spleen weight (mg of spleen/10 g of body wt.) was calculated. The spleen index was calculated by dividing the mean relative weight of the spleens of the experimental group by the mean relative spleen weight of the negative control. Indices of less than 1.30 are considered negative.

Cell numbers used in the experimental groups (expressed in millions) were 20, 30, and 50 for bone marrow cells; 10, 15, 20, and 50 for thymus and 5, 10, 15, 20, and 50 for spleen. When cell mixtures were tested (Table II), the proportions were as follows: (a) thymus and bone marrow: 10 of each, and 15 thymus plus 5 bone marrow cells; (b) spleen and bone marrow: 5 spleen plus 15 bone marrow, and 10 each; and (c) thymus and spleen: 10 each, and 15 thymus plus 5 spleen cells.

Results. Table I shows the spleen indices

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[1] American Legion Memorial Heart Research Professor of Pediatrics and Microbiology.

TABLE I. Spleen Indices Obtained by Injection of C3H/Bi Cells Intraperitoneally into (C3H × C57Bl/1)F1 Hybrids.[a]

Cell type:	Bone marrow			Thymus				Spleen				
Cell dosage (×10⁶):	20	30	50	10	15	20	50	5	10	15	20	50
	0.91	0.62	0.70	0.93	1.06	1.07	1.30	1.32	1.39	1.35	1.89	2.89
	0.99	0.92	0.89	0.95	1.12	1.12	1.72	1.50	1.77	2.04	2.60	3.00
	0.99	0.95	0.95	1.00	1.27	1.12	1.90	1.55	1.69	2.15	2.79	3.00
	1.00	1.01	1.03	1.00	1.39	1.20	1.90	1.80	2.50	2.25	2.90	3.10
	1.00	1.02	1.05	1.12	1.40	1.21	1.91	2.90	3.00	2.32	2.90	3.19
	1.02	1.05		1.14	1.43	1.24				2.40	2.94	
	1.02	1.05		1.16		1.33				2.72	2.95	
	1.06	1.07		1.21		1.40				2.80	2.99	
	1.10	1.10		1.24		1.63				3.01	3.00	
	1.15	1.10		1.26		1.97				3.17	3.12	
				1.30						3.20		
				1.52						4.16		
Mean	1.02	0.98	0.92	1.15	1.27	1.37	1.74	1.81	2.07	2.63	2.84	3.03
Positive indices /total tests	0/10	0/10	0/5	2/12	3/6	4/10	5/5	5/5	5/5	12/12	10/10	5/5

[a] All cell donors were 45-day-old females and the F1 hybrid recipients were 8 days old.

obtained after injection of different numbers of C3H/Bi bone marrow, thymus or spleen cells into (C3H × C57Bl/1F1 hybrids. Bone marrow cells in dosages ranging from 20 to 50 × 10^6 cells produced negative indices in all instances. Thymus cells in dosages ranging from 10 to 50 × 10^6 cells were effective in producing some positive indices (14 of 33 for all cell dosages tested). Spleen cells in dosages ranging from 5 to 50 × 10^6 cells produced positive indices in all instances.

Table II shows the spleen indices obtained with mixtures of cells of different origin.

TABLE II. Spleen Indices Obtained by Injection of Mixtures of Different Cell Types of C3H/Bi Origin into (C3H × C57Bl/1)F1 Hybrids.[a]

Cell type:	Thymus and bone marrow		Spleen and bone marrow		Thymus and spleen	
Cell dosage (×10⁶):	10T+10BM	15T+5BM	5S+15BM	10S+10BM	10T+10S	15T+5S
	0.93	0.82	0.69	1.20	1.30	1.28
	0.97	1.00	1.14	1.23	1.30	1.30
	1.00	1.03	1.17	1.42	1.89	1.36
	1.02	1.07	1.30	1.98	2.20	1.77
	1.05	1.12	1.45	2.75	2.21	2.55
	1.05	1.12	1.67			2.69
	1.06	1.15	1.70			
	1.07	1.19	1.77			
	1.10		1.79			
	1.13		1.80			
Mean	1.03	1.06	1.42	1.71	1.78	1.82
Positive indices /total tests	0/10	0/8	7/10	3/5	5/5	5/6

[a] All cell donors were 45-day-old females and the F1 hybrid recipients were 8 days old. Cell suspensions were injected intraperitoneally.

