PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 169, 295–300 (1982)

Old Mice: Marrow Response to Bleeding or Endotoxin[1] (41346)

MARVIN L. TYAN

Medical and Research Services, Veterans Administration, Wadsworth Medical Center, Los Angeles, California 90073, and The Department of Medicine, UCLA School of Medicine, Los Angeles, California 90024

Abstract. Peripheral blood hemoglobin, white cell, and lymphocyte levels decreased significantly with age in healthy female B6D2F$_1$ mice. However, these old mice maintained their hemoglobin levels in the face of chronic blood loss as well as did young mice by releasing younger than normal RBC into the peripheral circulation. In addition, before and after bleeding, the old mice had at least as many marrow and spleen CFU-S and CFU-C as did young mice. Old mice given endotoxin responded with greater elevations of peripheral blood lymphocytes and PMN than did young animals; the response of marrow CFU-S, CFU-C, and T-cell precursors to endotoxin was not impaired in old mice. Indirect evidence suggests that acute bleeding may increase the number of spleen CFU-S and T-cell precursors in old animals.

Advancing age in man and mouse is associated with declining levels of humoral and cellular immunity (1), hemoglobin (2, 3), and peripheral blood lymphocytes (4–6). It has been suggested that these changes may contribute to the increased vulnerability to certain infectious, autoimmune, and neoplastic diseases observed in the elderly (1). The etiology and pathogenesis of these changes are unclear, although evidence suggests that the decline in cells or function in part is secondary to diminished stem cell pools or to changes in the regulation of the marrow stem cells (7, 8).

In the present report it will be shown that despite the defects recounted above the marrow of old mice responds well to the stresses of endotoxin and chronic bleeding. That is, CFU-S,[2] CFU-C, and marrow T-cell precursors react to these stresses as well as do those in the marrow of young animals.

[1] This work was supported by USPHS Grant AG-00990 and VA Medical Research Funds.

[2] *Abbreviations used:* Hgb, hemoglobin; WBC, white blood cell; RBC, red blood cell; PMN, polymorphonuclear leukocyte; GOT, glutamic oxalacetic transaminase; GSSG-R, glutathione reductase; CFU-S, colony forming unit, spleen; CFU-c, colony forming unit, culture (granulocyte/macrophage); LPS, lipopolysaccharide.

Materials and Methods. *Mice.* Female B6D2F$_1$ mice, 4–33 months old, purchased from the Jackson Laboratory, Bar Harbor, Maine, were used. They were housed in a restricted access conventional colony, and they were given Purina Lab Chow and acidified water *ad lib.* These mice are considered to be mature at 12 months and old at 28 months.

Hematological data. Hemoglobin, hematocrit, and total peripheral blood white cell counts were determined by automated equipment (Coulter) using heparinized blood from the retroorbital plexus. Reticulocyte counts and white cell differential counts were done by standard methods (9).

Bone marrow and spleen cells. Only healthy donors were used. Any mouse found to have an enlarged spleen or other evidence of infection or malignancy was discarded. Cells were flushed from the marrow cavities with Hanks' solution: spleen cells were pressed through a fine mesh stainless steel screen. The cells were washed twice in Hanks' solution, and they were counted twice in a hemocytometer. Total cellularity was calculated for the two femurs and spleen obtained from each donor. Cell viability was assayed by the trypan blue exclusion test, and after adjustments in cell concentration, the appropriate number of viable cells was injected or placed in culture.

0037-9727/82/020295-06$01.00/0

Effects of chronic bleeding. In chronic bleeding studies groups of five 4- and 30-month-old female B6D2F$_1$ mice were bled weekly from the retroorbital plexus (0.3 ml), and Hgb, reticulocyte, GOT, and GSSG-R values were determined. In another experiment, the mice were killed after 7 weeks of weekly bleedings and their femurs and spleens were assayed for CFU-S and CFU-C.

Effects of endotoxin. Young and old mice were injected ip with sterile pyrogen-free saline or 8–10 μg of endotoxin (LPS *E. Coli* B5, Microbiological Associates) and bled three to four times over a 6-day period in order to follow peripheral blood hematological values. In one experiment they were killed on the sixth day and their femurs and spleens assayed for CFU-S, CFU-C, and the ability to repopulate the thymus.

