Influence of Thymus Cells on Erythropoiesis of Parental Marrow in Irradiated Hybrid Mice* (33334)

JOAN WRIGHT GOODMAN AND SARAH G. SHINPOCK
Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

Although the role of the thymus in immunobiology has been intensively and fruitfully investigated in the last ten years (1–5), comparatively little is known about the influence of this organ on erythropoiesis. Auerbach (6) found that thymus fragments cultured *in vitro* with embryonic spleen fragments had a beneficial effect on the subsequent growth of all elements of the splenic tissue. This effect was nicely confirmed *in vivo* by Metcalf (7), who found a 67% greater mass in splenic fragments grafted subcutaneously with thymus fragments than in splenic fragments implanted alone. This increased mass represented all splenic components and, as Metcalf pointed out, was in agreement with the converse finding of decreased splenic size of both follicles and red pulp after thymectomy in adult mice (8). That the thymus may be involved in control of red cell formatiion is further indicated by (a) the anemia subsequent to neonatal thymectomy of mice (3), (b) maturation arrest of erythroid cells in neonatally thymectomized opossum (9), and (c) the assocation of erythroid aplasia with an epithelial cell tumor of the thymus in man (10, 11).

While studying factors that modify the poor growth of parental marrow in irradiated F_1 hybrid mice, we observed that thymus, bone marrow, and liver cells were ineffective (in doses up to 3×10^7) in pretreatment experiments (12–14). Of various organs used to treat F_1 hybrid recipients two to three weeks before their irradiation, only those having a high proportion of immunocompetent cells (spleen and lymph nodes) augmented growth of subsequently transplanted parental marrow. However, when given as post-treatment to irradiated hybrids, parental bone marrow cells exposed to 10,000 R *in vitro* were the most effective cell type studied, spleen being second. Dead cells from lymph nodes and thymus produced only marginal improvement of poor growth, whereas liver and red cells had no effect. This report presents experiments in which viable thymus cells, injected into heavily irradiated hybrid mice treated with parental bone marrow, very clearly augmented erythropoiesis in the resultant chimeras.

Methods. Inbred parental strains and their F_1 hybrid crosses included C57BL (hereafter called B), C57BL/6 (or B6), C3H (or C3), DBA/2 (or D2), (B6♀ × D2♂)F_1 (or B6D2F_1), and (B ♀ × C3♂)F_1 (or BC3F_1). Recipients were 12–16 weeks old at the time of irradiation, and donors were 12–25 weeks old.

Mice were exposed to a single, 900-R whole-body dose of X-rays at a rate of around 160 R per min measured in air. Physical parameters were 250 kVp, 15 mA, 1 mm Al added filtration, hvl = 0.5 mm Cu, target-object distance = 60 cm. Parameters were the same for *in vitro* irradiation of cell suspensions, except that the target-object distance was 25 cm and the dose rate around 850 R per min measured in air.

Bone marrow plugs were suspended in phosphate-buffered saline by aspiration through 22-gauge and then 27-gauge needles. Thymus cells were suspended by aspiration through a 27-gauge needle after the whole organs, trimmed of fat and connective tissue, were pressed through a fine-mesh, stainless-steel screen. After erythrocytes were lysed by saponin, nucleated cells were enumerated on a Coulter counter. A viability estimate was made by counting, at a light microscope, the proportion of cells not stained by eosin. Yields in these experiments ranged from 50 to 110×10^6 eosin-excluding cells per thymus. Dilutions were based on eosin-excluding cell counts. Unless otherwise noted, cells, either

* Research sponsored by the U. S. Atomic Energy Commission under contract with the Union Carbide Corporation.

TABLE I. Effect of Viable Thymus Cells on Erythropoiesis in Irradiated $BC3F_1$[a] Hybrid Mice Given 10^6 Parental (B) Marrow Cells.

