Histological and immunological studies on congenitally thymusless mice
by Sister Ruth Helen Barron

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE Microbiology
Montana State University

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Histological sections of the lymph nodes and spleen showed a severe lymphocytic deficiency in the thymus-dependent areas.

The thymusless mice did not respond to an intraperitoneal dose of 10^8 sheep erythrocytes by producing rosette-forming cells, whereas littermate controls responded well. Non-immunized thymusless mice produced normal quantities of immunoglobulins IgG2 and IgM but they made less IgG1 and IgA than their littermate contrals.

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Signature Sister Ruth Barron

Date Aug. 11, 1971

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ABSTRACT

Total white blood counts, hematocrits, absolute counts of polymorphonuclear cells, lymphocytes, eosinophils, and monocytes were done on congenitally thymusless mice and their phenotypically-normal littermates. The thymusless mice were found to have a lowered lymphocyte count and an elevated polymorphonuclear count. Total white blood counts were compared between the heterozygous (+/nu) mice and the homozygous wild type (+/+) littermates and it was concluded that there was no significant difference between the two groups of mice.

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INTRODUCTION

In 1962 Miller (27) demonstrated that thymectomy of neonatal mice gave rise to a wasting syndrome consisting of weight loss, lethargy, ruffled fur, hunched posture, periorbital edema, diarrhea, and which led to their death about one week after onset of symptoms. In a germfree environment this wasting did not occur, suggesting that the wasting was caused by an overwhelming infectious process (29).

Mice thymectomized at birth had an impaired response to sheep erythrocytes (SRBC); rat erythrocytes; goose erythrocytes; Salmonella typhi H, O and Vi antigens; influenza A virus; T2 coliphage; diphtheria toxoid; human gamma globulin (HGG); ovalbumin; and bovine serum albumin (BSA) (14, 20, 31, 32). These represent the so-called thymus-dependent antigens. On the other hand the mice responded normally or near normally to tetanus toxoid; hemocyanin; Pneumococcus type III capsular polysaccharide; Salmonella flagellar antigen in it's polymerized form; ferritin; MS-2 bacteriophage; and polyoma virus (20, 31). These antigens are referred to as thymus-independent antigens.

At least four immunoglobulin classes have been identified in mice (11). These are 7S IgG_1 and IgG_2, 7S or 11S IgA and 19S IgM. Reports vary on the ability of neonatally thymectomized mice to form these immunoglobulins. Humphrey et al. (quoted in 31) using C3H/Bi

and (C57Bl x C3H/Bi)F_1 mice found a delay in the synthesis of immunoglobulins, but eventually similar levels were reached in neonatally thymectomized mice and their sham-operated controls. The IgA reported in this study was sometimes elevated in the thymectomized mice. Antibody responses to sheep erythrocytes and S. typhi H and O antigens were depressed. Fahey et al. (13) using C3HF/Lw mice found IgG_1 and IgG_2 normal except during severe wasting when they were low. IgM remained normal and IgA was normal or increased. Arnason et al. (3) repeated this work using Balb/c mice. They found that neonatally thymectomized mice reached normal adult levels of IgG and IgM, but had average IgA levels somewhat lower than controls, as determined by double diffusion in gel and with immunoelectrophoresis. At six months after thymectomy these mice failed to respond to ovalbumin and BSA, but produced antibody to Salmonella typhosa O antigen. They repeated the immunoglobulin work with C3H mice and found that these mice had a much lower IgA level than the Balb/c mice treated similarly.

Arnason et al. (1,2) also quantitated the immunoglobulins of Wistar rats using double diffusion in gel or immunoelectrophoresis. In the neonatally thymectomized rats IgG was normal, IgM was normal or elevated and IgA was low or sometimes depleted. The rats also had a lowered response to BSA and diphtheria toxoid, both of which, according to Arnason, provoke production of antibodies of the class

IgA.

The discrepancy in the results presented by these three groups is perhaps explained by the fact that the IgA of Humphrey (quoted in 31) and of Fahey (13) are not the same proteins as that of Arnason (1, 2, 3). This issue remains unresolved.

The response to grafts in neonatally thymectomized mice seemed impaired. These mice retained for prolonged periods homografts from donors related at the H-2 locus and donors of strains not closely related (31). Heterografts also were retained, including a rat leukemia and a human carcinoma of the cervix (31). In 1967 Miller (29) found that germfree mice which had been neonatally thymectomized were able to reject skin homografts, although the rejection was delayed. He suggested that the conventionally reared mice suffered from infectious processes which pre-empted the activities of the few available cells in the immune system leaving them unable to respond to the graft.