RBC enzyme assays. Red blood cell GOT activity is increased in young RBC and decreased in old RBC (10); GSSG-R activity does not change significantly with the age of the RBC (M. L. Tyan, unpublished results). Therefore, the level of activity of GOT in whole blood crudely reflects the mean age of circulating RBC. Blood was obtained from the retroorbital plexus of individual mice. White blood cells were removed by passing 100 μl of heparinized blood through a 1-ml column of cellulose made with 50% Sigmacell type 50 and 50% α-cellulose (Sigma Chemical Co., St. Louis, Mo.) in 0.9% NaCl. The eluted RBC were washed three times in saline and hemolysed in stabilizing solution (11). The assays were performed using the methods of Beutler (11).

CFU-S assay (12). Young B6D2F$_1$ recipients were given 900 rad ^{60}Co radiation, and they were injected iv with 10^5 marrow or 5 $\times$ 10^5 spleen cells from healthy young or old syngeneic donors. Eight days after the injection of the cells the host spleens were fixed in Bouin's solution and macroscopic surface nodules were counted.

CFU-C assay (13, 14). CFU-C were assayed in a standard soft gel culture system using an extract of mouse placenta as the source of colony stimulating factor. Cultures were performed in quintuplicate and

TABLE I. PERIPHERAL BLOOD VALUES IN HEALTHY YOUNG AND OLD FEMALE B6D2F$_1$ MICE

Age (months)	n	Hgb (g/dl ± SD)	WBC (10^3/ml ± SD)	PMN (10^3/ml ± SD)	Lymphocytes (10^3/ml ± SD)	Reticulocytes (% ± SD)	Serum iron (μg/dl)[a]
4	15	16.0 ± 0.4[b]	13.3 ± 2.9[c]	1.5 ± 0.3	11.1 ± 0.4[d]	1.1 ± 0.3	272
30	14	13.2 ± 1.0[b]	7.6 ± 2.2[c]	1.2 ± 0.5	5.7 ± 0.7[d]	2.8 ± 1.4	204

[a] μg/dl, performed on pooled serum.
[b,c,d] $P < 0.01$, Students' t test.

were initially seeded with 5×10^4 marrow or 5×10^6 spleen cells.

Thymus regeneration assay (7). It is generally accepted that only T-cell precursors repopulate the thymus of a lethally irradiated marrow- or spleen cell-reconstituted recipient. Therefore, lethally irradiated (900 rad) young mice were injected iv with 3×10^6 marrow or 10×10^6 spleen cells from young and old donors, and 19 days later the thymi were removed and weighed. This interval was selected because previous work has shown that repopulation of the thymus by donor cells is near maximum and host marrow recovery minimal at this time (7). Each cell preparation was injected into 5–10 hosts.

Results. *Hematological values* (*Table I*). Hemoglobin, total WBC, and lymphocyte levels decreased significantly with age. There were no significant age-related changes noted in PMN or reticulocyte counts in these mice.

Effects of chronic bleeding on peripheral blood RBC (*Fig. 1*). When groups of 4- and 30-month-old B6D2F$_1$ mice were bled weekly over a period of 56 days, mean hemoglobin values declined only slightly in each group (old mice were not bled after the eighth week because of increasing debility). Reticulocyte counts were elevated initially in old mice and they were raised further by bleeding. Young mice responded to the chronic bleeding with only a slightly less vigorous reticulocyte response. Chronic bleeding produced a modest increase in the GSSG-R activity of RBC from young mice and little or none in the RBC from old mice. On the other hand, GOT activity, already elevated in RBC from old mice, rose significantly in the RBC of both age groups suggesting that the mean age of the RBC was diminished (10).

Effects of chronic bleeding on marrow and spleen CFU-S and CFU-C (*Table II*). After having been bled eight times over a 49-day period groups of 5- and 33-month-old mice were killed and their femurs and spleens assayed for CFU-S and CFU-C. It was found that young and old mice have about equal numbers of CFU-S in their spleens and femurs and that chronic bleeding has little or no effect on this population. Old mice were found to have more total marrow CFU-C than young animals; chronic bleeding had no significant effect

