DEFECTS IN HEMATOPOIETIC DIFFERENTIATION IN NZB AND NZC MICE*‡

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The process of hematopoietic differentiation is an extremely complex one which involves a series of distinct stages with both humoral regulators and microenvironmental factors determining the direction of differentiation (1). The hematopoietic stem cell is the primordial cell of this system, and has been shown to be capable of differentiation into any of the mature hematopoietic elements. The analysis of the separate events or processes involved in differentiation has been greatly aided by the use of several mutant genes in the mouse which influence hematopoietic differentiation. Mutant alleles at the *W* locus result in intrinsic stem cell defects which manifest as grossly defective cloning capacities with impaired stem cell proliferation and differentiation. This impairment leads to a macrocytic anemia (2). A similar form of anemia results from action of mutant alleles at the *Sl* locus but in this condition the defect is in the hematopoietic microenvironment necessary for the induction of hematopoietic stem cell proliferation and differentiation (2–4).

In this report, we have analyzed the process of hematopoietic stem cell differentiation in two other mouse strains, which show clear genetic evidence of an abnormality affecting a later state of hematopoietic differentiation. Mice of the inbred strain NZB/BL spontaneously develop a syndrome of autoimmune hemolytic anemia which closely parallels the human disease (5–7). This mouse strain was developed in New Zealand by Bielschowsky and Bielschowsky from an outbred source. A series of other related strains were simultaneously developed, from the same source, with coat color being the major selective factor. The interrelationships of these strains has been previously described (8). None of the other inbred NZ strains show any evidence of hemolytic anemia. The hybrid of the NZB and NZC strains is unique amongst the NZ hybrid in that it shows a similar incidence and severity of Coombs'-positive hemolytic anemia to the NZB inbred strain (6, 9).

Hematopoietic differentiation can be most readily studied in the mouse by use of the in vivo spleen colony-forming assay (10), and this paper presents a detailed analysis of this process in NZB and NZC mice.

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Materials and Methods

Mice.—The NZB and NZC mice used in this study were originally derived from the colony of Dr. Marianne Bielschowsky at the 42nd and 79th inbred generation respectively. The strains were then maintained in the Institute by brother–sister mating and by the production of stock mice which are no further than three generations away from the pedigree line. Mice of other inbred NZ strains, NZW and NZO, were also from colonies in the Hall Institute originally derived from New Zealand. Normal mice of strains BALB/c, C57BL, CBA, DBA/2J, B10.D2 were from inbred colonies maintained in this Institute, or were obtained from the Jackson Laboratories, Bar Harbor, Maine. Hybrid crosses of NZB or NZC mice with other inbred strains were bred as required. All mice were housed six to eight to a cage and fed water and Barastoc mouse cubes ad libitum.

Hematological Tests.—Peripheral blood samples were taken from the tail vein for routine white cell and red blood cell counts using a hemocytometer. Mice were killed for bone marrow shaft counts which were performed on single femurs with the marrow plug being aspirated into 1 ml of Eisen's balanced salt solution (EBSS).[1] Spleens were weighed and processed for histological examination. Coombs' tests were performed as previously described (6) using both polyvalent anti-mouse immunoglobulin sera and antisera specific for different heavy chain types.

Irradiation.—Mice were irradiated with a Phillips RT 250 X-irradiation machine (N. V. Phillips Gloeilampenfabriechen, Eindhoven, Holland) at approximately 127 rads/min for the required dose.

Colony-Forming Unit (CFU) Assay.—The assay system of Till and McCulloch was performed as previously described (10). Briefly, cell suspensions of donor tissue sources for analysis were prepared in EBSS, and total cell counts performed. Suitable numbers of cells were then injected intravenously into adult recipient mice which had been irradiated 1–3 hr previously with 800 rads of X-irradiation. The recipient mice were killed 7 days later and spleens removed and placed in Bouin's fixative. Counts of macroscopically visible colonies were then made and related to the injected number of cells. In the routine assay, donor cell suspensions were assayed in syngeneic recipients, although in a few specific cases allogeneic recipients were also used and these are indicated in the text.

Endogenous Stem Cell Assay.—This was also performed as previously described (11) and simply involves the irradiation of mice to be examined, using a range of irradiation doses between 550 and 800 rads. Mice were then sacrificed 8–9 days later for spleen colony counts.

Other Genetic Tests.—Several series of backcross or intercross mice which were examined for stem cell activity were also assayed for their genetic type at other loci. Immunoglobulin allotypes were determined on serum samples by Ouchterlony test with appropriate alloantisera as previously described (12). Histocompatibility (*H-2*) type was determined by the red blood cell hemagglutination method of Stimpfling (13) with antisera prepared by intensive immunizations of BALB/c mice with either C57BL (anti-$H\text{-}2^b$) or CBA (anti-$H\text{-}2^k$) spleen cells. Mice were scored for both presence of agouti and coat color. In one NZC backcross series, mice were sacrificed and the kidneys macroscopically examined for evidence of hydronephrosis, a condition present in many inbred NZC mice and associated with a single recessive gene (14).

RESULTS

Hematological Examination of NZC Mice.—As many detailed descriptions have been previously published on the NZB mice this section will only be con-

[1] *Abbreviations used in this paper:* CFU, colony-forming units; EBSS, Eisen's balanced salt solution.

cerned with the inbred NZC mice. 18 300-day and 20 500-day old NZC mice (including both sexes) were Coombs' tested and found to be completely negative. As reported elsewhere (14), a high proportion (approximately 50–80%) of NZC mice over 4 months of age showed evidence of varying degrees of hydronephrosis on either palpation or on autopsy examination.

Peripheral red and white blood cell counts were made on 18 16–18-month old NZC mice. Values somewhat lower than those of normal mice of the same age were obtained, with a mean red cell count of $6.10 \times 10^6/mm^3$ being less than that of controls of the same age range ($9.6 \pm 0.7 \times 10^6/mm^3$), while the leukocyte count (mean $9.7 \times 10^3/mm^3$) was quite normal. Serum immunoglobulin levels, and the humoral antibody response to several antigens, was also in the normal range.