As reported by Parrott (36) the lymph nodes of neonatally thymectomized mice are depleted of lymphocytes in the thymus-dependent paracortical area. The periarteriolar area of the spleen was also found to be thymus-dependent (36).

In mice, the lymphocyte count and the lymphocyte:polymorphonuclear cell ratio normally rises progressively during the first

eight days of life to almost reach the normal adult level. In contrast, mice thymectomized at birth did not display this rise and their total white count in adult life was lower than normal due to a lymphopenia (3). As these mice became older the absolute lymphocyte counts became lower, and in mice dying of wasting the absolute lymphocyte count sometimes became as low as 500/cu mm. In neonatally thymectomized rats (1, 2, 31) there was a marked lowering of the absolute lymphocyte count. From this work on mice and rats (31) we can conclude that a certain portion of their small lymphocyte population is thymus-dependent.

The immune apparatus of mice thymectomized as adults and tested soon after thymectomy responded normally to antigenation (26). Such mice tested one to two months after thymectomy have a slightly lower lymphocyte count, 30 to 40% of normal, but a normal immune response (3, 26, 28). Adult rats tested two months after thymectomy had a thoracic duct lymphocyte count of 60% of normal (31). In mice, by three months post-adult thymectomy the immune response to BSA had begun to decline and by seven or more months the immune response to SRBC was markedly lower than the controls. These results were found in CBA (28, 45), (AK x T6)F_1 (28) and (AKR x C57B1)F_1 (26). Taylor (45) felt that the immune response of his CBA mice ceased declining after seven months. During this time the polymorphonuclear counts

were normal and in infectious processes they became elevated.

Adult mice which were thymectomized and then given wholebody irradiation and restored with a graft of normal bone marrow showed the same changes that have already been discribed for neonatally thymectomized mice (26, 28). (For a description of radiation effects and reconstitution see page 7.)

It would seem that the thymus matures long-lived small lymphocytes which seed to other lymphoid areas, such as the spleen and lymph nodes. Mice thymectomized after the adult level of lymphocytes has been attained respond normally at first due to the normal number of thymus lymphocytes. As these cells decrease in number or function they are not replaced, due to the absence of the thymus, which explains the slow decline of the lymphocyte count and of the immune response in mice thymectomized as adults. Whole body irradiation kills the thymic lymphocytes. If the thymus has been removed it will not be possible to replace the lymphocyte level and it will remain low as in the neonatally thymectomized rodent where the thymus was removed before the body could be seeded with lymphocytes.

Reconstitution experiments were attempted to further explore how the thymus affected the lymphocyte count and the immune response. It was found that thymus cells alone had to be given in huge amounts (i.e., 100-300 million cells) to restore immunological capacity or

prevent wasting in neonatally thymectomized mice (31). Thymus grafts implanted subcutaneously or under the renal capsule were, however, able to restore neonatally thymectomized mice to full immunological and hematological activity. It is of interest that within two weeks the small lymphocytes within the thymus were of host not donor type (9, 31). Spleen cells could restore the neonatally thymectomized mice with doses as low as five million cells although it was found that in three to seven months, apparently these cells had been used or had died, the mouse developed wasting disease and died (31).

Thinking that this reconstitution might be by a humoral factor, thymus tissue in a Millipore diffusion chamber was tried by a number of workers with inconsistent results. Fahey et al. (13), Levey et al. (quoted in 31), Trench et al. (quoted in 31), and Osoba (quoted in 31) claimed a restoration of immunological competence, while Wong et al. and Barclay noted no help form the thymus enclosed in the diffusion (quoted in 31). Another proof for a humoral factor was sought by Osaba, using neonatally thymectomized CBA mice bred to a T6 male. After delivery these females showed a normal response to allogenic skin grafts, to SRBC and they failed to develop wasting (31). They concluded that a humoral factor acts on the hosts lymphocytes, however, a migration of cells had not been completely excluded as the cause of the reconstitution either with the thymus in the

Millipore diffusion chamber or with reconstitution through pregnancy. Perhaps both cell-seeding and a humoral factor are required.