Experiment	Donor of 10^6 thymus cells	No. of mice	^{59}Fe uptake (% injected dose; mean ± SE) RBC	Spleen
A	B	31	7.2 ± 0.5	5.1 ± 0.2
	$BC3F_1$	28	2.5 ± 0.3	2.3 ± 0.2
	None	30	1.4 ± 0.1	1.6 ± 0.2
	Radiation control[b]	15	0.3 ± 0.04	0.3 ± 0.02
B	B	22	5.9 ± 0.7	3.1 ± 0.2
	C3	3	0.2 ± 0.1	0.3 ± 0.02
	$BC3F_1$	23	2.5 ± 0.4	1.7 ± 0.2
	$BC3F_1$ (+ no marrow)	5	0.2 ± 0.1	0.2 ± 0.1
	None	28	0.9 ± 0.1	0.5 ± 0.1
	Radiation control[b]	28	0.1 ± 0.01	0.3 ± 0.02
C	B	19	12.3 ± 0.7	5.6 ± 0.2
	B (+ no marrow)	3	1.1 ± 0.4	0.02 ± 0.00
	None	22	3.5 ± 0.4	2.2 ± 0.2
	Radiation control[b]	19	1.2 ± 0.3	0.03 ± 0.00
D	B	19	12.6 ± 0.9	5.9 ± 0.3
	B, i.p.[c]	21	2.1 ± 0.1	1.1 ± 0.1
	B (+ no marrow)	23	0.1 ± 0.01	0.4 ± 0.01
	None	25	2.0 ± 0.2	1.1 ± 0.2
	Radiation control[b]	9	0.3 ± 0.1	0.2 ± 0.02

[a] Recipients ♀ ♀ in Experiment A, ♂ ♂ in all others.

[b] Received no bone marrow, no thymus cells.

[c] Thymus cells given intraperitoneally; in all other cases, cells given intravenously.

marrow alone or marrow plus thymus, were given intravenously in a volume of 1 ml 18 – 22 hr after the recipients had been irradiated. When embolic death after injection of large numbers of thymus cells was anticipated, mice were given 50 units of heparin in 0.5 ml of saline intraperitoneally 10–20 min before receiving the intravenous inoculum.

Seven days after bone marrow was given, ^{59}Fe as citrate (0.5 μCi per mouse) was administered intravenously in a volume of 0.25 ml. Twenty-four hr later the mice were killed, and blood and spleens were removed for assessment of radioactivity in a well-type scintillation counter. Individual counts were recorded and are expressed as percentages of the injected ^{59}Fe dose. Erythrocyte values, obtained from the saline-washed red cells of a known volume of blood, were corrected to represent the mouse's total blood volume (15). Mean ^{59}Fe-uptake values for various groups of mice were treated by statistical tests of all comparisons among means, and differences were considered significant at the 5% level. In one experiment, spleen weight on day 8 after bone marrow was used as a criterion of graft proliferation (16).

Results. Viable cells from parental thymus produced a significant increase in erythropoiesis of grafted marrow (Tables I and II). Experiments A, B, and H illustrate a small but significant improvement resulting from viable F_1 hybrid (isogenic with the recipient) thymus cells. Thymus cells alone gave rise to no erythropoiesis, as shown by experiments B, C, and D. When thymus donors were of the second parental strain (C3 in experiment B), uptake values were indistinguishable from those of radiation control mice. High mortality in this group (8/11) and failure of the marrow transplant suggest that a graft-versus-graft (GVG) reaction of immunocom-

TABLE II. Comparison of Effect of Living[a] with Dead[b] Thymus Cells on Erythropoiesis in Irradiated Hybrids Given 10^6 Parental[c] Marrow Cells.

