Effect of Thymidine Suiciding on Colony Formation *in Vitro* by Mouse Hematopoietic Cells[1] (36175)

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The specific progenitors of granulocytes and macrophages [*in vitro* colony-forming cells (CFC)] can be assayed because of the capacity of these cells to proliferate in agar cultures and form colonies of granulocytes and/or macrophages (1, 2).

Cell cycle killing agents have been used to determine whether or not *in vitro* CFC are in active cell cycle in the body. Suiciding by tritiated thymidine or treatment with vinblastine or hydroxyurea have all been reported to kill a proportion of *in vitro* CFC but there is some disagreement regarding the size of the surviving fraction and whether the latter cells are in a G_0 state (3–6). These questions are of some importance in identifying and determining the cell cycle status of the human granulocytic leukemic cells which also form colonies *in vitro*.

The present experiments were undertaken to determine the reproducibility of *in vitro* suiciding of mouse CFC using ^{3}HTdR and to explore some possible reasons for variation in the proportion of CFC killed.

Materials and Methods. Mice used were of the inbred strains C57BL, BALB/c, NZB, CBA, germfree CBA and RF and were aged 3 months.

All cultures were prepared in 35 mm plastic petri dishes (Falcon Plastics, Los Angeles) using modified Eagle's medium in 0.3% agar as described in detail elsewhere (7). Colony formation was stimulated by incorporating in each 1 ml agar culture 0.1 ml of a 1:6 dilution of pooled serum from C57BL mice injected 3 hr previously with 5 μg of endotoxin. This serum stimulates the formation of maximum numbers of colonies from cultured hematopoietic cells and a high proportion are granulocytic or mixed colonies (8).

Pooled cell suspensions were prepared in Eisen's balanced salt solution of bone marrow cells from a single femur shaft from each of three donors or spleen cell suspensions from 3 pooled spleens. Assays for the incidence of CFC were performed in 6 replicate cultures for each cell suspension using 10,000 bone marrow or 100,000 spleen cells/culture. Colonies were scored after 7 days' incubation at 37° in a fully humidified atmosphere of 10% CO_2 in air. Colony size ranged from 100 to 2000 cells. Colony morphology was determined by removing 50 sequential colonies from each type of culture, staining with 0.6% orcein in 60% acetic acid and typing according to criteria described previously (9). In other studies, total aggregate counts were performed on replicate cultures at daily intervals by scoring all discrete aggregates containing 3 or more cells.

Thymidine suiciding was performed using an adaptation of the technique of Iscove *et al.* (4). Samples of 2–4 $\times$ 10^6 cells in 1 ml of Eisen's balanced salt solution (BSS) were added to 1 ml of Eisen's BSS containing 40 μCi of ^{3}HTdR (23 Ci/mmole; Amersham, England). Control samples of each cell suspension were added to Eisen's BSS. All suspensions were incubated for 20 min at 37° then washed three times with 10 ml volumes of ice-cold Eisen's BSS containing 100 μg/ml of unlabeled thymidine and 10% fetal calf serum. Autoradiographs were prepared from suicided cell suspensions by smearing cells on

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TABLE I. Reduction of Mouse Bone Marrow *in Vitro* Colony-Forming Cells by Thymidine Suiciding.

Strain	No. of expts.	Mean *in vitro* CFC/10^5 cells[a]	Mean percentage reduction of *in vitro* CFC[b]
C57BL	16	244 ± 53	48 ± 14
BALB/c	5	191 ± 57	39 ± 11
NZB	6	281 ± 106	36 ± 16
RF	6	273 ± 78	45 ± 16
CBA	6	229 ± 58	48 ± 18
CBA germfree	8	155 ± 52	46 ± 10

[a] Mean *in vitro* colony-forming cells per 10^5 cells in control bone marrow suspension ± standard deviations. Pool of 3 donors used in each experiment.