Thymus and bone marrow mixtures gave negative indices in all instances and no synergism could be detected using this test. These cell mixtures contained 10 or 15 $\times$ 10^6 thymus cells that per se were capable of producing some positive indices when injected alone (Table I). No synergism was observed when spleen cells were used in combination with either bone marrow or thymus cells. The number of positive indices in these tests was somewhat proportional to the numbers of spleen cells (5 or 10 $\times$ 10^6) present in the mixtures. The addition of bone marrow seemed to produce a diminution in the capacity of the competent cells present in either thymus or spleen cell suspensions to produce positive spleen indices.

Discussion. Our present results indicate that for the graft-versus-host reactions, as measured by the Simonsen assay (7), the response is porportional to the number of competent effector cells in the inoculum (i.e., thymus and spleen). No such competent reactive cells were detected in bone marrow. The addition of bone marrow to spleen or thymus cell suspensions had no clear effect on the reactivity of those cells. If anything, bone marrow seemed to exert an inhibitory effect on the capacity of thymus or spleen cells to exercise the graft-versus-host reaction.

The magnitude of graft-versus-host reactions is mainly related to antigenic differences between donor and host and to a minimum number of reactive competent cells in the inoculum (9). All the available data suggests that the graft-versus-host reaction can be considered as a single hit event started by a determined number of competent cells producing cell damage (10) and inducing an accompanying host response (11). Graft-versus-host reactivity is a thymus-dependent function in the sense that it is produced by cells that depend on the thymus for differentiation and development (5, 6). The absence of interactions between precursor and effector cells suggest a direct line of maturation of cells, produced or directed by the thymus, to become competent in the peripheral lymphoid organs. Thymus cells are relatively feeble in ability to express immunological reactions when compared to thymus-dependent cells in peripheral lymphoid tissues (12, 13). It takes approximately 10 times as many thymus cells as spleen cells to induce significant graft-versus-host reactions. Assuming that the immunologically reactive cells in both locations represent a single line of differentiation (derived probably from bone marrow precursors), these observations suggest that functional maturity is achieved after the cells leave the thymus.

This process of differentiation can be considered unidirectional and probably comprises precursor, intermediate, and effector compartments. Precursor and/or intermediate cells, under thymic influence, capable of exerting graft-versus-host reactivity in fetal liver and bone marrow using irradiated F1 hybirds as test system have been described (14). It will seem from our present experiments that, in contrast to certain antibody producing systems, the addition of precursor cells does not act synergistically with the effector cells in short term graft-versus-host reactions.

In relation to the synergistic effect of thymus and bone marrow cells described for the response to foreign red cells (1–4), several considerations must be kept in mind. Those experiments used heavily irradiated recipients as *in vivo* culture tests for the injected cells. The addition of bone marrow cells could increase the immune response by providing hemopoietic precursors and preventing depletion of the precursor pool or by a nonspecific effect of the bone marrow cells on the localization of antibody-forming cells in the spleen (15, 16). These effects were mainly manifested when bone marrow cells were associated with non-primed spleen cells (15). Absence of bone marrow–thymus synergism was observed when steroid-treated animals were used as cell receptors (17). These findings suggest that there may be something special related to the irradiation systems in which the synergism has been observed. These observations do not give an explanation to the fact that in the thymus–bone marrow interactions the antibody was made

