The in vivo reconstitution of congenitally thymusless mice
by Dale Darwin Isaak

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

Abstract:
Studies presented here confirm that congenitally athymic (nude) mice are deficient in their ability to produce plaque-forming cells, rosette-forming cells and serum antibodies specific for sheep red blood cells. They are also unable to reject skin homografts. These immune deficiencies were corrected by implanting neonatal Balb/c thymus glands into nude mice.

In contrast, the ability of nude mice to respond to sheep red blood cells or to reject homografts was not generated following the intraperitoneal implantation of millipore diffusion chambers containing thymus glands, pregnancy, or the injection of the polyanions dextran SO4 or poly acrylic acid. These experiments show that the immune impairment of nude mice can be repaired by thymus derived cells, but not by soluble factors of thymic origin or polyanionic chemicals. These results suggest that the site of action of such soluble factors and polyanionic chemicals is a thymus-derived cell rather than a cell derived directly from the bone marrow.

Statement of Permission to Copy

In presenting this thesis in partial fulfillment of the requirements for an advanced degree at Montana State University, I agree that the Library shall make it freely available for inspection. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by my major professor, or, in his absence, by the Director of Libraries. It is understood that any copying of publication of this thesis for financial gain shall not be allowed without my written permission.

Signature Dale Darwin Isaak

Date May 29th, 1973

THE IN VIVO RECONSTITUTION OF CONGENITALLY THYMUSLESS MICE

by

DALE DARWIN ISAAK

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree

of

MASTER OF SCIENCE

in

Microbiology

Approved:

William G. Walter
Head, Major Department

Norman D. Reed
Chairman, Examining Committee

Henry L. Parsons
Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

June, 1973

ACKNOWLEDGMENTS

I wish to express my sincere thanks to Dr. Norman D. Reed for his guidance and encouragement, without which these studies would not have been possible.

I also wish to acknowledge the cooperation and support of the Microbiology faculty, particularly Dr. John W. Jutila and Dr. David Stuart for their help in preparing this manuscript.

Financial support for this research was provided by Research Grant Number AI10384-01, 02 from the U. S. Public Health Service.

TABLE OF CONTENTS

LIST OF TABLES

ABSTRACT

Studies presented here confirm that congenitally athymic (nude) mice are deficient in their ability to produce plaque-forming cells, rosette-forming cells and serum antibodies specific for sheep red blood cells. They are also unable to reject skin homografts. These immune deficiencies were corrected by implanting neonatal Balb/c thymus glands into nude mice.

In contrast, the ability of nude mice to respond to sheep red blood cells or to reject homografts was not generated following the intraperitoneal implantation of millipore diffusion chambers containing thymus glands, pregnancy, or the injection of the polyanions dextran SO_4 or poly acrylic acid. These experiments show that the immune impairment of nude mice can be repaired by thymus derived cells, but not by soluble factors of thymic origin or polyanionic chemicals. These results suggest that the site of action of such soluble factors and polyanionic chemicals is a thymus-derived cell rather than a cell derived directly from the bone marrow.

INTRODUCTION

Though as early as 1900, Beard had described the involvement of the thymus in the seeding of lymphocytes into the lymphoid system of animals, the role of the thymus in the proper functioning of an animal's immune system was not recognized until much later, primarily because of the failure to detect antibody production within the thymus. Askonas and White (1) described the lack of plasma cell accumulation and antibody production within the thymus, in contrast to other lymphoid tissues such as the spleen and lymph nodes, of guinea pigs immunized with ova-albumin and then assayed in vitro. Further support for the lack of a thymic involvement in the proper functioning of the immune system was inferred from results obtained by Harris et al. (2). Using adult thymectomized rabbits immunized with sheep erythrocytes (SRBC) one day following thymectomy, Harris and coworkers found no significant differences in hemolytic antibody titers in thymectomized animals and control non-thymectomized animals. Similarly, Maclean et al. (3) could find no difference between the immune responses of control rabbits and thymectomized rabbits when assayed soon after thymectomy.