During the preparation of spleen and bone marrow cell suspensions for stem

TABLE I

Spleen Weights and Bone Marrow Cell Counts in NZC Mice

Strain	No. mice	Age range	Spleen weight	Femoral shaft count
		(*months*)	(*mg* ± SD)	(×10^6 ± SD)
Control*	22	3–10	135 ± 26	13.1 ± 3.8
(NZC × BALB/c)F_1	10	2–4	92 ± 9	12.9 ± 3.2
NZC	27	2–10	69 ± 22	11.6 ± 2.7
Control*	15	13–20	94 ± 22	19.5 ± 8.6
(NZC × BALB/c)F_1	12	17	142 ± 39	14.6 ± 3.2
NZC	20	14–28	98 ± 42	5.7‡ ± 5.9

* Includes mice of BALB/c and several F_1 hybrid strains.

‡ Includes six mice with nonpatent marrow shafts.

cell assays, it was noticed that the NZC spleens often appeared small in size and the marrow shaft counts were somewhat lower than normal. A quantitative determination of this was then made with both young-mature (2–10 months) and older (>13 months) mice. Control data were pooled from BALB/c and hybrid (BALB/c × NZB)F_1 and (C57 × NZB)F_1 strains. (NZC × BALB/c)F_1 hybrid mice were separately examined. The results are given in Table I and show that the NZC spleen weights are considerably lower than those of the control mice, with the (NZC × BALB/c)F_1 hybrids also being slightly smaller than normal. The NZC spleens (2–10 months) ranged from 46 to 81 mg in weight, whereas the smallest control spleen was 74 mg. The older NZC spleens were somewhat larger, although still being smaller than the controls. Histological examination of the NZC spleen showed a significant depletion of the red pulp area. Semiquantitative assessment of this from camera lucida drawings of spleen sections indicated that the NZC spleen had about 75% of the red pulp area of control mice (BALB/c). The cellular components of the spleen appeared

quite normal and no change in lymphoidal components was evident. This depletion appears confined to the erythroid and myeloid series.

Total femur shaft counts were within normal limits in NZC mice under 1 yr of age. In older mice (12–28 months) considerably lower values were obtained. 6 of 20 mice examined had totally occluded femur shafts with gross evidence of excess bone formation. Even when these 6 are excluded, the mean value of 8.3 is

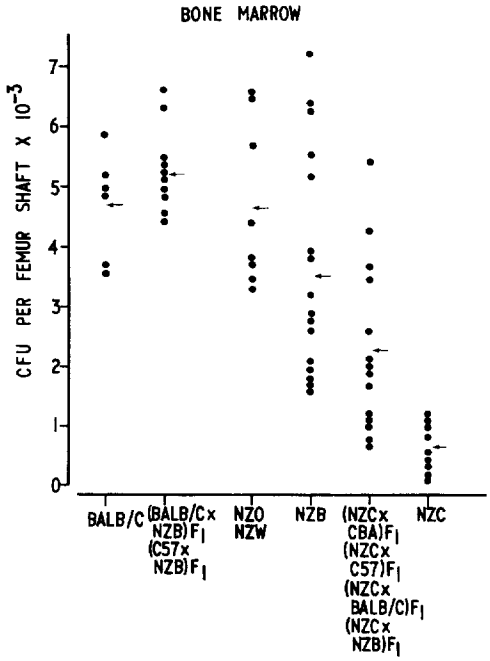

FIG. 1. CFU content of mouse femoral marrow for individual mice of several strains. Each point represents the CFU/femoral shaft of a single donor tested in a group of 6–8 recipients. The arrows give mean values for each of the strain groupings. Each donor cell suspension was tested in syngeneic recipients.

considerably lower than normal. The significance of the 6 mice with occluded marrow shafts is uncertain, as the mice had been fed for several months previously with cubes which contained an excessive amount of fluoride. It was noted, however, that similar bone marrow changes were not observed in mice of other strains in the same period of time.

Transplantation Assay for Hematopoietic Stem Cells.—Bone marrow and spleen cell suspensions were prepared from adult mice (3–6 months) of various inbred strains and from hybrids of either NZB or NZC with other normal strains. Each cell suspension was injected intravenously into 800 rad-irradiated

syngeneic mice, using a known cell number between 0.5 and 5.0 $\times$ 10^5 bone marrow cells or 1–5 $\times$ 10^6 spleen cells per recipient. From the spleen colony counts made 7 days later, the number of CFU/10^5 inoculated nucleated cells was determined. For bone marrow cells, this number was then related to the

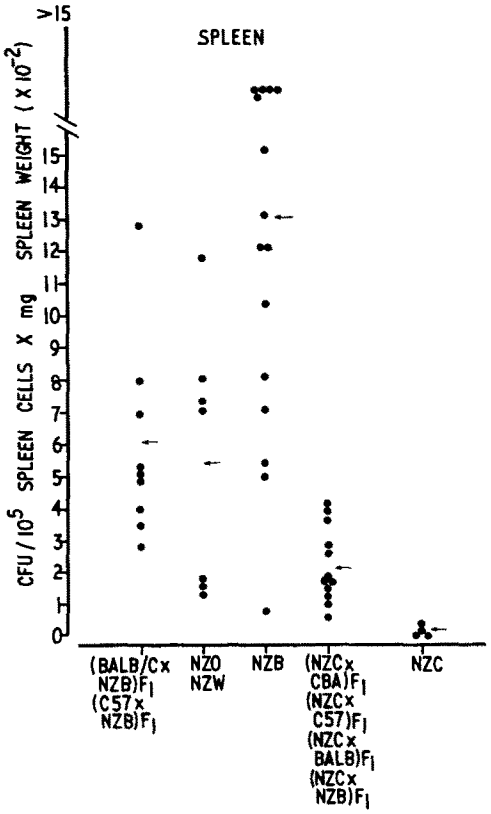

FIG. 2. CFU content of mouse spleen. Each point represents the CFU content of a single spleen cell suspension expressed as CFU/10^5 spleen cells multiplied by the weight of the spleen in milligrams. The arrows again indicate the means for the strain groups listed below. Each donor cell suspension was tested in syngeneic recipients.

total shaft count to give the CFU count per total femur. For spleen cells, an estimate of the total content of CFU in the spleen was made by multiplying the CFU/10^5 spleen cell value by the weight of the spleen in milligrams. Each inoculum was injected into groups of 5–8 mice, and the mean colony counts used for the calculations.