[b] Reduction of *in vitro* CFC compared with individual matching controls.

gelatin-coated microscope slides, dipping in NTB_2 emulsion, and exposing at 4° for 24 hr. Preparations were developed, fixed, and stained with Giemsa as a check on the efficiency of ^{3}HTdR uptake.

Results. The comparative effects of *in vitro* thymidine suiciding were determined on pooled bone marrow cell suspensions (3 donors/pool) from mice of 5 inbred strains. In 16 separate experiments using C57BL cells, the percentage reduction by suiciding varied from 33 to 77% with a mean value of 48 ± 14%. Essentially similar data were obtained using cells from BALB/c, NZB, RF, and CBA mice (Table I). The percentage reduction of CFC in germfree CBA mice was similar to that with cells from conventional CBA and other strains, despite the lower incidence of CFC in the bone marrow of germfree CBA mice.

Colony size in cultures of suicided marrow cells appeared smaller than in cultures of control bone marrow cells. However colony growth rates are potentiated by colony crowding (12) and when special cultures were prepared containing 20,000 suicided cells versus 10,000 controls cells, so that colony counts were equivalent, no significant difference in mean size was observed in detailed cell counts on sequentially analyzed 7-day colonies.

Analysis of colony morphology in cultures of suicided and control C57BL and BALB/c bone marrows cells indicated that the relative frequency of granulocytic, mixed, and macrophage colonies was not altered by suiciding (Table II).

The incidence of *in vitro* CFC is much lower in the spleen than in bone marrow and as the spleen is a more reactive organ to

TABLE II. Percentage Distribution of Colonies Formed by Suicided Bone Marrow Cells.

Exp.	Strain	Type of culture	Mean percentage of colonies[a]		
			Granulocytic	Mixed	Macrophage
I	C57BL	Control	35	21	44
		Suicided	35	30	35
II	C57BL	Control	25	27	48
		Suicided	32	23	45
III	BALB/c	Control	7	31	62
		Suicided	8	19	73

[a] Fifty sequential colonies analyzed from each type of culture.

TABLE III. Reduction of Mouse Spleen *in Vitro* Colony-Forming Cells by Thymidine Suiciding.

Strain	No. of Expts.	Mean *in vitro* CFC/10^5 cells[a]	Mean percentage reduction of *in vitro* CFC[b]
C57BL	10	4.8 ± 2.4	28 ± 18
CBA	5	10.8 ± 8.4	22 ± 15
NZB	4	28.5 ± 7.3	17 ± 11
CBA germfree	4	0.4 ± 0.3	—

[a] Mean *in vitro* colony-forming cells per 10^5 cells in control spleen suspension ± standard deviations. Pool of 3 donors used in each experiment.

[b] Reduction compared with matching control.

antigen-induced changes, it shows a greater interstrain variability in *in vitro* CFC content than bone marrow. The results of suiciding spleen cell suspensions were more variable than with bone marrow. However, comparison of the data from different strains suggested that the level of suiciding was not related to the absolute number of CFC/10^5 spleen cells. In general, the reduction in spleen *in vitro* CFC was lower following suiciding than with bone marrow cells (Table III). Comparison of the pooled bone marrow and spleen data from all strains indicated a mean reduction of 45 ± 14% for bone marrow cells but a significantly smaller reduction of 24 ± 17% for spleen cells ($t = 5.1$; $p < .01$). *In vitro* CFC incidence in germfree spleens was too low ($< 1/10^5$ cells) to allow measurement of the effects of suiciding.

One characteristic of bone marrow cultures in agar is the marked asynchrony of initiation of aggregate and colony formation (7), some cells remaining dormant for 3–5 days in culture before commencing proliferation. Analysis of events in the first 3 days of culture of suicided marrows, counting all discrete aggregates of 3 or more cells, showed (Fig. 1) that the percentage reduction in total aggregates compared with control cultures was uniform throughout the incubation period. This indicates that the lag before proliferation with normal marrow cells *in vitro* is not likely to be due to a delay in passing from a G_0 to a cycling state. Furthermore the percentage reduction in the number of total aggregates was always similar to the reduction in colony numbers. Thus the reduction in cluster-forming cells [cells forming aggregates of smaller size than colonies (7)] is similar in magnitude to the reduction in colony-forming cells.