Repeated clinical observations by Good (4) and Gafni et al. (5), however, strongly suggested a possible involvement of the thymus in antibody production. The repeated association of a condition characterized by total lack of antibody production, or agammaglobulinemia, and a second condition, thymoma, gave indirect evidence that impaired thymus function could lead to impaired antibody production.

Although these observations seemed to contradict the earlier results described by Harris (2) and Maclean (3), Glick and co-workers were able to partially resolve them. These studies (6) demonstrated that following the early surgical removal of the bursa of Fabricius, a lymphoid organ associated with the dorsal part of the cloaca in chickens, decreased antibody production to S. typhimurium occurred in later life. Similarly, Mueller, Wolfe and Meyer (7) were able to show decreased antibody production in chickens antigenized with bovine serum albumin, after the administration of 19-nortestosterone, a hormone which, when injected into the egg on the fifth day of incubation, causes a reduction in the size of the spleen and the thymus and a complete absence of the bursa. These observations stimulated renewed interest in the immunological role of the thymus. Experiments involving the early surgical removal of the thymus provided striking results.

In contrast to the lack of obvious immediate effects of adult thymectomy on the immune system as reported by Harris et al. and Maclean et al., neonatal thymectomy yielded animals with severely impaired immune systems. Miller (8) reported that mice thymectomized within 16 hours of birth were unable to reject homografts normally, showed a severe depletion of circulating peripheral lymphocytes along with an acute lack of germinal centers, and showed a reduced number of plasma cells. Similarly, Martinez et al. (9) reported the prolonged survival

of homografts in mice thymectomized within 24 hours of birth but not in mice thymectomized at 30 days of age. Associated with these immunological impairments in the absence of the thymus, Martinez and coworkers also reported a drop in the number of peripheral lymphocytes.

Although Miller's initial observations concerning the impaired immune response following neonatal thymectomy gave strong evidence for the importance of the thymus to the immune system, his observations concerning the severe depletion of circulating lymphocytes gave more direct evidence as to the role of the thymus in the immune system. Histological examinations of thymic tissue (10) revealed lymphocytic mitotic indexes to be 10 times those of subcutaneous lymph nodes and 5 times those of Peyer's patches and mesenteric lymph nodes. Similarly, using DNA turnover as an index of cellular proliferation, Andreasen and Ottesen (11) concluded that the greatest degree of lymphopoiesis takes place in the thymus, except in old age when the organ atrophies. Though many of these newly formed small lymphocytes are destined to die locally, others emigrate from the thymus. Murray and Woods (12), after injecting the thymus glands of guinea pigs with tritiated thymidine, looked for labeled lymphocytes in peripheral lymphoid tissue. Heavy labeling was associated with the mesenteric lymph nodes and the spleen, implying that a normal thymic function may be to generate lymphocytes which migrate to the peripheral lymphoid tissues.

A number of procedures are available for depleting animals of lymphocytes. When assayed these animals show varying degrees of immunological impairment. McGregor and Gowans (13) have shown that the removal of small lymphocytes via chronic drainage from a thoracic duct fistula severely impaired the ability of rats to respond to SRBC and tetanus toxoid. Similarly, Dougherty (14) has described the action of numerous chemicals and their role in causing the involution of lymphoid tissue and the disappearance of peripheral circulating lymphocytes. Jutila (15) reported that mice, given .2 mg. of cortisol acetate as neonates suffered a severe involution of lymphoid tissues, including the thymus and the spleen, and a decreased ability to respond to SRBC. A third method of depleting animals of their lymphoid cell populations has already been alluded to in the neonatal thymectomy work of Miller (8) which showed that in sham thymectomized mice the lymphocyte to polymorphonuclear leukocyte ratio increased progressively during the first 8 days after birth when near adult ratios were obtained. In mice thymectomized at birth, however, this ratio did not increase due primarily to an acute lymphopenia. Similarly, Mitchell and Miller (16) reported that thoracic duct cannulation of neonatally thymectomized mice yielded only 3-4% of the lymphocytes drained after 48 hours from sham operated mice, implying a severe reduction in the population of circulating small lymphocytes.