Experiment	Recipient	Donor of 10^8 thymus cells	No. of mice	^{59}Fe uptake (% injected dose; mean ± SE) RBC	Spleen
E	B6D2F$_1$ ♀ ♀	B6, dead	30	1.5 ± 0.2	1.2 ± 0.2
		None	23	1.2 ± 0.2	0.7 ± 0.1
		Radiation control[d]	14	0.6 ± 0.1	0.3 ± 0.02
F	B6D2F$_1$ ♀ ♀	B6, living	26	8.2 ± 0.7	4.9 ± 0.2
		B6, dead	29	1.6 ± 0.1	1.6 ± 0.2
		None	29	1.2 ± 0.2	0.6 ± 0.1
		Radiation control[d]	31	0.2 ± 0.03	0.4 ± 0.04
G	BC3F$_1$ ♂ ♂	B, living	19	6.6 ± 0.7	4.4 ± 0.3
		B, dead	21	3.5 ± 0.5	3.5 ± 0.7
		None	25	1.6 ± 0.2	1.3 ± 0.2
		Radiation control[d]	10	0.1 ± 0.01	0.2 ± 0.02
H	BC3F$_1$ ♀ ♀	BC3F$_1$, living	25	2.5 ± 0.3	2.4 ± 0.2
		BC3F$_1$, dead	26	1.7 ± 0.2	1.8 ± 0.2
		None	25	1.2 ± 0.1	1.3 ± 0.2
		Radiation control[d]	6	0.1 ± 0.03	0.3 ± 0.00

[a] Eosin-excluding.
[b] Exposed to 10,000 R *in vitro*.
[c] B donors for BC3F$_1$, B6 for B6D2F$_1$ recipients.
[d] Received no bone marrow, no thymus cells.

petent elements among the many (10^8) thymus cells resulted in rejection of the B marrow transplant.

In contrast to viable suspensions, heavily irradiated thymus cells gave variable ^{59}Fe-uptake results (Table II). In all experiments a small but significant increase in spleen values was recorded, but in only two cases, G and H, was the erythrocyte value significantly elevated above that of the corresponding group that received marrow only. One of these experiments involved F$_1$ hybrid thymus cells and therefore suggested that the small and inconsistent increase was nonspecific.

Figure 1 shows the dose-effect relationship of thymus cells with spleen weight used as a criterion of repopulation (16). These increases in spleen weight represent proliferation of all cell types normally found in the spleen and not simply GVH splenomegaly. In another experiment, groups of mice were given 0, 4, 8, or 16 × 10^7 parental thymus cells with no bone marrow, and spleen weights were recorded 8 days later (as in Fig. 1). Mean spleen weights increased from 20 mg (no thymus) to 40 mg at the highest thymus cell dose. Presumably the difference of 20 mg represents the maximum spleen weight increase to be found as a result of grafted thymocytes and their progeny en-

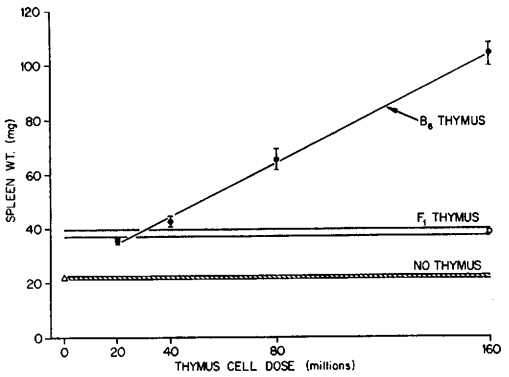


FIG. 1. Effect of thymus cell dose on spleen weight of irradiated B6D2F$_1$ females given B6 marrow (10^6 cells). Twenty-five to thirty mice per point.

gaged in GVH (17–19). Increases in excess of this in Fig. 1 must therefore be a consequence of hematopoiesis. Our assessment of erythropoiesis as well as histologic examination of splenic tissue attest to this interpretation. ^{59}Fe-uptake data for spleens and erythrocytes in this experiment paralleled those for the three highest spleen weight values, but ^{59}Fe data representing 2 and 4 $\times$ 10^7 parental thymus cells were indistinguishable from each other. Certainly between thymus cell doses of 4 and 16 $\times$ 10^7 spleen weight was a reflection of erythropoietic activity. The group given 16 $\times$ 10^7 isogenic (F_1) thymus cells in addition to parental marrow showed significant but slight augmentation consistent with data of Experiments A, B, and H. This high number of cells increased parental marrow growth to about the same extent as did a much smaller number (4 $\times$ 10^7) of parental thymus cells.

