Studies on the immunological recovery of neonatally thymectomized mice
by James Thomas Hunter

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
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Abstract:
A time-course study was made on the immunological responsiveness of Swiss Manor mice which had been thymectomized at birth. The production of hemolysins and hemagglutinins, tested 5 and 10 days after stimulation with sheep erythrocytes, was found to be depressed in mice immunized 30, 40, or 50 days after thymectomy. No age dependant increase in the ability to produce hemagglutinins or hemolysins was found as no titers above the subnormal levels generated by the 30 day old mice were found in either the 40 or 50 day old mice. The ability of spleen cells obtained from the neonatally thymectomized mice to induce graft-versus-host disease in newborn A/jax mice was found to be severIy impaired when 20 x 10 6 cells from 30-70 day old animals were used or when 40 x 10 6 cells from 30, 40, or 50 day old animals were used. However, substantial recovery of the ability to induce graft-versus-host disease was observed when 40 x 10 6 cells from 60 or 70 day old donors were given newborn A/jax mice. The ability of the thymectomized mice to reject skin homografts from A/jax donors was found to be normal at 30 days.

The mice used in these experiments suffered from a high incidence of post thymectomy wasting (33/. mortality). When spleen cells obtained from the thymectomized mice were used to induce graft-versus-host disease in A/jax recipients a high incidence of early death (prior to 6 days post-injection) was encountered in the recipient animals. The early deaths could be prevented by incubating the donor cells in media containing low levels of antibiotic prior to injection into the A/jax recipients. An infectious agent was postulated and subsequently Streptococcus faecalis was isolated from the spleens of two Swiss Manor mice undergoing post-thymectomy wasting disease.

Peripheral studies were conducted in which Swiss Manor mice were splenectomized within 24 hours of birth. Two out of fourteen of the animals were observed to die of a wasting like syndrome at 18 days of age.

The data are interpreted as supporting the concept that neonatalIy thymectomized mice tend to recover immunological competence with increasing age. Further, the data indicate that enteric bacteria may be associated with post-thymectomy wasting and that the spleen may be of some importance in the development or maintenance of the immunologic responsiveness of newborn mice.

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Approved:

Head, Major Department

Chairman, Examining Committee

Dean, Graduate Division

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## ABSTRACT

A time-course study was made on the immunological responsiveness of Swiss Manor mice which had been thymectomized at birth. The production of hemolysins and hemagglutinins, tested 5 and 10 days after stimulation with sheep erythrocytes, was found to be depressed in mice immunized 30, 40, or 50 days after thymectomy. No age dependant increase in the ability to produce hemagglutinins or hemolysins was found as no titers above the subnormal levels generated by the 30 day old mice were found in either the 40 or 50 day old mice. The ability of spleen cells obtained from the neonatally thymectomized mice to induce graft-versus-host disease in newborn A/jax mice was found to be severly impaired when $20 \times 10^6$ cells from 30-70 day old animals were used or when $40 \times 10^6$ cells from 30, 40, or 50 day old animals were used. However, substantial recovery of the ability to induce graft-versus-host disease was observed when $40 \times 10^6$ cells from 60 or 70 day old donors were given newborn A/jax mice. The ability of the thymectomized mice to reject skin homografts from A/jax donors was found to be normal at 30 days.

The mice used in these experiments suffered from a high incidence of post thymectomy wasting (33% mortality). When spleen cells obtained from the thymectomized mice were used to induce graft-versus-host disease in A/jax recipients a high incidence of early death (prior to 6 days post-injection) was encountered in the recipient animals. The early deaths could be prevented by incubating the donor cells in media containing low levels of antibiotic prior to injection into the A/jax recipients. An infectious agent was postulated and subsequently Streptococcus faecalis was isolated from the spleens of two Swiss Manor mice undergoing post-thymectomy wasting disease.

Peripheral studies were conducted in which Swiss Manor mice were splenectomized within 24 hours of birth. Two out of fourteen of the animals were observed to die of a wasting like syndrome at 18 days of age.

The data are interpreted as supporting the concept that neonatally thymectomized mice tend to recover immunological competence with increasing age. Further, the data indicate that enteric bacteria may be associated with post-thymectomy wasting and that the spleen may be of some importance in the development or maintenance of the immunologic responsiveness of newborn mice.