In addition to a severe reduction in the number of circulating small lymphocytes, Parrot et al. (17) have also given evidence for cellular depletion of peripheral lymphoid tissues in neonatally thymectomized mice. Histological examination of peripheral lymphoid tissues from these animals revealed a marked lymphocyte deficiency in the lymphocytic fields of the lymph nodes and the periarteriolar lymphocyte sheath of the spleen, the so called thymus-dependent areas.

Associated with the loss of the circulating pool of small lymphocytes in thymectomized animals are a number of immunological defects. Antibody production following antigenization of neonatally thymectomized animals has led to conflicting results, depending on the antigens used. Osoba and Miller (18) summarize that lower than normal levels of antibody are produced in neonatally thymectomized animals when immunized with SRBC, T_2 coliphage, diphtheria toxoid, ovalbunim, and certain serum proteins such as human gamma globulin and bovine serum albumin. On the other hand, neonatally thymectomized animals immunized with such antigens as MS-2 bacteriophage, ferritin and pneumococcus III capsular polysacharide yielded responses equal to their nonthymectomized counterparts. These conflicting results have led to classification of antigen into two groups, the thymus-dependent antigens and the thymus-independent antigens.

In addition to the immunological impairment associated with their responses to thymus-dependent antigens, neonatally thymectomized

animals have also been shown to be severely impaired with regard to cell-mediated immune responses such as the ability to induce graft-versus host reactions and the ability to reject homografts of both normal and malignant tissue. Good et al. (19) and Dalmasso et. al.(20) have shown that lymphoid cells from neonatally thymectomized mice are less able to induce graft-versus-host reactions than are similar cells from nonthymenctomized animals. In rats, Rieke (21) has observed that whereas two million thoracic duct lymphocytes from normal rats will induce a graft-versus-host reaction in an appropriate newborn host, as many as twenty million thoracic duct lymphocytes from neonatally thymectomized animals will not.

Mice thymectomized at birth, when grafted with either closely related skin (19) or with skin with major histocompatability differences, have been shown to be unable to reject the skin normally. Grafts either remained intact until the death of the animal or were rejected after prolonged survival periods. Fisher and Fisher (22) have shown that rats thymectomized at birth also have an impairment of their homograft rejection mechanisms.

Neonatally thymectomized mice have also been shown to be unable to reject tumor homografts. Martinez et al. (23) have described the inability of neonatally thymectomized mice to reject an allogeneic mammary adenocarcinoma of mouse origin, as opposed to their sham

thymectomized counterparts which rejected the tumor. Also, Osoba and Auersperg (24) have reported the establishment of a human cervical carcinoma in mice thymectomized at birth but were unable to establish the tumor in nonthymectomized controls.

Animals thymectomized as adults also show varying degrees of immunological impairment analogous to neonatally thymectomized animals but only under specific conditions. These animals, when challenged soon after surgery, were found to be immunologically capable of producing normal amounts of specific antibody when challenged with SRBC (25). That the thymus is still effective in lymphopoiesis in adult life, however, is strongly suggested by the studies of Cross et al. (26). Cross and co-workers reported that adult thymectomized mice exposed to a sublethal dose of irradiation exhibited a much prolonged recovery time as compared to non-thymectomized control animals. Similarly, adult thymectomized animals exposed to a potentially lethal dose of irradiation and subsequently protected with bone marrow cell inocula were rendered permanent immunological cripples. Miller and Osoba (18) summarized that the thymus is initially responsible during fetal life for the establishment of the long-lived recirculating population of immunologically competent small lymphocytes, and that even in adult life the thymus is necessary to maintain this population of cells.

A number of techniques, including the transplantation of thymic tissue, the inoculation of cell suspensions from various lymphoid sources, the administration of soluble thymic extracts, and the use of various chemicals have been employed to correct the immunological defects associated with neonatally thymectomized animals and adult thymectomized irradiated animals. Miller (27) has shown that the immunological defects associated with neonatally thymectomized animals can be corrected by thymus organ grafts. Thymus grafted animals recover their ability to reject homografts in both first and second set fashion and also their lymphoid tissues appear normal with the spleen, Peyer's patches and lymph nodes being well developed. Ducor et al. (28) have reported that in histological preparations thymic implants appeared to have regained normal thymic architecture within one week. The implant consisted largely of donor type cells until about two weeks when a replacement of donor cells by recipient lymphoid cells was observed. That these host type cells found in the implants are really lymphoid precursoral cells sequestered from the circulation by the thymus is suggested by the use of the T6T6 chromosomal marker in thymus grafting work (29), the use of radiation chimeras (30), and in parabiont studies (31). Dalmasso et al. (32), using spleen assays allowing them to distinguish host cells from donor cells in neonatally thymectomized mice grafted with allogeneic thymus tissue, showed that