The results from 63 donor mice are given in Figs. 1 and 2. Each point represents the mean result from a single donor cell suspension. The bone marrow

values are quite similar for BALB/c, (BALB/c × NZB)F_1, (C57 × NZB)F_1, NZO, NZW, and NZB mice, although a greater variability was observed in the last group. The values for NZC mice show a striking depression in CFU activity with the mean value for CFU per total shaft being only 15% of the control values. The NZC hybrids give somewhat intermediate values, being about 50% of the control.

The depression of CFU activity is even greater with spleen cell suspensions. Total CFU activity in NZC spleens gave a value of around 3% of that of controls. Individual mice showed more variability with spleen cell inocula, particularly in the case of NZB mice. This strain again showed a relatively normal

TABLE II

In Vivo Colony-Forming Units in NZ Mice

Strain	No. mice	Bone marrow		Spleen	
		CFU/10^5	CFU/femur	CFU/10^5	CFU/10^5 × spleen wt. (mg)
BALB/c	6	72 ± 19*	4680 ± 892	3.2 ± 1.5	352 ± 169
(BALB/c × NZB)F_1	4	50 ± 9	5010 ± 439	5.3 ± 1.2	779 ± 358
(C57 × NZB)F_1	6	59 ± 11	5480 ± 814	4.2 ± 0.6	445 ± 162
NZB	5	37 ± 13	3150 ± 1930	7.6 ± 2.6	1230 ± 719
NZB (Coombs' positive)	11	28 ± 11	3900 ± 1884	4.2 ± 2.7	1387 ± 851
NZO	6	38 ± 8	4660 ± 1510	2.3 ± 1.7	397 ± 339
NZC	9	7.4 ± 6.3	629 ± 413	0.2 ± 0.2	11 ± 11
NZC Hybrids‡	8	19 ± 11	2308 ± 1521	2.5 ± 1.2	233 ± 126
(NZC × NZB)F_1	6	18 ± 11	2235 ± 1420	1.6 ± 1.4	197 ± 113

* ±Standard deviations.

‡ Hybrids of NZC with CBA, C57BL, and BALB/c.

spleen CFU activity per 10^5 cells, but gave a marked elevation in total spleen content, as the spleens of NZB mice are considerably enlarged. The NZC hybrids again gave intermediate values of around 40% of controls. The results are summarized in Table II and give the mean values of CFU/10^5 cells and per total organ.

The results clearly show that bone marrow and spleen cell suspensions from NZC mice give considerably reduced numbers of spleen colonies when injected into irradiated NZC mice.

Total Cell Content and CFU Content of First Passage Colonies.—A group of 800 rad-irradiated NZC and (NZB × C57)F_1 mice were injected with syngeneic bone marrow cells in sufficient numbers to give several colonies per recipient spleen. At 12 or 14 days after injection, recipients were sacrificed and individual spleen colonies were dissected out from the spleen and a cell suspension was

made of each entire colony. Each cell suspension was counted and then assayed for its content of CFU by injection into a second group of irradiated syngeneic mice. These were then killed 7 days later and the number of colonies counted. The results in Table III give the total cell and CFU content of 15–18 separate first passage colonies from each strain. The NZC colonies contained significantly fewer total cells and fewer CFU than the control (NZB × C57)F_1 colonies: approximately 33 and 20%, respectively.

Spleen CFU Assays with Allogeneic Recipients.—Absence or considerable depression in CFU activity after syngeneic transfer may involve abnormalities in either the donor cells or the recipient spleen. In order to delineate between

TABLE III

Total Cell Content and CFU Content of First-Passage Spleen Colonies

NZC*				(NZB × C57)F_1*			
12 day‡		14 day		12 day		14 day	
Individual colonies§							
TC	CFU	TC	CFU	TC	CFU	TC	CFU
2.3	20	8.0	—	8.4	80	4.3	960
3.6	340	1.7	80	9.0	20	9.2	700
1.4	0	3.2	40	4.9	1000	5.9	120
5.8	29	2.8	0	17.0	40	4.3	60
2.4	0	7.4	160	10.7	—	16.3	140
2.4	—			27.0	520	16.0	460
5.2	—			7.4	20	12.8	1500
3.8	—			28.3	140	16.5	660
2.1	—			5.5	430		
1.9	—			6.3	260		

* CFU assayed in syngeneic recipients.

‡ Time at sampling after bone marrow inoculum.

§ Total cell content (TC) × 10^6 and CFU content of colonies.

these two possibilities, NZC stem cells were transferred into irradiated mice of other strains, and NZB cells were transferred into NZC mice. The results with NZC donor cell suspensions are shown in Table IV. Four separate donor NZC marrows were used, and in each case 1–2 × 10^5 cells were injected into 800 rad-irradiated recipients of different strains. NZC recipients showed uniformly poor colony development, whereas NZB and NZB hybrid recipients gave values within normal expected limits. In the last experiment, good colony numbers were also found in both DBA/2 and B10.D2, but not in C57BL mice. It is noteworthy that all recipients except (C57BL (H-2^b), are of histocompatibility locus H-2^d type. NZC mice have not been typed for H-2.

The effect of transfusion of NZB bone marrow cells into NZC mice is described in Table V. Whereas normal numbers of colonies were obtained in NZB

inbred and hybrid recipients, no colonies were obtained in NZC recipients. In considering NZB and NZC mice, it is therefore clear that bone marrow cells from either donor will give normal colony numbers in NZB recipients, but not in NZC recipients.

Test for Possible Linkage of Reduced Colony Number to Genetic Influence for Hemolytic Anemia.—(NZB × NZC)F_1 hybrid mice develop autoimmune hemolytic anemia at the same age and to a similar degree as do the NZB inbred mice. From this and other genetic studies, it is clear that the NZC mouse strain carries at least one gene which is associated with the development of red cell

TABLE IV

CFU Assay with NZC Bone Marrow Cells Transferred into Different Recipient Strains

Donor NZC bone marrow*	Mean spleen colony count in recipients‡						
	NZC	NZB	(NZB × BALB/c)F_1	DBA/2	C57BL	B10.D2	(NZB × NZC)F_1
10^5	<1	18.4	10.8	—	—	—	8.7
2 × 10^5	4.4	33.0	—	—	—	—	34.2
2 × 10^5	6.2	38.3	—	—	—	—	—
2 × 10^5	1.0	—	26.0	19.7	0	26.1	—

* Each line represents a separate experiment with a different NZC donor marrow.

‡ Background (endogenous value for no cells injected) value subtracted.

