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Contract number: AT(11-1)-3264

Title of project: INBORN ANEMIAS IN MICE

Investigators: Elizabeth S. Russell, Ph.D.
Senior Staff Scientist

Seldon E. Bernstein, Ph.D.
Senior Staff Scientist

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DISCLAIMER

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A. SCIENTIFIC BACKGROUND

The basic purpose of this contract is delineation of inborn anemias of the laboratory mouse, 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 13 single-gene induced 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 AEC Contract AT(11-1)-3264. We also have established an NZB/BlN colony susceptible to autoimmune disease.

We plan to analyze all presently known hereditary anemias of the mouse and to apply our findings towards 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 ♂♂); microcytic anemia (mk/mk).

Considerable effort has been devoted to establishing each mutant allele on a genetically homogeneous genetic background which 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.

At any one point in time, research on these anemic mice tends to center upon several distinct but inter-related questions. These questions shift gradually as progress is achieved, an approach is exhausted, or new problems encountered. Our yearly reports can give only a partial picture of these developmental processes. Inasmuchas the Atomic Energy Commission

expects an extensive report of progress every third year, we have chosen to present separately our findings relating each focus of interest, beginning with background information, following with a brief review of activities in years 16 and 17, and finally describing in more detail research in the current, or eighteenth, year of support.

B. COMPARISON AND CONTRAST OF W/W^v AND Sl/Sl^d MACROCYTIC ANEMIAS

The phenotypic manifestations of W/W^v and Sl/Sl^d mutant mice are nearly identical; they are both black-eyed white mice with severe macrocytic anemia and few if any primordial germ cells. W-anemic mice have a primary defect in their stem-cell compartment which leads to marked erythroid hypoplasia, especially after X-irradiation, and which prevents formation of macroscopically visible splenic colonies when W/W^v marrow cell suspensions are transplanted into lethally irradiated normal recipients. W/W^v mice are known to provide a normal environment for erythropoiesis, since implants of histocompatible normal (+/+) hemopoietic tissues containing normal stem cells, cure the anemia of W/W^v mice completely and permanently. We speak of W-anemic mice as being "stem-cell deficient."

By contrast, Sl/Sl^d mutant mice have normal numbers of stem cells in their hemopoietic tissues, as determined by the colony forming assay. Implants of marrow cell suspensions from Sl/Sl^d anemic mice form spleen colonies and cure the anemia of W/W^v mice. Injections of normal marrow or spleen cell suspensions are completely without effect in Sl/Sl^d recipients, even following heavy irradiation of the host. Sl/Sl^d mice seem to have normal hemopoietic cells, but are anemic because they provide a defective environment for erythropoiesis.

It has long been known that both W/W^v and Sl/Sl^d anemic mice respond very poorly to exogenous erythropoietin. W/W^v mice cured by implants of +/+ or Sl/Sl^d hemopoietic cell suspensions become normally responsive to erythropoietin. Capacity for production of erythropoietin seems to be normal in both W/W^v and Sl/Sl^d anemic mice. In collaboration with Dr. Geoffrey Keighley, studies of plasma erythropoietin levels in W/W^v and Sl/Sl^d mice subjected to erythropoietic stress (hypoxia, irradiation) were completed in years 16 and 17. Both W/W^v and Sl/Sl^d anemic mice can produce large amount of erythropoietin over prolonged periods, but are unable to respond effectively to this hormone. W/W^v mice remain anemic because of a genetic defect in red blood cell (RBC) precursors, and Sl/Sl^d mice remain anemic because of a genetic defect in some part of the internal environment in which the RBC must develop (Russell and Keighley, 1972). Both normally responsive stem cells and an environment encouraging erythropoiesis are necessary for an effective response to erythropoietin.

C. TISSUE LOCALIZATION OF THE INTERNAL ENVIRONMENTAL DEFECT IN $\underline{Sl}/\underline{Sl}^d$ ANEMIC MICE

Hemopoietic analysis of $\underline{Sl}/\underline{Sl}^d$ anemic mice parabiosed with normal (+/+) partners demonstrated that this fusion neither augmented erythropoiesis in the steel partner nor diminished it in the normal partner. The "environmental defect" in $\underline{Sl}/\underline{Sl}^d$ mice thus does not seem to be hormonal, nor due to deficiency of a circulating nutrient essential for erythropoiesis. Long-term treatment of $\underline{Sl}/\underline{Sl}^d$ anemic mice with injections of normal serum fortified with high concentrations of additional vitamins and minerals was also completely ineffective. In year 16 of this contract, Bernstein (1970) reported that transplantation of intact normal or $\underline{W}/\underline{W}^v$ spleens into $\underline{Sl}/\underline{Sl}^d$ recipients alleviates their anemia. The "environmental defect" of $\underline{Sl}/\underline{Sl}^d$ mice thus appears to be localized within hemopoietic tissue space, but extrinsic to erythroid stem cells: a microenvironmental or matrix defect. The nature of this defect was explored in year 17, in collaboration with Dr. A. C. Upton and co-workers (1971). $\underline{Sl}/\underline{Sl}^d$ mice bearing transplanted +/+ or $\underline{W}/\underline{W}^v$ spleens were lethally irradiated and injected with +/+ marrow cell suspensions. Splenic colonies appeared only in the +/+ and $\underline{W}/\underline{W}^v$ spleens, wherever they resided. $\underline{Sl}/\underline{Sl}^d$ spleens in $\underline{Sl}/\underline{Sl}^d$ or $\underline{W}/\underline{W}^v$ hosts did not support colony growth from injected marrow cells.

