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Report No. COO-3264-13

THE JACKSON LABORATORY
Bar Harbor, Maine 04609

1 May 1976

Comprehensive Progress Report to accompany
Twenty-first renewal proposal to the

Division of Biomedical and Environmental Research
Energy Research and Development Administration
Washington, D.C. 20545

Contract number: E(11-1)-3264

Title of project: INBORN ANEMIAS IN MICE

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Senior Staff Scientist

Period of report: 1 May 1973 to 30 April 1976

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Distribution

7 - Research Contracts Division
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The Jackson Laboratory

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1. MAIN RESEARCH ACCOMPLISHMENTS

A. Delineation of inborn anemias. 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 and parabiosis experiments; by analysis of reactions of normal and anemic mice to a variety of stressful stimuli, including X-irradiation and hypoxia; and by histological and biochemical study of normal vs. abnormal blood-forming tissue. At present 12 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 E(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 single-locus induced 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).

Each of these blood dyscrasias is caused by the action of a unique mutant gene, each of which determines the structure of a different intracellular molecule, and thus controls a different metabolic process. Our wide range of different hereditary anemias has considerable potential for uncovering many different aspects of hemopoietic homeostatic mechanisms in the mouse. This potential is enhanced when the effects of altering a single process (through substituting a mutant for the normal allele at one particular genetic locus) can be observed against one constant genetic background.

Considerable effort has been devoted to establishing each mutant allele on a genetically homogeneous genetic 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), can be compared with each other and with congenic hematologically normal $WBB6F_1$-+/+ mice.

B. 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 the

Jackson Laboratory's new Mammalian Genetics Laboratory (completed in 1975) where stocks are to be maintained with maximum environmental protection. We are well along in establishment, in this new facility, of 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 served as foster mothers to Caesarian-derived (hence aseptic) offspring from the stocks we plan to put into our Anemia Mutant Foundation Stocks. We also used WB/Re foster mothers, which were themselves descended from WB/Re mice introduced into our colony as Caesarian-derived aseptic neonatal mice.

By the end of April, 1976, we have established, in the Mammalian Genetics Laboratory, Foundation colonies of 30 of our 34 special anemia-mutant producing stocks (see 21st Annual Progress Report, item B, for detailed list). We hope to incorporate the remaining four stocks into MGL within the next six months.

C. Hematologic detection of the carrier state for recessive anemias. Study of the four hemolytic anemias, all recessively inherited, has become increasingly important in our program, and the potentialities of these mutants have attracted attention from many and diverse potential collaborators. Our efforts to push forward have been greatly hampered by delay in setting up matings producing homozygous (m/m) anemic offspring. Test-matings of mice of unknown (+/+ or m/+) genotype to known heterozygotes (m/+) have been required for identification of heterozygotes, causing considerable delay and consequent reduction of mutant mouse production. Due to two approaches pursued by Dr. Bernstein, we are now able to recognize at least 90 to 95 per cent of ja/+, nb/+, and sph/+ adult heterozygotes, and 85 per cent of ha/+ heterozygotes. His first method, discriminant function analysis, utilizes a combination of routine hematologic measurements (red cell numbers, hemoglobin concentration, and hematocrit percentage) and the second method involves differences between genotypes in rate of heme synthesis, as studied in collaboration with Dr. Shigeru Sassa of the Rockefeller University. Within the next six months we will have completed tests of the effectiveness of these methods for recognizing young carriers of anemia-producing mutant alleles. If the heterozygotes can be recognized at 50 days, test-matings can be eliminated, our mouse-producing facilities can be used more efficiently, and considerably larger numbers of mice with the various types of hemolytic anemia can be provided for research.

D. Studies of Hertwig's anemic (an/an) mice. Until very recently, studies of the etiology of Hertwig's macrocytic anemia (an/an) have been severely limited by limited postnatal viability of an/an mice. However, we have been able, since 1973, to make considerable progress due to the availability of fully viable WBB6F$_1$-an/an mice. These have the added advantage of being highly congenic (except for the specific anemia-producing loci) with our other two macrocytic anemic types, WBB6F$_1$-W/W^v and WCB6F$_1$-Sl/Sl^d. Working from neonates to very old mice, we have demonstrated at all ages reduced numbers of macrocytic erythrocytes, reduced numbers of leukocytes (2/3 of +/+ values); but practically normal numbers of platelets. The lifespans of WBB6F$_1$-an/an mice are shorter than those of normal littermates, with many dying from 18 to 24 months of age, often with reticulium cell carcinoma, Type A. Many of the physiologic responses of an/an mice are similar to those of W/W^v and Sl/Sl^d mice. They increase hematocrit