TABLE V

CFU Assay with NZB Bone Marrow Cells Transferred into NZC Recipients

Donor bone marrow	Recipient*			
	NZB	(NZB × BALB/c)	(NZB × NZC)F_1	NZC
4 × 10^4 NZB	24.6	20.6	13.8	<1

* Each value is the mean colony count for a group of 6–8 mice.

autoantibody. In order to test for possible association of this gene with that involved in poor CFU colony growth in NZC recipients, a backcross mating of (NZB × NZC)F_1 to NZC was prepared. 45 of the progeny backcross mice were then tested at 9–12 months of age by the Coombs' test. The sex and coat color of all mice were recorded, and all mice were then given 800 rads X-irradiation and were injected intravenously with 10^5 NZC bone marrow cells using a pool of six NZC marrows as donor. Spleen colony counts were taken 7 days later, and the distribution of these counts is shown in Fig. 3. The upper histogram shows the spleen colony counts from NZB or (NZB × NZC)F_1 recipients which were injected with 10^5 NZC cells. The range in count is from 11 to 24. The counts in progeny of the backcross mice, however, range from 2 to 28, with a high proportion being less than 10. If 10 colonies/spleen is considered to be the lower value

for normal, then $^{26}/_{45}$ of the progeny mice had lower than normal counts. The complete data is summarized in Table VI. Apart from the slightly lower than expected Coombs' incidence, the observed values fit very well with those expected. A chi-square test was performed for possible association of either Coombs' positivity, sex, or coat color with the depressed (<10) colony count. No significant association was found for any of these parameters.

Endogenous Colony Assays.—Groups of mice from both normal strains (BALB/c, B10.D2, DBA/2), NZB and NZC hybrids with normal strains (CBA,

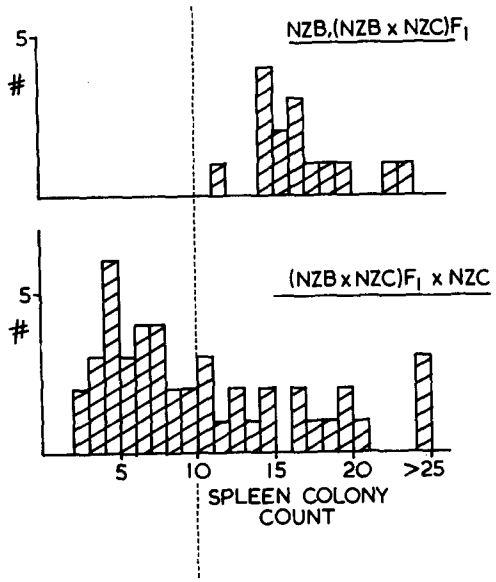

FIG. 3. Histogram of spleen colony counts in NZB and (NZB × NZC)F_1 mice (upper) or progeny of (NZB × NZC)F_1 × NZC (lower) injected 7 days previously with 10^5 NZC marrow cells. The dashed line indicates the lower limit of normal (upper) values.

B10.D2, BALB/c), and inbred NZB and NZC mice were irradiated with 650 rads of X-irradiation and endogenous spleen colonies were counted 8–9 days later. The results are summarized in Table VII and are shown individually in Fig. 4. Whereas normal mice and NZB and NZC hybrids with normal mice gave counts mainly between 0 and 6 with a mean of 3, both inbred NZB and NZC mice all had grossly elevated colony counts with a mean of around 20 colonies per spleen.

The effect of different radiation doses was then determined using both inbred NZB and NZC mice, four different NZB hybrids, and NZO and NZW mice. Groups of 6–8 mice were given X-irradiation at 50 rad increments between 550 and 750 rads. The mean endogenous colony counts are plotted in Fig. 5 and

show that the NZB and NZC strains show a marked elevation in colony counts at all radiation doses.

Genetic Control of Elevated Endogenous Colony Counts.—As all F_1 hybrids examined appeared to have normal endogenous colony numbers, it appears that at least one recessive gene is involved in determining this elevation. In order to assess whether only a single gene is involved, a group of F_2 and backcross mice were given 650 rads of X-irradiation and endogenous spleen colonies were

TABLE VI

Analysis for Association of Depressed Colony Count and Coombs'-Positive Reactions in (NZB × NZC)F_1 × NZC Mice

Progeny analysis	Expected	Observed
	(%)	(%)
Coombs' positive	50	33 ($^{15}/_{45}$)
Depressed CFU count	50	58 ($^{26}/_{45}$)
Sex (male)	50	58 ($^{26}/_{45}$)
Coat color (black)	50	49 ($^{22}/_{45}$)
Associations	Probability from chi-square test	
Depressed colony count: Coombs'	0.4	
Depressed colony count: sex	0.9	
Depressed colony count: black coat	0.9	

TABLE VII

Endogenous Spleen Colonies after 650 Rads X-Irradiation

Strain	No. mice	Mean No. colonies*
BALB/c, B10.D2, DBA/2	40	2.9 ± 3.1
Hybrids of NZB or NZC	54	3.1 ± 2.2
NZB	51	17.5 ± 9.8
NZC	16	25.4 ± 7.8

*±Standard deviations.

counted 9 days later. The results are shown in Figs. 6 and 7 and are summarized in Table VIII. The NZB strain was used for the F_2 cross and the NZC strain for the backcross experiment. In the F_1 (C57 × NZB) group and the backcross to the normal parent group ([CBA × NZC]F_1 × CBA), it would be expected that all mice would have a low colony count after 650 rads. This was indeed the case, with 0 and 4% respectively having colony counts greater than 6. Of the F_2 mice ([C57 × NZB]F_2), 30% gave elevated colony counts, as did 43% of the backcross group to the NZC parent. These values are very close to the 25 and 50% values, respectively, which would be expected on a single recessive gene hypothesis.

