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Progress Report ~~to accompany~~

~~Twentieth~~ renewal proposal to the

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Title of project: INBORN ANEMIAS IN MICE

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ABSTRACT

Hereditary anemias of mice have been the chief objects of investigation under ERDA Contract E(11-1)-3264. At present under study are four macrocytic anemias, four hemolytic anemias, nonhemolytic microcytic anemia, transitory siderocytic anemia, sex-linked iron-transport anemia, and the autoimmune hemolytic anemia of NZB. These anemias have been studied through: (a) characterization of peripheral blood values, (b) determinations of radiosensitivity under a variety of conditions, (c) measurements of iron metabolism and heme synthesis, (d) histological and biochemical study of blood-forming tissue, (e) functional tests of the stem cell component, (f) examination of responses to erythroid stimuli, and (g) transplantation of tissue between individuals of differently affected genotypes. Considerable effort is devoted to perfection of hematologic, cell culture, and transplant methods to make these techniques useful in dealing with special problems associated with abnormal function.

RESEARCH ACCOMPLISHMENTS

A. Delineation of inborn anemias

The basic purpose of this Research Contract is to delineate inborn anemias of the laboratory mouse. This is carried out by preparation of

genetically homogeneous stocks segregating only for anemia-producing genes, by descriptions of each condition at all stages in the life--history, by determination of tissue-sites of primary gene action through transplantation experiments, and by analysis of reactions of normal and anemic mice to a variety of stressful stimuli, including X-irradiation and hypoxia. At present 12 single-locus anemias are known in the mouse, plus one with multifactorial inheritance (the autoimmune hemolytic anemia of NZB inbred mice). Effects of anemia--producing mutant alleles at 10 of these loci (an; f; ha; ja; mk; nb; Sl and Sl^d; sla, sph; and W, W^v, W^J) are currently under investigation at the Jackson Laboratory with support from this contract. We also have established an NZB/BlNJ colony susceptible to autoimmune disease.

We are continuing to analyze all presently known hereditary anemias of the mouse and to apply our findings toward an increased understanding of the genetic control of hemopoiesis, regulation of gene action, mechanisms for erythroid homeostasis, and relations between erythropoiesis and myelopoiesis.

The anemias under investigation may be classified as follows:

Macrocytic anemias: dominant-spotting W-locus (W/W, W/W^v, and other double-dominant combinations); steel (Sl/Sl, Sl/Sl^d); Hertwig's anemia (an/an).

Hemolytic anemias: jaundiced (ja/ja); hemolytic (ha/ha); normoblastic (nb/nb); spherocytic (sph/sph); NZB (autoimmune hemolytic anemia).

Iron defect anemias: flexed (f/f) transitory siderocytic anemia; sex-linked anemia (sla/sla ♀♀, sla/Y ♂♂) with defect in transport of iron from the intestinal epithelium to their blood stream; and microcytic anemia (mk/mk), with a defect in iron uptake.

B. Establishment of foundation stocks with anemia-producing genes on genetically uniform backgrounds.

Considerable effort has been devoted to establishing each mutant allele on a genetically uniform background that allows some postnatal survival of affected individuals. Wherever possible, all mutant alleles have been transferred (by repeated crosses) to two specific genetic backgrounds, C57BL/6J and WB/Re, so that $WBB6F_1$-m/m individuals, congenic except for the differing mutant allele, m, could be compared with each other and with congenic hematologically normal $WBB6F_1$- +/+ mice.

One feature of this gene-maintenance should be stressed in this 20th progress report:

Foundation stock colonies. A serious concern of the Jackson Laboratory is long-term future availability for research of our mutant-bearing and congenic normal animals. In fact, this is the basic reason for the

construction of our new Mammalian Genetics Laboratory, where stocks are to be maintained with maximum environmental protection. We have begun to establish, in this new facility, foundation stocks of each of our anemia-producing congenic lines. The first step in this process was building up a colony of very clean C57BL/6J$_{fh}$(J67) mice descended from C57BL/6J mice fostered in 1967 on descendants of germ-free hand-reared mice maintained in a special Jackson Laboratory animal health colony. Females from this special C57BL/6J$_{fh}$(J67) stock will serve as foster mothers to Caesarian-derived (hence aseptic) offspring from the stocks we plan to put into our Anemia Mutant Foundation Stocks. We will also use WB/Re foster mothers, which are themselves descended from WB/Re mice introduced into our colony as Caesarian-derived aseptic neonatal mice. So far we have built up in the Mammalian Genetics Laboratory a colony of more than 30 mated pairs of C57BL/6J$_{fh}$(J67) mice, and 8 pairs of WB/ReJfb mice, and have successfully introduced the W mutant allele in the form of WB/Re-W/+. We estimate it will take approximately one year to complete introduction of the 28 anemia-mutant bearing stocks now considered essential for future research on mouse hereditary anemias.