In year 18, Dr. Bernstein has sought to identify some connective tissue defect in $\underline{Sl}/\underline{Sl}^d$ spleens by histochemical methods. The microenvironmental defect of $\underline{Sl}/\underline{Sl}^d$ spleens could conceivably involve defect in stem cell connective tissue interaction, either through direct cell-to-cell contact, or through abnormality of a cell-mediated transformation of some metabolite, stimulus, or inhibitor, that has a very short half-life, so that producing and utilizing cells must be in close proximity. In the search for an aberrant morphological component in the $\underline{Sl}/\underline{Sl}^d$ erythroid environment, histological sections of +/+, $\underline{W}/\underline{W}^v$, and $\underline{Sl}/\underline{Sl}^d$ spleens were processed specifically with the strains indicated in Table 1.

TABLE 1. LIST OF HISTOCHEMICAL PROCEDURES EMPLOYED IN MORPHOLOGICAL STUDIES OF THE SPLEEN.

Staining Procedure	Specific Purpose
Alcian Blue	Acid mucopolysaccharide, mucin, muco substances
Alcian Blue - Kernectrot	" " " " " "
Alcian Blue - Pas	Acid mucopolysaccharide, mucin, muco substances and carbohydrates
Alcian Blue - Pease	Acid mucopolysaccharide, mucin, muco substances
Aldehyde Fuchsin	Elastic tissue
Aldehyde Fuchsin - Halmi	Elastic tissue
Aldehyde Fuchsin - Van Gieson	Elastic tissue and collagen
Feulgen	Nucleic acid, DNA
Gallocyanin	Nucleic acid, DNA
Gallocyanin - Eosin	Nucleic acid, DNA
Gomori Iron	Ferric iron
Gridley Reticulum	Reticulum
Hematoxylin & Eosin	Routine morphology
Lepehne	Hemoglobin
Lepehne - Lawson	Hemoglobin
Luxol Blue - Cresyechtviolet	Myelin
Masson	Connective tissue, collagen
Methyl Green - Pyronin	Nucleic acids, DNA, RNA
Palmgren	Nerve fibers
Phosphotungstic Acid - Hematoxylin	Connective tissues
Sudan Black - Kernechtrot	Myelin and other fatty substances
Van Gieson	Collagen

In all cases except one, no differences associated with genotype were observed. The exception was the methyl-green pyronin reaction for RNA, which showed a greater concentration of cells with pryroninophilic granules in the red pulp of $\underline{Sl/Sl^d}$ spleens than in +/+ or $\underline{W/W^v}$ spleens. No attempt was made to count affected cells. Treatment of spleen sections with ribonuclease removed the pyronin-positive material, suggesting it was probably some kind of RNA. Unfortunately, classical quantitative chemical analyses of the spleens for DNA, RNA, and protein did not reveal any significant differences in RNA/DNA concentration (Table 2).

TABLE 2. QUANTITATIVE ANALYSIS OF MUTANT SPLEENS FOR THEIR RNA, DNA, AND PROTEIN CONTENT. (IN COLLABORATION WITH DR. HARRY CHEN, THE JACKSON LABORATORY.)

	Genotype of Spleen		
Substance	+/+	$\underline{W/W^v}$	$\underline{Sl/Sl^d}$
DNA	22.8 mg/g	24.8 mg/g	25.5 mg/g
RNA	3.97	3.88	4.34
Total Protein	86.5	80.5	86.5

Either the macrochemical methods were not sufficiently sensitive, or the histochemical results were misleading.

A recent report (McCuskey and Meineke, 1972) suggested that histochemically identifiable sulfated acid mucopolysaccharide is elevated in $\underline{Sl/Sl^d}$ as compared to +/+ spleens. Despite considerable effort, we could not repeat this finding. As yet, histochemical methods have failed to provide convincing evidence that defective connective tissue elements are responsible for microënvironmental defects in $\underline{Sl/Sl^d}$ spleens.

Some light on the nature of the normal environmental support for erythropoiesis has resulted from spleen retransplant studies. We have previously shown that suspensions of isolated normal erythroid stem cells are easily transplanted and retransplanted, curing successive $\underline{W/W^v}$ (or lethally irradiated normal) recipients. However, the normal "environment" (in the form of an intact +/+ or $\underline{W/W^v}$ spleen) apparently can be transplanted only once. Retransplanted spleens may provide normal stem cells to secondary recipients, but they fail to support effective erythropoiesis initiated either from host stem cells or from stem cells which came into the secondary recipients with the implanted spleen. These findings imply that the components of the normal erythroid environment include at least one type of cell which has limited survival or transplantability.

D. ATTEMPTS AT THERAPY OF W/W^v AND Sl/Sl^d ANEMIA WITH LYMPHOID CELLS

Search for pluripotent cell. Lymphoid cells make up a large part of the spleen, and can thus be considered as part of its microënvironment. Also, the concept of a pluripotent hemopoietic stem cell is generally accepted. This cell, not yet morphologically identified, is a common ancestor of all types of leukocytes and of the committed erythroid stem cell. Could it be possible to find lymphocytes or lymphocyte-like cells in the spleen which have a capacity to differentiate in the erythroid direction? Cure of W/W^v anemia by implanted lymphoid cells would be an excellent test of this possibility.