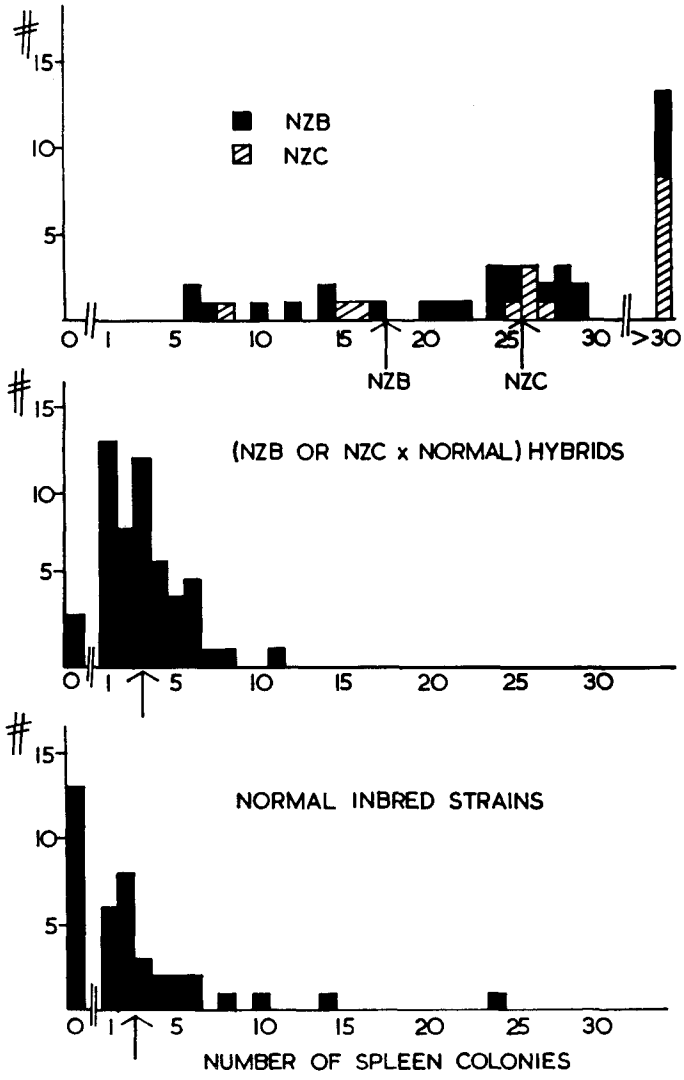

FIG. 4. Histogram of number of endogenous spleen colonies in mice irradiated 8–9 days previously with 650 rads X-irradiation. The upper graph is of NZB and NZC inbred mice, the middle of NZB and NZC hybrids with either BALB/c, CBA, or B10.D2, and the lower (normal strains) of BALB/c, B10.D2, and DBA/2 mice. The number of mice with the designated number of colonies is plotted, with the arrows indicating the mean colony count for the group: 17 for NZB, 26 for NZC, 3 for F_1, and 2 for control inbreds.

Analysis for Association of Elevated Endogenous Colony Activity to Other Genetic Markers and to Coombs' Positivity.—The F_2 and backcross series of mice used in the previous section were also analyzed for immunoglobulin allotypes, histocompatibility types, and coat color, where appropriate. The association of

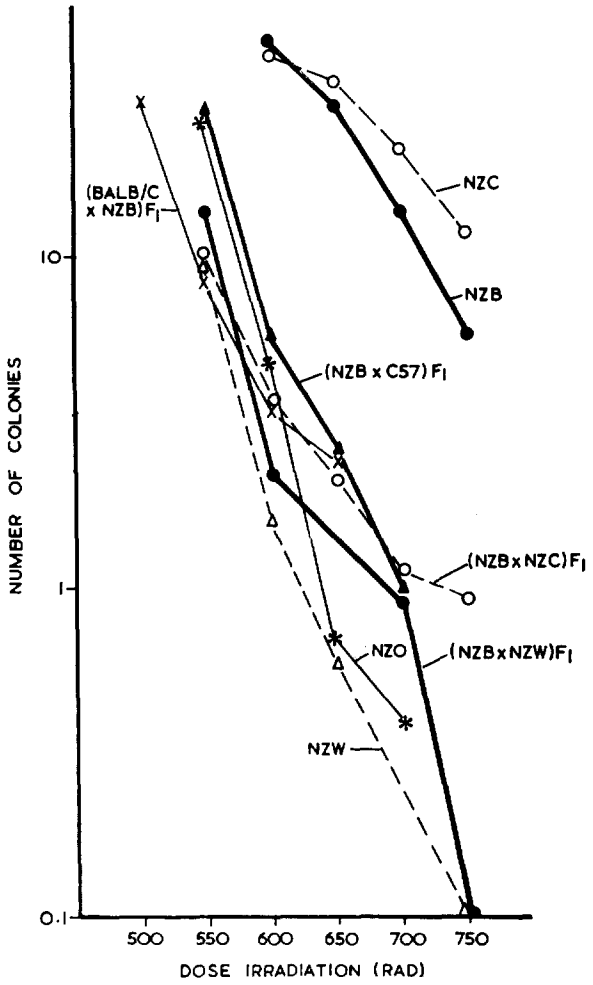

FIG. 5. Mean number of endogenous spleen colonies in mice previously given varying doses of X-irradiation. Each point is the mean colony count of 6–8 mice given the designated radiation dose.

these genetic types with endogenous spleen colony formation is shown in Tables IX and X.

The (NZB × C57)F_2 progeny mice were tested for the presence of *H-2*^{b} and for serum immunoglobulin allotypes *Ig-1*^{b} and *Ig-1*^{a}. No correlation between

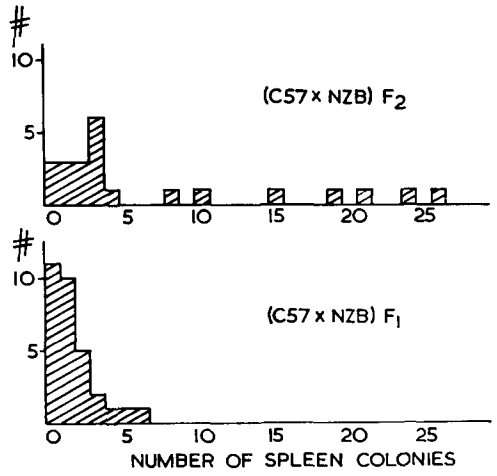

FIG. 6. Histogram of number of endogenous spleen colonies in F_1 and F_2 mice of C57 and NZB, previously given 650 rads of X-irradiation.