host cells primarily were responsible for immunological activity in these mice, suggesting that the grafted thymus acts to mature the host lymphoid precursoral cells which passed through it and then out into the peripheral lymphoid system.

Dalmasso et al. (32) also give evidence that lymph node cell inocula and spleen cell inocula were effective in preventing wasting syndromes associated with neonatal thymectomy and in restoring immunological competence as measured by rejection of skin homografts and the ability to induce graft-versus-host disease, presumably because these cell inocula contained immunologically mature lymphocytes of donor thymus origin. In contrast to the effectiveness of lymph node and spleen cell preparations in establishing immune competence, thymus cell preparations were much less effective, suggesting that full immune competence of thymus derrived lymphocytes is not reached until they leave the thymus.

In addition to the cellular contribution of the thymus, a number of investigators have looked at the possibility of a thymus-derived soluble substance being involved in the immunological reconstitution of neonatally thymectomized mice. Levey, Trainin and Law (33) reported that the implantation of cell-impermeable millipore diffusion chambers (MDC) containing neonatal thymus glands into the peritoneal cavity of neonatally thymectomized mice would prevent wasting

syndromes and would establish near normal levels of peripheral blood lymphocytes and normal numbers of lymphocytes in lymphoid tissues, including the spleen, Peyer's patches and lymph nodes. Osoba and Miller (34) reported that neonatally thymectomized mice bearing MDC containing newborn thymus tissue were capable of rejecting skin homografts, whereas their non-implanted counterparts were not. Further work by these investigators (34) confirmed that neonatally thymectomized mice bearing MDC containing thymic tissue did not loose weight, were able to produce normal levels of 7S agglutinins to SRBC, rejected skin homografts within 25 days and showed varying degrees of lymphoid cellular reconstitution in lymph nodes, spleen and Peyer's patches. Their neonatally thymectomized non-implanted counterparts showed none of these characteristics. Histological examination of the thymic tissue in the MDC at the termination of the experiment revealed only fibroblasts, fibrous tissue and epithelial cells. These epithelial cells were postulated to have produced some hormonal substance that acted on lymphoid precursoral cells present in the tissues of neonatally thymectomized mice to trigger their differentiation into immunologically competent cells.

Osoba (35) has also described the reconstituting effect of this thymic hormone in a second system. Neonatally thymectomized female mice were raised to adulthood and bred. After delivering, these mice were

assayed and found capable of rejecting skin homografts and of producing anti-SRBC responses. Neonatally thymectomized females which had never given birth were found to be immunologically incompetent in each of these assays. Presumably the transplacental passage of some thymic humoral factor from the developing fetuses to the mother was responsible for her newly acquired immunological competence.

Attempts to reconstitute neonatally thymectomized animals with thymus extracts stem from an early report by Metcalf (36) in which he described the action of a lymphocytosis stimulating factor obtained from a thymus extract. De Somer et al. (37) have reported that in neonatally thymectomized mice, a single intraperitoneal (I.P.) injection of calf thymus extract (CTE) restored a normal white blood cell count within three days and prevented wasting syndromes. In addition to the establishment of normal peripheral blood pictures, CTE has also been found to restore skin and tumor homograft immunity in neonatally thymectomized mice and in adult thymectomized irradiated mice (38). Small and Trainin (39) also reported that CTE partially restored the ability of neonatally thymectomized mice to form antibody in a primary response against SRBC.