levels during prolonged exposure to hypoxia, but are much less responsive to exogenous erythropoietin than are +/+ mice. (The limited response of polycythemic an/an mice to graded doses of erythropoietin is very close to that of polycythemic W/W^v mice.) Hertwig's anemic mice definitely have a defect in hemopoiesis indigenous to their blood-forming tissue, since transplants of an/an marrow cells into W/W^v recipients take, but do not cure, the anemia of the recipient. The marrow and spleens of an/an mice contain stem cells which can proliferate at a normal rate when transplanted to W/W^v recipients, and can lead to formation of macroscopic splenic colonies in lethally irradiated normal mice; the absolute number of these stem cells may, however, be reduced. Hemopoietic activity is higher than normal in spleens of an/an mice.

In addition to all of these investigations of the anemia of mice with Hertwig's anemia, we are also studying their germ-cell defect, which is in many ways similar to that of W/W^v and Sl/Sl^d mice, though slightly less extreme. In all three, the germ cell defect is clearly apparent by the 12th day of prenatal development. One different feature in young $WBB6F_1$-an/an females is a high incidence of mating at an early age, followed by pregnancy (one to three fetuses) but inability to deliver their full-term offspring.

E. Results of parabiosis between mice of differently affected genotypes. In all cases where mice with severe macrocytic anemia have been joined in parabiosis with normal mice, an apparent cure has been achieved, at least temporarily. However, in some combinations the cure is not maintained after separation. Experiments by Dr. Bernstein during 1973 to 1976 have demonstrated clearly that the mechanisms of parabiotic cure vary greatly according to the specific genotypes combined. His results have provided considerable insight as to gene action responsible for each of the anemias. His studies have involved not only blood counts, but also histological studies (on Millipore filters) of erythroid activity in conjoined mice. In parabiosis, an/an stem cells invade normal hemopoietic microenvironments but retain their indigenous differentiation defect. Also, an/an mice may not be permanently cured by parabiotic union and implant of normal stem cells. $WCB6F_1$-Sl/Sl^d mice provide perfectly normal stem cells to their parabiotic partners, but do not foster growth of stem cells from their partners. They are not permanently cured by parabiosis. Instead, Sl/Sl^d anemics, whether parabiosed to +/+, W/W^v, or Sl/Sl^d partners, display a reduction to near zero in erythroid activity in their own marrow and spleen. Neither Sl/Sl^d nor an/an mice appear to elaborate an inhibitor of erythropoiesis; nor does presence of a normal partner enhance an/an or Sl/Sl^d erythroid activity. $WBB6F_1$-W/W^w mice, with their primary defect in the erythroid stem cell compartment, possess a fully normal hemopoietic microenvironment, and are completely cured by implant of +/+ or Sl/Sl^d stem cells. Mice of all three anemic genotypes appear to have normal capacity to produce erythropoietin. In all parabiotic unions involving +/+ and anemic mice, the normal partner shows a rapid increase in erythropoiesis, particularly in the spleen. These responses almost certainly result from circulating erythropoietin coming from the anemic partner.

F. Characterization of the splenic hemopoietic microenvironment. Studies with Sl/Sl^d mice have demonstrated clearly that their hemopoietic stem cells are completely normal, and that their defective hemopoiesis must be attributed entirely to faulty function of their hemopoietic microenvironment. Since 1973 we have attempted in a variety of ways to identify the nature of this defect.

One approach has been histochemical study of adult $\underline{S1/S1^d}$ vs. +/+ spleens, which failed to show any clear-cut difference. In collaboration with Dr. David Chui of McMaster University in Canada, we have studied prenatal blood formation in $\underline{S1/S1}$ vs. +/+ fetal liver using electron microscopy. The proportion of early to late stages of erythropoiesis indicated clearly that erythroid differentiation was impeded in $\underline{S1/S1}$ fetuses, but detailed study of the liver stroma and of connections between hemopoietic cells and stroma did not disclose any structural differences between +/+, $\underline{S1/+}$ and $\underline{S1/S1}$ fetal livers. Tests of the radiosensitivity of the splenic microenvironment (how much radiation will destroy the capacity of the implanted spleen to provide a favorable site for erythropoiesis by $\underline{S1/S1^d}$ stem cells) have likewise been frustrating. The nature of the $\underline{S1/S1^d}$ defect in hemopoietic microenvironment remains a mystery.