To answer the question we grafted subcutaneously +/+ lymph nodes to stem-cell-deficient W- anemic mice. Each of 12 W/W^v mice were grafted with an intact blood-free +/+ thymus and, in addition, each of 12 W/W^v anemics received the entire complement of intact blood-free lymph nodes from a normal mouse (with the exception of the deep thoracic, cervicals, inguinals, and sciatic nodes).

These experiments were repeated with grafts transplanted to two additional sites, under the kidney capsule and under the splenic capsule. During the 6 to 8 month observation period, there was no hematological evidence in any of the graft recipients of a therapeutic effect, even though later histological examination revealed that the lymph nodes were grafted successfully. We concluded that either lymph node lymphocytes were not pluripotent under the conditions of these experiments, or the number of pluripotent stem cells in normal lymph nodes is too small for them to be effective in curing W/W^v anemia.

Lymphocytes as helper cells. Even if lymphocytes are not pluripotent in themselves, is it possible that they participate in erythroid differentiation? Several different approaches to this question were deemed possible. Studies included destruction of lymphoid elements in cured anemics by antisera directed against lymphocytes and lymphocyte transformation by phytohemagglutinin.

The first attempt was to abrogate, differentially with rabbit anti-mouse lymphocyte serum, the curative property of successfully grafted normal spleens residing in steel recipients. Theoretically, sera of this sort could work adversely both on the graft and on the host if the pluripotent stem cell is either lymphoid in nature or shares surface antigens with lymphocytes, or if the lymphoid cell is an essential "neighbor or helper" cell. Certainly others have already demonstrated cross reaction of anti-lymphocyte sera with hemopoietic stem cells (Field and Gibbs, 1968).

In our experiments, doses of rabbit anti-mouse lymphocyte serum, ranging from 0.1 ml given on a single occasion to 1.5 ml injected over a 3-day period, were administered to five mice of each of the following types: Sl/Sl^d mice successfully grafted with a +/+ spleen; Sl/Sl^d mice grafted with a W/W^v spleen; W/W^v grafted with a +/+ spleen; and W/W^v mice grafted with a Sl/Sl^d spleen. All mice were classified as "cured"

prior to treatment with antiserum. Following treatment the hematocrits of all mice declined to about 90 per cent of pretreatment values. But the responses were unrelated to the doses administered. Lacking a reasonable dose response curve we were unwilling to conclude that anti-lymphocyte serum had any effect. In a parallel experiment with rabbit antithymocyte serum the hematocrits did not vary significantly from normal.

Phytohemagglutinin. Another approach was suggested by the work by B. B. Lozzio (1969) in which he induced in mice spleen follicle hyperplasia and the proliferation of splenic hemapoietic foci by means of a single injection of phytohemagglutinin - P. This substance is known to provoke lymphopenia, but also appears to initiate the appearance of large undifferentialed cells (possibly stem cells?) in the spleen. Phytohemagglutinin P transforms lymphocytes in vitro to blast forms (and possibly changes their developmental potential). It seemed desirable to test this hemagglutinating substance in W/W^v and Sl/Sl^d hypoplastic anemic mice for its ability to transform lymphocytes and augment hemopoiesis. To this end, 15 +/+, 15 Sl/Sl^d, and 15 W/W^v mice were injected with 10 mg phytohemagglutinin - P/100 g bodyweight/week for 10 weeks. Analysis of peripheral blood values prior to treatment, during the 10-week treatment period, and for 11 weeks following termination of treatment, failed to reveal any long term significant deviation from erythrocyte counts, hematocrits, or total leucocyte counts typical of untreated individuals.

Thymocytes. Finally, in this study of cellular factors which contribute to the hemopoietic environment, we studied the possibility that normal thymocytes enhance bone marrow growth, and that in one or another of these anemics a thymocyte defect might account for the hypoplastic state. A prime candidate would be the Sl/Sl^d anemic mouse, since these mice have been reported by Dr. E. D. Murphy of the Jackson Laboratory to have small thymi (about 35 per cent of normal weight at weaning). Salinas and Goodman (1972) recently reported a synergistic interaction between bone marrow cells and thymocytes, confirming Goodman's earlier findings. In their hands a thymocyte to marrow cell ratio of 16 normal thymocytes to one bone marrow cell yielded optimal results in their X-irradiation transplantation study. They suggested that a cell to cell interaction phenomenon accounts for their results. They further suggested specifically that blastic lymphoid cells of the thymus were responsible for augmentation of bone marrow growth.

We tried to extend their observations by injecting suspensions of normal thymocytes and normal bone marrow cells into steel anemic recipients which were either non-irradiated or had just received 100 R of whole-body X-irradiation. The ratio of thymocytes to bone-marrow cells ranged from 17 to 1 (8.5×10^5 thymocytes with 5×10^4 bone marrow cells), to 95 to 1; 680 to 1; and 950 to 1 (4.75×10^7 thymocytes with 5×10^4 bone marrow cells). In no case did any of the Sl/Sl^d recipients respond in any detectable manner to this treatment. Either we have not yet devised a proper way to administer needed thymocytes to the right spot in Sl/Sl^d recipients, or thymocytes are not the defective element in the Sl/Sl^d hemopoietic environment.