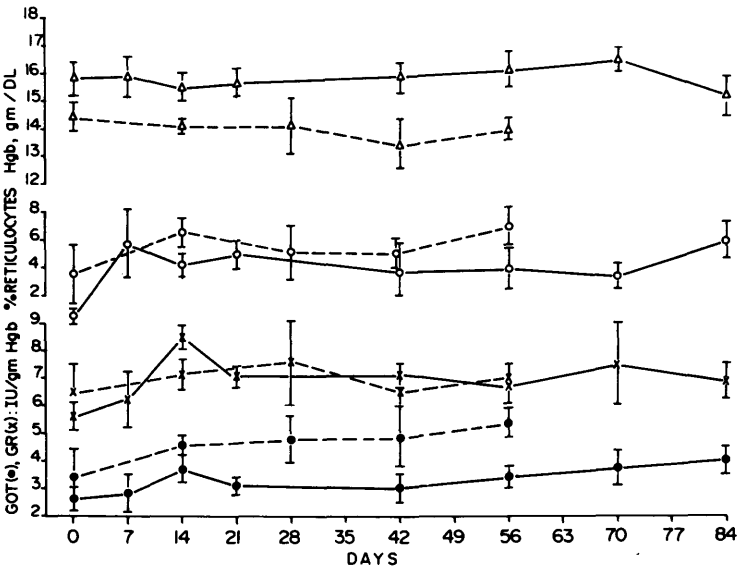

FIG. 1. Changes in erythrocyte GOT, GSSG-R, hemoglobin, and reticulocytes (mean ± SD) produced by bleeding (0.3 ml) 4-month-old (solid lines) and 30-month-old (broken lines) healthy female B6D2F$_1$ mice weekly. There were five mice in each group.

TABLE II. EFFECTS OF CHRONIC BLEEDING ON MARROW AND SPLEEN CFU-S AND CFU-C IN YOUNG AND OLD FEMALE B6D2F$_1$ MICE

Days bled	Age (months)	n	Day killed	Total CFU-S ($\times 10^3$)		Total CFU-C ($\times 10^3$)	
				Two femurs	Spleen	Two femurs	Spleen
0	6	5	0	5.4 ± 1.3	1.3 ± 0.2	145 ± 16[b]	0.5 ± 0.2
	32	5	0	7.1 ± 2.2	0.6 ± 0.2	243 ± 21[b]	0.8 ± 0.2
8[a]	5	5	49	5.4 ± 0.8	0.7 ± 0.5	135 ± 7[c]	1.0 ± 0.1
	33	5	49	6.7 ± 1.9	2.6 ± 2.3	229 ± 36[c]	0.5 ± 0.6

[a] Bled 0.3 ml once weekly for 8 weeks.
[b] $P < 0.01$, Students' t test.
[c] $P < 0.01$.

upon total CFU-C in young or old marrow or spleen.

Response of peripheral blood lymphocytes and PMN to endotoxin (Fig. 2). Healthy young and old mice were given 10 μg LPS and their absolute lymphocyte and granulocyte counts were followed for 7 days. It was found that initially the lymphocyte counts were lower in old mice and that by 6 hr after the injection of endotoxin the counts had dropped precipitously in young and old mice. On Days 1, 2, and 4 lymphocyte counts were higher in the old mice but by Day 7 they had returned to preinjection levels. A similar situation prevailed with respect to granulocytes with the exception that counts were slightly higher in the old animals in the resting state.

Effects of endotoxin on CFU-S, CFU-C, and T-cell precursors (Table III). Young and old mice were injected with saline or 8 μg of endotoxin, bled three times over a 6-day period in order to follow peripheral blood hematological values, and on the sixth day they were killed and their femurs and spleens assayed for CFU-S, CFU-C, and the ability to repopulate the thymus. It was found that (a) saline-injected young and old mice had equal numbers of femoral CFU-S, and splenic CFU-S were increased in both groups over the values found in unbled mice (Table II) but to a greater degree

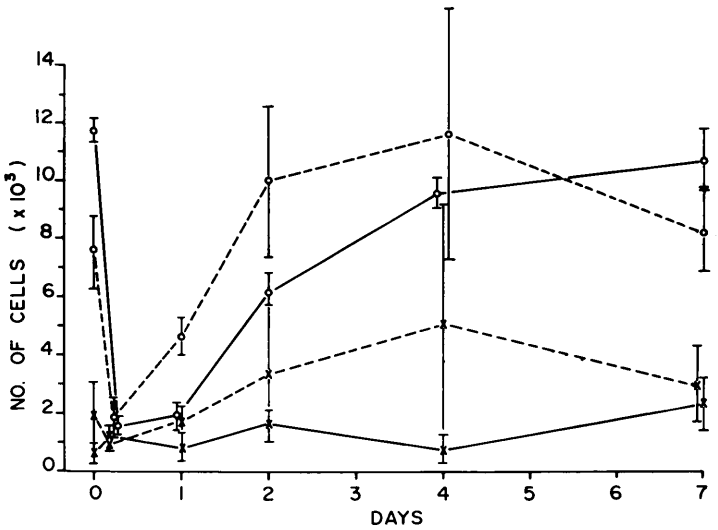

FIG. 2. Effect of 10 μg LPS *E. Coli* B5 on peripheral blood lymphocyte (○) and PMN (X) counts of 7-month-old (——) and 32-month-old (– – –) B6D2F$_1$ female mice. There were five mice in each group.