G. Regeneration of intestinal crypt cells in $\underline{W/W^v}$ and $\underline{S1/S1^d}$ mice. Cells of the intestinal crypts must divide rapidly particularly following whole-body radiation. In collaboration with Dr. Arthur Hopper of Rutgers University, we tested the regenerative capacity of intestinal crypt cells, following 700 R whole body radiation, in $WBB6F_1$-$\underline{W/W^v}$, $WCB6F_1$-$\underline{S1/S1^d}$, and $WBB6F_1$-+/+ mice. We concluded that not all rapidly proliferating tissues of the mouse are adversely affected by action of mutant alleles at the $\underline{W}$ and $\underline{S1}$ loci. In contrast to greatly impeded proliferation of blood-cell precursors, primordial germ cells, and melanocyte precursors in $\underline{W/W^v}$ and $\underline{S1/S1^d}$ anemic, sterile, black-eyed white mice, normal proliferation of intestinal mucoosa 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 ($\underline{W/W^v}$ and $\underline{S1/S1^d}$) mice. The intestinal mucosa of the $\underline{W/W^v}$ and $\underline{S1/S1^d}$ 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.

H. Stem-cells in circulating blood, including transplantation through transfusion. Hemopoietic stem cells were shown to circulate in the blood of normal mice and of mice of six tested anemic genotypes ($\underline{ha/ha}$, $\underline{ja/ja}$, $\underline{mk/mk}$, $\underline{nb/nb}$, $\underline{sph/sph}$, and $\underline{S1/S1^d}$). Circulating stem cells of each genotype appear capable of implanting in $\underline{W/W^v}$ mice following blood transfusion. Very small doses of jaundiced or normablastic blood contained sufficient stem cells to convert $\underline{W/W^v}$ mice from macrocytic to hemolytic anemia, while intermediate doses of blood from hemolytic ($\underline{ha/ha}$) anemics were required, and large doses of spherocytic ($\underline{sph/sph}$), $\underline{S1/S1^d}$, and microcytic ($\underline{mk/mk}$) blood.

I. Localization of defects responsible for hemolytic anemias. Over the years we have succeeded in determining, via the intergenotype transplantation technique, that all of hemolytic anemias ($\underline{ha/ha}$, $\underline{ja/ja}$, $\underline{nb/nb}$, $\underline{sph/sph}$) represent intrinsic defects of erythroid stem cells or their differentiated progeny. We have been able to eliminate hemoglobinopathy, immunopathy, and glycolytic cycle aberrancies (Hutton and Bernstein, 1973) as possible etiologic agents responsible for the short-lived, lysis-prone erythrocytes encountered in these diseases, but until recently have not been able to find positive evidence of membrane defects. Within the past year several major breakthroughs have occurred. Techniques developed by others during the past 2 years have permitted us to examine more critically the erythrocyte membranes from mice with each type of hemolytic anemia. In collaboration with Dr. Alfred Greenquist of the University of

California Medical School at San Francisco, Dr. Bernstein has shown that the erythrocytes of spherocytic (sph/sph) anemic mice have a spectrin defect. In collaboration with Dr. Harry Chen of the Jackson Laboratory, Bernstein has shown lower-than-normal concentrations of cholesterol in the red cell membranes of spherocytic mice and of mice with the other three types of hemolytic anemia. No specific quantitative defect in spectrin synthesis has been encountered in normablastic (nb/nb) anemia. These single-gene determined biochemical differences in red-cell membranes provide us with an excellent opportunity to assess the role of spectrin, of membrane cholesterol, and of membrane-bound enzymes in the hemolytic process.

2. PLANS FOR CONTINUATION OF PRESENT OBJECTIVES

We have now delineated extensively 10 of the 12 single-locus induced anemias of the mouse, and have the capacity to study each of these further by such methods as intergenotype transplant, biochemical analysis, and histological analysis at the electron microscope level. We will place special emphasis in the near future on four problems:

A. Analysis of membrane defects, cholesterol levels, and membrane-bound enzyme aberrations in mice of each of our four genotypes (ha/ha, ja/ja, nb/nb, sph/sph) with hemolytic anemia. In doing this we will take full advantage of increased mutant animal supplies made possible by direct detection of heterozygous carriers to be used in breeding (see item C above). We will both continue existing collaborative arrangements (with Chen, Eppig, Greenquist, Jacob, and Sassa) and expand collaboration to include many other investigators eager to study erythrocyte membrane defects.