by cells derived from the bone marrow (4). It is clear, however, that in the mouse the immediate precursor of antibody-forming cells may be derived from antigen insensitive stem cells present in the bone marrow and other tissues and following immunization with foreign red cells such precursor cells proliferate and differentiate giving rise to a specific antibody-forming progeny (18–23). Moreover, the cells derived from the thymus or differentiated under thymic influence do not participate directly in antibody synthesis (24). Another possibility is a highly nonspecific activation of progenitor cells of thymic origin by antigen with liberation of agents capable of influencing adjacent cells (25). One of these effects could be on "bursa"-dependent progenitor cells with potential to react with the antigen concerned. The thymus-dependent "helper" cells will thus facilitate the reaction with certain antigens by the "bursa"-dependent effector cells. Obviously the proportion of "helper" and "effector" cells may be variable in different organs. The thymus–bone marrow synergism may be characteristic for the response to antigens on red cell or other cell surfaces.

In the graft-versus-host reaction, as measured by the Simonsen assay (7), no synergism is observed due to the fact that the reaction has a threshold number of competent effector cells and is due directly to the impact of the thymus-dependent immunologically competent parental cells on the F1 hybrid (9, 10). Therefore, addition of precursor cells which might eventually differentiate into competent cells under thymic influence does not modify the short-term events measured by the graft-versus-host spleen assay. Preliminary studies also indicate absence of synergism between thymus and bone marrow cells for expression of graft-versus-host reactions in long term experiments using irradiated recipients (26).

The present findings indicate that thymic-dependent immunological functions (i.e., graft-versus-host reactions) do not operate through cell synergism but are more directly a function of the differentiative influence of the thymus on hemopoietic precursors.

Summary. Bone marrow cells were ineffective in producing significant graft-versus-host reactions as measured by a spleen assay test. No synergism between bone marrow and thymus was observed in the graft-versus-host reactions.

1. Claman, H. N., Chaperon, E. A., and Faser Triplett, R., Proc. Soc. Exptl. Biol. Med. **122**, 1167 (1966).

2. Claman, H. N., Chaperon, E. A., and Faser Triplett, R., J. Immunol. **97**, 828 (1966).

3. Miller, J. F. A. P. and Mitchell, G. F., Nature **216**, 659 (1967).

4. Mitchell, G. F. and Miller, J. F. A. P., Proc. Natl. Acad. Sci. U. S. **59**, 296 (1968).

5. Good, R. A., Dalmasso, A. P., Martinez, C., Archer, O. K., Pierce, J. C., and Papermaster, B. W., J. Exptl. Med. **116**, 773 (1962).

6. Dalmasso, A. P., Martinez, C., and Good, R. A., Proc. Soc. Exptl. Biol. Med. **110**, 205 (1962).

7. Simonsen, M., Engelbreth-Holm, J., Jensen, E., and Poulsen, H., Ann. N. Y. Acad. Sci. **73**, 834 (1958).

8. Stutman, O., Yunis, E. J., Martinez, C., and Good, R. A., J. Immunol. **98**, 79 (1967).

9. Simonsen, M., Cold Spring Harbor Symp. Quant. Biol. **32**, 517 (1967).

10. Simonsen, M., Brit. Med. Bull. **21**, 129 (1965).

11. Davies, A. J. S. and Doak, S. M. A., Nature **187**, 610 (1960).

12. Billingham, R. E. and Silvers, W. K., J. Exptl. Zool. **146**, 113 (1961).

13. Sosin, H., Hilgard, H., and Martinez, C., J. Immunol. **96**, 189 (1966).

14. Tyan, M. L., J. Immunol. **100**, 535 (1968).

15. Perkins, E. H. and Makinodan, T., J. Immunol. **92**, 192 (1964).

16. Radovich, J., Hemingsen, H., and Talmage, D. W., J. Immunol. **109**, 756 (1968).

17. Craddock, C. G., Winkelstein, M., Matsuyuki, Y., and Lawrence, J. S., J. Exptl. Med. **125**, 1149 (1967).

18. Trentin, J. J., Wolf, N., Cheng, V., Fahlberg, W., Weiss, D., and Bonhag, R., J. Immunol. **98**, 1326 (1967).

19. Doria, G. and Agarossi, G., Proc. Natl. Acad. Sci. U. S. **58**, 1366 (1967).

20. Osoba, D., Proc. Soc. Exptl. Biol. Med. **127**, 418 (1968).

21. Gregory, C. J. and Lajtha, L. G., Nature **218**, 1079 (1968).

22. Kennedy, J. C., Siminovitch, L., Till, J. E., and McCulloch, E. A., Proc. Soc. Exptl. Biol. Med. **120**, 868 (1965).

