POSTTHYMECTOMY WASTING ASSOCIATED WITH AUTOIMMUNE PHENOMENA*

I. Antiglobulin-Positive Anemia in A and C_{57}BL/6 Ks Mice

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The wasting syndrome of neonatally thymectomized mice shows similarities to the syndrome observed in mice experiencing a graft-versus-host reaction. Among the consistent features of both disorders are emaciation, diarrhea, and skin lesions. Atrophy of the lymphatic tissues and impairment of immunological functions are also found in mice after neonatal thymectomy. The lymphoid atrophy which occurs in the thymectomized animals could be the result of defective lymphoid cell production or differentiation, whereas, in the graft-versus-host reactions the deficiency of lymphocytes seems to be consequent to an immune reaction of the donor cells against the host lymphoid organs. The thymus has become suspect in the pathogenesis of autoimmune disorders, mainly because of clinical association between pathological changes in the thymus and diseases with autoimmune signs such as myasthenia gravis (1, 2) and systemic lupus erythematosus (3). Thymomas, hypertrophied Hassall's corpuscles, and germinal centers have been observed in the thymus in association with these apparent autoimmune processes. The importance of the thymus to the development of autoimmune disorders received support from findings reported in New Zealand black mice. These mice developed autoimmune hemolytic anemia, positive L.E. tests, and lupus nephritis and were found to have thymic abnormalities (4–9). The etiology of the wasting syndrome occurring in neonatally thymectomized mice remains conjectural, but infection, most likely viral (10, 11), increased susceptibility to injury from the animal's normal microbial flora (12), and the autoimmunity theory (13–16) have emerged as leading hypotheses.

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It is the purpose of this paper to present a comprehensive analysis of the anemia occurring in neonatally thymectomized mice of the A and C_{57}BL/6 Ks strains. These observations establish that a true hemolytic process, as well as bone marrow suppression, occurs in association with a positive antiglobulin reaction of the erythrocytes in these animals. It will be shown that the red blood cells of neonatally thymectomized mice are coated with IgM immunoglobulin. Similarly, it will be shown that the anemia which occurs spontaneously in the New Zealand black mice of our colony is associated with the presence of both IgM and β_{1C} but not IgG on the surface of their red blood cells.

Materials and Methods

Mice.—Inbred mice of the A, C_{57}BL/1, C_{57}BL/6 Ks, C_3H, (C_3HxA)F_1 hybrid, and New Zealand black (NZB) mice were used. The A, C_3H, and C_{57}BL/1 mice were separated from the mouse colony of the late Dr. John Bittner in 1956 and have been maintained by rigorous inbreeding since that time (17). The C_{57}BL/6 Ks mice were obtained from Jackson Laboratory (Bar Harbor, Me.) and maintained in our colony for five generations. The NZB[1] mice were obtained from Dr. Edward W. Hook of Cornell Medical Center in New York. The latter obtained them from Dr. M. Bielschowsky in New Zealand. This strain has been maintained in our laboratories for 24 months (four generations) by standard inbreeding methods.

Surgical Procedures.—Thymectomies were performed by the technique of Sjodin et al. (18). In sham operations the thorax was opened but the thymus was left intact.

Observations.—All animals were observed at least every 4th day from 25 to 150 days of age. Weights and clinical characteristics were recorded (diarrhea, hunching of the back, and skin lesions).

Hematological Studies.—Blood samples were drawn from the tail veins of control and experimental mice. Microhematocrit and leukocyte counts with differential leukocyte and reticulocyte counts were determined by standard methods.

Red Cell Fragility Studies.—These studies were performed according to the method of Cohen (19). The following NaCl solutions were prepared: 0.80, 0.66, 0.56, 0.52, 0.48, 0.44, 0.40, and 0.32%, with a pH between 5.5 and 7.0. 10 ml of each solution was pipetted into 15-ml conical centrifuge tubes. Blood was collected in amounts of 0.5 ml in tubes containing 0.05 ml of heparin (20 mg/ml) per tube. 0.02 ml of blood was pipetted into each tube of NaCl solution and mixed gently. The tubes were left at room temperature for 1 hr and then they were centrifuged at 2000 rpm for 10 min.[2] The amount of hemoglobin in the supernatants was determined colorimetrically. The per cent hemolysis was calculated by dividing the hemoglobin concentration in the supernatant by the hemoglobin concentration of the blood sample and multiplying by 100.

Preparation of Mice with Reticulocytosis.—Mice of the A and NZB strains were bled daily for 10 days via the tail veins. From 15 to 20 drops of blood were removed daily. This procedure induced the reticulocyte count to increase to more than 40% of the erythrocytes.

Preparation of Red Cell Suspensions for Absorption Studies.—Blood was collected from normal A, C_{57}BL/6 Ks, and NZB mice at 2 months of age and from mice with induced reticulocytosis in separate 15.0-ml centrifuge tubes to which 10 mg of heparin (0.5 ml) had been added. The pools of erythrocytes were washed 6 times in lactate–Ringer's solution and centrifuged after each washing at 2000 rpm.[2] After the last centrifugation, the supernatant was carefully

[1] In this paper NZB refers to the NZB/B1 strain of mice.

[2] International clinical centrifuge, model CL, International Equipment Co., Needham Heights, Mass.

removed with a capillary pipette. The packed red cells were then used for absorption of anti-mouse sera.

Anti-Mouse Sera.—Anti-mouse serum and anti-mouse gamma globulin of goat origin were obtained commercially.[3] Anti-mouse β_{1C} of rabbit origin was obtained from Dr. Müller-Eberhard. These antisera were found to contain a variable amount of mouse (species) specific antibodies which were detected by direct agglutination of normal mouse (A, C_3H, and $C_{57}BL/6$ Ks) erythrocytes at 25°C. All sera were absorbed by incubation for 1 hr at room temperature by mixing equal volumes of the antiserum and packed normal erythrocytes from A mice. Two absorptions were usually sufficient to remove mouse specific antibodies.

The sera were tested in serial double dilutions up to 1/512 against reticulocyte-rich red cells obtained from mice of the A and NZB strains. The titers varied from 1:4 to 1:8. Because of this, the absorbed sera were again absorbed with an equal volume of packed A and NZB reticulocyte-rich red cells. One absorption was sufficient to remove the anti-reticulocyte–reacting antibody. This was confirmed by repeated tests.

The absorbed goat anti-mouse serum produced many precipitin arcs with normal mouse serum in immunoelectrophoresis. In particular, antibody activity against IgG, IgM, and β_{1C} was demonstrated. The goat anti-mouse gamma globulin absorbed with red cells showed a single band characteristic of IgG when reacted against normal mouse serum in immunoelectrophoretic analysis. This antiserum was also shown to react with L chains derived from mouse IgG. The rabbit anti-mouse β_{1C} was monospecific on immunoelectrophoretic analysis.

These absorbed sera were tested, undiluted, against 2% suspensions of red cells obtained from healthy mice of the A, $C_{57}BL/6$ Ks, C_3H, and NZB strains. The mixtures were incubated at 25° and 37°C for 1 hr and examined for agglutination. None of these antisera produced agglutination.

The Antiglobulin Reaction.—Mouse blood obtained from the tail veins was suspended in 0.9% saline, washed 4 times in 0.9% saline and reconstituted to a 4% erythrocyte suspension. 2 drops of this suspension were centrifuged and the supernatant was discarded. To the button of washed red cells was added 1 drop of either undiluted and absorbed anti-IgG or anti-β_{1C} or undiluted anti-mouse serum absorbed with reticulocyte-rich erythrocytes. (These sera did not show a prozone phenomenon.) The mixtures were then incubated for 5 min at 25°C and centrifuged at 3000 rpm in a serofuge[4] for 15 sec and examined for the presence of agglutination which was verified by microscopic examination.