When thymus cells were given one or two days before or after, instead of with the marrow inoculum, they were also effective in increasing erythropoiesis. The results of this experiment are graphically presented in Fig. 2. Spleen ^{59}Fe-uptake values were greatest for the group that received thymus cells two days before and least for the one given thymus cells two days after marrow. The degree of augmentation was positively correlated with the length of time between thymus cell administration and assessment of ^{59}Fe uptake. The red cell values, however, a better indication of activity in the entire erythron, followed a slightly different pattern, with the largest value belonging to the group treated with thymus and marrow on the same day. The data of Fig. 2 make it clear that viable thymus cells are effective even when given a relatively long time before or after marrow.

Discussion. Recent studies of Claman *et al.* (20, 21) have shown a synergistic effect of thymus and bone marrow cells on immune response that may be analogous to our results. Using two assay methods, they were able to demonstrate in irradiated mice that ability to react immunologically to sheep erythrocytes was greater in recipients treated with adult thymus plus marrow than could be accounted for by summing results from recipients of the individual cell types given separately. They thought that the inability of a subcutaneous graft of whole thymus to act synergistically suggested that the effect seen when cell suspensions were injected was not a humoral one. As these authors point out, there are difficulties with this interpretation, since a humoral agent elaborated at a relatively distant site might fail to achieve the required concentration in lymphatic tissues. The complete lack of effectiveness of thymus given intraperitoneally (Expt. D, Table I) indicates that localization of injected cells is critically important in our own experiments. That the distribution of transfused lymphocytes may vary with the tissue of origin as well as with donor-host relationship has been shown experimentally (22).

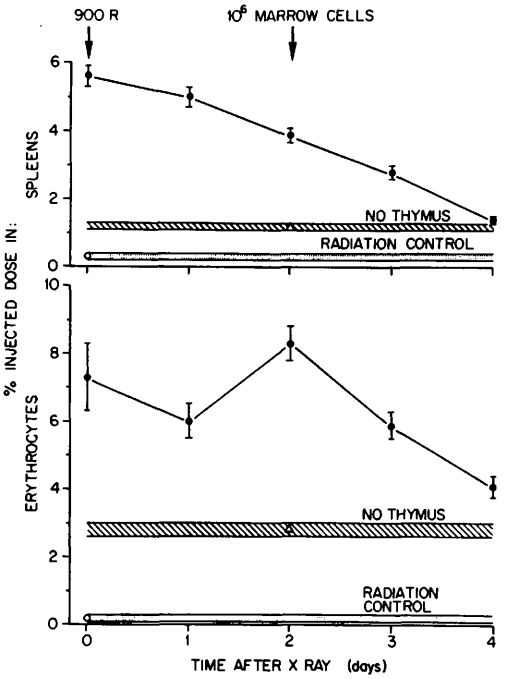


FIG. 2. Twenty-four-hour ^{59}Fe uptake as a function of time of B6 thymus cell (10^8) administration in B6D2F$_1$ males. Twenty-eight to thirty-one mice per point except for day zero, which had 16, and radiation control group, which had 7. All mice except radiation controls received 10^6 B6 marrow on day 2.