It seems to us that the promising findings of spectrin deficiency in spherocytic (sph/sph) erythrocytes, plus the absence of such defect in normoblastic (nb/nb) erythrocytes, plus observations of low levels of cholesterol in red cell membranes in all four types of lysis-prone erythrocytes, all combine to suggest an analyzable system where identification of the site and nature of action of each mutant gene (ha, ja, nb, or sph) will provide valuable insight on mechanisms of membrane synthesis and maintenance. These studies may also increase understanding of certain human hemolytic anemias due to membrane defect.

B. Continued studies of Hertwig's anemia. This third macrocytic anemia with pleiotropic effects on germ cell number clearly has an etiology different from that of either W/W^v or Sl/Sl^d macrocytic anemia. We hope that further studies of stem cell numbers in an/an marrow and spleen, combined with more detailed studies of erythroid differentiation from an/an stem cells, will increase our understanding of the hemopoietic defect intrinsic to blood-forming tissues of an/an mice. In particular, we will try to determine why they depend more upon splenic erythropoiesis than do other mice. We will follow for prolonged periods the blood pictures of lethally-irradiated +/+ mice whose lives have been saved by implants of an/an marrow cell suspensions.

We will also continue analysis of the leukopenia characteristic of an/an mice. Particularly intriguing is the sex-difference in leukocyte numbers in $WBB6F_1$-+/+ mice (males higher at all ages), and the absence, or at least great reduction, of this sex difference in leukocyte numbers in $WBB6F_1$-an/an mice. Some resistance to a hormonal effect may be involved.

The hormonal situation of an/an mice should also be more extensively studied, since they become pregnant but cannot deliver offspring, a situation very different from that in W/W^v and Sl/Sl^d mice.

C. Studies of the nature of the Sl/Sl^d defect in hemopoietic microenvironment must be continued, along with attempts to find reliable ways to provide effective therapy of macrocytic anemia through implant of normal hemopoietic stroma. In this line of endeavor, both electron microscopic and histochemical methods should be further explored. Implants of normal stromal cells would be facilitated by development of methods for in vitro culture of hemopoietic stroma, and by determination of optimal sites for implantation and function of transplanted normal stroma.

D. Methods of in vitro culture of hemopoietic cells and of supporting stromal cells must be perfected. We must be able to observe typical erythroid differentiation in vitro from stem cells from mice of each anemic genotype. We also need to develop methods for in vitro cultivation and multiplication of hemopoietic stem cells. The problem in culture of hemopoietic tissue has always been maintenance of its differentiation. Fibroblasts are wonderful, but not for the study of gene-action determining pattern of hemopoietic differentiation.

3. GRADUATE STUDENTS TRAINED SINCE 1973

Although our research program does not typically support doctoral research, Ralph Stern worked towards his Ph.D. degree in our laboratory in 1973-74, studying embryonic hemoglobin genetics in the mouse and (in a separate project) finding genetic variant in carbonic anhydrase II in the mouse, and determining its close genetic linkage to the locus for carbonic anhydrase I. Dr. Stern received his Ph.D. from Johns Hopkins in 1975.

In line with Jackson Laboratory custom, we have since 1973 sponsored 9 summer or academic year college-level research trainees and 5 precollege summer trainees.

We have also since 1973 sponsored the following as Visiting Research Investigators working in our laboratory on problems related to E(11-1)-3264:

Dr. George J. Brewer of the University of Michigan,
Dr. David Chui of McMaster University, and
Dr. Shigeru Sassa of the Rockefeller University.

4. BIBLIOGRAPHY OF TITLES ASSOCIATED WITH THIS CONTRACT

The following is a bibliography of publications during the past three years associated with this contract.

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

KREIMER-BIRNBAUM, M., R. BANNERMAN, E. S. RUSSELL, and S. E. BERNSTEIN. 1972. Pyrrole pigments in normal and congenitally anaemic mice. Comp. Biochem. Physiol. 43A:21-30.

ALTUS, M. S., S. E. BERNSTEIN, E. S. RUSSELL, A. L. CARSTEN, and A. C. UPTON. 1972. Defect extrinsic to stem cells in spleens of steel. Proc. Soc. Exp. Biol. Med. 138:985-988.

RUSSELL, E. S., and G. KEIGHLEY. 1972. The relation between erythropoiesis and plasma erythropoietin levels in normal and genetically anemic mice during prolonged hypoxia or after whole-body irradiation. Brit. J. Haematol. 22:437-452.

BANNERMAN, R. M., J. A. EDWARDS, M. KREIMER-BIRNBAUM, E. C. McFARLAND, and E. S. RUSSELL. 1972. Hereditary microcytic anemia in the mouse: studies in iron distribution and metabolism. Brit. J. Haematol. 23: 319-329.