# INTRODUCTION

The thymus is a comparatively large lymphoid organ located in most animals in the ventral anterior mediastinum of the chest in close association with the pericardium and great veins at the anterior aspect of the heart. Prior to 1961, the role played by the thymus was uncertain, although the work of numerous early investigators, most notably Beard (1900) and Hammer (1921), had implicated the thymus as being important in the physiology of the lymphoid system. In 1961 a dramatic change in the impetus of research on the thymus was initiated by the independent demonstration by Miller (1961, 1962a) and Good's group (Martinez et al., 1962; Archer et al., 1962) of the profound immunological effects of neonatal thymectomy. The work of Miller demonstrated that thymectomy of mice in the immediate neonatal period was associated with severe lymphopenia and an impaired ability to reject homografts in the adult animal (Miller, 1961, 1962a). Simultaneous work by Good's group demonstrated that skin homograft survival in neonatally thymectomized mice was prolonged in certain donor host combinations (Martinez et al., 1962) and that the ability of neonatally thymectomized rabbits to respond to stimulation with soluble antigens was markedly impaired (Archer et al., 1962). Experiments dealing with thymic extirpation had been carried out prior to the work of Miller and Good's group, but these investigations were conducted in mature animals and the results were equivocal. Such experiments are typified by those reported by Fichtelius, Laurell, and Phillipsson (1961) using adult male guinea pigs. Since the first discoveries on the effects of neonatal thymectomy an enormous volume of

research has been done and the thymus has come to occupy a central role in immunobiological thought particularly in regard to the ontogeny of the immune system.

The impairment of the adult immune system brought about by perinatal thymectomy of various facets is now well documented. A number of studies exemplified by the experiments of Good et al. (1962) and those of Waksman et al. (1962) have established that thymectomy of newborn animals is related to a diminished lymphocyte population in the adult animal. Generally, the level of humoral antibody response in adult neonatally thymectomized animals is greatly depressed. Subnormal levels of antibody have been reported to be produced in response to challenge with many antigens such as sheep erythrocytes (Fahey, Barth and Law, 1965), Salmonella typhi H, O and Vi antigens (Humphrey, Parrott and East, 1964) and bovine serum albumin (Arhason et al., 1964). However, the situation is not clearly defined as normal or near normal levels of response to other antigens such as ferritin, hemocyanin (Fahey, Barth and Law, 1965) and tetanus toxoid (Hess, Cottier and Stoner, 1963) have been reported. Such discrepancies may be due to a multiplicity of variables such as sex variations (Balner and Dersjant, 1966) or to time elapsed between immunization and serum collection (Sinclair, 1965). There is also conflicting evidence concerning the ability of neonatally thymectomized animals to mount a primary and a secondary immune response. One worker has reported a depressed primary response and a normal secondary response (Sinclair, 1967a, b) while another group has reported a normal primary

response and a depressed secondary response (Hess, Cottier and Stoner, 1963). Again, the discrepancy may be due to a wide number of uncontrolled variables. Numerous reports indicate that neonatal thymectomy impairs cell-mediated immune reactions. In neonatally thymectomized mice there is, generally, marked impairment of ability to reject homografts and in some cases heterografts (Dalmasso, Martinez and Good, 1962a; Miller, 1962b; Miller, Marshall and White, 1962). The same situation has been reported in other species such as rats (Arnason, Jankovic and Waksman, 1964) and hamsters (Sherman, Adner and Dameshek, 1964). Various reports such as that of Martinez, Dalmasso and Good (1962a) and that of Perri et al. (1963) indicate that allogenic tumor grafts are accepted by thymectomized animals. Neonatal thymectomy has been reported to diminish the effects of hypersensitivity reactions such as those associated with systemic lymphocytic choriomeningitis infection as reported by Rowe, Black and Levey (1963). Cells from the lymphoid organs of neonatally thymectomized mice were found to be less capable of eliciting graft-versus-host reactions than were similar numbers of cells from normal mice (Dalmasso, Martinez and Good, 1962b; Miller, Marshall and White, 1962). Still further evidence for the long range immunosuppressive effects of neonatal thymectomy can be inferred from the development of a wasting disease following neonatal thymectomy. Mice thymectomized at birth may develop a syndrome in latter life characterized by lethargy, ruffled fur, hunched posture, weight loss, diarrhea and, ultimately, death (Miller, 1962b, 1964). Wasting, however, does not occur in all

all strains of mice and onset may begin anywhere from 4 weeks to 4 months after thymectomy depending on the strain of mouse involved (Parrott, 1962). The pathogenesis of the post thymectomy wasting syndrome is not definitely established, but there is a preponderance of evidence favoring an infectious etiology (Azar, 1962; McIntire, Sell and Miller, 1964).