Preparation of Globulin Fractions.—IgG was prepared from A strain mouse serum by chromatography on DEAE-cellulose using 0.0175 M phosphate buffer, pH 6.3 (20). IgM was obtained from A strain mouse serum by gel filtration on Sephadex G-200 in 0.16 M saline–borate buffer, pH 8.0. The protein fractions were concentrated by pervaporation and tested for purity by microimmunoelectrophoretic analysis (21). The IgG preparation showed no contaminants. The IgM fraction was free of IgG but contained small amounts of transferrin and traces of β_{1C}. Mouse β_{1C}-globulin was obtained from Dr. Müller-Eberhard. IgG, IgM, and β_{1C}-globulin were adjusted by spectrophotometry to contain 12 mg/ml of protein for blocking experiments. Heavy and light chains were isolated from mouse IgG as described by Fleischman et al. (22).

Blocking Experiments.—Erythrocytes from thymectomized A and $C_{57}BL/6$ Ks mice and from 12-month old anemic NZB mice which gave a strong agglutination reaction with goat anti-mouse serum were chosen in a first group of blocking experiments. The titer of the anti-mouse serum with erythrocytes from thymectomized mice was 1:16 and with NZB erythrocytes was 1:512. The anti-β_{1C} gave a titer of 1:32 with NZB erythrocytes. To 0.05 ml of washed 4% erythrocytes was added 0.05 ml of solution of globulin (β_{1C}, IgG, IgM, globulin

[3] Hyland Laboratories, Los Angeles, Calif.

[4] Adams serofuge, Clay-Adams, Inc., New York.

fraction containing the three proteins or light chains obtained from IgG) in concentration of 12 mg/ml of protein. Then 0.05 ml of anti-mouse serum was added. Agglutination was recorded after 5 min incubation at 25°C and centrifugation for 15 sec in a serofuge.

In a second group of experiments, an agglutination inhibition experiment was done using amounts of anti-mouse serum (1:100 dilution in physiologic saline) and anti-mouse gamma globulin (1:100 dilution in physiologic saline) containing 4 hemagglutinating units as determined by testing with erythrocytes obtained from NZB mice. In each of a series of tubes, 0.05 ml of a washed 4% red cell suspension obtained from NZB mice were added with decreasing amounts of IgG or IgM (0.3–0.019 mg), in a total volume of 0.05 ml of saline to 0.05 ml aliquots of diluted anti-mouse or anti-gamma globulin serum. Agglutination was recorded after 5 min incubation at 25°C and centrifugation for 15 sec in a serofuge.

Erythrocyte Survival Studies.—Animals 25–30 days old were tested according to the method of Oliner et al. (23). Radioactive counts were performed in a well scintillation counter on samples obtained at 3, 6, 10, 13, and 17 days following injection of ^{51}Cr-labeled erythrocytes from syngeneic animals. Corrections for background and for body weight changes (during the developmental stage of these determinations we found that correction of body weight changes was more reliable than correction of hematocrit changes) were made and the counts plotted on semilogarithmic paper. The T/2 (half-life) of the labeled erythrocytes was estimated by graphic analysis.

Preparation of Erythrocyte Eluates.—Eluates were prepared from erythrocytes of mice that had a positive antiglobulin reaction by the method of Kochwa and Rosenfield (24). The eluates were concentrated by negative pressure dialysis and the protein concentration estimated spectrophotometrically. They were then studied for their specificity against red blood cells from normal 2-month old A, C_3H, $C_{57}BL/6$, and NZB mice by the antiglobulin reaction using anti-mouse serum.

Bone Marrow and Spleen Imprints.—The histology of the animals in these experiments will be reported elsewhere. Bone marrow smears and spleen imprints were prepared from both normal mice and neonatally thymectomized mice having wasting disease either with or without a positive antiglobulin test. The animals studied were 45–60 days of age. The bone marrow smears were obtained from left iliac bone and the spleen imprints from the upper portion of the spleen (from a fragment approximately 3 mm thick) and were stained with Wright-Giemsa stain. The left femur was fixed in 10% formalin and decalcified before preparing histological sections.

Statistical Evaluation.—Mean values, standard deviations, standard error, and "*t*" tests were calculated by standard methods.

RESULTS

Clinical Findings.—The incidence of the wasting syndrome in neonatally thymectomized A strain mice was 84% (63 of 75 males and 75 of 89 females). Similarly, 77% of neonatally thymectomized $C_{57}BL/6$ Ks mice developed wasting disease (Table I). The period of intensive study for the A mice was from age 25–150 days, and for $C_{57}BL/6$ Ks, 25–60 days. The first signs of disease in either strain were rarely seen before 34 days of age, but usually occurred before 60 days. The animals with postthymectomy syndrome were hunched and had ruffled fur. Diarrhea was present in 18% of A mice and 8% of $C_{57}BL/6$ Ks mice. The animals with wasting reached a maximum weight of 9.9–18.2 g between 28 and 109 days which was followed by 11–36% loss of body weight. Some of these mice (19%) never grew normally and had (from the earliest period of observation) a deficient weight until sacrifice or death. Their maximum

body weight varied from 8.1 to 10.8 g. Of the 138 neonatally thymectomized mice of the A strain showing wasting disease, 76 died spontaneously and 62 were sacrificed before death. 26 neonatally thymectomized mice appeared healthy at the end of the observation period (14 females and 12 males). In the C_{57}BL/6 Ks strain, 12 of 17 male and 15 of 18 females acquired the wasting syndrome, an over-all incidence of 77% (27/35). Of these 35 mice, 18 died spontaneously before 60 days of age and 17 were sacrificed at 60 days of age (7 males and 10 females). Nine of the latter group had wasting disease and eight appeared normal.

Antiglobulin Reaction.—The incidence of positive antiglobulin reactions (goat

TABLE I

Incidence of Positive Antiglobulin Reaction and Wasting Syndrome in Neonatally Thymectomized Mice of Different Strains

Strain	Wasting syndrome	Positive antiglobulin	
		Neonatal thymectomy	Wasting syndrome
	%	%	%
A	84.1 (138/164)	60.7 (68/112)	71.2 (62/87)
C_{57}BL/6 Ks	77.1 (27/35)	80.0 (8/10)	100.0 (7/7)
C_3H	98.5 (196/199)	9.4 (8/85)	9.4 (8/85)
($C_3H \times A$) F_1	65.3 (32/49)	—	55.5 (5/9)
C_{57}BL/1	69.2 (27/39)	—	9.0 (1/11)

anti-mouse serum) in neonatally thymectomized mice with wasting was 71% in strain A (62 of 87) and 100% in C_{57}BL/6 Ks (7 of 7). Eight of eight anemic NZB mice of both sexes gave positive reactions at 1 yr of age. 25 neonatally thymectomized A mice without signs of wasting were sacrificed at 120 or 150 days of age. None had positive antiglobulin reactions. However, six of these 25 mice had given positive antiglobulin reactions between the ages of 30 or 65 days but they had become negative when tested at 80 and 120 days. Normal mice (35 A, 19 C_{57}BL/6 Ks, and 12 NZB) of 30 and 60 days of age were tested and gave negative antiglobulin reactions. Positive antiglobulin tests were also observed in neonatally thymectomized C_3H, C_{57}BL/1, and (C_3HxA)F1 mice. The incidence of positive antiglobulin tests, however, was much lower in the C_3H and C_{57}BL/1 strains than in the A or C_{57}BL/6 Ks strains. However, the incidence of positive antiglobulin reactions in (C_3HxA)F_1 hybrid mice was higher than in C_3H mice and approached the incidence observed in the A mice. These results are summarized in Table I.

Table II summarizes the age and sex distribution of positive antiglobulin tests in neonatally thymectomized A mice having wasting disease. The antiglobulin reactions recorded in the table are the results of the last tests made on

the animals. The largest group of animals studied in this experiment were between 50–60 days of age because of the large number of neonatally thymectomized animals having wasting disease in that age group.

Hematocrit Levels and Antiglobulin Reactions.—The data in Tables III and IV

TABLE II

Age and Sex Distribution of Neonatally Thymectomized A Mice with Wasting Disease and Positive Antiglobulin Reaction

	Age in days of mice dying or sacrificed with wasting disease					Total	%
	30–40	40–50	50–60	60–70	70–100		
Female	2/4*	10/13	21/26	2/4	1/3	36/50	72
Male	4/6	6/7	14/19	1/2	1/3	26/37	70.2
Total	6/10	16/20	35/45	3/6	2/6	62/87	71.2

* Number of positive over number of mice tested for antiglobulin reaction.