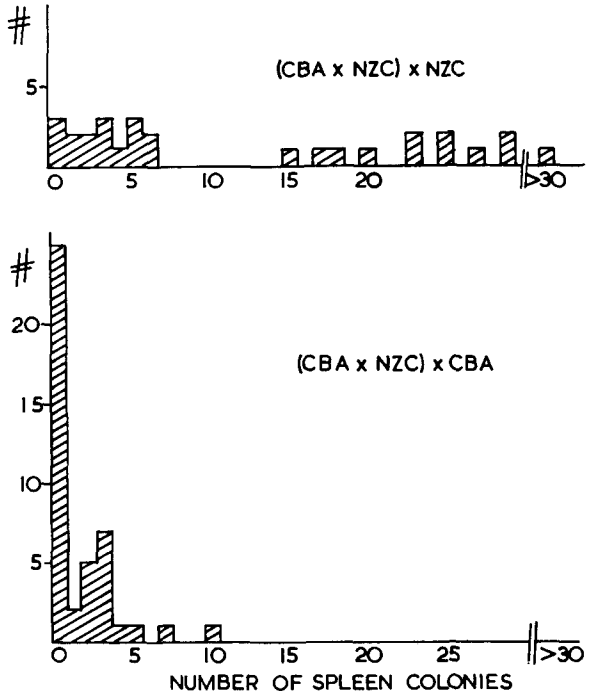

FIG. 7. Histogram of number of endogenous spleen colonies in backcross mice of CBA and NZC, previously given 650 rads of X-irradiation.

either of these loci and elevated spleen colony formation was evident (Table IX) the small numbers involved making the absence of elevated colonies in the *Ig-1*^{e} homozygous group of dubious significance. The (CBA × NZC)F_1 × NZC backcross mice were analyzed for the presence of *H-2*^{k}, agouti coat color, and for hydronephrotic kidneys, the latter being present in a very high proportion of inbred NZC mice and being controlled by a single recessive gene. The results in Table X clearly indicate that the elevated endogenous colony activity is not

TABLE VIII

Endogenous Spleen Colony Counts in F_2 and Backcross Mice

Strain	No.	Spleen colony count*	
		0–6	>6
		(%)	(%)
(C57 × NZB)F_1	31	31 (100)	0 (0)
(C57 × NZB)F_2	23	16 (70)	7 (30)
(CBA × NZC)F_1 × NZC	28	16 (57)	12 (43)
(CBA × NZC)F_1 × CBA	44	42 (96)	2 (4)

* After 650 rads irradiation.

TABLE IX

Lack of Association of H-2 or Ig-1 with Elevated Endogenous Colony Formation

Strain	Range	Endogenous Colony Count				
		*H-2**		*Ig-1*‡		
		dd	bd or bb	bb	be	ee
(C57 × NZB)F_2	0–6	3§	13	4	9	3
	>6	4	4	3	5	0

* Histocompatibility-2 locus: mice assayed for *H-2*^{b} by red cell hemagglutination.

‡ Immunoglobulin-1 locus (*IgG2a*): mouse serum assayed by Ouchterlony test.

§ No. of mice with indicated characteristics.

associated with either the *H-2* type, agouti or coat color types, or with the NZC hydronephrosis syndrome.

For analysis of possible linkage of Coombs' positivity to the elevated endogenous colony formation, 140 progeny mice from the intercross of (BALB/c × NZB)F_1 × (NZB × NZC)F_1 were tested at 9–12 months of age for their Coombs' reaction. Coat color and sex were recorded and the mice were then given 650 rads of X-irradiation and 8 days later endogenous spleen colonies were counted. A histogram of the colony counts is shown in Fig. 8, in which it is quite evident that although a large proportion of mice have colony counts in the expected control range of 0–6, a significant proportion of mice have high colony

TABLE X

Lack of Association of Elevated Endogenous Colony Formation with H-2, Coat Color, or Hydronephrosis in NZC Backcross Mice

Colonies	No.	Color*				Kidneys‡		*H-2*	
		aB§	AB	aCh	ACh	N	HN	dd	dk
0–6	17	5‖	2	6	4	11	8	4	7
>6	13	7	2	3	1	6	5	5	7

* A = agouti, a = nonagouti, B = black, Ch = chocolate.

‡ HN = at least one kidney showing definite hydronephrotic changes; N = normal kidneys.

§ Endogenous colonies in (NZC × CBA)F_1 × NZC backcross progeny.

‖ No. of mice with indicated characteristics.

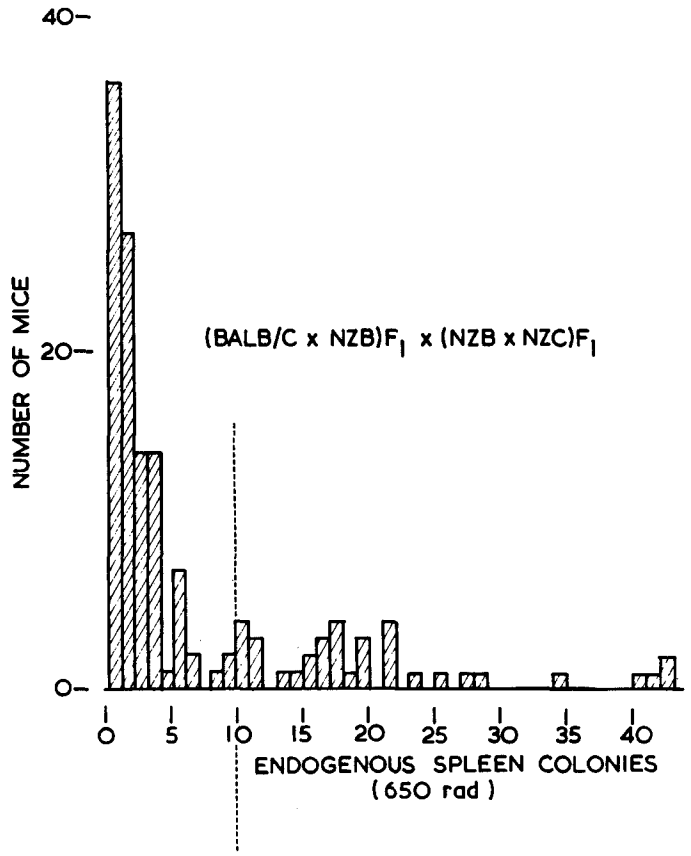

FIG. 8. Histogram of the number of endogenous spleen colonies in mice of (BALB/c × NZB)F_1 × (NZB × NZC)F_1 previously given 650 rads X-irradiation. The dashed line indicates an approximate maximum value observed for normal mice.

counts. The results for all tests are given in Table XI. The expected value for elevated colony count based on the single recessive gene data would be 25%, and indeed 24% of elevated counts were found. The expected values for the other parameters recorded were also closely approximated by the results. Analysis for association of elevated endogenous colony counts with the other analyzed parameters clearly showed there to be no association with the Coombs'-positive reaction. This was particularly evident in the female mice which comprise the majority of the Coombs'-positive and elevated colony mice.