Simple thymectomy of adult animals has not been associated with immediate immunological defects (Harris, Rhoads and Stoakes, 1948; Fichtelius, Laurell, and Phillipsson, 1961). However, when antigenic stimulation was delayed until several months post-thymectomy deficient antibody responses were detected. Taylor (1965) reported depressed responses to bovine serum albumin immunization and Metcalf (1965) reported lowered responses to immunization with sheep erythrocytes following thymectomy of adult mice. The capacity of spleen cells from thymectomized adult mice to induce graft-versus-host reactions degenerated gradually over a period of time (Miller, 1965). Another line of evidence indicates that the thymus can function to restore the immunological competence of adult animals whose immune mechanism has been impaired or destroyed. Numerous studies typified by the work of Globerson and Feldman (1964), Miller, Doak and Cross (1963) and Jeejeebhoy (1965) indicate that the recovery of the ability to respond to various antigens following total-body irradiation is impaired or completely suppressed by thymectomy in adult animals. Thymectomy of mature animals made tolerant to bovine gamma-globulin at birth has been reported to delay the abrogation of the tolerant state (Claman and Talmage, 1963; Hunter and Jutila,

1964). From the evidence accumulated by experiments dealing with the effects of neonatal and adult thymectomy it appears that the thymus functions to generate or assists in generating the immune system during the perinatal period and that it also functions to maintain or regenerate the immune system throughout the life of the animal. How the thymus accomplishes this function is still a matter of controversy and investigation.

A number of approaches have been used in attempts to elucidate thymic function. They include either grafting of thymus tissue, implantation of thymus tissue in diffusion chambers, injection of cell suspensions or injection of thymus extracts into thymectomized mice. Also, embryological studies, and studies involving the use of chromosomal markers or radioactive labeling have served to identify cellular components of the thymus which participate in the generation of the immune response. Various studies have established that lymphopenia and loss of immunological functions following neonatal thymectomy can be precluded by the grafting of either syngeneic or allogeneic thymic tissue into thymectomized animals (Miller, 1961, 1962b; Dalmasso et al., 1963). The same results have been reported in the case of adult thymectomized irradiated mice (Miller et al., 1964; Leuchars, Cross and Dukor, 1965). A number of workers such as Dukor et al. (1965) have found that the implanted thymuses are composed of donor type cells initially but that gradually these cells are replaced by host lymphocytes and in the case of homografts and graft is rejected by the host. These results have

been interpreted as indicating a directive influence by the thymus upon the lymphoid precursors of the host as opposed to a repopulation of the host by precursor cells arising in the grafted thymus (Miller and Osoba, 1967). This interpretation is supported by the observation that diffusion chambers containing thymic tissue restore the immunological competence of neonatally thymectomized animals, (Levey, Trainin and Law, 1963; Levey et al. 1963; Osoba and Miller, 1963; Aisenberg and Wilkes, 1965). Similar results have been reported using thymus tissue enclosed in diffusion chambers to restore competence to thymectomized irradiated adult mice (Miller et al., 1964). However, the restoration of lymphocyte levels by implantation of thymus tissue in diffusion chambers has not been a consistent finding (Levey, Trainin and Law, 1963). The diffusion chamber experiments suggest that the thymus elaborates a humoral factor or factors which act on lymphoid precursor cells causing them to become immunologically competent. Several investigators have questioned the validity of results obtained with diffusion chambers by indicating that the chambers may have leaked, that the experiments were inadequately controlled, and that the porosity of the diffusion bariers used was large enough to allow the passage of cells (Auerbach, 1964; Weissman, 1967). It is possible to reconstitute the immune mechanism of neonatally thymectomized mice by injecting lymph node cells or spleen cells obtained from normal mice (Dalmasso et al., 1963; Cross, Leuchars and Miller, 1964). Cells from neonatally thymectomized mice or bone marrow or liver cells from normal mice failed to protect