The effect reported here on erythropoietic activity of transplanted parental bone marrow is a large one. Red cell ^{59}Fe-uptake val-

ues obtained in mice given 10^8 thymus cells plus 10^6 marrow cells are roughly equivalent to those obtained from comparable F_1 hybrid recipients given 6 to 8 $\times$ 10^6 parental marrow cells alone or to isogenic parental recipients given 5 $\times$ 10^5 cells (unpublished data). This 6- to 8-fold increase in effectiveness results from the presence of thymus cells or from their products. The data available do not allow us to distinguish between two obvious possibilities: (a) cell surface interaction (or syngeneic preference) phenomena (23) and (b) humoral factors, either the same as or different from those previously reported for the thymus with respect to immunological activity (24, 25).

The small effect provided by living F_1 cells might represent a "sparing" of stem cells in the donor marrow; i.e., a diversion from immunological commitment to red cell formation. We have no experimental evidence that this was the case and believe it possible that the hybrid cells functioned in a completely nonimmunologic way. Whatever their mode of action, hybrid cells were considerably less effective than parental cells. The evidence strongly suggests a strain-specific effect.

It is not known whether a GVH reaction resulting from transplanted thymus cells contributed in some way to the augmentation of erythropoiesis. The fact that four separate experiments (unpublished data) in which parental lymph node cells in doses of from 4 to 100 million (together with parental marrow) were injected into irradiated hybrids gave rise to no augmentation indicates that GVH activity alone is not the factor responsible for the present results. Hybrid recipients of intravenously injected parental lymph node cells exhibited gross signs of secondary or GVH disease, terminating in death within 2 weeks of their treatment. The additional fact that parental thymus cells given without marrow to F_1 hybrids produced no real splenomegaly within 8 days (16 $\times$ 10^7 cells increasing spleen weight only 20 mg above that of irradiated untreated controls) suggests that the augmentation does not result directly from florid GVH reaction.

The relative inability of dead thymus, in contrast to dead marrow or spleen cells (13), to improve poor growth in these parent-to-hybrid combinations may reflect a problem in proximity or cellular localization like that suggested above for cells given intraperitoneally.

Further studies utilizing additional donor-host combinations and other experimental approaches, including Millipore diffusion chambers, should help us answer some of the questions raised by these results and to determine whether this effect is widespread or merely represents another peculiarity of the parent-F_1 situations in which poor growth is found.

Summary. Living parental thymus cells injected into heavily irradiated F_1 hybrid recipients augmented erythropoiesis resulting from transplanted bone marrow from the same, but not the second, parental strain. The augmentation increased as parental thymus cell dose was increased. Thymus cells alone, regardless of origin, gave rise to no erythropoiesis. When recipient spleen weight, 8 days after marrow transplantation, was used as a criterion of marrow growth, the data for parental cell doses 4 $\times$ 10^7 and above paralleled those from ^{59}Fe-uptake studies in the same mice. Parental thymus cells were effective even when administered 1 to 2 days before or after the marrow transplant.

Viable F_1 hybrid thymus cells produced a much smaller, but significant augmentation in the same parent-to-F_1 hybrid combination, one in which poor growth of marrow is well documented. When thymus cells, either parental or F_1 hybrid, were heavily irradiated (10,000 R), only a very small, apparently nonspecific augmentation was seen.

1. Defendi, V. and Metcalf, D., eds., "The Thymus," Wistar Inst. Symp. Monograph No. 2. Wistar Press, Philadelphia, Pennsylvania (1964).

2. Good, R. A. and Gabrielsen, A. E., eds., "The Thymus in Immunobiology." Harper, New York (1964).

3. Metcalf, D., "The Thymus." Springer, New York (1966). Recent results in cancer research vol. 5.

4. Wolstenholme, G. E. W. and Porter, R., eds., "The Thymus." Little, Brown, Boston (1966).

5. Miller, J. F. A. P. and Osoba, D., Physiol. Rev. 47, 437 (1967).

6. Auerbach, R., Natl. Cancer Inst. Monograph **11**, 23 (1963).

7. Metcalf, D., *in* "The Thymus," (V. Defendi and D. Metcalf, eds.) p. 53. Wistar Press, Philadelphia, Pennsylvania (1964). Wistar Inst. Symp. Monograph No. 2.