23. Playfair, J. H. L., Papermaster, B. W., and Cole, L. J., Science **149**, 998 (1965).
24. Davies, A. J. S., Leuchars, E., Wallis, V., Marchant, R., and Elliott, E. V., Transplantation **5**, 222 (1967).
25. Burnet, F. M., Nature **218**, 426 (1968).
26. Cole, L. J. and Davies, W. E., Jr., Exptl. Hematol. **16**, 21 (1968).

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The Effect of 6-Mercaptopurine on the Synthesis and Action of Interferon (33671)

JOSEPH W. ST. GEME, JR., DIANA S. HORRIGAN, AND PATRICIA S. TOYAMA
Department of Pediatrics, UCLA School of Medicine at Harbor General Hospital, Torrance, California 90509

Since other antimetabolites which disrupt nucleic acid and protein synthesis also inhibit the synthesis and action of interferon (1–7), it seemed plausible that 6-mercaptopurine (6-MP) may function in a similar fashion. Previous experiments demonstrated that 6-MP inhibits viral multiplication *in vitro* (8). However, infection of weanling mice with herpes simplex virus and simultaneous administration of 6-MP enhanced the synthesis of virus and interferon in the liver and brain, compared to untreated controls, suggesting that 6-MP fails to inhibit the elaboration of interferon (9).

These observations were clarified by study of the effect of 6-MP on both the synthesis and action of interferon *in vitro*. Inactivated chikungunya virus was employed to evoke satisfactory titers of interferon in chicken fetal cells (1). Alteration of the system by 6-MP was evaluated with the inclusion of actinomycin D as an inhibitory control. As anticipated, actinomycin fully inhibited the synthesis and action of interferon while 6-MP failed to alter significantly either phase of interferon biology.

Methods. Cells. Chicken fetal cell cultures were prepared from decapitated 10–11-day-old red rooster fetuses by trypsinization (0.2%) of minced, rinsed tissue and seeding of 16 × 125 mm glass tubes with 10^6 cells. Cells were plated in growth medium (GM) consisting of Eagle's basal amino acid and vitamin supplement in buffered Hanks' balance salt solution (BSS), 90 vol, and bovine serum, 10 vol. Cells were nourished during experiments with a similar maintenance medium (MM), substituting only bovine fetal serum, 2 vol.

Human diploid cells were derived by similar methods from a 6-week-old embryo.[1]

Virus. Chikungunya virus, strain S-27, obtained from American Type Culture Collection as intracerebral passage 173 in infant mice, was passed in the same manner twice more in our laboratory. The virus pool of suspended 10% brain was assayed in chicken fetal cells and yielded a titer of $10^{7.5}$ infectious doses (ID_{50})/ml.

Sindbis virus was obtained as the first mouse brain and subsequent second chicken allantoic fluid passage from Dr. Marcus Jensen. Two additional intracerebral passages were performed in infant mice, first, strain Swiss albino and next, strain C_3H/eB. The virus pool of suspended 5% brain was assayed in chicken fetal cells and produced a titer of $10^{8.8}$ ID_{50}/ml.

Interferon. Chick fetal cell cultures were inoculated with inactivated chikungunya virus (37°, 24 hr) at a multiplicity of 1.0 and culture fluids were harvested after 24 hr of incubation at 35°. Fluids were acidified to pH 2 with 1 *N* HCl and stored for 1 week at 4° before restitution to pH 7.2 with 1 *N* NaOH. Spent media from uninfected chicken fetal cell cultures were also harvested and

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