neonatally thymectomized recipients against immunological defects (Cross, Leuchars and Miller, 1964). These results suggest that replenishing the supply of immunologically competent cells in a thymectomized animal will protect that animal. However, Trainin, Law and Levey (1965) have reported a 20% incidence of death in animals protected with spleen or lymph node cells when the animals were 3 to 7 months old. This would seem to indicate that cells alone are not sufficient to protect thymectomized animals over extended time periods. Although the previously mentioned experiments utilizing thymus grafts, diffusion chambers and cell suspensions have been construed as indicating a noncellular thymus factor capable of restoring immunological reactivity in thymectomized animals (Miller and Osoba, 1967), attempts to isolate and demonstrate the effects of a thymus humoral factor have been inconclusive. Metcalf's lymphopoiesis stimulating factor (Metcalf, 1956) is often referenced by thymus researchers but attempts to restore immunological competence to thymectomized animals utilizing thymus extracts obtained according to the procedure of Metcalf have proved unfruitful (Dalmasso et al., 1963; Miller, 1964). Other workers have reported isolating thymus humoral factors which stimulate the lymphoid system (Comsa and Bezssonoff, 1958; Jankovic, Isakovic and Horvat, 1965; Comsa, 1966; Brunkhorst and Herranen, 1967), but definite proof of the existence of an effective thymus humoral factor produced under physiological conditions is still lacking (Miller and Osoba, 1967).

Embryologically the thymus arises as an outgrowth of the third and fourth branchial pouches (Bloom and Fawcell, 1964). The thymus is, therefore, of epithelial origin. However, considerable doubt remains as to the origin of the lymphoid cells found in the thymus. The thymus. The experiments of Auerbach (1960, 1961) strongly suggest that the thymic lymphoid cells arise directly from the epithelial elements of the thymus with mesenchymal tissue exerting a morphogenetic effect. Auerbach's experiments with mice are supported by those of Ackerman and Knouff (1964, 1965) whose results suggest that the thymic lymphocyte of embryonic chicken and hamster have an epithelial origin. On the other hand, the work of Smith (1965) has indicated that lymphoblasts may already have migrated to the epithelium of the branchial pouches prior to the outgrowth of the pouches to form the thymus. Although the transformation of thymic epithelial cells to lymphocytes remains an open question it is well established that, ontogenetically, the thymus is the first lymphoid organ to develop in the embryo (Archer et al., 1964; Good and Papermaster, 1964). It has been postulated that lymphocytes formed in the thymus during embryogenesis are seeded to the peripheral lymphoid tissues (Auerbach, 1961). If the thymic epithelium does give rise to lymphoid cells during embryonic development this situation must be modified to some extent soon after birth. In this regard, studies carried out using chromosomes markers in conjunction with grafted thymuses (Harris et al., 1964), with bone marrow grafts (Tyan and Cole, 1965), and with parabiosis (Harris and Ford, 1964; Brumby and Metcalf,

1967) have convincingly demonstrated an afferent flow of cells to the thymus from myeloid tissues. However, there is also in the adult animal a low rate of efferent flow of cells from the thymus to peripheral lymphoid tissues as indicated by radio-labelling studies (Nossal, 1964a, 1964b; Weissman, 1967) and studies with chromosome markers (Harris et al., 1964; Harris and Ford, 1964). The low rate of efferent flow of lymphocytes from the thymus is puzzling in view of the high mitotic activity found in the thymus which is 4 to 6 times that found in the lymph nodes or spleen in the case of young mature animals (Andreasen and Christensen, 1949). Thus, it seems that many lymphocytes flowing into the thymus or being born there must die locally in a short time. This led Burnet (1962) to postulate that the thymic epithelium may give rise to progenitors of immunologically competent cells and may "censor" those cells or aberrant cells coming in from the "peripheral" lymphoid organs destroying those cells capable of reacting against "self" antigens.

The concept that the thymus may "censor" self reacting clones of cells has led to experiments dealing with the role of the thymus in controlling immunological tolerance. Several series of investigations have been carried out (Isakovic, Smith and Waksman, 1965; Tolluett and Waksman, 1966) but there is not sufficient evidence to support the thesis that tolerance is induced by interaction of antigens with the thymus (Miller and Osoba, 1967).