Chemical reconstitution attempts involving polynucleotides such as polyadenylic-polyuridylic acid (poly A:U) have stemmed from early work implicating the importance of an RNA and or RNA-antigen

complex involved in the activation of lymphocytes. Fishman and Adler (40, 41) isolated an RNA-rich fraction from immune macrophages which could, in vitro, cause nonimmunized lymphocytes to undergo blast transformation and to begin to synthesize antibody specific for the bacteriophages used to sensitized the macrophages. Braun and Cohen (42) postulate that the uptake of this activating RNA by lymphocytes may be controlled by specific antibody like receptor sites on lymphocytes. The antigen antibody-like reaction between lymphocyte-bound antibody and the antigen fragments detected in the RNA fraction (43) may serve to facilitate the entry of the RNA into the lymphocytes. Cone and Johnson (44) reported that the administration of poly A:U to neonatally thymectomzied mice restored their ability to produce rosette forming cells (RFC) specific for SRBC and to reject homografts, presumably because of latent thymus cell (T cell) activation by the poly A:U. Diamanstein et al. have also demonstrated an adjuvant effect of polyanionic chemicals such as polyacrylic acid (PAA) (45) and dextran SO_4 (46), which they found to increase the plaque-forming cell (PFC) response and the hemolytic antibody titers of mice challenged with SRBC. Also, they report that PAA and dextran SO_4, when administered to adult thymectomized, lethally irradiated mice, would reconstitute their response to SRBC (47).

Although studies involving characterization of T-cell depleted animals and attempts to reconstitute them have yielded much information

about the normal functioning of the thymus, there are a number of drawbacks involved in the use of T-cell depleted animals. Humphrey et al. (48) have suggested the possibility that early in the ontogeny of the animal, various cell clones are seeded from the thymus so that prior to the time of parturation the animal may have present in its peripheral lymphoid system small numbers of thymus-derived cells. Similarly, the use of adult thymectomized, lethally irradiated, bone marrow reconstituted animals has the dual disadvantage of T-cell survival following irradiation and the unavoidable introduction of low numbers of T-cells present in bone marrow preparations used in reconstitution attempts (49). Because of the presence of these T-cells, results obtained from neonatally thymectomized animals or adult thymectomized, irradiated, bone marrow reconstituted animals may not be completely valid when interpreted as having happened in an environment free of T-cells.

With these drawbacks in mind, the most useful animal for studying thymus participation in the immune response may not be the neonatally thymectomized or adult thymectomized animal, but rather the nude mouse described initially by Flanagan (50). Pantelouris (5) has characterized this mouse as being congenitally athymic in that the development of the thymus is arrested genetically at 14-15 days of in utero development, with the organs appearing as narrow strips of

tissue devoid of the lymphoid cells commonly associated with the development of normal thymus glands.

Though Raff and Wortis (52) have indicated the presence of very low numbers of thymus-derived, θpositive cells in nude mice, these mice are completely defficient in responses requiring thymus-derived lymphocytes. Responses to thymus-dependent antigens such as sheep red blood cells are very low (53, 54, 55, and 56) but responses to thymus independent antigens such as E. coli lipopolysaccharide and type III pneumococcal polysaccharide are normal (57). Also, cell-mediated immune responses are severely impaired in nude mice. Rygaard (58) has described the inability of nudes to reject either primary or secondary rat heterografts and Pantelouris (59) and Kindred (53) have given evidence that nudes fail to reject homografts. Povlsen and Rygaard (60 and 61) have reported the successful transplantation of human adenocarcinoma to nude mice and Giovanella et al. (62) have reported that human melanoma cells injected into nude mice develope into invasive tumors which are not rejected.

In addition to this lack of cell-mediated immunity, de Sousa et al. (63) have reported that nude mice have a lack of lymphocytes in the thymus-dependent areas of the peripheral lymphoid organs and a decreased number of circulating lymphocytes.

Attempts to correct these immunological deficiencies in nude mice have met with varying degrees of success. Wortis et al. (64) and

Pantelouris (65) have reported that the grafting of thymus organs will promote the rejection of homografts. Also, Kindred has reported that the injection of related thymus cells (66) or spleen, lymph node and educated thymus cells (67) will restore their ability to produce hemagglutinins and PFC specific for sheep red blood cells.