TABLE III

Hematocrit and Antiglobulin Reaction in Normal and Neonatally Thymectomized "A" Strain of Mice

	Hematocrit % 30–60 days age	*P**	No. mice with anemia
Normal (72)‡	45.12 ± 1.62	—	0
Neonatal thymectomy (68):		7800	
Group I§ (45)	35.47 ± 5.21	<0.001	41
Group II‖ (15)	42.33 ± 4.05	N.S.¶	7
Group III** (8)	43.60 ± 3.07	N.S.	2

Nos. in parenthesis = Nos. of mice in each group.

* Thymectomized compared to normal.

‡ 24 intact mice of 60 days and 11 of 30 days of age were tested with anti-mouse serum and all gave negative results.

§ Wasting disease, positive antiglobulin reaction.

‖ Wasting disease, negative antiglobulin reaction.

¶ Not significant.

** Without wasting disease negative antiglobulin reaction. Six of these mice had positive tests until 65 days of age.

show that a significant degree of anemia was present among neonatally thymectomized wasting A and $C_{57}BL/6$ Ks mice with positive antiglobulin reactions. 41 of 45 A mice with wasting disease showed hematocrits which were lower than normal. 7 mice had severe, 29 moderate and 5 mild anemia. Anemia of a significant degree was not found in mice with negative Coombs' tests. Comparable

results were obtained in eight mice of NZB strain at 12 months of age giving a 35.2% ± 3.3[5] hematocrit as compared to healthy 2-month old NZB mice with hematocrit of 45.0% ± 2.96.

Reticulocyte Levels in Normal and Neonatally Thymectomized A Mice.—Table V summarizes the results of reticulocyte determinations of normal A mice

TABLE IV

Hematocrit and Antiglobulin Reactions in Normal and Neonatally Thymectomized "$C_{57}BL/6$ Ks" Strain of Mice

	Hematocrit % (60 days)	*P*	No. mice with anemia
Normal (19)*	46.72 ± 2.42	—	0
Neonatal thymectomy (17)			
Group I‡ (9)	34.52 ± 4.55	<0.001	9
Group II§ (8)	44.39 ± 2.59	N.S.¶	2

Nos. in parentheses = Nos. of mice in each group.
* 12 normal mice gave negative red cell antiglobulin reaction.
‡ Wasting disease, positive antiglobulin test in 7 of 7 animals tested.
§ Without wasting, one out of 3 mice tested gave positive antiglobulin reaction.
¶ Not significant.

TABLE V

Reticulocyte Count in Normal and Neonatally Thymectomized A Mice

Condition	Antiglobulin test	No. of mice	Reticulocyte % ± SD (30-60 days of age)	*P* value
Normal	—	32	3.08 ± 0.524	—
Neonatal thymectomy	Positive	26	2.80 ± 1.63	N. S.
	Negative	7	2.84 ± 1.17	N. S.

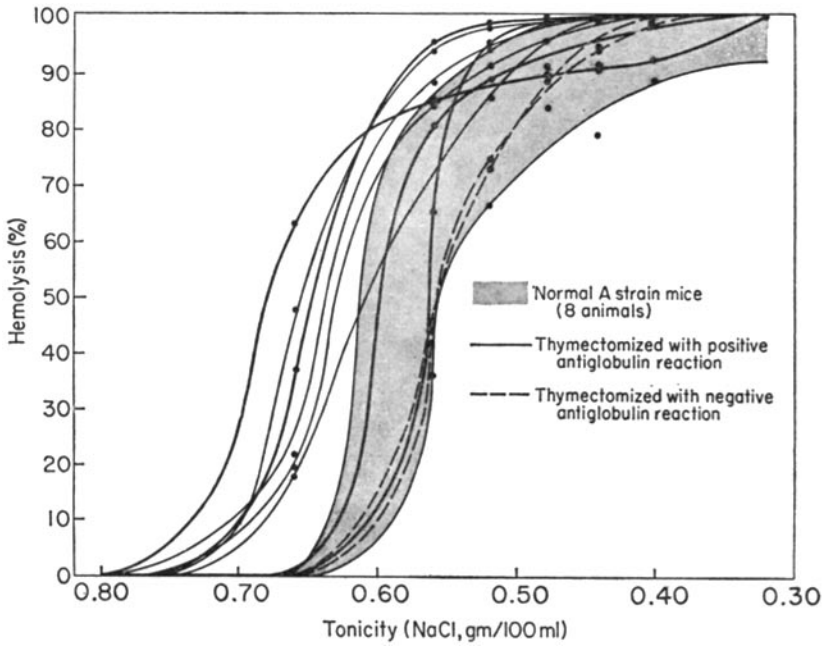
N.S., not significant.

and two groups of neonatally thymectomized A mice with positive or negative antiglobulin tests. No significant differences are seen. Reticulocytosis was not observed among the two groups of neonatally thymectomized A mice. By contrast, the eight anemic NZB mice studied showed reticulocyte values ranging from 9 to 72% (35.2 ± 10.32) as compared to 12 normal NZB mice of 2 months of age (3.38 ± 1.12).

Red Cell Fragility Studies.—Text-fig. 1 shows the fragility studies of neonatally thymectomized A mice with positive and negative Coombs' test and of

[5] Standard deviation from the mean. All subsequent ± figures indicate one standard deviation from the mean.

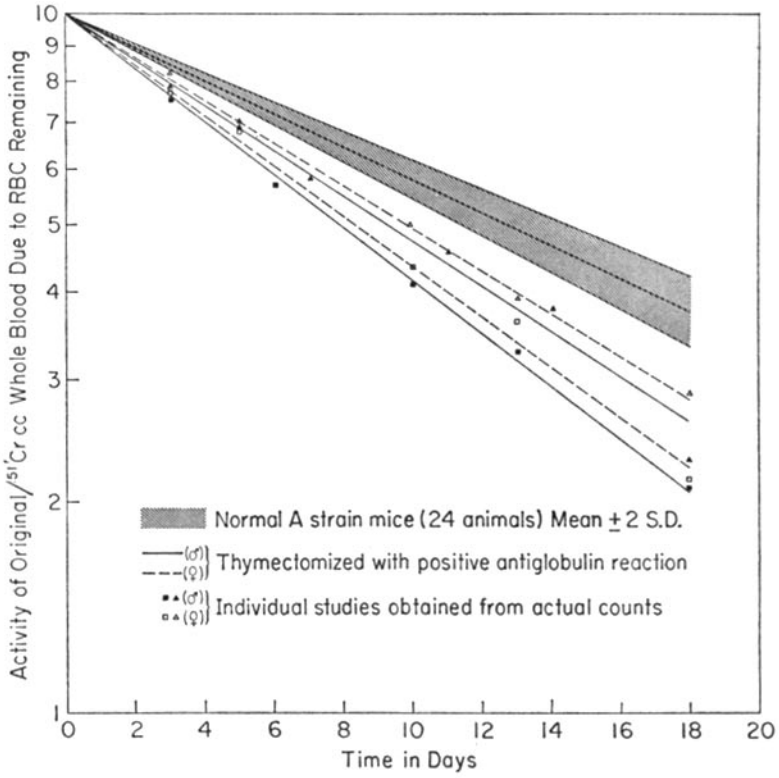
normal 2-month old A mice. Studies from eight normal A mice (four males and four females) comprise the shaded area. 50% hemolysis of red cells occurred when the NaCl concentration ranged from 0.62 to 0.56%. No red cells of normal mice were lysed when the concentration of the NaCl solution was higher than 0.86%. By contrast, six of eight neonatally thymectomized A mice with positive antiglobulin test had red blood cells that were fragile in hypotonic NaCl solution. Two mice with negative Coombs' test had normal red cell fragility.



TEXT-FIG. 1. Osmotic fragility of red cells from normal and neonatally thymectomized A strain mice. The shaded area comprises eight normal animals (4 males and 4 females).