Discussion. The reduction in bone marrow

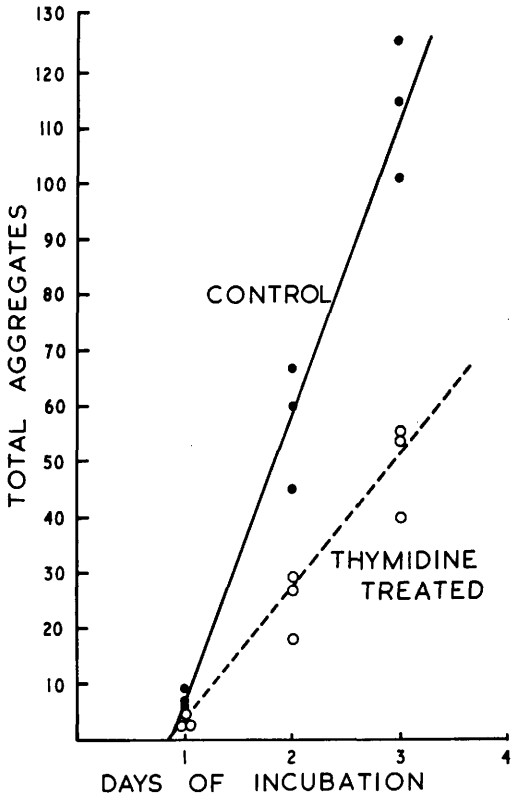

FIG. 1. Rise with time of total aggregates in cultures of normal and suicided C57Bl bone marrow cells: Each point represents data from a single replicate culture of 10,000 cells.

content in granulocytic and monocytic precursors (*in vitro* CFC) by thymidine suiciding suggests that most of these cells are in cell cycle, although, as suggested by others (4), a minority population could be in a G_0 state. Although cell separation techniques can partially separate cluster-forming from colony-forming cells (13) and also granulocytic colony-forming cells from macrophage colony-forming cells (14), suiciding caused a similar reduction in these cells and the cell cycle status of these various cells seems to be similar. In contrast to the data for bone marrow CFC, spleen CFC appear to have a longer cell cycle or, alternatively, a large fraction of spleen CFC may be in a G_0 state. *In vitro* CFC are a heterogeneous population (5, 13, 14) and it is not known whether the spleen selectively retains CFC with a certain pattern of proliferative activity or whether the spleen microenvironment can influence the cell cycle status of CFC.

No significant interstrain variation was noted between the five mouse strains tested which is of interest in view of the variable content of *in vitro* CFC in these strains, particularly in the spleen. Of special interest was the normal reduction observed with NZB mice which are subject to hemolytic anemia and with RF mice which have a high susceptibility to spontaneous and irradiation-induced granulocytic leukemia.

Since antigenic stimulation increases the level of granulopoiesis and the number of *in vitro* CFC in the bone marrow and spleen (10, 11) it is curious that germfree CBA mice exhibit the same reduction in CFC by suiciding as conventional CBA mice, despite the lower numbers of CFC in germfree mice.

The present *in vitro* suiciding technique has been used successfully with normal and leukemic human *in vitro* CFC (Metcalf, D. and Moore, M.A.S., umpublished data) but the differences reported here between bone marrow and spleen CFC make it necessary to interpret with care apparent differences between leukemic CFC (usually grown from peripheral blood) and normal CFC (grown from bone marrow cells).

Summary. In vitro suiciding of mouse *in vitro* granulocytic and macrophage colony-forming cells (CFC) using tritiated thymidine caused an average reduction of bone marrow colonies by 45% in 5 different mouse strains but only a 24% reduction in colony-forming cells from the spleen. Most bone marrow CFC appear to be in active cell cycle but many spleen CFC appear to have longer cell cycles or to be in a G_0 state.

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