Because of the incompleteness of the data on reconstitution studies in nude mice, the following experiments were done in order to gain more information on the normal functioning of the thymus in the immune system as well as the nature of the defect in nude mice.

MATERIALS AND METHODS

Animals

Nude mice (nu/nu) and their phenotypically normal litter mates (+/nu or +/+) were the offspring of heterozygous animals obtained by crossing Re+/+nu males (supplied by R. C. Roberts and D. S. Falconer at the University of Edinburgh, Scotland) with females from our specific pathogen free (SPF) Baylor Balb/c colony and were used as the principle experimental animals throughout the study. Inbred Baylor Balb/c mice, obtained initially in 1968 from Baylor Medical School and subsequently maintained by brother-sister matings under SPF conditions were used as donors of thymus glands and lymphoid cell inocula. CBA/J mice, obtained initially in 1971 from Bar Harbor Laboratories and subsequently maintained by brother-sister matings under conventional rearing conditions, were used as donors of thymus glands and also as donors of skin in homograft immunity studies. These animals differ from the Balb/c and nude lines at the H-2 histocompatability locus.

All animals were given sterilized Purina 5010C and acidified chlorinated water (68).

Millipore Diffusion Chambers

Millipore Diffusion chambers (MDC) constructed from Scotch plastic film (number 471) and millipore membrane filters (cat.

GSWP-013-00 with 0.22 μ pore size and # VCWP-013-00 with 0.1 μ pore size) according to the method described by Bartlett and Prehn (69) were used throughout the study. Immediately preceeding implantation, the chambers were exposed to ultraviolet light at a distance of 3 inches for 7-8 minutes to reduce the microgial flora. After sealing the tape edges, the chambers were surgically implanted in the peritoneal cavity of anesthetized nude mice. A series of interrupted silk sutures were used to sew up the inner fascia and then the outer integument layers.

Chemicals

Chemicals used in attempts to stimulate antibody production included polyadenylic-polyuridylic acid (poly A:U), poly acrylic acid (PAA), and dextran SO_4. Poly A:U was formed by mixing equal volumes of polyadenylic and polyuridylic acid (lot numbers 71 and 78 respectively) obtained from Miles Laboratories Inc. and was administered intraperitoneally (I.P.) in 0.08 mg amounts immediately preceeding antigen administration. Polyacrylic acid (K and K Laboratories of Hollywood Calif. Inc.) was administer I.P. in 1.3 mg/.25ml of phosphate buffered saline amounts 2 hours preceeding the antigen. Dextran SO_4 (General Biochemicals lot number 51593, M. W. 500,000) was given I.P. in 0.5mg/.25ml phosphate buffered saline amounts ½ hour prior to antigen administration. No attempt was made to assure sterility of the solutions used.

Neonatal thymectomy

Newborn littermate animals were anesthetized by gentle cooling at -10^{o} C for 7-8 minutes in the freezing compartment of a common household refrigerator or until the animals had lost their pink color. Neonatal thymectomy was performed within the next 5 minutes by the method described by Hunter (70). Briefly, the newborns were taped to a chilled petri dish lid in crushed ice such that the head was bent sharply downward and toward the operator. A single incision through both the skin and the sternum was made to expose both glands which were then removed by gentle suction through a pasteur pipette attached to a sink aspirator. One or two interrupted silk sutures through the skin were used to close the wound and to draw the sternum together. The animals were placed under a warming light and returned to the mother following recovery from surgery. Completeness of thymectomy was determined by visual observation of the mediastinal cavity following experimental procedures.

Assays for Immunological Competence

Throughout this study, assays for the response of mice to a single dose of 1×10^{8} SRBC or 0.25 ml of a 10% suspension of SRBC consisted of the detection of specific plaque-forming cells (PFC), rosett-forming cells (RFC) and humoral antibody titers, including

hemagglutinating (HA) titers and hemolytic (HL) titers 5 days after the I.P. administration of the SRBC.

Specific PFC were detected by the slide modification of the Jerne plaque assay (71). Briefly, single celled suspensions from the spleens of immunized mice were prepared in Dutton's balanced salts. After suitable washing, varying numbers of cells were added to 0.5% agarose containing a 1:15 dilution of SRBC. Specific PFC were counted as definite zones of lyses.