^{51}Cr Red Cell Survival Studies on Normal and Neonatally Thymectomized A Mice.—Text-fig. 2 shows ^{51}Cr red cell survival studies in 24 normal 2-month old A mice (12 males and 12 females). The mean survival is 12.98 $\pm$ 0.78 days. Also shown are representative ^{51}Cr red cell survival times in four neonatally thymectomized mice in which erythrocytes a positive antiglobulin reaction was detected. All four animals showed a decreased erythrocyte survival. Data on ^{51}Cr red cell survival in neonatally thymectomized A mice are summarized in Table VI. The mean red cell survival in 14 neonatally thymectomized A mice was 10 days. Nine of the mice with decreased red cell survival died spontaneously between 45 and 119 days of age. The remaining five were living at 120

days; three clinically normal and two wasting. The neonatally thymectomized A mice that had a negative antiglobulin test were found to have a normal ^{51}Cr red cell survival. A significant difference in the ^{51}Cr red cell survival data ($P = <0.001$) was found when results in 24 normal A mice and 14 neonatally thymectomized A mice with positive antiglobulin reactions were compared.



TEXT-FIG. 2. Chromium 51-labeled red blood cell survival study in normal and neonatally thymectomized A strain mice. The shaded area was obtained from the mean ± 2 SD of 24 normal animals. Four representative individual studies are shown.

Specificity of Eluates from Red Cells with Positive Antiglobulin Reaction.— Eluates prepared from the red blood cells of A, C_{57}BL/6 Ks, and NZB mice with positive antiglobulin reactions were tested against normal red cells from A, C_{57}BL/6 Ks, NZB, and C_3H mice and also against human red cells. All eluates tested revealed specificity for mouse red cells by Coombs testing with rabbit anti-mouse serum. None reacted with human red cells.

Characterization of the Protein "Coating" the Erythrocytes of Neonatally Thymectomized A, C_{57}BL/6 Ks, and NZB Mice (12 months old) Having Positive Anti-

globulin Reactions.—Table VII shows the results of Coombs' reactions of several mice of A, $C_{57}BL/6$ Ks, and NZB strains. The red cells of neonatally thymectomized A, and $C_{57}BL/6$ Ks mice gave negative reactions with goat anti-mouse IgG and rabbit anti-mouse β_{1C}. In contrast, the erythrocytes of anemic

TABLE VI

^{51}Cr Red Cell Survival Studies in Normal and Neonatally Thymectomized A Mice

Condition	Antiglobulin reaction	T/2 (days ± SD)	Spontaneous death following wasting (age in days)	Date and condition when sacrificed (negative antiglobulin reaction)
Neonatally thymectomized	Positive*	7.8	55	
		8.4‡	45	
		8.7‡	48	
		9.3	52	
		9.9	119	
		10.2	57	
		10.3	52	
		10.6	70	
		11.5‡	82	
		9.1		120 (Normal)
		9.3		120 (Normal)
		10.5		120 (Normal)
		11.0		120 (Wasting)
		13.4		120 (Wasting)
		10.00 ± 1.42		
		$P = 0.001$		
	Negative	13.7	53	
		13.7	57	
		13.1		120 (Normal)
Normal	Negative	12.98 ± .78§	—	—

* All mice gave a positive antiglobulin reaction at 20–25 days and 30–35 days. The ^{51}Cr study was started at 30–35 days of age. Samples were obtained at days 3, 6, 10, 13, and 17. At day 10 after beginning survival study and at end of study these animals gave positive antiglobulin reaction.

‡ Mice whose ^{51}Cr study was calculated with 3 or 4 samples only.

§ This group included 24 healthy A strain mice from 35 days of age at start of experiment.

NZB mice gave positive reactions with antisera containing specificities for β_{1C}, IgG, and IgM. The positive reactions with anti-mouse serum were not due to transferrin antibodies since activity persisted after absorption of the antiserum with reticulocyte-rich blood.

The nature of the globulin coating was clarified by blocking experiments. In the tests involving A or $C_{57}BL/6$ Ks cells, only IgM produced blocking. Since

only anti-mouse serum that contains antibodies directed against IgM heavy chains agglutinates cells and antiserum that reacts with IgG or β_{1C} but lacks

TABLE VII

Characterization of Globulin Coat of Erythrocytes of Neonatally Thymectomized A and $C_{57}BL/6$ Ks and Nonthymectomized NZB Mice

Antiserum	Blocking agent	Strain of test red cells												
		A						$C_{57}BL/6$ Ks				NZB		
Anti-IgG	—	—*	—	—	—	—	—	—	—	ND‡	ND	4+	4+	4+
Anti-β_{1C}	—	—	—	—	—	—	—	—	—	ND	ND	2+	2+	3+
Anti-mouse serum (abs. with reticulocytes)	—	4+	4+	3+	3+	2+	2+	4+	3+	2+	1+	3+	4+	4+
Anti-mouse serum	β_{1C}	4+	3+	3+	3+	ND	ND	3+	3+	ND	ND	4+	4+	4+
" "	IgG	3+	3+	2+	2+	ND	ND	3+	1+	ND	ND	2+	1+	1+
" "	IgM	—	—	—	—	ND	ND	—	—	ND	ND	+	+	+
" "	IgG, IgM, and β_{1C}	—	—	—	—	ND	ND	—	—	ND	ND	—	—	—
" "	L chains (IgG)	+	+	ND	ND	ND	ND	ND	ND	ND	ND	+	+	ND
Anti-IgG	L chains (IgG)	—	—	ND	ND	ND	ND	ND	ND	ND	ND	—	—	ND

* Negative agglutination reaction.

‡ Not done.

TABLE VIII

Blocking of Antiglobulin Reaction of Erythrocytes of NZB Mice by IgG and IgM

Antis rum		Mg of protein added (IgM or IgG)						
		0.6	0.3	0.15	0.075	0.038	0.019	0 (saline)
0.05 ml anti-mouse serum containing 4 hemagglutinating units	Agglutination of red cells after blocking with IgM	—*	—	—	—	+	2+	2+
	Agglutination of red cells after blocking with IgG	+	+	+	+	2+	2+	2+
0.05 ml anti-mouse IgG containing 4 hemagglutinating units	Agglutination of red cells after blocking with IgM	—	—	—	±	2+	3+	3+
	Agglutination of red cells after blocking with IgG	—	—	—	±	2+	3+	3+

* Negative agglutination reaction.

specific IgM antibodies does not result in agglutination, it seems clear that the globulin coat on A or $C_{57}BL/6$ Ks is IgM alone. Since anti-mouse serum containing specificities for IgG, IgM, and β_{1C}, as well as antiserum directed only against IgG or β_{1C}, produces agglutination, it would appear that the NZB eryth-

rocytes possess on their surfaces IgG, IgM, and β_{1C}. That inhibition of the multispecific antiserum is obtained when all three inhibiting agents (IgG, IgM, and β_{1C}) are used supports this notion. However, when a specific antiserum directed against IgG is employed, complete inhibition is obtained by prior treatment with L chains or IgM (Tables VII, VIII). Thus, the agglutination with anti-IgG is due to the known cross-reaction of IgG and IgM because of common

TABLE IX

Bone Marrow and Spleen Imprint Differential Counts of Normal A Mice at 2 Months of Age (500 Cells Counted)

Megakaryocytes can be found easily							
Hematocrit, %	45.4	42.8	46.2	47.4	44.6	42.2	46.0
Reticulocyte, %	1.2	3.8	4.3	1.7	2.0	4.5	3.4
Bone marrow							
Normoblasts, %	27.0	21.0	26.0	23.8	28.0	22.8	32.0
Stem cells, %	0.6	1.2	1.7	1.6	3.2	2.4	2.2
Myeloid, %	40.4	54.6	46.3	33.6	42.8	18.6	32.6
Lymphocytes, %	30.6	22.0	24.3	39.2	20.0	52.6	29.0
Monocytes, %	1.2	0.8	1.7	1.4	0.2	0.4	2.8
Eosinophiles, %	0.2	0.4	Rare	0.4	0.8	3.2	1.2
Plasma cells	Rare	Rare	Rare	Rare	Rare	Rare	0.2
Megakaryocytes can be found							
Spleen imprint							
Normoblasts, %	5.0	8.8	5.8	2.4	15.2	14.2	10.2
Stem cells, %	0.4	0.8	0.0	0.2	0.0	0.4	0.2
Myeloid, %	2.2	5.2	2.2	2.2	2.6	6.2	3.4
Lymphocytes, %	90.0	83.2	90.8	92.8	78.0	76.2	84.0
Monocytes, %	2.4	1.8	1.2	1.0	4.2	2.8	1.6
Eosinophiles, %	Rare	Rare	Rare	Rare	Rare	Rare	Rare
Plasma cells, %	Rare	0.2	Rare	1.4	Rare	0.2	0.6

L chain determinants and not the presence of IgG molecules in the coating. The antiglobulin reaction of the NZB erythrocytes is therefore due to β_{1C} and IgM. It should be noted that apparently the cross-reaction does not involve the IgM coating of A and C_{57}BL/6 Ks erythrocytes. This point will be considered later.