E. ATTEMPTS AT THERAPY OF W/W^v AND $S1/S1^d$ ANEMIAS WITH CORTICOSTEROIDS

The human Blackfan-Diamond macrocytic anemia syndrome shows many similarities to W/W^v and $S1/S1^d$ mouse anemia; corticosteroid therapy has been remarkably successful in therapy of some Blackfan-Diamond cases. The mechanism of action is not known, but could be triggering of stem-cell differentiation, or abolition of an immune reaction. We administered two drugs to +/+, $S1/S1^d$, and W/W^v adult mice. Cortisone was injected intraperitoneally for 7 days in doses of 10 to 40 micrograms per day. No changes in hematocrit were observed during the 14-day post-treatment period. In an analogous experiment we injected hydrocortisone sodium succinate at dose levels of 0, 10, 20, and 40 micrograms per day and following the last injection looked for altered 24 hr Fe^{59} uptake in blood, spleen, and femur. Such treatments were totally without observable effect. Thus, cortisone and hydrocortisone neither augmented erythroid differentiation nor decreased erythropoiesis.

One weakness in these experiments was use of corticosteroids that are not native to the mouse. Possibly better results could be obtained with the native corticosterone.

F. ANALYSIS OF RADIOSENSITIVITY OF W/W^v AND $S1/S1^d$ ANEMIC MICE

Both W/W^v and $S1/S1^d$ anemic mice are extremely radiosensitive (top of Table 3), though for different reasons. For mice of each of these genotypes, radiosensitivity is attributable to their erythropoietic defect, since the type of hemopoietic implant (normal cell suspension or intact normal spleen) that alleviates their anemia also elevates their resistance of X-rays to nearly normal levels. In earlier publications, we showed that successful implantation of normal erythroid stem cells simultaneously cures anemia and increases radiation resistance to normal levels (middle of Table 3). Now we have demonstrated that in addition to suspensions of +/+ spleen or bone marrow cells, other sources of histocompatible erythroid stem cells may be successfully employed, for example intact whole +/+ spleens, or even $S1/S1^d$ whole spleens (bottom, Table 3). All appeared to enhance radiation resistance with about equal efficiency.

$S1/S1^d$ anemic mice can only be "cured" of their anemia by the engraftment of a normal hematopoietic microënvironment, i.e., a whole +/+ spleen, or a W/W^v spleen inherently devoid of erythroid colony forming units. $S1/S1^d$ mice "cured" in this fashion have near normal $LD_{50/30}$'s (550 to 670 R) suggesting that in the surgically unmanipulated state their defective "erythroid environment" is largely responsible for their extreme radiation sensitivity ($LD_{50/30}$ = 129 R).

TABLE 3. GENOTYPIC DIFFERENCES IN RADIOSENSITIVITY BEFORE OR AFTER HEMOPOIETIC TISSUE ENGRAFTMENT.

Genotype of recipient	Genotype of donor	Type of graft	No.	$LD_{50/30}$*	95% confidence limits Lower	Upper
+/+	None	None	112	68	659	704
W/W^v	None	None	30	237	209	269
Sl/Sl^d	None	None	82	129	119	140
W/W^v	+/+	B.M.S.**	28	688	663	714
W/W^v	Sl/Sl^d	B.M.S.	27	667	635	701
Sl/Sl^d	+/+	spleen***	63	669	575	777
Sl/Sl^d	W/W^v	spleen	34	550	516	585
W/W^v	Sl/Sl^d	spleen	51	724	642	815

* Lethal dose for 50% of the animals in 30 days. Determined by computer generated best curve fit using probits and log dose values.

** Bone marrow suspensions injected intravenously.

*** Whole spleen grafted to the peritoneal wall.

To recapitulate, $\underline{W/W^v}$ mice are deficient in their stem cell compartment but have normal erythropoietic microënvironments. $\underline{Sl/Sl^d}$ are deficient in their erythroid microënvironments, but have normal stem cells. +/+ mice are not deficient in either stem cells or environment. And, since all of these phenotypes readily accept hematopoietic grafts from one another, it is possible to analyze various constituents of the erythropoietic system through an exchange of tissues between genotypes and a determination of the extent of complementation which results therefrom.

One of the questions which concerns us is to what extent radiation sensitivity of a given tissue is affected by its immediate surroundings at the time of, or subsequent to, X-ray exposure. To determine this, spleens were irradiated in the donor and then either removed immediately, or permitted to remain in the irradiated donor for various times (up to 96 hr) before removal and transplantation to unirradiated anemic recipients for assessment of their curative potential. Differences in time of post-irradiation residence of the future spleen graft in the donor might result in differences in curative powers of the graft, which could be interpreted and classified under the topics of repair, out-migration, recruitment, or inhibition of the restorative processes. Investigations along these lines were started several years ago with small numbers of animals. While they are not yet complete, they now involve sufficient numbers to make possible some estimates of confidence limits. The data obtained thus far appear in Tables 4 and 5 and in Figure 1.

These experiments with spleens transplanted after irradiation demonstrate that:

1. Even following 610 R wholebody radiation, each +/+ spleen contains enough stem cells to cure the anemia of 50 per cent of $\underline{W/W^v}$ recipient mice, and this curative power is only slightly affected by its time of residence (0.5 to 96 hr) in situ in the irradiated normal donor. The curative power of a +/+ spleen transplanted after irradiation to a $\underline{W/W^v}$ mouse compares favorably with that of a +/+ spleen present before irradiation of the recipient. The curative power of a +/+ spleen transplanted after irradiation to a $\underline{Sl/Sl^d}$ mouse is less than that of a +/+ spleen present before irradiation of the recipient (Table 4).