8. Metcalf, D., Brit. J. Haematol. **6**, 324 (1960).

9. Miller, J. F. A. P., Block, M., Rowlands, D. T., Jr., and Kind, P., Proc. Soc. Exptl. Biol. Med. **118**, 916 (1965).

10. Fisher, E. R., *in* "The Thymus in Immunobiology," (R. A. Good and A. E. Gabrielsen, eds.) p. 676. Harper, New York (1964).

11. Roland, A. S., Am. J. Med. Sci. **274**, 719 (1964).

12. Goodman, J. W. and Wheeler, H. B., Nature **211**, 1420 (1966).

13. Goodman, J. W. and Wheeler, H. B., *in* "Advance in Transplantation" (J. Dausset, J. Hamburger, and G. Mathé, eds.) p. 427. Munksgaard, Copenhagen (1968).

14. Goodman, J. W. and Wheeler, H. B., Transplantation **6**, 173 (1968).

15. Hodgson, G. S., Blood **19**, 460 (1962).

16. Popp, R. A., Congdon, C. C, and Goodman, J. W., Proc. Soc. Exptl. Biol. Med. **120**, 395 (1965).

17. Vos, O., de Vries, M. J., Collenteur, J. C., and van Bekkum, D. W., J. Natl. Cancer Inst. **23**, 53 (1959).

18. Congdon, C. C and Duda, D. B., Arch. Pathol. **71**, 311 (1961).

19. Yunis, E. J., Hilgard, H., Sjodin, K., Martinez, C., and Good, R. A., Nature **201**, 784 (1964).

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

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

22. Fichtelius, K. E. and Bryant, B. J., *in* "The Thymus in Immunobiology" (R. A. Good and A. E. Gabrielsen, eds.), p. 274. Harper, New York (1964).

23. Hellström, K. E. and Möller, G., Progr. Allergy **9**, 158 (1965).

24. Levey, R. H., Trainin, N., and Law, L. W., J. Natl. Cancer Inst. **31**, 199 (1963).

25. Osoba, D. and Miller, J. F. A. P., Nature **199**, 653 (1963).

Received March 21, 1968. P.S.E.B.M., 1968, Vol. 129.

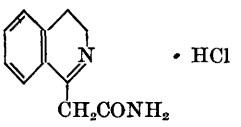
The Antiviral Activity of 3,4-Dihydro-1-isoquinolineacetamide Hydrochloride *in Vitro*, *in Ovo*, and in Small Laboratory Animals (33335)

E. GRUNBERG AND HERBERT N. PRINCE

Department of Chemotherapy, Hoffmann-La Roche, Inc., Nutley, New Jersey 07110

Recent years have witnessed the development of antiviral substances which have been either somewhat group specific in their effect or which have possessed a slightly broader antiviral spectrum. Examples of the former type are the isatin thiosemicarbazones for the pox virus group (1–3), iododeoxyuridine for DNA viruses, especially herpes (4, 5) and amantadine hydrochloride for influenza A (6, 7). Examples of the latter type are 2-dehydroemetine (8) and 1,1,3,3-tetracyanopropene (9) both of which exerted activity *in vivo* against Columbia SK, herpes and Coxsackie virus B1. The present report describes the results of experiments with a new substance, 3,4-dihydro-1-isoquinolineacetamide hydrochloride (10) (DIQA), showing activity *in vivo* against both RNA and DNA viruses but a very narrow spectrum of activity *in vitro*.

Materials and Methods. 3,4-Dihydro-1-isoquinolineacetamide hydrochloride (DIQA) is an odorless, white crystalline solid having the following structural formula:



The substance is highly soluble in water (20% at 20°) and alcohol (10% at 23°). Aqueous solutions are acidic, stable, and unchanged by heat.

In all the present experiments the test compound was dissolved in distilled water and prepared fresh before each experiment.