The amount of protein required to neturalize 0.05 ml of anti-mouse serum (tested with 0.05 ml of 4% red cells from A strain mice which gave a 4+ agglutination) was 0.03 mg of IgM preparation. Blocking is obtained in the NZB system with approximately 0.075 mg of IgM or IgG (Table VIII).

Microscopic Findings

Bone Marrow.—The bone marrow of neonatally thymectomized A mice in hematoxylin-eosin sections appeared normal in cellularity. The megakaryocytes

also appeared to be normal in number and morphology. Tables IX, X, and XI show the bone marrow differentials performed on smears stained by Wright-Giemsa of normal 60-day old A mice and neonatally thymectomized A mice with positive and negative antiglobulin tests. The corresponding individual hematocrit and reticulocyte counts are also shown. As pointed out above, no difference between the reticulocyte values of the three groups was observed. However, normoblast and lymphocyte percentages in the bone marrow in neonatally thymectomized A mice are decreased when compared to the normal

TABLE X

Bone Marrow and Spleen Imprint Differential Counts of Neonatally Thymectomized A Mice (Positive Antiglobulin Reaction Group)

	Megakaryocytes can be found easily												
Hematocrit, %.....	28.0	31.0	28.0	32.0	38.0	35.5	22.0	34.0	44.0	38.5	26.0	33.0	41
Reticulocyte, %.....	2.9	0.7		2.8		2.3	4.1		4.9	0.5		6.2	5.4
Bone marrow													
Normoblasts, %....	0.8	2.2	4.6	5.0	7.8	9.0	10.0	12.8	12.8	14.0	24.0	30.8	35.2
Stem cells, %.....	1.2	2.4	1.6	2.2	4.8	3.8	4.8	2.0	3.2	6.2	3.8	7.0	0.4
Myeloid, %.....	91.8	86.0	79.8	78.2	78.0	72.0	69.2	73.0	73.6	64.0	54.0	48.4	33.4
Lymphocytes, %...	4.0	7.4	12.2	11.8	6.0	10.0	12.8	9.8	9.2	12.0	16.0	8.2	22.2
Monocytes, %.....	2.0	2.0	0.8	2.8	2.2	1.2	2.2	2.0	1.0	0.8	2.0	2.4	4.0
Eosinophiles, %....	0.2	0.0	1.0	0.0	1.2	4.0	1.0	0.4	0.2	3.0	0.2	3.2	4.8
Plasma cells, %....	Rare	Rare	—		Rare	Rare	Rare	Rare	Rare	Rare	Rare	Rare	Rare
	Megakaryocytes can be found												
Spleen imprint													
Normoblasts, %....		61.0		10.4	42.6	41.4	21.0	25.6		20.0	41.0	40.4	51.0
Stem cells, %.....		2.6		0.6	1.6	3.0	3.6	1.4		4.6	0.4	0.4	1.0
Myeloid, %.....		27.4		4.6	7.4	10.6	14.6	14.4		17.0	11.0	10.2	24.6
Lymphocytes, %...		7.2		82.0	45.6	44.0	59.4	56.0		54.4	46.0	47.0	18.4
Monocytes, %.....		0.6		2.4	2.4	0.6	1.4	1.6		3.4	1.6	2.0	3.6
Eosinophiles, %....		0.8		0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	1.4
Plasma cells, %....		0.4		0.0	0.4	0.4	Rare	1.0		0.6	Rare	Rare	0.0

controls. In 15/20 anemic mice a depression of normoblasts was observed in association with normal reticulocyte counts. Bone marrow smears of normal mice (Fig. 1) and of neonatally thymectomized A mice (Fig. 2) show a deficiency of normoblasts and lymphocytes in the thymectomized animals.

Spleen Imprints.—Tables IX, X, and XI also summarize data from differential counts of spleen imprints in normal neonatally thymectomized A mice. Most cells are lymphocytes, but myeloid and erythroid elements are relatively frequent. Plasma cells are rare. The spleen imprints of neonatally thymectomized A mice by contrast show a large proportion of normoblasts and myeloid elements. These relationships are illustrated in Figs. 3 and 4.

DISCUSSION

The findings presented here are of interest from several points of view. They are in agreement with the observations of Sutherland et al. (14) and Kellum

et al. (15) that rabbits subjected to neonatal extirpation of "central lymphoid tissue" are unusually prone to develop Coombs positivity of the red blood cells. Similar are the findings of de Vries et al. (13) who discovered that neonatally thymectomized mice develop wasting disease, anemia, and lupus erythematosus-like phenomena. These investigators, indeed, have attributed the wasting of neonatally thymectomized mice to the development of autoimmune disorders.

TABLE XI

Bone Marrow and Spleen Imprint Differential Counts of Neonatally Thymectomized A Mice (Negative Antiglobulin Reaction Group)

Megakaryocytes can be found easily							
Hematocrit, %	39.5	39.5	44.9	38.5	40.0	38.2	46.0
Reticulocyte, %	2.8		3.4		4.9	1.6	2.1
Bone marrow							
Normoblasts, %	0.8	2.8	3.8	10.8	11.2	18.2	20.4
Stem cells, %	3.6	4.0	4.8	3.8	3.0	1.4	2.6
Myeloid, %	82.4	74.2	76.0	68.0	67.6	65.0	52.4
Lymphocytes, %	2.2	15.8	12.2	7.4	11.0	10.2	20.0
Monocytes, %	1.8	2.2	2.2	4.8	6.2	4.8	3.2
Eosinophiles, %	9.2	1.0	1.0	5.2	1.0	0.4	1.4
Plasma cells, %	Rare	Rare	Rare	Rare	Rare	Rare	—
Megakaryocytes can be found							
Spleen imprint							
Normoblasts, %	43.4		27.0		24.6	38.6	8.4
Stem cells, %	4.4		1.2		1.0	0.4	2.0
Myeloid, %	24.0		17.2		29.4	14.6	6.6
Lymphocytes, %	9.0		52.0		43.4	45.0	82.4
Monocytes, %	3.0		2.2		1.0	1.4	0.6
Eosinophiles, %	16.2		Rare		0.6	Rare	0.0
Plasma cells, %	Rare		0.4		Rare	Rare	Rare

In the studies reported here, it has been demonstrated that an antiglobulin positive anemia occurs in high frequency in the A strain and C_{57}BL/6 Ks strains of mice. The Coombs positivity and anemia were correlated with the wasting syndrome in our neonatally thymectomized mice, but these findings were not limited to the wasting animals. Coombs-positive anemia was also observed in neonatally thymectomized C_3H and C_{57}BL/1 mice. However, the incidence of this consequence of neonatal thymectomy was much lower in such animals than in the mice of the A and C_{57}BL/6 Ks strains. Hybrids of C_3H and A strain mice thymectomized in the neonatal period showed an incidence of Coombs-positive

anemia approximately equal to that occurring in neonatally thymectomized A strain animals. In these studies, no sex differences were observed.

The finding of a large proportion of neonatally thymectomized mice with anemia and positive antiglobulin reaction suggests the existence of autoimmune disorders in these mice. Evidence of a hemolytic process is provided by a decreased ^{51}Cr red cell survival and increase of red cell fragility to hypotonic solutions in some neonatally thymectomized mice with positive antiglobulin reaction.