C. Comparison of Hertwig's anemia with other hypoplastic anemias

Hertwig's (an/an) anemic mice, discovered by Paula Hertwig in 1942, have only recently come into proper focus after more than 10 years of genetic manipulation in our laboratory, where the an gene was first coupled with the closely linked coat-color marker gene B^{lt}, following which the coupled pair of genes were backcrossed for more than 10 generations onto the now standard inbred strains WB/Re and C57BL/6J. The viability of an/an mice is much greater on the WBB6F$_1$ hybrid background than on any previously studied. These efforts have made it possible to undertake hematological studies comparing an/an macrocytic hypoplastic anemia with the now classical macrocytic anemias of W/W^v and Sl/Sl^d mice, with all three mutants segregating upon the same genetic background. We used young adults for comparison with similar-aged W/W^v and Sl/Sl^d mice and found that an/an mice are less severely anemic (see Table 1), with mean RBC 69% of normal, mean hematocrit 87%, mean cell volume 123%, and mean hemoglobin constant 82% of normal. Peripheral white counts in WBB6F$_1$-an/an mice tend to be depressed (65% of normal), but we have found no selective loss of any of the peripheral leucocyte types. Platelet counts, on the other hand, are normal to slightly elevated. The reduction of leucocyte counts in WBB6F$_1$-an/an mice is not as great as that observed by us in an/an mice congenic with other genetic backgrounds (BAN/Re, BANB6F$_1$), and since the platelet counts are high, our judgement now is that if Hertwig's anemia can be classified at all as a pancytopenia, on the WBB6F$_1$ background, some features, at least, are well compensated. There is evidence of mild stem-cell deficiency as measured by the number of colonies formed (CFU-S) in the X-irradiated spleen assay. (Table 1, 9 rather than 18 CFU-S/femur). Perhaps the relation of femoral nucleated cellularity and red cell number is the best indication of the functional state of an/an marrow, although the an/an femur has only 50% of the cells

seen in +/+ femora, and contains only one-quarter as many CFU-S. The circulating erythrocyte level of an/an mice is not as deficient as one might expect (65% of normal), and this mouse apparently compensates for its marrow deficit by increasing its splenic erythroid activity.

The proportion of red cell precursors was significantly higher in an/an spleens (mean, 2.66 per cent) than in +/+ spleens (mean, 1.29 per cent), quite in contrast to the finding of lowered proportions of red cell precursors in W/W^v (mean, 0.60 per cent) and Sl/Sl^d (mean, 0.72 per cent) spleens. However, no splenic enlargement was observed in these particular an/an mice. It seems clear that the an/an mice tested had not reached their limits of compensation.

TABLE 1. COMPARATIVE HEMATOLOGY OF THREE HEREDITARY HYPOPLASTIC ANEMIAS ALL ON THE SAME GENETIC BACKGROUND

	+/+	W/W^v	Sl/Sl^d	an/an
Peripheral blood values				
RBC × 10^6/mm^3	9.66 ± 0.13	6.16 ± 0.04	4.23 ± 0.10	6.70 ± 0.17
HT (%)	49.1 ± 0.3	40.4 ± 0.2	31.7 ± 0.3	42.8 ± 0.5
MCV (μ^3)	50.9 ± 0.5	65.6 ± 0.5	73.6 ± 0.5	62.5 ± 0.8
MCHC (%)	32.8 ± 0.3	33.7 ± 0.5	32.2 ± 0.4	32.5 ± 0.3
Hemoglobin (g/100 ml)	16.1 ± 0.2	13.2 ± 0.3	10.2 ± 0.2	13.2 ± 0.4
Reticulocyte (%)	3.37 ± 0.21	3.79 ± 0.37	5.99 ± 0.63	4.71 ± 0.26
WBC × 10^3/mm^3	11.11 ± 0.44	14.49 ± 0.33	12.43 ± 0.57	8.72 ± 0.24
Platelets × 10^6/mm^3	1.13 ± 0.04	1.09 ± 0.04	1.10 ± 0.03	1.55
Bone marrow				
Nucleated cells × 10^7/femur	2.31 ± 0.11	1.92 ± 0.10	1.47 ± 0.07	1.50 ± 0.11
CFS-S/10^5 nucleated cells	18.35 ± 1.86	< 0.8	21	9.29 ± 1.25
CFU-S/femur	4329	< 150	3087	1393
RBC precursors (%)	6.75 ± 0.41	5.05 ± 0.33	5.01 ± 0.35	6.80 ± 0.52
Erythroid cells × 10^6/femur	1.56	0.97	0.74	1.02
Spleen				
Size (mg)	103 ± 4.0	86 ± 4.1	123 ± 6.2	85.8 ± 3.5
RBC precursors (%)	1.29 ± 0.28	0.60 ± 0.14	0.72 ± 0.13	2.66 ± 0.47