This latter observation of a marked sex association with colony count was

TABLE XI

Analysis of Possible Linkage between Elevated Endogenous Colony Formation and Other Markers in Intercross (BALB/c × NZB)F_1 × (NZB × NZC)F_1 Mice

Test	Endogenous spleen colony count		Probability of association
	Normal	Elevated	
Coombs' positive	41*	20	0.05
Coombs' negative	65	14	
Agouti	61	24	0.20
Nonagouti	45	10	
Black coat	67	21	0.90
Chocolate coat	39	13	
Male	53	4	<0.001
Female	53	30	
Females, Coombs' positive	26	19	0.20
Females, Coombs' negative	27	11	

* No. of mice with indicated characteristics.

unexpected, 30 of the 34 elevated colony counts being in females. No association with coat color or agouti was observed. A comparison of the observed values for several parameters was made with the values expected on the basis of previous genetic studies with these mice. The total expected incidence per observed incidence of the following parameters was found: Coombs' positive 50%/44%, elevated endogenous colony formation 25/24%, male sex 50/41%, black coat color 50/63%, and agouti coat 50/60%.

Recovery of CFU Activity after Irradiation.—8–10 mice of strains NZB, NZC, and C57, and 3 (BALB/c × NZB)F_1 mice were given 200 rads whole body X-irradiation. 48 hr later marrow cell suspensions were prepared from the femurs and were each injected into 6–8 syngeneic recipients with the exception of the NZC marrows which were injected into NZB recipients. Spleen colonies were

counted and the mean CFU/10^5 donor cells is given in Fig. 9. The circled points represent the mean control values for unirradiated mice. Both the C57 and the (BALB/c × NZB)F_1 mice 48 hr after irradiation show a mean depression in CFU activity of the marrow to 15–19% of the control. The irradiated donor NZC and NZB mice, however, give 53% of the control CFU activity, although considerable variation from donor to donor was observed.

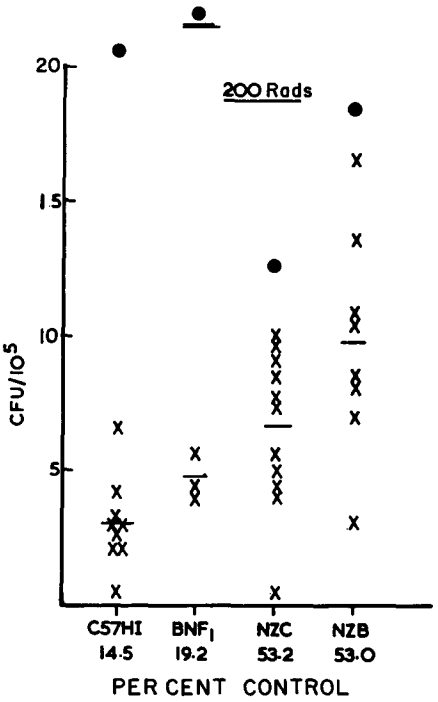

FIG. 9. CFU activity of femoral marrow suspensions of mice irradiated 48 hr previously Each point (×) is the CFU value of marrow for a donor preirradiated mouse, tested in syngeneic lethally irradiated recipients, with the exception that NZC donor cells were assayed in NZB recipients. The circles (●) are mean values of CFU activity for marrows from unirradiated donors. The bars (—) represent mean values for the irradiated donor groups. This mean value is expressed below as a percentage of the control mean value.

DISCUSSION

Differentiation of hematopoietic stem cells into mature red cells, granulocytes, platelets, or immunocompetent cells depends on two basic components, namely, a functional intact stem cell and a suitable microenvironment for induction of differentiation. Experimental analysis of this process in NZ mice has revealed two unexpected abnormalities; NZC mice show a defective capacity to support spleen colony formation after exogenous marrow grafting whereas in

the endogenous spleen colony assay a striking increase in spleen colony formation was found in these mice. This latter abnormality was also found in the NZB mouse.

The activity of hematopoietic stem cells can be readily quantitated by the CFU assay of Till and McCulloch (10). This assay usually involves syngeneic recipients and, when all the available NZ strains were tested in this manner, it was found that NZC mice showed a unique and severe reduction in colony formation. This was evident when both bone marrow and spleen were used as donor cells. When allogeneic recipients were used however (Tables IV and V), NZC marrow cells were found to be capable of giving normal spleen colony numbers providing the recipients were of the *H-2*^{d} type. In contrast, neither NZB nor NZC stem cells could form colonies in NZC recipients. This clearly indicates that NZC stem cells are fully functional and that the defect in spleen colony formation is in the environment of these mice which impairs colony-forming capacity of transfused stem cells. As an incidental point, studies with allogeneic recipients, particularly B10.D2 compared to C57BL, strongly indicate that NZC mice are of the *H-2*^{d} type.

Studies of F_1 hybrids of NZC mice show that with both spleen and bone marrow inocula, below normal spleen colony numbers occurred in the transplantation assay in syngeneic recipients. The depression observed was, however, by no means as marked as that found in the inbred NZC mice. Analysis of the irradiated backcross progeny of (NZB $\times$ NZC)$F_1 \times$ NZC injected with NZC cells (Table VI) showed that 58% of recipients had a very low (NZC type) number of colonies. This is very close to the 50% value expected on the basis of a single recessive gene controlling this phenomenon. The moderate degree of reduction in colony number observed in the F_1 hybrid suggests that in normal animals whatever process is controlled by this gene involves a limiting amount of gene product, and that a reduced amount available in the heterozygous state (of NZC) may therefore lead to the lower values observed.