2. Immediately after 430 R of wholebody irradiation, a $\underline{Sl/Sl^d}$ spleen contains enough stem cells to cure the anemia of 50 per cent of $\underline{W/W^v}$ recipients. This is impressive, since the $LD_{50/30}$ of intact $\underline{Sl/Sl^d}$ mice is only 130 R (Table 4). The erythroid potential of $\underline{Sl/Sl^d}$ stem cells diminishes with postirradiation residence in the steel environment (Table 5), either via out-migration of $\underline{Sl/Sl^d}$ stem cells from the spleen to other hemopoietic sites, or through death or reduced capacity of stem cells within the spleen.

TABLE 4. COMPARISON OF DOSE OF X-RAYS LETHAL FOR A SPLEEN GRAFT IRRADIATED IN THE DONOR VS. DOSE OF X-RAYS LETHAL FOR AN ENTIRE ANIMAL CONTAINING PREVIOUSLY IMPLANTED SPLEEN OF THE SAME GENOTYPE.

Genotype of spleen donor	Genotype of recipient	LDg 50/180 d with CL95(1) (Lethal dose for graft) in roentgens	LD 50/30 d with CL95(2) (Lethal dose-whole animal) in roentgens
+/+	$\underline{W/W^v}$	610 (550 - 680)	680 (660 - 730)
+/+	$\underline{Sl/Sl^d}$	356 (200 - 600)	669 (575 - 777)
$\underline{Sl/Sl^d}$	$\underline{W/W^v}$	433 (355 - 528)	724 (642 - 815)
$\underline{W/W^v}$	$\underline{Sl/Sl^d}$	625 (500 - 700)	550 (516 - 585)
0	+/+		681 (659 - 704)
0	$\underline{W/W^v}$		237 (209 - 269)
0	$\underline{Sl/Sl^d}$		129 (119 - 140)

(1) Dose of X-irradiation which will destroy curative power of the spleen graft for 50% of the recipients determined 180 days following X-irradiation. In parenthesis, 95% confidence limits.

(2) Dose of X-irradiation which will kill 50% of the recipients within 30 days. In parenthesis, confidence limits.

TABLE 5. EFFECTS OF IRRADIATION AND POST-IRRADIATION MICROENVIRONMENT ON CURATIVE POWER[a] OF SPLEEN TRANSPLANTS.

spleen donor ---> recip.	success rate, 0 R	0.5 hr residence in X-irrad. donor			96 hr residence in X-irrad. donor		
		No.	$LD_g50/180$[b]	95CL[c]	No.	$LD_g50/180$[b]	95CL[c]
A. Whole spleen used as source of stem cells, transplanted into stem-cell deficient non-irradiated recipient.							
+/+ ---> W/W^v	22/25 = 88%	15	610 R	(550 - 680)	16	550 R	(406 - 607)
S1/$S1^d$ ---> W/W^v	38/39 = 97%	34	433 R	(355 - 528)	36	281 R	(200 - 360)
B. Whole spleen used as favorable erythroid environment, transplanted into environmentally deficient non-irradiated recipient.							
+/+ ---> S1/$S1^d$	26/30 = 87%	28	356 R	(200 - 600)	24	550 R	(400 - 700)
W/W^v ---> S1/$S1^d$	18/20 = 90%	36	625 R	(500 - 700)	33	642 R	(607 - 678)

a. Measured as that dose of total body X-irradiation by donor which destroys the curative power of its spleen for 50% of the recipients to which that spleen is subsequently grafted.

b. $LD_g50/180$ = 50% lethal dose for the graft as measured by its functional capacity at 180 days. Computer estimated mean and limits based on best curve fit using probits and log doses.

c. 95 CL = 95% confidence limits.