D. Tests of implantation therapy using Hertwig's anemic mice

We have been able to improve the hematologic status of WBB6F$_1$-an/an anemic mice, with a mild stem cell deficit (see above), by implantation of +/+ bone marrow cells (Table 2). The success of this treatment suggests that the an/an erythroid microenvironment is nearly normal, since implanted +/+ stem cells can function for more than a year in an/an mice. Additional evidence supporting the hypothesis of intrinsic an/an stem cell deficit was obtained from the transplantation of an/an bone marrow, containing a hemoglobin marker, to stem cell deficient W/W^v mice. In contrast to previous results with implants of Sl/Sl^d stem cells which "cure" W/W^v recipients, implants from an/an donors did not result in "cured" W/W^v recipients with normal hematocrit levels. However, a year later, blood of the W/W^v recipient mice contained only donor type hemoglobin. It appears that an/an stem cells took up residence in the W/W^v hemopoietic environment and overgrew the indigenous W/W^v erythroid tissue (with its lessened stem cell capacity, < .005 CFU-S/10^5 for W/W^v vs. 9.5 CFU-S/10^5 for an/an), but that these implanted cells, despite their increased activities, still produced only macrocytic erythrocytes and, despite the apparent normality of the erythroid environment, still produced an insufficient number of circulating erythrocytes.

TABLE 2. THERAPEUTIC EFFECTIVENESS OF NORMAL (+/+) BONE MARROW CELLS GIVEN INTRAVENOUSLY TO HERTWIG'S ANEMIC MICE (an/an). VALUES PRESENTED ARE MEANS AND STANDARD ERRORS OF ALL MICE TREATED.

Days after grafting	RBC × 10^6/mm^3	Hematocrit (%)	Mean cell volume (μ^3)
0	5.48 ± 0.18	34.4 ± 0.7	63.8 ± 1.2
15	5.76 ± 0.18	38.3 ± 0.7	67.1 ± 1.7
30	7.06 ± 0.22	43.0 ± 0.4	55.3 ± 6.3
45	7.68 ± 0.16	45.8 ± 0.6	59.7 ± 1.1
60	8.15 ± 0.30	44.7 ± 0.8	55.3 ± 1.5
180	8.99 ± 0.29	48.4 ± 1.1	53.9 ± 0.7

E. Pilot studies on erythropoietin in mice with Hertwig's anemia

The obvious differences observed between WBB6F$_1$-an/an mice and highly congenic mice (W/W^v and Sl/Sl^d) with other macrocytic anemias led us to question the basic nature of gene action causing their anemia and to ask whether an/an mice can respond to erythroid regulatory agents, or produce erythropoietin, or respond to it.

To answer the first question, an/an, an/+, and +/+ mice were injected with doses of exogenous erythropoietin with the dose schedules and times indicated in Table 3. The observed increase in red cell mass of an/an mice in response to human urinary erythropoietin show that they are at least partially responsive to this hormone. They also produce erythropoietin. Urine from untreated an/an mice was analyzed for erythropoietin content in the standard plethorized mouse assay. The data appear in Table 4, where it will be seen that injections of urine from untreated an/an mice induced at least as much erythropoiesis as did that of +/+ or W/W^v mice, but not as much as that of Sl/Sl^d mice. In each case, the stimulatory substance was probably erythropoietin, since its activity was greatly diminished by rabbit anti-erythropoietin.

TABLE 3. RESPONSE OF NON-PLETHORIZED an/an MICE TO EXOGENOUS ERYTHROPOIETIN

Genotype	Dose/d	Days	Hematocrit	
			pretreatment	terminal
+/+	0.25 U	17	47.2	48.7
+/an	0.25 U	17	45.3	48.3
an/an	10.0 U	17	39.5	49.0
+/+	10.0 U	21	47.3	75.8
+/an	10.0 U	21	47.9	73.7
an/an	10.0 U	21	39.5	57.0

TABLE 4. ASSAY OF ERYTHROPOIETIN CONTENT IN URINE OF UNTREATED NORMAL AND ANEMIC MICE. IN EACH CASE, 0.4 ml URINE WAS INJECTED INTO EACH POLYCYTHEMIC NORMAL RECIPIENT, WITH OR WITHOUT THE ADDITION OF RABBIT ANTI-ERYTHROPOIETIN. PER CENT UPTAKE OF Fe^{59} MEASURES STIMULATION OF ERYTHROPOIESIS.

Genotype of urine donor	Percentage uptake Fe^{59}	
	no anti-EP	with anti-EP
$WBB6F_1$- +/+	3.39	0.44
$WBB6F_1$-an/an	4.27	0.97
$WBB6F_1$-W/W^v	3.73	0.86
$WCB6F_1$-Sl/Sl^d	10.42	1.08