Radiation of laboratory animals is a useful tool in immunological work and will now be briefly discussed to provide information necessary for an interpretation of thymus experiments to be discussed later. The destruction to a mouse is dependent upon many factors which should be taken into account. Some of these are the strain of mouse, age, diet, presence or absence of bacteria and radiation dose.

A dose of radiation of 100 rads to 1000 rads is enought to destroy the ability of some mice to make a primary immune response. The response is dose dependent at this level. At 600-900 rads bone marrow death occurs. Mice dying from this dose of irradiation can be saved by an inoculation of bone marrow or spleen or fetal liver, or by shielding a portion of their bone marrow or spleen. Death from about 1000-10,000 rads is termed gut death. In this range the damage is dose independent and causes damage to the intestines. Radiation greater than 10,000 rads causes what is termed CNS death (16).

For immunologic work on mice it is customary to use radiation doses of 750-900 rads as this causes bone marrow death and death to the cells of the immune system thus rendering the animal a living test tube into which groups of cells can be placed to see how they

interact or replace the lost immune system. Experiments concerning the function of the thymus which have utilized irradiated animals will now briefly be described.

If the thymus was intact, bone marrow or spleen cells could be used to slowly reconstitute the immune system (8), but if the mouse was also thymectomized, bone marrow alone would not work whereas bone marrow plus thymus cells would effect a response to antigen (8, 30). In a thymectomized animal reconstituted with a thymus graft genetically different from the bone marrow given it was found that the bone marrow cells passed through the thymus becoming the immunologically competent thymus-derived cells. Other bone marrow cells which probably did not pass through the thymus became the cells which actually produced the antibody (8). It was found by repeating this work in vitro that three cells were required for a normal immune response to SRBC, the above two and a third, the macrophage (44). In the in vivo system the importance of this cell was not noticed because the macrophage is resistant to irradiation. Hence, for one immune response to an antigen such as SRBC, the macrophage, the thymus derived lymphocyte, and the bone marrow lymphocyte all interact before the bone marrow lymphocyte differentiates into the antibody producing cell.

It is known that the intact immune system can be rendered unresponsive or tolerant. By using lethally irradiated mice and reconstituting such mice with cells from a tolerant animal or from a normal animal Chiller et al. (7) were able to show that both bone marrow cells and thymus cells could be made tolerant, but that whereas the bone marrow tolerance varies with the dose of the antigen and was present only from day seven to day forty-nine, thymus lymphocyte tolerance was present by day two, remained for long periods, and was induced by smaller doses of antigen.

In 1966 a mouse appeared in a closed, but not inbred, albino stock of N. R. Grist of Glascow, Scotland which gave a new tool to help in this work of assigning a place to the thymus in the immune response. The mutant mouse was first noticed because of its hairlessness. It was found to have small ovaries and its sperm were often immotile so that it was with difficulty that a colony of these mice could be maintained . For this reason the autosomal recessive nude gene was bred into two strains with genetic markers, the Rex and Trembler strains of Flanogan et al. (15), and the heterozygous mice were used for breeding. Rygaard (41) introduced the nude gene into a Balb/c strain, while Wortis (47) used a CBA strain. For all of these, the heterozygous mice were used for breeding. The new mouse mutant, termed nude (nu), died of infection early in life. In

searching for the cause of the high infection rate Pantelouris in 1968 (33) reported that the nude mouse was also congenitally thymus-less. It had a thymic anlage present in the fetus which develops up to day 14, but never was populated with lymphoid-like cells (34). These animals then made it possible to check out the work performed in neonatally thymectomized mice in an animal which has never had a functioning thymus.

Total lymphocyte counts reported by Pantelouris (33) on his conventionally reared nude mice were very low. He also reported that the heterozygous mice had a low count even though they had a normal thymus. With the CBA-nude cross Wortis (47) also reported lower agranulocyte counts along with normal granulocyte counts. In contrast to Pantelouris, he did not report a difference in count between the heterozygous mice and the wild-type littermates.

The lymphoid tissues in the nude mouse, as compared to normal littermates, were studied by De Sousa et al (10). They found lymphoid depletion in the thymus-dependent area of the lymph nodes in the nude mouse as compared to the control. The spleen of the nude mouse had a greater red/white pulp ratio, an unusual number of mega-karyocytes in the red pulp as compared to controls, but it was difficult to see a difference in the thymus-dependent area as many sections appeared normal. The Peyer's patches of the nude had no

germinal centers, but well defined primary nodules were present. They concluded that primary nodules and plasma cells are thymus-independent.