These findings are also similar to those described in immunologically induced runt disease by Oliner et al. (23) and by Helyer et al. (8, 9) in NZB mice. A large proportion of neonatally thymectomized mice of A and $C_{57}BL/6$ strains exhibited excessive extramedullary hematopoiesis in the spleen. The bone marrow showed myeloid hyperplasia and normoblastic and lymphoid hypoplasia. Similar observations have been reported as occurring in graft-versus-host reactions (25). Thus, erythroid hypoplasia of the bone marrow, perhaps associated with an immunological assault similar to that occurring on the peripheral erythrocytes, may result in a shift of erythropoiesis from the bone marrow to the spleen. Even though increased erythropoiesis is observed in spleen, the development of anemia suggests suppression of compensatory erythropoietic capacities in these animals. Two factors seem to be important in the anemia of neonatally thymectomized mice which have a positive Coombs' test: (*a*) increase of red cell destruction as demonstrated with decreased ^{51}Cr red cell survival and increase of the red cell fragility to hypotonic solutions, and (*b*) a decrease of red cell production as demonstrated by bone marrow hypoplasia and absence of reticulocyte response. The same factors are also involved in the production of anemia in homologous disease. Evidence of hemolysis in homologous disease was shown by Oliner et al. (23), normoblastic hypoplasia of the marrow by Bain (25), and lack of reticulocyte response together with normoblastic hypoplasia by Cornelius et al.[6] Decrease of red cell production in some strains of neonatally thymectomized mice and in graft-versus-host reactions may be explained by immunological reactivity against hemopoietic tissue. Recently, two patients with erythroblastopenia (red cell aplasia with thymoma and one with acute erythroblastopenia) were described in which an erythropoietic inhibitor was found in their plasma. It was suggested that the inhibitory effect of these plasmas might be due to the formation of antibody directed against erythrocyte stimulating factor or erythropoietin (26). The shift of erythropoiesis from bone marrow to the spleen and the lack of reticulocyte response was present in both groups of neonatally thymectomized mice either

[6] Cornelius, E., E. Yunis, and C. Martinez. Depression of Erythrocyte Maturation as a Result of the Graft-versus-host Reaction. Unpublished experiments.

with positive or negative antiglobulin reaction. These results suggest that some factors other than a direct attack of antibody against red blood cell precursors may be operating. Such factors could include antibody directed against erythropoietin, or erythropoietin deficiency as a direct result of thymic extirpation. In contrast to the findings in the thymectomized animals, the Coombs-positive hemolytic anemia of NZB mice is associated with reticulocytosis (9). The mechanisms of the anemia in the two conditions thus seem to result from differing causes.

The autoantibodies eluted from anemic NZB mice or from neonatally thymectomized A and C_{57}BL/6 Ks with wasting disease show no strain specificity but are specific for mouse erythrocytes. In both groups of diseased animals, the immunoglobulin attached to the red cells is IgM and not IgG; of interest is the finding that β_{1C} can be detected on the red cells of NZB mice having positive antiglobulin reactions. However, β_{1C} is not present on the red cells from neonatally thymectomized mice.

Our data seem clearly to show, in agreement with Mellors (27), that the immunoglobulin on the red blood cells of NZB mice is IgM and not IgG. Perhaps the contrary conclusions of Norins and Holmes (28) can be explained on the basis of cross-reactivity of light chain antigenic groups of IgM and IgG. Further, it has also been shown by East, de Sousa, and Parrott (29) that elevated macroglobulin levels are found in the serum of NZB mice affected with autoimmune hemolytic anemia.

Although IgM was present on the cells from both thymectomized animals as well as on the erythrocytes of NZB mice, a positive Coombs reaction was elicited with anti-L chain antibodies only in the NZB system. The reason for this variation is unclear but may be the absence of sufficient L chain determinants in the IgM coat or the unavailability of the L chain antigenic groups in the case of cells from the thymectomized mice. It has been observed that L chain groups are not available for antigenic reaction in γA-myelomas (30). It is also possible that the reaction with complement components in the case of the NZB cells may have resulted in configurational changes which exposed L chain determinants. Finally, it has been shown that hemagglutinating antibodies have limited heterogenicity and a tendency toward monoclonality (31). It is, therefore, possible that the IgM population comprising the antibody covering was deficient in antigenic determinants capable of reacting with the antibodies present in the anti-IgG antiserum. This difference in reactivity of the IgM coatings, considered with the observation that β_{1C} is present only on the NZB erythrocyte, suggests a difference in mechanisms that results in IgM deposition on the erythrocytes. One wonders if the presence of the complement component is more suggestive of an immune mechanism in the NZB mice while the coating of erythrocytes with IgM globulin in animals could be due to other causes. It

could be that certain other IgM antibodies besides rheumatoid factor do not fix complement.

How do the observations recorded here reflect on the pathogenicity of autoimmune diseases? It seems entirely possible that the imbalance of the immunological apparatus produced by neonatal thymectomy interferes with the self-recognition processes as we (14–16) and de Vries et al. (13) have suggested earlier. Such an autoimmune pathogenicity could then contribute to the anemia, disturbed histopathology, and the wasting syndrome itself. Thus, as in parabiosis intoxication and in homologous disease (32), the immunological assault, here on the host itself, may be basic.

However, equally attractive seems the possibility that in the circumstances described here, and even in the NZB mice, deficiencies of thymic function could foster invasion by microorganisms or activation of infections not yet defined. In this respect, a filtrable agent, derived from NZB malignant lymphoma, has recently been reported to induce an earlier appearance of glomerulonephritis when injected into preweanling NZB mice (33). Virus-like particles were identified by electron microscopy in old NZB mouse tissues and cells, including malignant lymphoma cells (33). These infections then could induce the production of cross-reacting antibodies which either assault the host tissues directly or operate through antigen antibody complexes to produce disease.

SUMMARY

1. Mice of A and C_{57}BL/6 Ks strains, thymectomized at birth acquire wasting disease in 84.1% (A) and 77.1% (C_{57}BL/6 Ks) of the cases. There is no sex predelection.

2. Anemia in these animals is characterized by shortened red cell survival and increased fragility to hypotonic salt solutions. Among thymectomized A mice reticulocytosis is absent and extramedullary hematopoiesis is found in the spleen in the presence of bone marrow hypoplasia for the erythroid and lymphocyte series.

3. Positive antiglobulin tests of the red cells were observed in all the thymectomized C_{57}BL/6 Ks (7/7) and 71.2% of the A strains (62/87). Normal mice do not show positive Coombs' tests.

4. The globulin coat on the A strain consists of IgM, whereas β_{1C} and IgG are not detectable. By contrast, red cell coats of NZB mice developing spontaneous autoimmune hemolytic anemia show IgM and β_{1C}, but these erythrocytes do not react with anti-gamma chain antibodies. Another difference in the globulin coats of the two types of erythrocytes is that the IgM on NZB red cells has available light chain determinants but these are apparently hidden or absent in the case of sensitized erythrocytes. The difference in antibody coating,

association with a component of complement in one but not the other, suggests a different mechanism for the immune surface phenomenon in each instance.

5. Anemia in NZB mice is associated with reticulocytosis while that in thymectomized A mice is not.

6. Thymectomy appears to initiate a chain of events leading to a series of autoimmune phenomena which may be due to alteration in host response consequent to loss of thymic tissue and thymic dependent functions or alternatively to infection to which increased susceptibility exists as a result of thymic extirpation.