F. Effects of age on expression of Hertwig's anemia

The availability of fully viable $WBB6F_1$-an/an mice, along with littermate an/+ and +/+ mice, has made it possible to follow, through the entire lifespan, the course of Hertwig's anemia which is characterized by reduction in number of both erythrocytes and leucocytes. Hematologic status of like-sexed $WBB6F_1$-an/an, an/+, and +/+ mice, set aside in groups of 5, has been evaluated at 3 month intervals, with sizeable groups (25 to 50) now more than 15 months old (Table 5). Some deaths were observed between 12 and 15 months, particularly in an/an mice. Red cell numbers and hematocrit levels show clearly that the macrocytic anemia of an/an mice, conspicuous at all ages, becomes more severe with advancing age. At all ages the an/an mice have lower total leukocyte numbers than do their normal littermates, and these levels differ little between sexes or with advancing age. By contrast, the higher leukocyte numbers of normal (an/+ and +/+) females decrease slightly with advancing age, and leucocyte counts of normal (an/+ and +/+) males are always higher than those of same-aged normal females. (The high leukocyte counts of these normal males may or may not be realted in some way to the grouping of 5 males together.) These results demonstrate conclusively that reduction in leukocyte number is an intrinsic part of the Hertwig's anemia syndrome.

TABLE 5. CHANGES IN HEMATOLOGIC STATUS WITH ADVANCING AGE.

Measurement	Age in months	an/an ♀♀	an/an ♂♂	an/+ ♀♀	an/+ ♂♂	+/+ ♀♀	+/+ ♂♂
mean red cell number, millions/mm^3	3	7.62	7.44	9.92	10.66	10.00	10.02
	6	7.24	7.01	10.39	9.84	9.73	10.18
	9	7.17	7.21	10.04	10.04	10.22	9.84
	12	7.06	7.01	9.69	9.54	9.74	9.86
	15	6.77	6.76	9.93	9.44	10.68	9.53
mean hematocrit percentage	3	41.4	92.4	48.3	51.0	48.7	48.0
	6	41.2	41.0	50.4	50.7	47.1	48.4
	9	41.0	40.7	48.9	47.7	49.2	46.9
	12	39.9	39.2	47.8	46.8	48.0	48.5
	15	39.5	38.1	48.2	48.0	46.8	48.1
mean cell volume, μ^3	3	56.8	59.4	48.8	51.4	48.8	51.8
	6	56.9	60.2	50.5	51.7	51.8	49.4
	9	57.4	58.3	48.8	48.9	49.9	51.0
	12	56.6	56.4	48.2	49.2	49.4	51.2
	15	57.9	58.8	50.5	51.1	51.1	52.6
Leukocyte number, thousands/mm^3	3	9.8	12.4	14.2	20.0	16.0	16.9
	6	10.8	12.2	14.3	19.0	14.6	17.8
	9	10.8	12.1	14.4	15.2	14.8	12.5
	12	12.0	11.1	12.5	14.7	11.8	15.4
	15	10.0	12.0	11.8	15.6	11.7	15.6

G. Regeneration of intestinal epithelium in $\underline{W}/\underline{W^v}$ and $\underline{Sl}/\underline{Sl^d}$ anemic mice

In collaboration with Dr. Arthur Hopper of Rutgers University, we have studied the proliferation of intestinal crypt cells as well as the response to radiation (700 R, wholebody) in untreated normal $WBB6F_1$- +/+, anemic $WBB6F_1$-$\underline{W}/\underline{W^v}$, and anemic $WCB6F_1$-$\underline{Sl}/\underline{Sl^d}$ mice.

Both $\underline{W}/\underline{W^v}$ and $\underline{Sl}/\underline{Sl^d}$ anemic mice are known to be extremely radiosensitive. The $LD_{50/30}$ for $\underline{W}/\underline{W^v}$ in 250 R (Bernstein, 1962), and the $LD_{50/30}$ for $\underline{Sl}/\underline{Sl^d}$ is 130 R (Bernstein, unpublished). While it is probable in each case that the hemopoietic defect is sufficient to account for this radiosensitivity, we felt it would be desirable to test whether these same mice might also have a defect in regenerative capacity of the intestinal mucosa. An additional reason for undertaking this study was that several types of cells, which in normal mice proliferate very rapidly, fail to do so in $\underline{W}/\underline{W^v}$ and $\underline{Sl}/\underline{Sl^d}$ anemic mice. There is a delay in marrow regeneration following sublethal irradiation (Russell et al., 1963). Also, there is a failure of multiplication of primordial germ cells between 8 and 12 days of prenatal development (Mintz and Russell, 1957), and a failure of melanocyte proliferation of hair follicles (Mayer and Green, 1968; Mayer, 1973). It seemed desirable to see if proliferation in another rapidly dividing cell population, the intestinal crypt cells, might also be impaired in $\underline{W}/\underline{W^v}$ or $\underline{Sl}/\underline{Sl^d}$, or both.