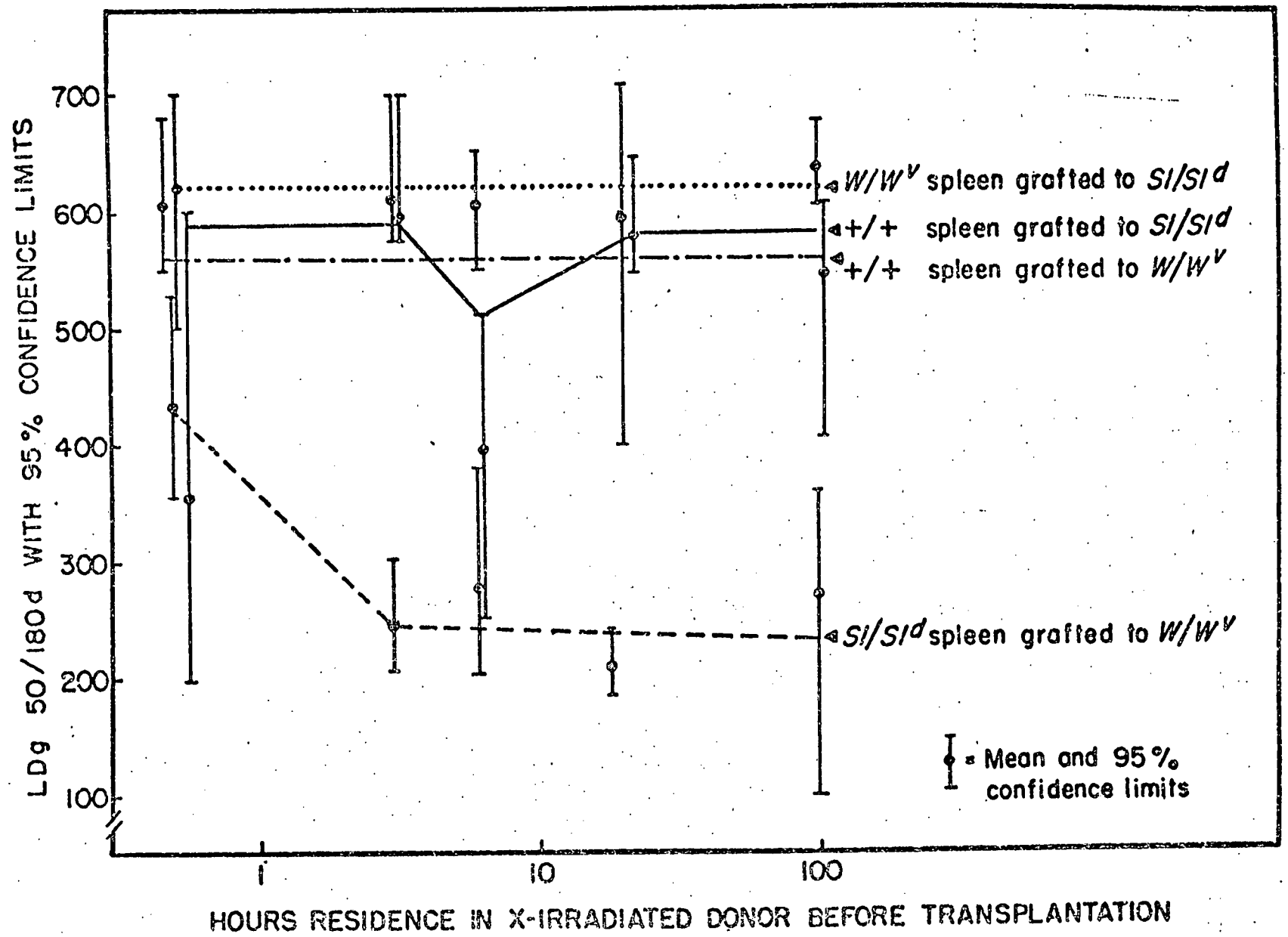

FIGURE 1. Effects of pretransplant irradiation of spleens on subsequent curative power ($LD_{g\ 50/180d}$).

3. When $\underline{Sl}/\underline{Sl^d}$ spleens are irradiated in the $\underline{Sl}/\underline{Sl^d}$ environment and subsequently transplanted as a source of stem cells, their stem cells are more radiosensitive (est. $LD_{50/180}$ = 430 R, vs. 610 R for +/+, Tables 4 and 5) than are +/+ stem cells irradiated in the +/+ environment, and similarly transplanted as a source of stem cells.

4. The data in Table 5B demonstrate unequivocally that heavy X-irradiation damages +/+ and $\underline{W}/\underline{W^v}$ spleens so that, when transplanted in $\underline{Sl}/\underline{Sl^d}$ recipients, they provide a less favorable environment for invading stem cells, the LD_{50} for damage sufficient to prevent cure of 50 per cent of recipient $\underline{Sl}/\underline{Sl^d}$ mice is in the neighborhood of 600 R. However, remaining in the radiated host does no further damage. Thus the curative quality of these grafts is neither noticeably modified by repair of presumably damaged elements, nor is it downgraded by conditions in the irradiated milieu.

G. ERYTHROPOIESIS IN S1/S1 ANEMIC AND LITTERMATE NORMAL FETUSES

In collaboration with Dr. David Chui of McMaster University, we have undertaken a study of erythropoiesis in S1/S1 and littermate normal fetuses during the current year. S1/S1 fetuses are lethal at or shortly after birth, and can be recognized unequivocally at an earlier stage in prenatal development than can S1/S1 fetuses. Previous studies by others have suggested a defective "hemopoietic microenvironment" in the adult S1/S1^{d} spleen and bone marrow. The present investigation examined the effect of S1 genes upon primitive (yolk sac) and definitive (hepatic) red cell production in 13- to 17-day fetuses (gestation 21 days). The number of circulating nucleated erythorcytes, of yolk sac origin, is not significantly different at all stages studied between S1/S1 and +/+ littermates. By day 13, however, S1/S1 embryos have fewer circulating definitive, non-nucleated red blood cells than do their normal (+/+) littermates. This difference increases during fetal development. Erythroblasts of the fetal liver are the precursors of the circulating non-nucleated erythrocytes. There are fewer hepatic erythroid precursor cells in the S1/S1 embryos than in +/+ embryos from at least day 13, although the appearance of erythroblasts in the mutant and normal fetal livers are identical by both light and electron microscopic criteria. The small mutant erythroid cell population is attributable to a reduced number of hemoglobinized precursors (polychromatophilic and orthochromatic erythroblasts); the number of immature, nonhemoglobinized precursors (pro- and basophilic erythroblasts) is not different in mutant and normal fetal livers.

These observations suggest: (1) That the S1 mutation affects erythropoiesis of the definitive fetal and adult cell lineages. The primitive (yolk sac) lineage, which differs from the definitive cell line with respect to morphology and hemoglobins synthesized, also is distinguished by its insensitivity to the S1 gene. (2) That the mutant "hemopoietic microenvironment" fails to support the normal rate of differentiation from immature to hemoglobinized erythroblasts.