TABLE III. EFFECTS OF LPS *E. Coli* B5 ON MARROW AND SPLEEN CFU-S, CFU-C, AND T-CELL PRECURSORS FROM YOUNG AND OLD B6D2F$_1$ FEMALE MICE

LPS (8 μg)	Age (months)	*n* (donors)	Thymus regeneration[a]		CFU-S ($\times 10^3$)[b]		CFU-C ($\times 10^3$)[b]	
			Two femurs	Spleen	Two femurs	Spleen	Two femurs	Spleen
No	5	8	308 ± 63[c]	419 ± 126[d]	5.1 ± 1.0[e]	2.4 ± 1.3[f]	133 ± 30[h]	2.8 ± 2.3
Yes	5	5	446 ± 70[c]	349 ± 133	6.9 ± 1.1[e]	2.1 ± 0.7[g]	204 ± 62[h]	1.5 ± 1.0
No	31–36	6	342 ± 66	716 ± 216[d]	5.2 ± 0.8	7.3 ± 4.8[f]	132 ± 60[i]	10.0 ± 9.2
Yes	31–36	5	433 ± 181	527 ± 170	5.5 ± 2.7	9.1 ± 4.4[g]	186 ± 16[i]	3.3 ± 6.2

Note. The assays were performed 6 days after the injection of 8 μg LPS.
[a] Thymus wt (mg) × total cells in bone marrow or spleen: number of cells given.
[b] Total CFU-S or CFU-C in two femurs or one spleen.
[c,g] $P < 0.001$.
[d,e] $P < 0.002$.
[f,h,i] $P < 0.04$.

in the old animals (this may represent a response to acute bleeding), (b) endotoxin increased CFU-S in young marrow only, (c) saline-injected young and old mice had equal numbers of marrow and spleen CFU-C (in contrast to results in Table II where old mice had greater numbers of marrow CFU-C) and both groups had increased marrow CFU-C after LPS, and (d) thymus regeneration was similar in hosts given marrow cells from young and old saline-injected mice (a previous report demonstrated that thymus regeneration is decreased in recipients of marrow from old donors) (7) and LPS produced a slight increase which was proportionately greater in young animals; thymus regeneration was increased significantly in recipients of spleen cells from saline-injected old donors.

Discussion. These studies have confirmed earlier observations that in healthy mice there is an age-associated decline in peripheral blood hemoglobin and lymphocyte levels, the latter primarily the result of a decrease in T cells (2–6). In addition it has been shown that marrow from old mice, although apparently not able to compensate rapidly for acute loss (15), is able to maintain slightly reduced levels of hemoglobin in the face of chronic blood loss by releasing relatively young RBC into the circulation. As previously reported, old mice were found to have as many marrow and splenic CFU-S as do young animals (7, 16, 17), and chronic bleeding produced no significant changes in the numbers of these stem cells.

When healthy old mice were challenged acutely with a moderate to high dose of endotoxin, peripheral blood lymphocyte and PMN counts increased to a significantly greater degree than did those in young mice indicating that old mice have little difficulty producing and releasing these cells under certain conditions. Further, the response of marrow CFU-S, CFU-C, and T-cell precursors to endotoxin was at least equal to that observed in young animals.

It has been reported that in mice there is an age-related decline in the number and/or proliferative capacity of T-cell precursors in the marrow and spleen (7). In the present studies it was found that when bled three to four times within one week marrow from

old mice appeared to have the same potential to regenerate the thymus as did marrow from young animals, and spleen from old mice gave evidence of an increased potential. Further, the number of splenic CFU-S was increased in young mice which had been bled acutely (Table III vs Table II) and this was even more pronounced in the case of old mice. Although indirect, these data suggest that acute bleeding may result in at least a transient increase in the numbers of marrow and splenic T-cell precursors in old mice and in splenic CFU-S in both young and old animals.

Finally when viewed together, these experiments suggest that the decline with age in immune competence, hemoglobin, and peripheral blood lymphocytes cannot be attributed easily to a loss of stem cells or to gross defects in the quantitative responses of precursor populations to moderate physiological stimuli.

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Received September 2, 1981. P.S.E.B.M. 1982, Vol. 169.