Immunoglobulins of the nude mouse were studied by Rygaard (41) using immunoelectrophoresis. He reported minimal values in the nude mice. This immunoelectrophoresis has been repeated by Wortis (47). Using isoelectrofocusing of acrylamide gels the nude mice were found to have a wide range of gamma globulin values some of which were much lower than the littermate controls.

The response to SRBC was checked by Pantelouris (35) using a hemolysin assay. He found that although nude mice did develop a hemolysin titer, it was lower than normal. The response to SRBC was tested by Wortis (47) using the Jerne plaque technique. He found that the nude mice responded to a dose of 4×10^7 SRBC, but the response was lower than that observed for normal controls.

Neonatally thymectomized mice were able to retain skin homografts and heterografts so Rygaard (41) injected nude mice with a highly differentiated mucoid producing adenocarcinoma derived from a human. All nudes grew tumors, whereas all controls rejected the grafts. He grafted rat skin heterografts on the nude mice. All grafts healed and were intact when the mice died of wasting. Pantelouris (35) gave homografts to nude mice which lasted the life of the mice whereas the controls had all rejected their grafts by day 13. He injected thymus

cells, or grafted a thymus, and then homografted mouse skin which was rejected by day 7. Wortis (47) repeated this using C57Bl skin. All nudes retained the grafts until death even as late as day 54. All controls rejected the grafts by day 10. Since neonatally thymectomized mice are able to reject homografts under germfree conditions (29), and since grafting puts stress on the animal bringing on wasting, it would be useful to repeat this graft work with the nude mouse under germfree conditions to see how long healthy thymusless mice can retain the grafts.

The present work was designed to provide additional information on the immunobiology of congenitally thymusless mice. Particular emphasis has been placed on hematological observations and on the determination of the serum levels of the various classes of immunoglobulins. An attempt was made to determine if blood counts can be used as a marker for the selection of heterozygous mice as current information concerning the value of blood counts in the selection of heterozygotes is conflicting. Studies reported on the levels of immunoglobulins have not involved quantitative techniques. In addition, this study involved a histological evaluation of congenitally thymusless mice, and a preliminary investigation of their immune capacity.

MATERIALS AND METHODS

Mice. The mice used in these experiments were derived from a breeding nucleus obtained from Prof. D. S. Falconer and Dr. R. C. Roberts of the Institute of Animal Genetics, University of Edinburgh, Edinburgh, Scotland. These mice came from a closed, but not inbred, colony in which in addition to the autosomal recessive nude (nu), the dominant marker Rex (Re) was also carried in the VIIth linkage group (15). $\left(\frac{Re\ +}{+\ nu}\right)$ males were mated with Balb/c females. The Balb/c mice were from our inbred colony, derived from breeding stock obtained from J. J. Trentin at Baylor Medical School. The offspring bearing the Re marker were discarded. The $\left(\frac{+\ +}{+\ nu}\right)$ F_1 mice were mated together to obtain litters of about 25% homozygous nu $\left(\frac{+\ nu}{+\ nu}\right)$, 50% heterozygous nu $\left(\frac{+\ +}{+\ nu}\right)$; and 25% homozygous wild $\left(\frac{+\ +}{+\ +}\right)$. The colony was maintained under specific pathogen free (SPF) conditions, except for a small nucleus of F_2 mice which was bred under conventional (CR) conditions. All cages, watering bottles, and San-i-cell bedding were autoclaved before use. The mice received autoclavable Purina Laboratory Chow 5010 which had been sterilized, and acidified-chlorinated water (25) ad libitum with occasional feedings of sterilized Quaker Rolled Oats.

Hematology. Total white blood counts were done by the conventional method (46). Differential counts were made from air dried blood smears stained with Wright's stain (46). The white cells were

classified as lymphocytes, monocytes, neutrophils and eosinophils. The absolute counts were calculated by multiplying the total white blood count by the proper percents.

The blood for the hematocrits was drawn up in micro hematocrit tubes, spun eight minutes and read in an Adams Autocrit centrifuge. It is reported in volume percent.

Platelet counts were done from blood smears stained with Wright's stain (46). Capillary coagulation times were done by the conventional method (46).