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

HARRISON, D. E., and E. S. RUSSELL. 1972. The response of $\underline{W/W^v}$ and $\underline{Sl/Sl^d}$ anaemic mice to haemopoietic stimuli. Brit. J. Haematol. 22:155-168.

RUSSELL, E. S., R. BLAKE, and E. McFARLAND. 1972. Characterization and strain distribution of four alleles at the hemoglobin α-chain structural locus in the mouse. Biochem. Genet. 7:313-330.

BERNSTEIN, S. E. 1973. Hemopoietic tissue transplantation via blood transfusion. Exp. Hematol. 1:281-286.

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MOLINARI, P. F., P. BULAT, and S. E. BERNSTEIN. 1974. Effect of testosterone proprionate on the erythropoietin tissue of three congenital anemias in mice. Biomedicine 21:111-114.

BLAKE, R., R. T. GRILLO, and E. S. RUSSELL. 1974. Increased taurine excretion in hereditary hyperprolinemia of the mouse. Life Sci. 14:1285-1290.

CHUI, D., and E. S. RUSSELL. 1974. Fetal erythropoiesis in steel mutant mice. I. A morphological study of erythroid cell development in fetal liver. Develop. Biol. 40:256-269.

HOPPER, A. F., and E. S. RUSSELL. 1975. Post-irradiation regeneration of intestinal epithelium in $\underline{W/W^v}$ and $\underline{Sl/Sl^d}$ genetically anemic mice. Radiat. Res. 63:310-319.

5. PRESENT STATE OF KNOWLEDGE REGARDING MOUSE HEREDITARY ANEMIAS

Mouse single-locus induced hereditary anemias provide extremely valuable insight into problems of hemopoietic differentiation; etiology of constitutional diseases of man as well as of the mouse; of gene action in mammals; and of genetic aspects of aging processes. Our studies of the etiology of $\underline{W/W^v}$ and $\underline{Sl/Sl^d}$ macrocytic anemias have attracted considerable attention, and many other investigators are currently working with these mice. Hertwig's ($\underline{an/an}$) anemia has considerable potential for broadening understanding of control of differentiation of both erythroid and myeloid tissue, indicating that future research utilizing this mutant has special value. We are just beginning to get good "handles" for analysis of the hemolytic anemias and foresee that studies of these valuable mutants will provide insight into membrane synthesis and structure, applicable especially, but not exclusively, to red cell membranes.

6. PRESENT DIVISION OF FEDERAL SUPPORT OF PHYSIOLOGICAL GENETICS RESEARCH PROGRAM

See attached financial interplay budget sheet.

INTERPLAY OF E(11-1)-3264 AND OTHER FINANCIAL ASSISTANCE, 1 AUGUST 1975 TO 31 JULY 1976

	Time % hrs	The Jackson Laboratory[1]	E(11-1) -3264	NIH CA-01074	NIH HD-00254	ACS VC-58	The Jackson Laboratory[2]	TOTAL
Salaries								
Prin. Invest., E. S. Russell	90%	1,860	12,720	10,470		3,650	4,110	32,810
Co-Prin. Invest., S. E. Bernstein	100%		7,650	15,300	6,090		1,560	30,600
Res. Assoc., to be appointed	100%			5,040		9,360		14,400
Res. Asst., E. C. McFarland	40 hrs		3,362	3,363		6,725		13,450
Res. Asst., S. A. Deveau	40 hrs		4,680		4,680			9,360
Res. Asst., D. B. Chapman	18 hrs				4,850			4,850
Res. Asst., M. S. Norwood	24 hrs			5,970				5,970
Res. Asst., May Keighley	10 hrs			2,400				2,400
Res. Asst., A. M. Grindle	40 hrs			4,515		4,414		9,030
Res. Asst., M. L. Muise	5 hrs			975				975
Secretarial			350	725	350	275		1,700
		1,860	28,762	48,758	15,970	24,525	5,670	125,545
Indirect Costs at 62.4		1,161	17,947	30,425	9,965	9,018	9,824	78,340
Supplies and Materials			2,000	6,400	3,000	2,900		14,300
Equipment			300	1,000	1,000	300		2,600
Publication Costs			300	600	300	300		1,500
Travel			500	800	500	400		2,200
Other								
Employee benefits (17%)		316	4,890	8,289	2,715	4,170	963	21,343
Animal Care Service (1,950)			3,600	32,400	8,400	2,400		46,800
Laboratory Services			700	2,300	2,000	300		5,300
Mice			500	1,000	1,000	500		3,000
TOTAL COST		$3,337	$59,499	$131,972	$44,850	$44,813	$16,457	$300,928

continued