Rosette-forming cells (RFC) specific for SRBC were detected by a slight modification of the technique described by Biozzi (72). Briefly, 0.1 ml of a 5% suspension of SRBC plus 0.1 ml of a mouse spleen cell preparation containing approximately 3-6 x 10^6 cells were incubated in 0.8 ml of phosphate buffered saline, at 4^o C overnight. RFC were counted the following day in a hemocytometer. Cells were considered to be positive rosette-formers when a minimum of 8 SRBC were found adhering to their surfaces. The actual number of spleen cells in the reaction mixture was then detected by lysing the red cells in 2% acetic acid. RFC were expressed as the number contained in 10^6 spleen cells.

Serum humoral hemagglutinating antibody levels were determined by the method described by Adler (73). After bleeding from the retro-orbital sinus, twofold serial dilutions of serum samples were prepared in modified barbitol buffer (74). Agglutinin (HA) titers were read

after centrifugation, following which the SRBC were resuspended and 0.1 ml of a 1:10 dilution of guinea pig complement was added to each tube. Hemolysin (HL) titers were read after 1½ hours incubation at 37° C.

In addition to the response to SRBC, immune competence was also measured as the ability to reject skin homografts. All haired recipients and donor mice were clipped and Nair was applied to remove residual hair at least 24 hours prior to grafting. Grafting procedures were adapted from Billingham and Silver (75). Briefly, full thickness grafts were prepared from previously shaved and "naired" 6 week old male CBA/J mice by skinning off the back of the donor, pinning the skin down to a disecting board and then scraping off the underlying membraneous layers. The resulting layers were then cut into circular pieces 10 mm in diameter which were then rinsed twice in phosphate buffered saline (PBS) and suspended in a final bath of PBS. Recipient mice were anesthetized with appropriate amounts of nembutal (76) and circular graft beds were cut on the dorsal area of the thorax above the rib cage. Open fit beds cut with a circular scissors were used and donor skin was placed directly on the open bed. Following the application of appropriate dressings (75), mice were allowed to recover and were returned to their cages. Casts were removed on day 8 following primary grafting procedures and on day 6 following secondary grafting procedures. Graft rejection was monitored by the development of inflamed

centers of necrosis. When the bed was 100% involved in necrosis, rejection was considered to be complete.

Thymus grafting

Twenty-four hour old Balb/c or CBA/J mice were used as thymus gland donors. Immediately preceding transplantation, donors were killed and their thymus glands were harvested into Hanks balanced salts solution. Recipients were anesthetized with nembutal and a small incision through both the dermal and the inner fascia layers was made in each axillary region. One gland was placed in each incision and 2-3 interrupted silk sutures were used to close up the incision. Animals were assayed for immune competence 2-4 weeks after thymus transplanting.

Thymus cell suspensions

Thymus cell suspensions were made by gentle screening of 24 hour old Balb/c thymus glands through stainless steel mesh until single cell suspensions were obtained in Hanks balanced salts solution. Cells were quantitated and given I.V. by tail vein injection after trypan blue exclusion viability studies were done.

RESULTS

Homograft immunity and anti-SRBC response of nudes grafted with thymus glands and sham-operated nudes.

The grafting of (CBAxAKR) F1 thymus glands to nude mice (65) has given evidence for a reconstitution effect of the glands on nude mice, but the numbers of animals involved and the results obtained did not lead to complete and satisfying conclusions. The first experiments dealing with the grafting of Balb/c thymus glands to the axillary regions of nude mice were done in an attempt to firmly establish the effect of normal thymus glands on nude mice. Experimental nude animals were given one neonatal Balb/c thymus gland in each axillary area (i.e., one donor mouse was used for each recipient) and control animals were sham operated. After a period of 2 to 4 weeks, both groups were given CBA skin grafts which differ from Balb/c at the H-2 histocompatability locus. Table I shows the results of these experiments. Animals grafted with thymus glands were able to reject the histoincompatable CBA skin in 18.7 days after primary challenge and in 10.9 days after secondary challenge, implying that an active functioning cellular immune system was operating in nudes grafted with thymus glands. As early as two weeks post operative, animals were fully capable of rejecting skin homografts as rapidly as those animals which were assayed one month following thymus gland grafting. In contrast to this, sham-operated nudes never rejected their skin homografts and all grafts remained healthy until the death of the animals or the termination of the experiment. Hair growth from the graft was taken as absolute evidence of graft acceptance. In addition to skin homograft immunity,