The results of our study will appear in a paper, "Post-irradiation regeneration of intestinal epithelium in $\underline{W}/\underline{W^v}$ and $\underline{Sl}/\underline{Sl^d}$ genetically anemic mice," to be published in Radiation Research before the end of 1975. Our conclusions are that not all rapidly proliferating tissues of the mouse are adversely affected by action of mutant alleles at the W- and Sl loci. In contrast to greatly impeded proliferation of blood-cell precursors, primordial germ cells, and melanocyte precursors in $\underline{W}/\underline{W^v}$ and $\underline{Sl}/\underline{Sl^d}$ anemic, sterile, black-eyed white mice, normal proliferation of intestinal mucosa was observed in mice of these same genotypes. Determinations of number of cells per intestinal crypt, labeling index, mitotic index, and uptake of tritiated thymidine per mg of mucosa, showed no significant differences between values for untreated normal (+/+) and anemic ($\underline{W}/\underline{W^v}$ and $\underline{Sl}/\underline{Sl^d}$) mice. The intestinal mucosa of the $\underline{W}/\underline{W^v}$ and $\underline{Sl}/\underline{Sl^d}$ mice responded to radiation insult in the same manner as did that of +/+ mice. Mice of all three genotypes were equally able to increase proliferative activity in the crypts, and to restore normal cell numbers.

The same problem of intestinal regeneration has also been studied by Torok and Boggs (American Journal of Physiology, scheduled for the July, 1975, issue), with somewhat different conclusions, since they feel that intestinal regeneration is defective in $\underline{W}/\underline{W^v}$ mice. This discrepancy was discovered during the critical reviews of our manuscript. We have corresponded and talked with Torok and Boggs, and will discuss our differences in the Radiation Research paper. We still feel that our original conclusion of normal intestinal regeneration in irradiated $\underline{W}/\underline{W^v}$ and $\underline{Sl}/\underline{Sl^d}$ mice was warranted.

H. Fetal erythropoiesis in steel mutant mice·

The comparison of defective fetal liver erythropoiesis of $\underline{Sl/Sl^d}$ fetuses and normal erythropoiesis in (-/+) fetuses has been made in collaboration with Dr. David Chui of McMaster University, with special attention to numbers of each kind of precursor cells at each developmental stage, and with a search for differences in morphology (at the EM level) between corresponding cells from normal and defective fetal livers. The results (as published in Developmental Biology 40:256-269) are summarized as follows:

A method of definitive identification of mutant ($\underline{Sl/Sl^d}$) and wild--type (+/+) mouse embryos in segregating litters is described, based on the total number of circulating erythrocytes in a unit volume of embryonic blood and the relative proportion of nonnucleated vs. nucleated red blood cells. Evidence is presented that, from days 13 to 17 of gestation, $\underline{Sl/Sl^d}$ embryos have many fewer fetal liver derived nonnucleated erythrocytes whereas the number of yolk sac-derived nucleated red blood cells is similar between $\underline{Sl/Sl^d}$ and +/+. Erythroid precursor cells at various stages of maturation in mutant fetal livers are studied by light and electron microscopy and their fine structure is found to be identical to those present in normal embryos. The number of hemoglobin-containing mature erythroblasts in mutant fetal livers is far fewer than that in the normal livers, whereas the number of immature erythroid precursors present in a unit area of fetal liver is not significantly different between $\underline{Sl/Sl^d}$ and +/+. It is suggested that the mutant Sl gene product(s) interferes with or fails to support the differentiation of immature erythroid precursors into hemoglobin synthesizing cells.

I. Short term cultures of mutant erythroid tissue

The ability to analyze erythroid activity at the cellular level would add great power to our studies of the physiology of interactive elements in normal erythropoiesis and our attempts to determine which elements have been pathologically modified by mutant gene action affecting erythroid development or proliferation. In an intact organism it is difficult to assess whether an essential nutrient like cobalamine, or a regulatory substance like erythropoietin, has gotten ot erythroid tissue in the proper form or in sufficient concentration. One questions whether the materials under study were synthesized, incorporated, or transported before they were inactivated or excreted. In the system in vitro one can be quite certain that the cells comin in contact with any added agent.

During the past two contract years we have attempted to maintain bone marrow cells in suspension cultures for short times, hoping to measure directly the impact of exogenous erythropoietin on the rate of heme synthesis, especially in cells which in the intact organism are refractory to this agent (i.e., $\underline{W/W^v}$ bone marrow cells). Our efforts have been frustrating, even though we carefully followed the published procedures of Kranz, Molinari, Cohen, and others, and scrupulously

followed the recipes and directions of our other colleagues in the field (personal communications). Results have been inconsistent and erratic. We did obtain synthesis of heme, but histochemical and morphological examination showed most of the cells to be of the granuloid variety. Cell enumerations were difficult because of cellular adhesions to surfaces and to each other. Many of the cultivated cells were proliferating actively, and those in the eosinophil pathway were especially rich in heme-containing cytoplasm (i.e., not all heme goes to erythropoiesis). The value of the various media and the conditions of cultivation came into serious doubt because of the ever-present populations of moribund and necrotic cells. Karyorrhexis of the erythroblastic series was an especially notable finding in all media. These complicating findings drove us to try to find a set of conditions in vitro which would give rise to cell suspensions comparable to those obtained with fresh bone marrow. We compared cultures using three commercially prepared media and three synthetic media which Dr. Charity Waymouth of the Jackson Laboratory devised. We varied the concentration and source of serum components, employing media without serum, or with fetal calf serum, calf serum, bovine serum, human serum, complete mouse serum, or lipid-free mouse serum. Gassification of the cultures was carried out with levels of CO_2 ranging from 0 to 10 per cent. Moreover, four types of tissue culture vessels, both glass and plastic, were employed in these studies. We are currently studying the influences of incubation temperature, of levels of both "cold" and "hot" iron, and of erythropoietin.