The characteristics of the thymus thus far mentioned suggest that it is a "central" lymphoid organ controlling the development of cells found in the "peripheral" lymphoid structures. However, investigations have been conducted which indicate that the thymus may not be the only "central" lymphoid organ participating in the ontogeny and maintainence of the lymphoid system. Work conducted as early as 1956 has indicated that the bursa of Fabricius in fowl is an organ as important or nearly as important as the thymus in the ontogeny of the immune system (Glick, Chang and Jaap, 1956). Investigations carried out using rabbits have shown that the cecum may be analogous to the bursa of Fabricius (Archer, Sutherland and Good, 1963; Archer, Papermaster and Good, 1964). Recent work in mice has intimated that there may be a second central lymphoid organ in those animals (Dukor, Dietrich and Rosenthal, 1966; Rogister and Lejeune, 1964; Rogister, 1965; Sinclair and Millican, 1967; Takeya and Nomoto, 1967a, b). The bursa of Fabricius is a lymphoid organ in fowl which develops from an outpouching of the hind gut and bears some histological similarity to the thymus. The experiments of Glick, Chang and Jaap (1956) and Chang, Rheins and Winter (1957) showed that its removal early in life resulted in marked diminution of antibody response to soluble antigens. Subsequent work wherein hormonally bursectomized chickens were used demonstrated that there was a dissociation of immunologic responsiveness in the chicken. Bursectomy did not inhibit skin homograft rejection while it did inhibit response to soluble antigens and reduced delayed hypersensitivity reactions. Hormonally induced

atrophy of the thymus cortex and bursa inhibited response to soluble antigens, mitigated delayed hypersensitivity reactions and delayed the rejection of skin homografts, but the ability of blood cells to produce lesions on chorio-allantoic-membranes was still present (Warner, Szenberg and Burnet, 1962; Warner and Szenberg, 1964). Surgical removal of the bursa, or thymus or both in young chickens followed by sub-lethal irradiation allowed the recognition of two morphologically distinct cell systems in the peripheral lymphoid system. The thymus evidently governs cellular immunity by seeding small lymphocytes to the circulation whereas the bursa governs the production of immunoglobulins by seeding large lymphocytes to the circulation (Cooper et al., 1966). Studies conducted with rabbits have shown that the appendix of the animal may be analogous to the bursa in fowl. Archer, Sutherland and Good (1963) reported that rabbits responded atypically to neonatal thymectomy in that recovery of the immune system following neonatal thymectomy was not as great as nor as general as the impairment following neonatal thymectomy of mice, and that by 14 weeks of age the rabbits appeared to have recovered normal levels of lymphocytes in the peripheral blood. Of the lymphoid organs they examined only the development of the appendix was not hindered by neonatal thymectomy. Further studies by this group (Sutherland, Archer and Good, 1964) showed that removal of both the thymus and the appendix from neonatal rabbits severely impaired the ability of the animals to respond to stimulation with soluble antigens and to reject homografts. Further,

they found no apparent recovery of the peripheral lymphocyte level by 14 weeks of age. Recent work has indicated that mice may recover immunologic responsiveness spontaneously over a period of time following neonatal thymectomy. Rogister and Lejeune (1964) reported an increase in ability to respond with hemagglutinins to sheep red cell immunization in neonatally thymectomized mice when tested at 60 days whereas they failed to report 30 days post thymectomy. Rogister (1965) latter extended these findings and reported an increased ability to reject homografts as a function of time post thymectomy as well as increased numbers of peripheral lymphocytes which reached near normal levels by 150 days post thymectomy. Extensive studies by Dukor, Dietrich and Rosenthal (1966) indicated that by 9 - 13 weeks post thymectomy colony bred Swiss mice recovered their ability to form hemagglutinins and hemolysins following injection of sheep erythrocytes. They also reported that antibody-plaque-forming ability had increased over a 13 week interval post thymectomy, but they found no increase in the numbers of peripheral lymphocytes. On the other hand, they reported no spontaneous reconstitution in the case of neonatally thymectomized highly inbred CBA mice. Their work is substantiated to some extent by that of Sinclair and Millican (1967) who reported a delayed development of the ability to respond with hemolysins to immunization with sheep erythrocytes in neonatally thymectomized, colony bred Swiss mice during a post immunization time-course study involving 10 days to 6 months. Takeya and Nomota (1967a) have reported increasing ability to respond

with hemolysins in neonatally thymectomized inbred mice of the SL strain following stimulation with sheep red cells over a period of 120 days post thymectomy. They also reported decreasing survival times for homografts and increasing numbers of antibody plaque forming cells over the course of the observation period although the mice had not reached control levels by the end of the experimental period. Of possible significance is a report by Kalpaktosoglou, Yunis and Good (1967) which indicated that neonatal splenectomy depresses the lymphocyte levels of C3H mice and results in a considerable incidence of wasting disease. They suggested that the spleen may be important in the ontogenetic development of hematopoietic tissue in mice.