Histology. Nude and control animals were killed by cervical dislocation and appropriate tissues taken for histological examination. These were embedded in paraffin, cut, and stained by the conventional hematoxalin and eosin method (22). Bone marrow was washed out of the long bones using a syringe attached to a 26 gage hypodermic needle and containing 0.85% sodium chloride. The eluate was smeared on slides, air dried and stained with Wright's stain using twice the times required for a good stain with peripheral mouse blood.

Rosette Technique. Nude and phenotypically-normal littermates were immunized intraperitoneally with 1×10^8 sheep erythrocytes (SRBC). On the fifth day after immunization they were sacrificed and their spleens removed. The spleens were teased apart in phosphate

buffered saline (PBS) and the cells used according to the method of Biozzi et al (5). One tenth milliliter of a spleen cell suspension, three times washed in PBS, was added to 0.1 ml of 5% SRBC or 0.1 ml of 2% chicken erythrocytes (CRBC) in a 15 x 100 mm tube containing 0.8 ml of PBS. The tubes were capped with rubber stoppers and incubated at $4^{o}C$ overnight. All procedures were carried out in the cold. The next day the pellet of cells was gently resuspended by rotating 16 times/min for 8 min and total spleen cells per ml was determined by making a 2% acetic acid dilution to lyse out the red cells, and counting on a hemacytometer. The total rosettes per ml were determined by counting the undiluted mixture on a hemacytometer. Spleen cells surrounded by eight or more SRBC or five or more CRBC were considered rosettes. Results were expressed as rosette forming cells x 10^3 per 10^6 nucleated spleen cells.

Single Radial Immunodiffusion. A modification of the technique of Masseyeff and Zisswiller was used to assay immunoglobulins (23). One percent Special Noble Agar (Difco) prepared in barbital buffer pH 8.6 (1.743 gm barbituric acid, 10.309 gm sodium barbital, distilled water qs ad one liter), was layered 1.5 mm deep in Hyland immunodiffusion plates. From this, discs 10 mm in diameter were cut with an appropriate sized cork borer, exposed to a drying environment for 30 min, then 30 microliters of antiserum specific for mouse

immunoglobulin G_1, G_2, A or M (Melpar, Falls Church, Virginia) diluted to an appropriate amount was applied to each disc. After 18 hours, the antiserum had diffused into the discs. A two mm well was then cut in the center of each disc with a blunted 13 gage hypodermic needle. Five microliters of the appropriate dilution of the serum to be tested was added to the well. After 36-48 hours incubation the discs were washed with three changes of 0.85% sodium chloride solution over a twenty-four hour period. These discs were then stained with acid fuchsin stain (6) (Dissolve 2 gm acid fuchsin in 500 ml absolute methyl alcohol; add 400 ml distilled water and 100 ml acetic acid) for 30 min. The discs were decolorized until the rings became readable using frequent changes of washing solution (prepared as for acid fuchsin with omission of the dye). The diameter of the precipitin rings was read with a Hyland Immunodiffusion Reader. (See Figure 1.) A standard concentration curve was prepared using Balb/c serum. Serum immunoglobulin concentrations for nude mice and their littermate controls are expressed as percent of the Balb/c normal serum values. Serum immunoglobulins of classes IgG_1, IgG_2, IgM and IgA were quantitated using this procedure.

Immunoelectrophoresis in Gel. One percent Purified Agar (BBL) prepared in barbital buffer pH 8.2 (6) was poured onto slides in a Gelman holder. Appropriate wells were cut on the slides and the sera

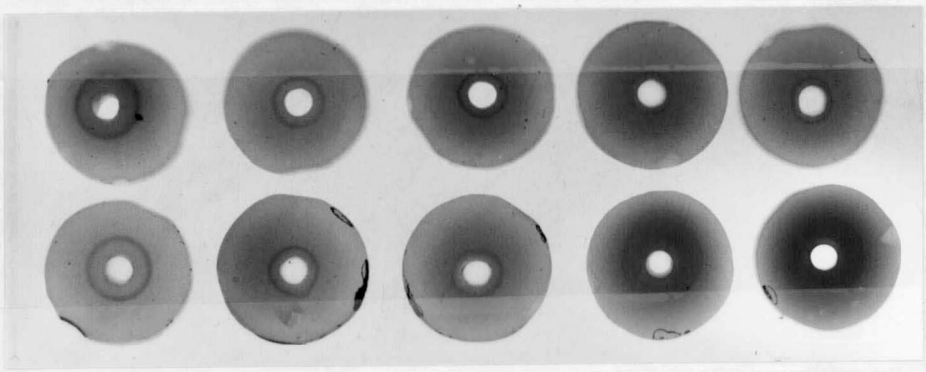

Figure 1. Radial immunodiffusion discs showing rings.