We compared cultures semi-objectively by using a composite score based on values obtained from determinations of cell clumping, presence and quality of erythroblasts and mature red blood cells, stainability of erythroid elements with benzidine peroxidase and with Wright-Giemsa stain and appearance of granulopoietic elements, the extent of dyserythropoiesis, occurrence of foamy cytoplasm in hematopoietic cells, and the decrease of cell numbers with time. From all this, one particular medium, designated by Dr. Waymouth as MAB87/3 (Table 6), emerged as most promising. For the cultivation of bone marrow cells, we add either heat inactivated calf serum (Gibso), or normal C57BL/6J mouse serum, to bring the final serum concentration to 9 per cent. Cohen and others report that mouse serum is not a satisfactory source of transferrin for binding Fe^{59} in the measurement of heme synthesis in culture. Pending experimental verification of this report, we are incubating human serum with Fe^{59} Cl_3 for 1 hour and neutralizing with 10 per cent sodium bicarbonate. Bone marrow is extracted aseptically from mouse femora with warm MAB87/3 medium, as prepared above, and cell concentrations of 0.8 to 1.2×10^7 per ml give good results. No ice is employed at any time. We use rubbered-stoppered Carrel flasks, each containing 1.5 ml of medium and 1 µg Fe per ml.

Under the conditions described above, we lose less than 30 per cent of all the nucleated cells put into culture, no cell clumping occurs, red cells remain intact, and numerous erythroblasts (90 to 112% of initial values) stain strongly with Lepehne's stain for heme. Dyserythropoiesis is minimal but unfortunately some cytoplasm is still seen.

TABLE 6. COMPOSITION OF TISSUE CULTURE MEDIUM MAB 87/3

Vitamins	mg/L	Amino acids	mg/L	Salts	mg/L
Ascorbic acid	17.5	L-alanine	11.2	NaCl	6000
Thiamin HCl	10	L-arganine HCl	75	KCl	150
Ca pantotheate	1	L-asparagine	24	$CaCl_2.2H_2O$	120
Riboflavin	1	L-aspartic acid	60	$MgCl2.6H_2O$	240
Pyridoxin HCl	1	L-cystine.HCl	75	$MgSO_4.7H_2O$	100
Folic acid	0.5	L-cystein	15	$Na_2H\ PO_4$	300
i-inositol	1	L-glutamic acid	150	$KH_2\ PO_4$	208
nicotinamide	1	L-glutamine	350	$NaH\ CO_3$	2240
B_{12}	0.2	glycine	50	$Cu\ SO_4.5H_2O$	0.05
Brotin	0.02	L-histidine.HCl	150	$Zn\ SO_4.7H_2O$	0.03
		L-isoleucine	25	$Mn\ SO_4.H_2O$	0.02
Other Substances					
		L-leucine	50	$CO\ Cl_2.6H_2O$	0.02
Insulin	8	L-lysine.HCl	240	$Fe\ SO_4.2H_2O$	0.45
Glutathione	15	L-methionine	50	$(NH_4Mo\ 0_{24}.4H_2O$	0.02
Choline	250	L-phenylalanine	50		
Hypoxanthine	25	L-proline	50		
Thymidine	8	L-serine	12.8		
Hydrocortisone	0.2	L-threonine	75		
Dexamethasone PO_4	0.2	L-tryptophane	40		
Penicillin G Na K Salt	315	L-tyrosine	40		
Dihydrostreptomycin SO_4	200	L-valine	65		

In view of the types of experiments which we expect to carry out in the search for erythropoietin inhibitors and the development of contact between cells of various genotypes, we must be able to devise cell free media, conditioned by cells previously grown in it. Because of this restriction, we have studiously avoided the use of plasm clots, methyl cellulose, or semi-solid media which had been so successfully employed with human bone marrow cells.

J. Improvements in stem cell assay

The modification and improvement in the existing splenic colony forming unit (CFU-S, hereafter simply CFU) assay methods have become necessary with the need to determine the stem cell content of material which contains low numbers of such cells or where there are only extremely small amounts of material available for analysis, making critical the survival of each assay mouse. To be more specific, determination of CFU content of blood from normal +/+ or CFU-poor donors (i.e., mk/mk) usually requires a volume of injected material too large (0.7 ml - 4 ml) to be given by the usual intravenous route. Theoretically the material could be administered over a prolonged period, but this would present additional difficulties in analysis if there are differences in colony generation time. Alternatively, the material could be given intraperitoneally, though equivalency values for intravenous (iv) vs. intraperitoneal (ip) assays have yet to be worked out. Studies carried out here during the past year indicate that the slopes of the iv and ip dose response curves are similar, but that the iv route gives higher colony counts for each cell dose. This route of administration would still be useful if it could be shown that values obtained using the ip method were always a constant fraction of the iv values, and if ip injection provides a gain in feasible dose greater than reduction in colony counts. Comparative studies under way with normal and mutant donor materials look very promising but the data are still too sparse for proper analysis. This project, however, should be completed within the next 6 months.