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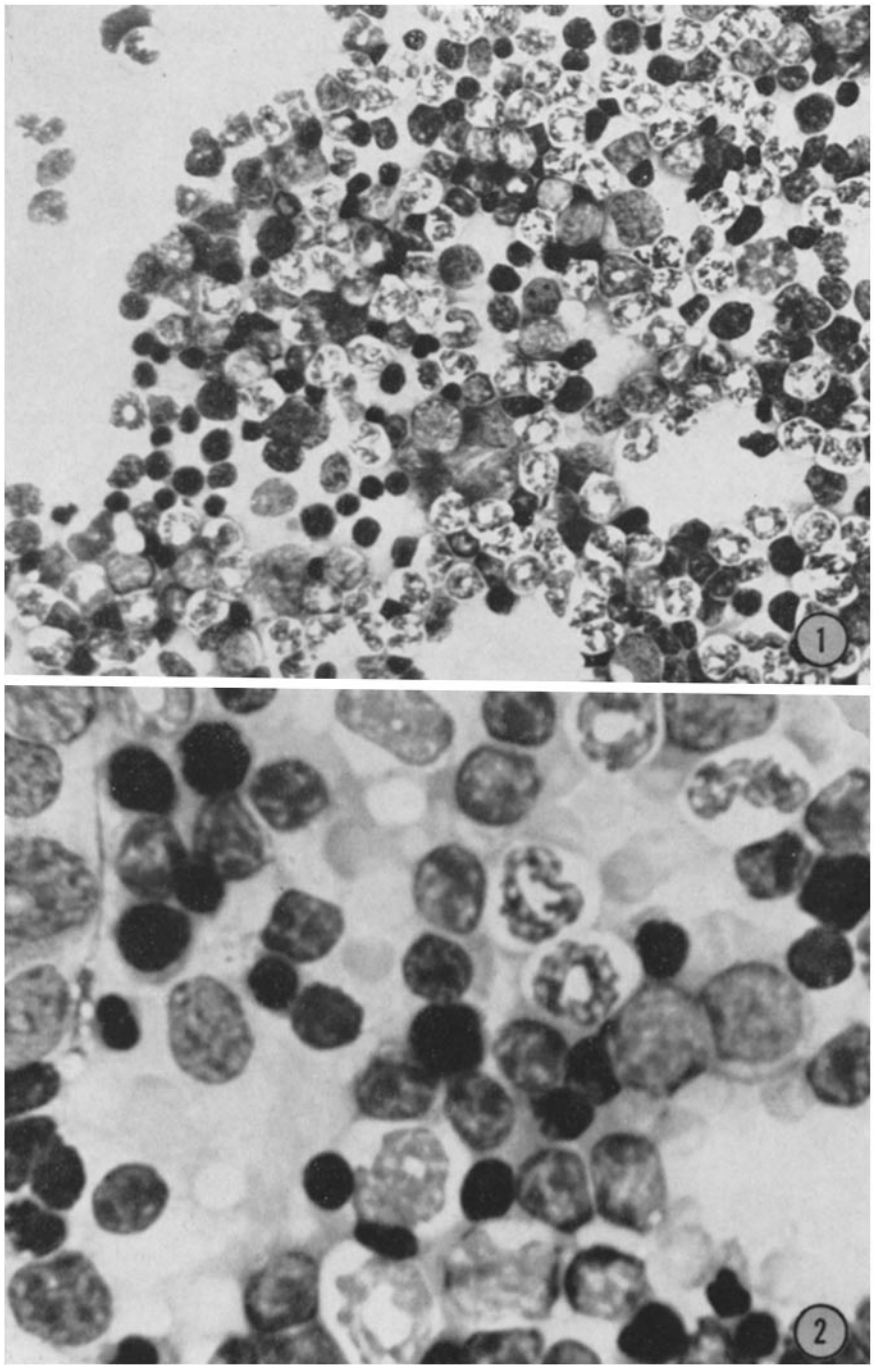
EXPLANATION OF PLATES

PLATE 100

Fig. 1. Wright-Giemsa stain of normal iliac bone marrow smear from 2-month old A strain mouse. There is normal proportion of erythroid, myeloid, and lymphoid elements. ×500.

Fig. 2. High power view of a portion of Fig. 1. ×1000 (oil immersion).

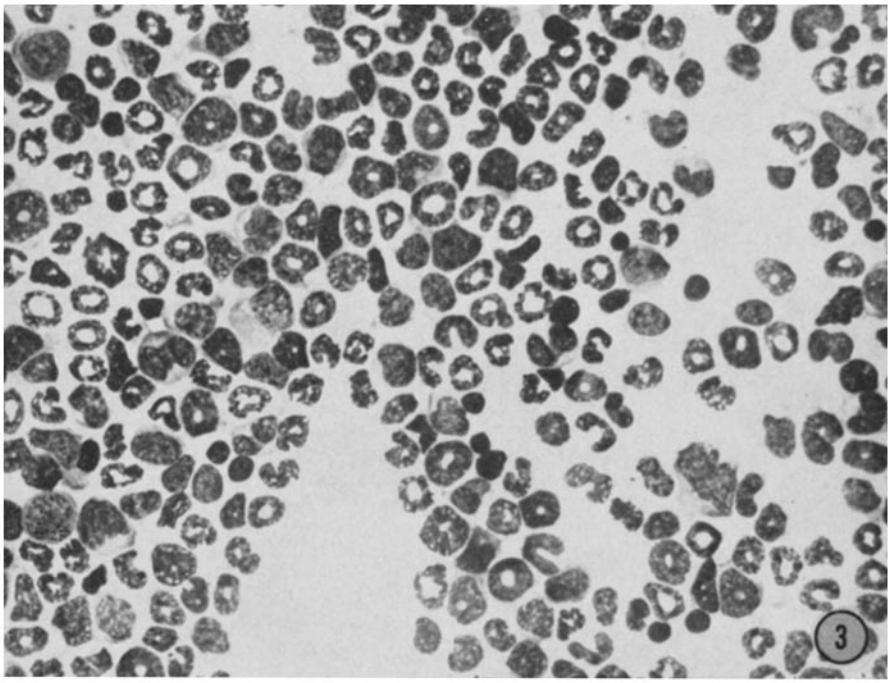


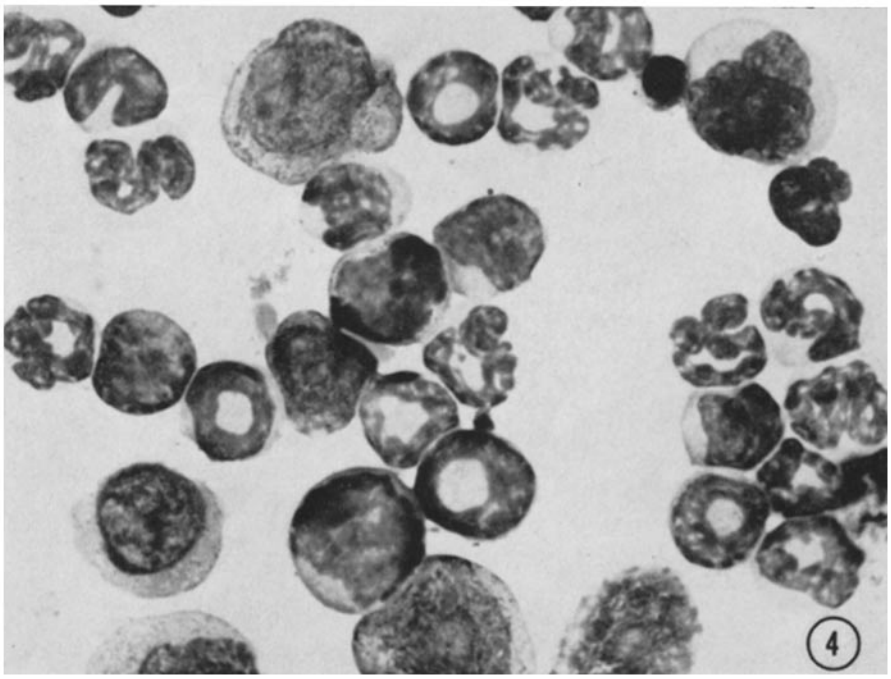
(Yunis et al.: Postthymectomy wasting and autoimmune phenomena)

PLATE 101

Fig. 3. Wright-Giemsa stain of iliac bone marrow smear from 56 day old neonatally thymectomized A strain mouse with wasting disease, positive Coombs' test and anemia. Note the large number of myeloid cells and a decrease of erythroid and lymphoid elements. ×500.

Fig. 4. High power view from the same preparation from which Fig. 3 was taken. ×1000 (oil immersion).