Mutant alleles affecting hematopoiesis in the laboratory mouse have been described at more than 11 different loci (1, 2). Most mutants are detected by the appearance of anemia of varying degrees of severity, and by impaired survival. The hematopoietic abnormality in NZC mice produces only a mild anemia and there is little evidence of impaired survival, although data on this point is complicated by the frequent presence of chronic hydronephrosis (14). The nature of the defect in spleen colony formation in the NZC mouse would appear to be a defect in the hematopoietic microenvironment of the spleen and marrow rather than an intrinsic abnormality of the hematopoietic stem cell, and thus is superficially similar to the defect produced by mutations at the highly mutable Steel locus in mice. The NZC abnormality can be distinguished from the pleiotropic effects of mutant alleles of the Steel locus since the latter invariably produce, in addition to severe macrocytic anemia, sterility with gonads completely devoid

of germ cells in the homozygote. No impairment of fertility has been noted in our NZC colony. The extreme sensitivity to irradiation noted in Sl mice (2, 3) ($LD_{50}/30$ = 100–200 R) is likewise not manifest in NZC mice.

If NZC spleens cannot support the growth of transplanted stem cells, it would be expected that endogenous colony formation would also be considerably reduced. It was found however that both NZB and NZC mice gave markedly elevated numbers of spleen colonies at all radiation doses used. Thus, not only did NZC mice have endogenous colony formation, but it was far in excess of normal values. Again NZW and NZO mice gave normal values as did all F_1 hybrids of NZB and NZC studied. When F_2 (NZB and C57) and backcross mice (NZC and CBA to NZC) were tested for endogenous colony formation, the per cent of mice with grossly elevated colony numbers was 30 and 43%, respectively, which closely approximates the 25 and 50% values expected on the basis of a single recessive gene. Analysis for linkage to several available markers showed no linkage of this gene to coat color (black), agouti, hydronephrosis of NZC mice, *H-2* or immunoglobulin allotype *Ig-1*.

The reason for high endogenous colony numbers in the spleens of NZC and NZB mice remains obscure. Recovery of CFU 48 hr after 200 R of irradiation was much higher in NZC and NZB mice than in other strains, suggesting a greater radioresistance of CFU in these mice, although the alternative possibility of accelerated regeneration cannot be excluded. The paradox of impairment of proliferation of injected CFU in NZC spleens in contrast to apparent normal splenic proliferation of endogenous CFU, suggests that the lesion involves impairment in homing of injection of CFU to the spleen rather than proliferation once within the splenic microenvironment. Elevated endogenous colony formation may therefore eventuate from surviving CFU present within the spleen at the time of irradiation. High endogenous colony formation, usually associated with increased postirradiation survival, may be induced by the administration of a variety of nonspecific agents including foreign plasma, phytohemagglutinin and various antigens, or more specific factors such as 19S alpha macroglobulin (15–18). There are also reports of considerable elevation in endogenous colony formation in a semiinbred hairless strain of mice where cutaneous inflammatory foci may have presented a chronic nonspecific stimulus to hematopoiesis, increasing splenic hemopoiesis, stem cell mobilization, and hemopoietic regeneration (19). Presence of chronic inflammatory reactions in NZC and NZB mice may precede or be associated with the development of autoimmune manifestations, and play some role in the development of elevated endogenous spleen colony formation.

These studies were originally commenced with a view to ascertaining whether the autoimmune hemolytic anemia of NZB mice was associated either primarily or secondarily with a defect in hematopoiesis. In this context, it must be stressed that most of the studies reported in this paper were made with 3-month old mice

which do not show autoimmune hemolytic anemia. A particular analysis of NZC mice was made, as genetic tests indicate that a minimum of two genes in NZB mice are involved in the autoimmune hemolytic anemia and one of these (which is recessive) is also carried by NZC mice (Warner, N. L. Unpublished observations).

The observation that both NZB and NZC mice have elevated endogenous colony-forming activity might therefore indicate that an excessive drive to hematopoietic differentiation is present in these mice which in some manner predisposes them to the production of anti-red cell autoantibodies. However, genetic analysis for association of hemolytic anemia (as shown by Coombs' test) to the heightened endogenous spleen colonies showed there to be no such association (Table XI). This was determined in the progeny of (BALB × NZB)F_1 × (NZB × NZC)F_1 mice in which 39% of mice with normal colony numbers were Coombs' positive and 59% of those with elevated colony numbers were positive. Although the increased per cent which were Coomb's positive did ineed occur in those with elevated colony numbers, a statistical analysis gave a significance probability of 0.05, which perhaps best indicates that a larger group needs to be analyzed to determine if this is indeed a significant correlation. It is also relevant to note that in the backcross of (NZB × NZC)F_1 × NZC, no association was found between depressed CFU and autoimmune hemolytic anemia.

By genetic testing the NZC mice therefore appear to carry at least four separate recessive genes associated with abnormalities: (*a*) hydronephrosis, (*b*) predisposition to autoimmune hemolytic anemia, (*c*) elevated endogenous spleen colonies, and (*d*) inability to support spleen colony formation by transfused stem cells. To date, no significant interconnection between any of these activities has been found.

Further experiments are in progress to ascertain the nature and mechanism of these two genetically controlled defects in hematopoietic differentiation. Studies on these mice should therefore considerably aid our understanding of the function of separate components which are involved in the complex process of hematopoietic differentiation.

SUMMARY

Hematopoietic stem cell activity in inbred NZB and NZC mice has been determined by transplantation and endogenous spleen colony assays. Whereas NZB mice show normal colony-forming unit (CFU) activity in the transplantation assay, they show markedly elevated endogenous CFU. NZC mice also show this markedly elevated endogenous CFU activity, but in the transplantation assay show only about 5–10% of normal CFU counts. When NZC stem cells are tested for CFU activity in irradiated recipients of the *H-2*^{d} type, almost normal colony numbers occur. NZB stem cells however also cannot form colonies in

NZC mice. These results suggest that NZC mice have a defect in the microenvironment of the spleen which renders them incapable of allowing transplanted CFU to form colonies.

Genetic analysis of both the NZC defect as a CFU recipient, and the elevated endogenous count in NZB and NZC, shows that both are controlled by single recessive genes which are not linked to either coat color, agouti, *H-2* or *Ig* loci. Of even more relevance is the finding that these hematopoietic abnormalities are not linked to the genes involved in controlling autoantibody formation to red cells in the NZB mice. These mice therefore appear to show two distinct hematopoietic abnormalities, the analysis of which may be of considerable value in understanding the detailed events of hematopoietic stem cell differentiation.

Addendum.—The demonstration of elevated endogenous stem cell activity in NZB mice provides experimental confirmation of the recent report of a high resistance to the effects of lethal doses of ionizing radiation (Morton, J. I., and B. V. Siegel. 1971. *Proc. Nat. Acad. Sci. U.S.A.* **68**:124.) in these mice.

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