H. STUDIES OF MICROCYTIC ANEMIA

Microcytic (mk/mk) mice have an odd type of anemia. Their erythrocytes are so microcytic and hypochromic that the mice are anemic even though their blood contains considerable higher than normal numbers of erythrocytes. Characteristically they show very high percentages of reticulocytes. In year 16, in collaboration with Dr. S. A. Landaw, we showed that their erythrocyte lifespan is considerably shorter than normal, with a subpopulation of red cells destroyed in the spleen almost immediately after they enter the circulation (Landaw et al., 1970). They have both increased free erythrocyte protoporphyrin and moderately increased fecal urobilinogen, suggesting an element of increased breakdown combined with defective hemoglobin synthesis (Kreimer-Birnbaum, Bannerman, Russell, and Bernstein, 1972). In another collaborative

project with Dr. Bannerman and co-workers (1972), evidence of iron deficiency was found in the form of depleted body stores, and increased total iron-binding capacity in plasma. No iron deposits can be visualized in the spleen. However, failure to find either rapid clearance and high utilization of tracer doses of ^{59}Fe or a complete response to parenteral iron treatment indicated that simple iron deficiency was not the cause of the anemia.

Making use of specially matched highly congenic stocks, one carrying mk/mk anemia, the other W/W^V anemia (see section A), Bernstein (1972) demonstrated that there is an intrinsic defect in the hemopoietic tissue of microcytic mice. Bone marrow suspensions from mk/mk mice changed the hematological values of recipient W/W^V mice from macrocytic non-mochromic levels typical of W/W^V mice to the microcytic, hypochromic values typical of mk/mk mice. There is, however, much evidence to suggest that other tissues also have difficulty taking up iron, so that microcytic mice may have a generalized defect in iron metabolism. Investigations in this area continue.

I. TISSUE LOCALIZATION OF OTHER GENETIC DEFECTS BY INTERGENOTYPE TRANSPLANTATION OF HEMOPOIETIC TISSUE

Once highly congenic lines carrying different anemia-producing mutants have been established, transplants can be made between them. In year 17, Bernstein (1972) described the potentiality of the W/W^V anemic recipient to serve as an assay mouse which may be used to distinguish defects intrinsic to erythroid stem cells from extrinsic defects imposed upon them. He transplanted histocompatible marrow from mice with jaundice (ja/ja), or hemolytic anemia (ha/ha), or normoblastosis (nb/nb), or Hertwig's anemia (an/an) into $WBB6F_1$-W/W^V mice. In each case the hematologic phenotype of the implanted W/W^V mouse was converted completely to that of the donor. For example, a W/W^V mouse with implanted ja/ja marrow had a hemolytic anemia. These results suggest that, in mice of genotypes an/an, ha/ha, ja/ja, and nb/nb, the hematologic defect is intrinsic to the erythroid precursor cells.

J. HEMOPOIETIC TISSUE TRANSPLANTATION VIA BLOOD TRANSFUSION

W-anemic mice (W/W^V) are not exact models of human hypoplastic anemias (Blackfan-Diamond Syndrome) but they do resemble them in enough detail to make them the most useful extant model. In the W/W^V mouse, as previously indicated, an intrinsic defect in the stem cell pool has been demonstrated and such mice readily accept histocompatible grafts. Occasionally human cases of Blackfan-Diamond anemia have spontaneously remitted following routine blood transfusions, but it has not yet been determined if remissions are transfusion induced.

With the knowledge that "stem cells" circulate, and that colony forming units are present in the circulating blood of mice, we undertook a set of experiments to determine the relative efficiency of whole blood transfusions for curing W-anemics and for ascertaining whether the blood from different donors varied in their capacity to transform hematologically the W/W^v recipient. Graded doses of fresh whole blood from histocompatible mice of genotypes +/+, ha/ha, ja/ja, mk/mk, nb/nb, sph/sph, or Sl/Sl^d were injected into untreated W/W^v recipients and their $ED_{50/90}$'s were determined. Our preliminary observations are presented in Table 6.

TABLE 6. DIFFERENCES IN EFFECTIVENESS OF WHOLE BLOOD OBTAINED FROM VARIOUS ANEMIC MUTANTS FOR PERMANENTLY CHANGING THE HEMATOLOGICAL PHENOTYPE OF $\underline{W}/\underline{W^v}$ RECIPIENTS.

Genotype of Donor	No. Obs.	Calculated $ED_{50/90d.}$			Comparative effectiveness based on:	
		Volume (ml)	No. nucleated cells X 10^6	Human volume equivalents (pints)	Volume	No. nucleated cells
+/+	39	1.2	16.2	7.1	1.0	1.0
ha/ha	36	0.2	4.8	1.2	6.0	3.37
ja/ja	17	< 0.1	< 49.3	< 0.6	> 12.0	< 0.33
mk/mk	40	> 2.3	> 14.1	> 13.6	< 0.52	> 1.14
nb/nb	39	0.2	37.6	1.2	6.0	0.43
sph/sph	38 c.a.	2.7	c.a. 6.8	16.0	0.44	0.24
$\underline{S1}/\underline{S1^d}$	38	> 3.2	> 43.3	> 18.9	< 0.38	> 0.37

It will be seen that blood from hemolytic anemic mice (ha/ha) and normoblastic anemic mice (nb/nb) are equally effective in changing the phenotype of W-recipients (the $ED_{50/90d}$ for both is 0.2 ml), yet they differ markedly (nearly 8-fold) in the number of nucleated cells present in whole blood (24×10^6/ml for ha/ha versus 188×10^6 per ml for nb/nb). One may postulate: (1) that these genotypes both contain the same number of "stem cells" but such cells are not readily distinguishable morphologically, (2) that ha/ha mutants have a considerably higher proportion of stem cells in the nucleated population in their blood and the stem cells are potentially equally curative, or (3) that the proportion of stem cells to differentiated elements in ha/ha is the same or lower than in nb/nb, but ha/ha stem cells have a proliferative capacity much greater (about 7-fold greater) than that of nb/nb. At this reporting we have not been able to distinguish between these possibilities, but it is conceivable that CFU analysis on blood which is high in curative potential could be most informative. Such considerations could also apply to the other genotypic differences encountered in these experiments. Jaundiced (ja/ja) whole blood is uncommonly curative. It is more than 12-times as effective as +/+ blood on a volume basis, yet it is only about 1/3 as effective on a nucleated cell basis. Other findings indicate that both Sl/Sl^d and mk/mk blood with their low nucleated cell contents seem to contain very few cells capable of seeding W/W^V marrow. Similarly, sph/sph blood, although it contains huge numbers of nucleated cells, does a particularly poor job of modifying the hematological phenotype of the W/W^V recipient.