Where small amounts of material are to be analyzed (i.e., yolk sac cells from individual embryos within a segregating litter, or biopsied material from one mouse), it appears that non-irradiated W/W^V because they are stem cell deficient but have normal hematopoietic inductive environments, would make excellent assay animals. Since there would be no losses due to radiation sickness, all assay animals, regardless of the cell doses used, should survive and provide useful CFU data. This has been found to be the case. Good sized readable colonies are found in their spleens within 9 days of the injection of bone marrow or blood cells. A comparison of CFU counts obtained from irradiated normal assay mice and non-irradiated W/W^V mice is presented in Table 7.

TABLE 7. COMPARISON OF CFU-S COUNTS ASSAYED IN X-IRRADIATED NORMAL (+/+) AND UNIRRADIATED W/W^V RECIPIENTS. WHOLE BLOOD WAS EMPLOYED AS THE SOURCE OF STEM CELLS.

Genotype of donor	CFU-S ± S.E./ml whole blood assayed in X-irradiated +/+[1][2]	W/W^V[2]	Regression coefficient ± S.E.[3]	Coefficient of determination (r^2)
+/+	29.0 ± 1.9	41.6 ± 2.3	1.26 ± 0.16	0.94
ha/ha	76.0 ± 2.9	81.0 ± 0.5	1.02 ± 0.14	0.93
ja/ja	778 ± 9.3	764 ± 25.1	1.10 ± 0.25	0.99
nb/nb	267 ± 3.1	414 ± 3.9	1.21 ± 0.03	0.77
sph/sph	206 ± 3.8	198 ± 1.1	0.93 ± 0.25	0.93

(1) +/+ mice total body X-irradiated with 900 R

(2) All blood administered iv. Spleen colonies larger than 0.5 mm were enumerated on day 9.

(3) Paired spleen colony counts obtained from +/+ recipients (X values) were compared with those obtained from W/W^V recipients (Y values) concurrently injected with the same pool of donor blood. N > 50 for each category.

The analysis indicated clearly that unirradiated W/W^V recipients are suitable assay animals, yielding CFU values comparable to those encountered in conventional assays. Note that the W/W^V test system may be used for assay of CFUs in blood obtained from animals of a variety of abnormal as well as normal genotypes. The observation that the average regression coefficient (ratio between CFUs in irradiated +/+ recipients and CFUs in W/W^V recipients) is a bit elevated (1.10 ± 0.06) suggests, moreover, the possibility that the unirradiated W/W^V environment may be a bit more conducive to colony development than is that encountered in irradiated recipients.

K. Radiation sensitivity of the erythroid microenvironment

The normal process of hemopoiesis involves a complex set of interactions between different hemopoietic cells, humoral regulators, essential nutrients, and stromal cells. Much is known about the radiation sensitivity of hematopoietic stem cells but little is yet known about the

sensitivity of the stromal elements.

In past years, with the support of the Energy and Research Development Administration, it has been possible for us to have contributed to an understanding of the X-ray sensitivity of genetically diverse murine erythroid stem cells. Using anemic mutant mice ($\underline{W}/\underline{W^v}$, $\underline{Sl}/\underline{Sl^d}$, $\underline{f}/\underline{f}$, we have studied the radiation sensitivities of intact mice bearing one or more of the anemia-producing genes, the same mutants modified by transplantation with foreign erythroid stem cells (bone marrow grafts) and, similarly, mutants which have been grafted with foreign stromal elements (whole spleen grafts). We have also determined the influence of cellular environment during X-irradiation on the subsequent vitality of erythroid stem cells. Briefly, we have discovered that transplants of normal bone marrow (+/+) to stem-cell-deficient anemics ($\underline{W}/\underline{W^v}$) raises their $LD_{50/30}$ from 235 R to 688 R, and grafts of normal stromal elements (+/+ intact spleen) to stroma-deficient recipients ($\underline{Sl}/\underline{Sl^d}$) raises their $LD_{50/30}$ from 130 R to 670 R.