to be tested were added. A current of 175 volts was run through the agar for three hours. Appropriate troughs were cut in the agar and the developing antisera were added. This was allowed to diffuse for 14-16 hours at room temperature and 24 hours in the refrigerator and pictures were taken of the results.

Statistics. Calculations of standard deviation and P values according to Student's t test were performed on a Sigma 7 computer.

RESULTS

Hematology. The mean total white counts for the three groups of mice (Table I) did not differ markedly. Specific pathogen free (SPF) nude mice had a mean of 5.1×10^3, conventionally reared (CR) nude mice 6.6×10^3, with the mean for the 20 mice 5.9×10^3. The mean for the 22 littermate control mice was 5.8×10^3. The range variation was much greater with the CR nu than either of the other groups. The mice with the extremely low counts were wasting and the mice with extremely high counts had diarrhea or external infected areas.

The absolute polymorphonuclear (PMN) count of both nude groups was higher than the littermate controls, but the CR nude mice had a marked elevation in their PMN count as well as an extreme variation between mice. The absolute lymphocytes on the other hand were markedly lower in both the SPF and CR nude mice. The absolute eosinophils, monocytes and the hematocrits were not markedly different hence ranges are not shown. One control mouse had a hematocrit of 38 and a blood picture of a microcytic anemia.

In order to establish whether white blood counts could be used as a genetic marker to determine the mice which were heterozygous, the littermates of four litters of F_2 mice were used. Initial blood counts were done on the six week old mice drawing blood from the tail after warming the tail in water. The mice were then bred with known heterozygotes to determine whether or not they carried the nude gene. Two

Table I. Hematological values on congenitally thymusless mice and their phenotypically normal littermate controls.

			Cells x 10^3				
		Hct.	Total WBC	Abs. PMN	Abs. Lymph	Abs. Eos.	Abs. Mono.
SPF nude	10[a]	51	5.1[b] (2.8-71)	2.2 (0.9-6.0)	2.6 (1 0-5.2)	0.035	0.204
CR nude	10	48	6.6 (1.4-13.9)	5.3 (0.9-13.2)	1.0 (0.2-2.2)	0.018	0.325
Average of above		49	5.9 (1.4-13.9)	3.8 (0.9-13.2)	1.8 (0.2-5.2)	0.026	0.265
Littermates	22	51	5.8 (3.6-10.8)	1.1 (0.1-2.7)	4.5 (1.9-8.4)	0.025	0.101

[a] Number of mice.

[b] Mean. Range is indicated in parenthesis.

SPF - Specific pathogen free. CR - Conventionally reared.
WBC - White blood cell count. Abs. - Absolute.
PMN - Polymorphonuclear cell. Lymph - Lymphocyte.
Eos. -Eosinophil. Mono. - Monocyte
Hct. -Hematocrit, mean of packed cell volume.

months after the initial blood counts the counts were repeated drawing blood from the tail without heat. One month later a few of the counts were repeated using the retro-orbital plexis as a source of blood. This latter procedure did not add new information so was not done with all the mice. The mean and range of each group are given in Table II. As can be seen there was no significant difference between the mean for the two groups and the range was identical.

The nude mice appeared to bleed abnormally when injured. For this reason platelet counts and capillary coagulation times were done. The platelet counts all were around 6×10^5/cu mm except on one mouse which had a count of 1.5×10^5/ cu mm. The capillary coagulation times were all very short and did not differ from controls. These procedures were not done on sufficient mice to draw any conclusions.

Histology. As seen above the nude mice had a low absolute lymphocyte count. This was also mirrored in the histological sections of lymph nodes and spleen stained with hematoxalin and eosin. There was a marked difference between the lymph nodes of the nude animals and their littermate controls in the paracortical area. As can be seen in Figures 2 and 3 the paracortical area in the lymph nodes of the nude mice was devoid of lymphocytes whereas the paracortical area in control lymph nodes was packed with lymphocytes. The difference