The work presented in the following pages consists of a study of the immunological recovery of Swiss Manor mice thymectomized at birth. This study was carried out in order to confirm and extend the reports on the immunological recovery of neonatally thymectomized mice. Further, the work was undertaken to provide data for comparison with a separate study being conducted in this laboratory dealing with the immunological recovery of Swiss Manor mice whose immune system was suppressed at birth by treatment with cortisol acetate.

# MATERIALS AND METHODS

## Experimental Animals

The non-inbred Swiss Manor mice used in this study were originally obtained from the germ-free stock of Manor Farms, Staatsburg, New York in 1964. A conventionally reared and colony bred stock of these mice has since been maintained in the Department of Botany and Microbiology, Montana State University.

The inbred A/jax mice used in these experiments were originally obtained from the Jackson Memorial Laboratories, Bar Harbor, Maine in 1964 and have since been maintained here with frequent brother-sister matings.

All stock animals were fed Purina Laboratory Chow while breeding mice were maintained on Purina Mouse Breeder Chow. Experimental animals were fed Purina Mouse Breeder Chow supplemented with Quaker Rolled Oats and water ad libitum.

## Surgical Procedures

### Anesthesia

New born mice were anesthetized by a cooling technique modified from the procedure described by East and Parrott (East and Parrott, 1962). Newborn mice were gently separated from their mother and placed three at a time into a 50 ml. plastic beaker. The beaker containing the mice was then placed into the freezing compartment of a domestic refrigerator and held at -10° C for 8 minutes. At the end of 8 minutes the mice had ceased to breathe, did not respond to tactile stimuli and had lost all

pink color. The mice were then considered fully anesthetized and were operated on within the next 8 minutes. Animals were revived by gentle handling and warming under a lamp.

Sodium pentobarbital (Nembutal-Abbott Laboratories) was used for anesthesia of adult mice. Mice to be anesthetized were weighed to the nearest tenth of a gram and were injected intraperitoneally with .02 ml of a 1:10 aqueous dilution of pentobarbital per gram of body weight. Recovery of anesthetized animals was facilitated by warming them under a lamp.

Thymectomy

Thymectomy of new born mice was performed using an amalgamation of techniques modified from those described by several groups of investigators (Gross, 1959; Miller, 1960; East and Parrott, 1962). Newborn Swiss Manor mice (less than 24 hours old) were anesthetized by the cooling technique above described and were taped to the surface of a chilled glass operating platform with Scotch tape. Taping was done so that the mouse was positioned on its back with its head sharply extended toward the operator. Extension of the head was necessary in order to draw the thymus forward in the mediastinal cavity.

A sternal splitting incision was made utilizing a pair of angle point irridectomy scissors one point of which was inserted at the manubrial notch and pushed, with the point held high, to the level of the fourth rib. The sternum was then split with a single stroke of the

scissors and the incision was spred with a pair of curved fine point forceps to expose the thymus. The thymus was removed by gentle aspiration at its anterior apices with a pasteur pipette attached with flexible rubber tubing to a vacuum pump. The incision was closed with one to two interupted 4-0 silk autures placed through the skin with no attempt being made to approximate the edges of the sternum. A drop of collodion was applied to the wound in order to seal it, prevent pneumothorax, and to reduce maternal cannibalism. The young were returned to their mother upon recovery from the effects of anesthesia. Sham thymectomy was performed by duplicating all operative procedures but no suction was used and the thymus was left in place.

Initially, immediate operative mortality was extreme but with practice mortality was reduced to less than 5%. However, maternal cannibalism was high with approximately 25% of all operated litters being lost.

Completeness of thymectomy was determined by sacrificing the mice at the beginning or end of experimental procedures and checking the mediastinal cavity macroscopically for evidence of thymic tissue. Suspicious tissue was removed for histological examination. Animals presenting thymus remnants were used as partially thymectomized controls.

Splenectomy

Newborn Swiss Manor mice (less than 24 hours old) were anesthetized by the cooling technique and were taped to a chilled glass operating

platform in such a way as to expose the left lateral surface of the animal. A transverse incision was made over the region of the stomach with irridectomy scissors. The stomach was extruded through the wound and turned slightly to expose the spleen which was adherent to the dorsal surface of the stomach. The spleen was then removed by aspiration with a pasteur pipette attached to a vacuum pump with flexable rubber tubing. Following removal of the spleen, the stomach was gently pushed back into the abdominal cavity and the incision was closed with three to four interupted 4-0 silk sutures. A drop of collodion was applied to the wond to help prevent maternal cannibalism and the young were returned to their mother. Sham splenectomy was performed by duplicating all operative procedures except that no suction was used and the spleen was left intact and in situ.