Ascertaining the radiation sensitivity of the stromal elements has proven to be a more difficult undertaking than originally imagined. Our early studies utilized spleens of varying genotypes irradiated in the donor and subsequently transplanted to stroma-deficient ($\underline{Sl}/\underline{Sl^d}$) recipients. Although all recipients benefited from implants of non-irradiated spleens, only 50 per cent were cured by +/+ spleens which had received 375 R in situ or by $\underline{W}/\underline{W^v}$ spleens which had received approximately 400 to 600 R. Difficulties arose from the necessity of transplanting the irradiated graft into suitable recipients rapidly and without technical failure. Potential grafts regained curative potential rapidly if they remained in the donor prior to transplant (for +/+ grafts transplanted at 0.5 hrs, LD_{50} = 375 R; at 18 hrs, LD_{50} = 675 R, slope = 4.39, intercept = 496, r^2 = 0.78). Further, such grafts require a healing-in process characterized by variations in cell death, establishment of new vascular connections and, eventually, stromal regeneration. We have little real control over these variables. Our uncertainties are compounded by the necessity (for probit analysis) of artificial dichotomizing the recipients into "cured" and "not cured" on the basis of hematocrit response. Similar difficulties characterize work in this field by W. Fried (University of Chicago), W. Knospe (Rush Medical School, Chicago), and many, if not most, other workers.

In an attempt to obviate these difficulties, we have begun a study in which the spleen is exteriorized, the body is lead-shielded, and the spleen alone is X-irradiated. It is then returned to its proper site in the abdominal cavity. This removes from further consideration all of the difficulties inherent in the grafting procedures. Twenty-one days later, when recovery is generally believed to be complete (Takada et al., Radiation Research 43:210,1970) the spleens are removed and the percentage of heme-containing Lepehne's positive erythroid cells undergoing development is determined. This provides us with an objective estimate of the extent to which the irradiated stroma is able to support erythropoiesis. Employing the exteriorized spleen technique with different doses of X-rays,

it should be possible to determine the dose of irradiation that will deplete erythroid activity by 50 per cent.

An extension of the investigation to the role played by stromal cells in the elaboration of acid mucopolysaccharide and its postulated function in regulating erythropoiesis (Meinike and McCuskey, University of Cincinnati) is presently under study, now that Dr. Meinike has provided us with his histochemical protocols. If there is a relationship between stromal cell mucopolysaccharide secretion and erythroid activity, radiation damage to these cells should be correlated with changes in this cellular product and with erythroid activity. Histological sections of exteriorized spleen have been prepared but quantitation has not yet been undertaken.

In our preliminary investigations we have exposed exteriorized $\underline{Sl}/\underline{Sl}^d$ spleens to X-ray doses ranging from 0 to 850 R and after 21 days have observed no differences between irradiated and sham-irradiated spleens in the distribution of Lepehne positive cells. This is a surprising finding in view of the fact that stromally-deficient but not stem-cell-deficient $\underline{Sl}/\underline{Sl}^d$ mice are extremely radiosensitive ($LD_{50/30}$ = 135 R). Recall that $\underline{Sl}/\underline{Sl}^d$ mice become radioresistant if provided with intact normal (+/+) splenic tissue, and that $\underline{Sl}/\underline{Sl}^d$ erythroid stem cells confer normal radioresistance to stem cell-deficient ($\underline{W}/\underline{W}^v$) recipients. Several possibilities have been considered: (1) Our dosimetric devices may have been malfunctioning. (We now have evidence to substantiate this possibility.) (2) The radiosensitivity of stroma from mice of this genotype may be well above 850 R. Jenkins et al. report that the microenvironment (stroma) in +/+ mice is resistant to 10,000 R (Radiation Research 43:212, 1970), or (3) We may have selected the wrong fixed post-irradiation interval to study the effects of radiation, perhaps inadvertently choosing a recovery period before stabilization has taken place (during an overshoot). Whatever the explanation, perturbations in the shielded bone marrow were not encountered as might be expected if extensive replacement of splenic erythroid elements were dependent on recruitment from bone marrow reserves.

L. Transitory transplantable polycythemia

Our experiences with transplantation of blood-forming tissue from an $\underline{an}/+$ mouse with a spontaneous polycythemia have been interesting but frustrating. Until February, 1975, we had observed polycythema in untreated mice in our colony only rarely, and associated with hepatoma. The finding of a 6-month-old mouse which maintained a hematocrit level over 75 per cent for more than 1 month was completely unexpected. Implants of blood and of marrow cell suspensions from this polycythemic mouse had no effect on hematologic values of normal recipients but routinely increased hematocrit levels of $\underline{W}/\underline{W}^v$ anemic recipients at least to 48 to 50 per cent, and made many of these formerly anemic mice polycythemic (4 of 8 $\underline{W}/\underline{W}^v$ recipients of marrow cells, and 6 of 8 $\underline{W}/\underline{W}^v$ recipients of spleen cells from the primary polycythemic mouse). A second

generation of transfer was also moderately successful. Polycythemia developed in 8 of 18 $\underline{W}/\underline{W^V}$ recipients of first generation transplant marrow cells and in one of 19 recipients of first generation spleen cells. Unfortunately, third and fourth generation transplants have been much less successful, and we now fear that we have lost this interesting cell line, which may have carried an infectious agent affecting the hemopoietic stem cell line.