Freezing and thawing, or sonication of the blood prior to transfusion, destroys its curative action. The hemoglobin type of the recipient is changed if the transfused blood is of a type different from that of the recipient. Both sets of observations suggest that intact viable cells are required for hematological transformation.

In general, the data presented above, even though preliminary in nature, rather clearly indicates that W/W^V anemic mice can be cured via blood transfusion of normal (+/+) whole blood, that genetic differences do exist, and that unbelievably small amounts of blood may be required, in some cases the equivalent of but a fractional USP unit in man. Further analysis reveals that there is no correlation between the number of nucleated cells administered and the curative effect of the transfusion. In the mutants employed here either "stem cells" are not a constant proportion of the nucleated cell population in the whole blood or there are great differences in the proliferative capacity of the various mutant stem cells which do circulate or both. Much remains to be done to relate curative action to CFU or to the presence of a particular cell type.

K. STUDIES OF HERTWIG'S ANEMIA

With the final realization of our efforts to place the an gene on a common genetic background with W, S1, and other hematological mutant genes, we have seen the desirability of quantitatively establishing new hematological baselines. This necessity becomes clear when we recall that the expression of all genes is dependent on the expression of other genes distributed in the genome. We see, for example, that on the $WBB6F_1$ hybrid background that the anemia is slightly less severe than on its original background. The red blood cell numbers are elevated from 5.48 ± 0.18 to $6.70 \pm 0.17 \times 10^6$ per mm^3. Its hematocrit is raised from 34.44 ± 0.73 to 42.77 ± 0.52 per cent, its hemoglobin level is raised from 11.6 ± 0.4 to 13.2 ± 0.35 g/100 ml. Its total leukocyte count is still low (8.7×10^3 per mm^3) but extremely variable (coeff. variability 26.8 per cent). Experiments in progress include the characterization and the distribution of erythroid cells in femoral marrow and spleen; a set of determinations of serum erythropoietin levels and physiological responses to hypoxia, in collaboration with Dr. Geoffrey Keighley; evaluation of the immunological status in collaboration with Dr. Richard Stoner of Brookhaven National Laboratory, and an analysis of erythrocyte lifespan and the degree of "ineffective erythropoiesis" with Dr. Stephen Landaw of the Donner Laboratory in Berkeley.

Although Hertwig's anemia (an/an) is macrocytic and shows many similarities to the anemias of W/W^V and $S1/S1^d$ mice, it differs from these in not involving a pigmentory defect. Dr. Russell is using the new viable $WBB6F_1$-an/an mice to see if the an mutation, like the W and S1 mutant alleles, has a pleiotropic effect on germ cell number. The study of histological sections of ovaries from 4- to 10-week old females indicates considerable reduction in number of primary oocytes, developing follicles, and corport lutea in ovaries of $WBB6F_1$-an/an anemic mice, compared with those of $WBB6F_1$-an/+ and - +/+ littermates. Evan at 10 weeks, however, a few developing follicles were seen in an/an ovaries. Similar observations were made on $WBB6F_1$-an/an, - an/+, and - +/+ testes. All of the an/an testes were smaller than those of normal littermates. While 100 per cent of seminiferous tubules showed active spermatogenesis in an/+ and +/+ testes, only a small proportion (8 to 40 per cent) showed limited spermatogenesis in an/an mice. Thus the an gene substitution does have a deleterious pleiotropic effect on germ cells.

L. PUBLICATIONS IN YEARS 15, 16, AND 17

The following papers acknowledged support from AEC AT(11-1)-3264:

BERNSTEIN, S. E. 1970. Tissue transplantation as an analytic and therapeutic tool in hereditary anemias. Amer. J. Surg. 119:448-451.

LANDAW, S. A., E. S. RUSSELL, and S. E. BERNSTEIN. 1970. Splenic destruction of newly formed red blood cells and shortened erythrocyte survival in mice with congenital microcytosis. Scand. J. Haematol. 7:516-524.

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RUSSELL, E. S., D. J. NASH, S. E. BERNSTEIN, E. L. KENT, E. C. McFARLAND, S. M. MATHEWS, and M. S. NORWOOD. 1970. Characterization and genetic studies of microcytic anemia in the house mouse. Blood 35:838-850.

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KREIMER-BIRNBAUM, M., R. M. BANNERMAN, E. S. RUSSELL, and S. E. BERNSTEIN. 1972. Pyrrole pigments in normal and congenitally anemic mice (+/+, W/W^{v}, ha/ha, nb/nb, mk/mk, f/f, and sla). Comp. Biochem. Physiol. 43:21-31.

*BERNSTEIN, S. E. 1972. Chimerism induced by intergenotype transplantation of mouse bone marrow. Exper. Haematol. 22:69-71.

*Seven reprints of each of these papers enclosed.