Immediate operative mortality was nil and mortality due to maternal cannibalism was less than 20%.

Completeness of splenectomy was determined by sacrificing the mice at the end of the experimental period and examining the abdominal cavity for the presence of splenic tissue. Animals having splenic remnants were discarded from the experiment.

Skin grafting

Skin grafting of adult Swiss Manor mice was performed using a modification of the technique developed in E. J. Eichwald's laboratory (Eichwald, 1967). To prepare the graft beds, mice were first anesthetized

with pentobarbital and then pinned to an operating board in such a fashion as to expose and stretch the left dorsolateral skin of the midsection. The area was wetted with 70% ethanol and shaved. A square section approximately one centimeter on a side was then removed using a razor blade. Into the graft bed was placed a close fitting graft or autologous or allogeneic tail skin. The mouse was subsequently removed from the board and a strip of transparent polyester plastic approximately 8 x 2 cm was wrapped tightly around the animal's midsection and secured with heat resistant Scotch tape. This dressing permitted visulization of the graft, prevented scratching of the graft by the animal or its cage mates and held the graft securely in place without the need of sutures. The dressing was changed on the 3rd and 6th day post-graft and removed on the 9th day post-graft.

Grafts were prepared from the tail skin of anesthetized or sacrificed donor animals. The tail was amputated with a razor blade and the skin was split down the midline of the tail. The skin was then peeled free and cut into appropriately sized grafts. The proximal portion of the tail was not used as it was usually severely traumatized during the stripping procedure. Grafts were routinely placed on recipients within 15 minutes of removal from the donor and were kept moist on saline soaked gauze prior to use.

Graft rejection was evaluated by macroscopic examination of the graft daily after the 9th day post-grafting. Rejection was considered to have occurred when the graft had become brown and dry.

## Production of Graft versus Host Disease

Thymectomized or sham-thymectomized Swiss Manor mice 30 to 70 days of age served as spleen cell donors. Following sacrifice by cervical dislocation the spleen was aseptically removed from each donor and placed in a small sterile petri dish (60 mm in diameter) containing 1 ml of sterile phosphate buffered saline pH 7.2 (PBS). The spleen was then gently ground apart using a sterile plunger from a 10 ml syringe. Coarse debris was removed from the splenic preparation and a 1:1,000 dilution in 4% acetic acid was made from an aliquot of the preparation. The nucleated cells were counted with a standard hemocytometer and the cell concentration of the splenic suspension was adjusted to contain $20 \times 10^6$ or $40 \times 10^6$ nucleated cells per .1 ml. To the preparation were added 500 units of dihydro-streptomycin sulfate (Nutritional Biochemicals Corporation) and 1,000 units of penicillin "G" (Nutritional Biochemicals Corporation) per 1 ml. The preparation with the antibiotics was incubated at 37° C for 30 minutes prior to being injected into mice.

A dose of either $20 \times 10^6$ or $40 \times 10^6$ spleen cells from neonatally thymectomized or sham thymectomized Swiss Manor mice was injected intraperitoneally into neonatal A/jax mice within 24 hours of birth. The dose was administered with a 1 ml tuberculin syringe fitted with a 25 gauge needle through the thigh muscle into the peritoneal cavity in order to minimize leakage. Mice which leaked excessively were discarded from the experiment.

## Criteria of Graft versus Host Disease

The criteria used for judging graft versus host disease in mice injected with allogeneic spleen cells within 24 hours of birth were failure to gain weight normally and ultimately death which commonly occurred within 21 days post-injection. Mice dying before day 6 post-injection were considered to be victims of injection trauma or maternal cannabilism.

Other symptoms noted during the course of graft versus host disease were diarrhea, a hunched posture, high-stepping gait and alopecia.