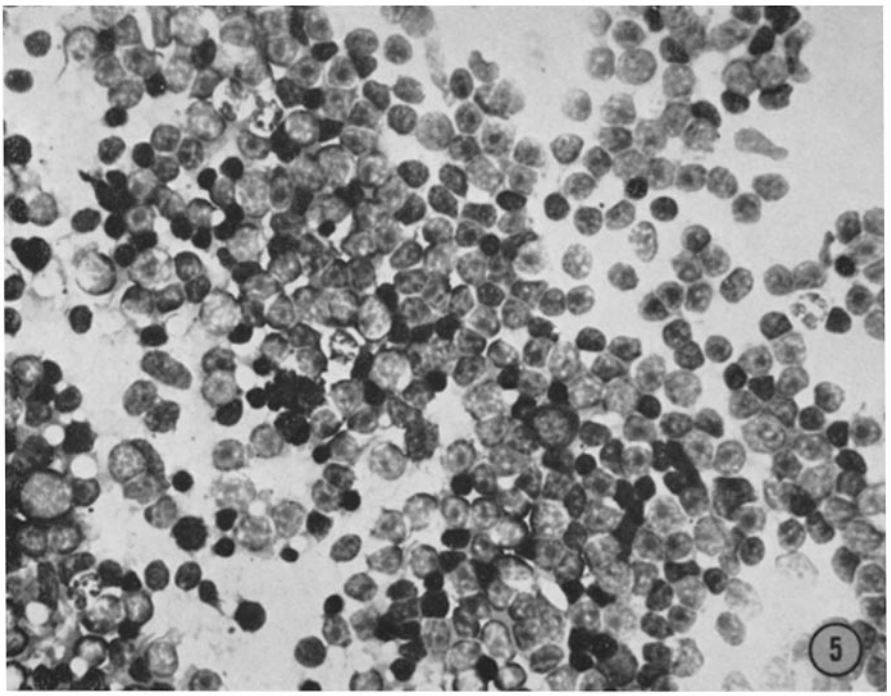


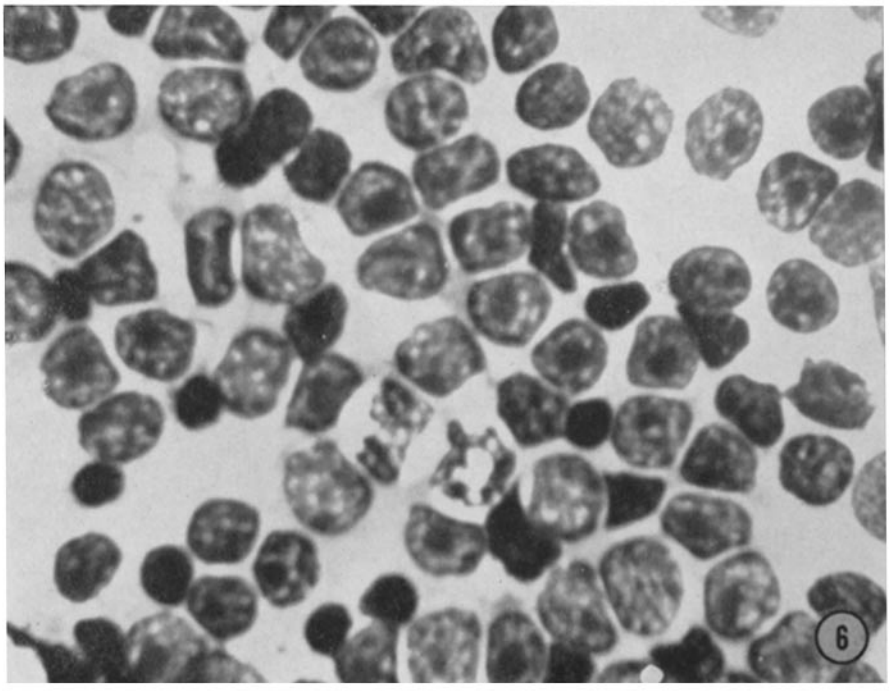
(Yunis et al.: Postthymectomy wasting and autoimmune phenomena)

PLATE 102

Fig. 5. Wright-Giemsa stain of spleen imprint from 2 month old A strain mouse. Note the large proportion of lymphocytes and the rare myeloid and erythroid elements. ×500.

Fig. 6. High power view of a portion of Fig. 5. ×1000. (oil immersion).



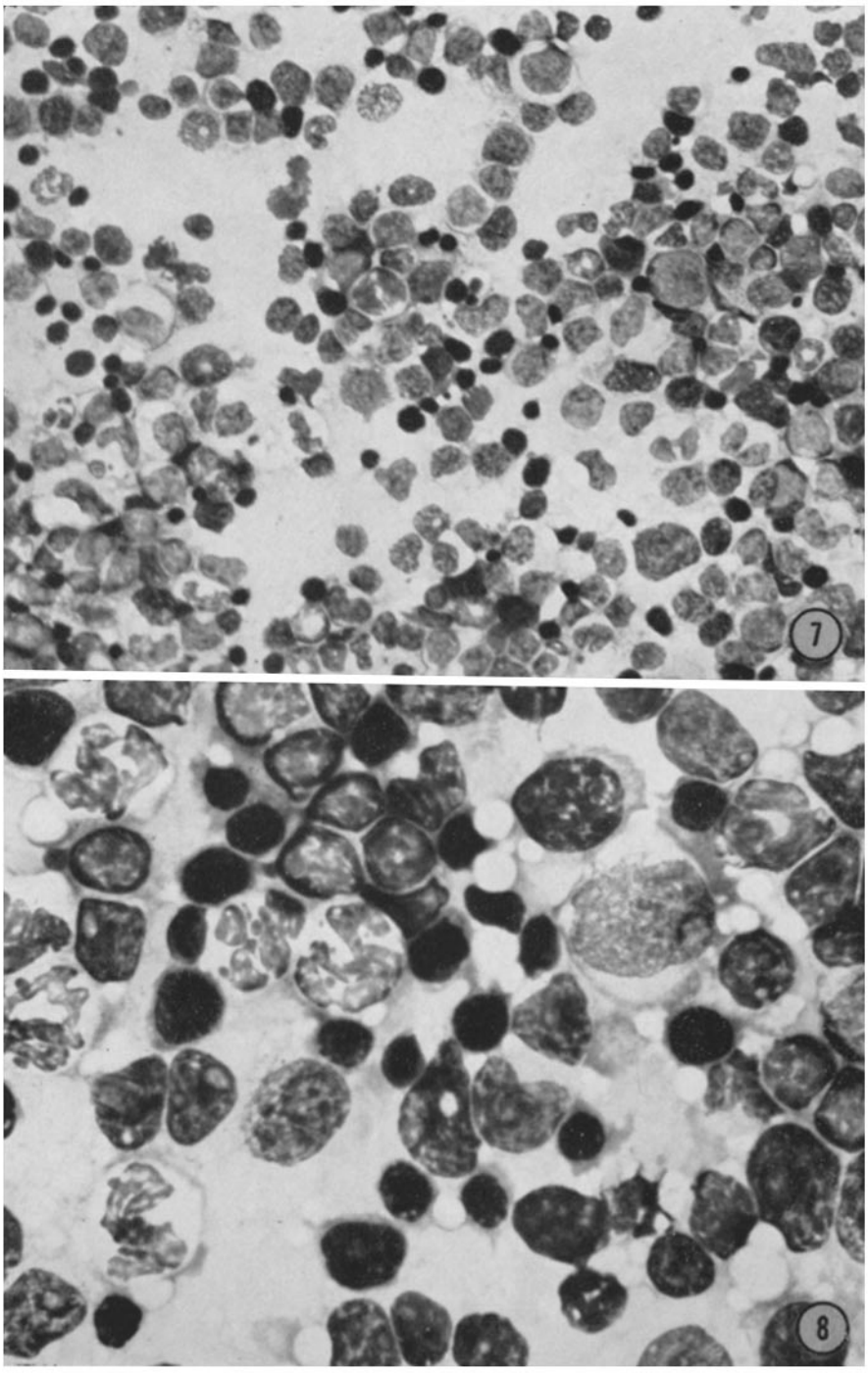


(Yunis et al.: Postthymectomy wasting and autoimmune phenomena)

PLATE 103

Fig. 7. Wright-Giemsa stain of spleen imprint from a 52 day old neonatally thymectomized A strain mouse with wasting disease. Note the large proportion of erythroid and myeloid elements and a decreased number of lymphocytes.

Fig. 8. High power view of a portion of Fig. 7. ×1000 (oil immersion).



(Yunis et al.: Postthymectomy wasting and autoimmune phenomena)