## Immunization with Sheep Erythrocytes

Sterile sheep blood preserved in Alsever's solution was thrice washed with saline prior to injection. A dose of 0.1 ml of a 10% solution of packed erythrocytes in saline was injected by the intraperitoneal route into 30, 50, and 70 day old Swiss Manor mice which had been thymectomized, sham-thymectomized or partially thymectomized at birth. The mice were bled from the tail vein 5 and 10 days post-injection. Ten drops of blood were collected in 0.5 ml of saline giving a serum dilution of approximately 1:5. The blood was allowed to clot and the sera were collected following low speed centrifugation. The sera were stored at 5° C and hemolysin and agglutinin titers for sheep erythrocytes were determined within two weeks of the bleeding date.

## Antibody Titration

Endogenous complement was inactivated by incubating the sera at 56° C for 30 minutes. The sera were then serially diluted 1:1 starting with the 1:5 dilution and ending at a dilution of 1:2560. To each tube was added 0.1 ml of thrice washed 1% sheep erythrocytes in saline and the tubes were incubated at 37° C for 30 minutes. Hemagglutination was read following low speed centrifugation of the reaction tubes. A titer of 0 was arbitrarily assigned to sera producing no detectable hemagglutination at a dilution of 1:5. Hemagglutination patterns were scored 4+ to 1+ and final titer was expressed as the serum dilution in the last tube showing 1+ hemagglutination.

Hemolysin titrations were carried out in the same reaction system as that employed on agglutination titrations. Following reading of the hemagglutination patterns one drop of guinea pig complement (Difco) diluted 1:5 in saline was added to each tube. The tubes were agitated and incubated at 37° C for 30 minutes. The sera were held at 10° C overnight and the titers were read the following morning. A limiting dilution giving complete hemolysis was considered the titer of the serum. An arbitrary value of 0 was assigned to those sera having a titer of 1:10 or less.

## Bacteriologic Methods

Attempts were made to isolate microorganisms from the spleens of thymectomized or sham-thymectomized 30 day old Swiss Manor mice. Mice

were killed by cervical dislocation and their spleens were aseptically removed. The spleens were placed into small petri dishes containing 1 ml of sterile saline and ground apart with a sterile plunger from a 10 ml syringe. Freshly prepared blood agar plates, 5% sheep blood in blood agar base (Difco), and thioglycolate (BBL) were inoculated with 0.3 ml each of the aseptically prepared spleen suspension from each mouse. One series of blood agar plates was incubated anaerobically and the other aerobically. All incubations were carried out at 37° C and media was held for at least 10 days at the temperature before considered negative for growth.

In six cases, the spleen suspension was freeze-thawed twice using a bath of acetone and dry ice before being inoculated into the media. This was done in order to release possible intracellular organisms. Organisms isolated from splenic tissues were characterized by standard bacteriological procedures.

## Histological Procedures

Suspected thymic remnants were removed from the mediastinal cavity of supposedly thymectomized mice for histological examination. Immediately after removal from the animal the tissue samples were placed in buffered (pH 7.6) formalin and held there until processed for sectioning. The tissues were processed according to standard histological procedures. Dehydration and clearing was carried out in a series of alcohols and toluene and infiltration was with paraffin at 52° C. Sections

were cut at 10 micra and stained with a standard hematoxylin and eosin procedure.

# RESULTS

## Time-Course Studies on the Ability of Spleen Cells from Neonatally Thymectomized Mice to Induce Graft-versus-Host Disease

Time-course studies on the ability of spleen cells from neonatally thymectomized Swiss Manor mice to ellicit fatal graft-versus-host (GVH) disease in newborn A/jax mice were carried out according to the procedure previously described (Materials and Methods). The data presented in Table I shows that a recovery of the ability of spleen cells from neonatally thymectomized Swiss Manor mice to ellicit fatal GVH disease in newborn A/jax recipients was not detected when a dose of $20 \times 10^6$ cells from 30 to 70 day old donors was used. The over-all mortality in the above group was 9% whereas the mortality in the control mice which received $20 \times 10^6$ spleen cells from 30 to 70 day old neonatally sham-thymectomized Swiss Manor donors was 78%. On the assumption that $20 \times 10^6$ cells was too small a dose to allow detection of recovery, the number of cells injected was increased to $40 \times 10^6$ cells. When this dose was used a partial recovery of the ability of cells from 60 and 70 day old donors was detected (Table I). The degree of mortality among A/jax mice receiving $40 \times 10^6$ spleen cells from neonatally thymectomized Swiss mice in the 60 and 70 day old groups was 83% and 67% respectively, as compared to 100% mortality, regardless of age, when spleen cells from neonatally sham-thymectomized donors were used (Table I).