both experimental and control groups were assayed for the ability to produce specific PFC, RFC, and HA and HL antibodies when immunized with SRBC. When all animals had clearly rejected their grafts or had grown large patches of hair from the graft sites, 1×10^8 SRBC were administered I.P. Five days later, the animals were bled for serum, plaqued for specific PFC and assayed for specific RFC. Table I shows the results of these studies. An increase in the number of PFC is evident in the experimental group as compared to the sham-operated group. Similarly, grafted animals showed a sharp increase in the number of RFC when challenged with SRBC, as opposed to the sham-operated animals which remained at a low background level similar to that described by Reed and Jutila (57). Table I also shows that following immunization with SRBC, the experimental thymus-grafted group was able to form much higher levels of HA and HL antibodies, as compared to similarly treated sham animals.

When nude animals were grafted similarly with CBA/J thymus glands and then challenged three weeks later with CBA skin grafts, the animals were not able to reject the skin grafts, as shown in Table II. Three weeks after skin grafting, both the experimental thymus-grafted nudes and the control sham-operated nudes were immunized with 1×10^8 SRBC. Five days later, serum was harvested and titrated for both HA and HL antibody levels. Table II shows that the thymus-grafted animals

TABLE I

HOMOGRAFT RESPONSE AND ANTI-SRBC RESPONSE OF NUDES GRAFTED WITH BALB/C THYMUS GLANDS OR SHAM-OPERATED NUDES

GROUP	HOMOGRAFT SURVIVAL TIME OF DAYS		PFC/10^6		PFC/SPLEEN		SERUM Ab		RFC/10^6
	1^o	2^o	D[d]	I[e]	D	I	HA	HL	SPLEEN CELLS
THYMUS-GRAFTED GROUP[a]	(14)[b]	(10)	(9)	(9)	(9)	(9)	(9)	(9)	(6)
	18.7	10.9	260	312	18,689	21,350	812	1498	1510
SHAM OPERATED NUDES	(12)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(4)
	37.1[c]	21.6[c]	37	27	2463	1913	36	71	41

a. Animals were grafted with one neonatal Balb/c thymus gland in each axillary region. Two to four weeks later they were grafted with CBA/J skin. Following complete rejection or acceptance of the 1^o and 2^o homografts, 1×10^8 SRBC were given I.P. Five days later, specific PFC, RFC and humoral antibody levels were determined.
b. The number of animals in the group.
c. All primary and secondary skin grafts on the sham-operated nudes were healthy and had large areas of hair growth at the termination of the experiment.
d. Direct plaque forming cells.
e. Indirect plaque forming cells.

TABLE II

HOMOGRAFT RESPONSE AND ANTI-SRBC RESPONSE
OF NUDES GRAFTED WITH CBA/J THYMUS GLANDS

GROUP	No. OF ANIMALS	MEAN GRAFT SURVIVAL TIME	SERUM ANTIBODY HA	SERUM ANTIBODY HL
thymus grafted nudes[a]	5	indefinite[b]	(3)[c] 67	(3)[c] 373
sham operated nudes	3	indefinite[b]	(2) 30	(2) 120

a. Animals with either sham-operated or grafted with 24 hour old CBA/J thymus glands. Three weeks later both groups were given CBA/J skin homografts and inspected daily for signs of graft rejection.

b. None of the animals in either group showed signs of graft rejection so all surviving animals were given 1 x 10^8 SRBC. Five days later, serum was harvested and levels of specific antibodies were determined.

c. Number of animals in group.

produced higher levels of both the HA and the HL antibody types, but this increase is small when compared to the increases observed in Table I when nudes were grafted with Balb/c thymus glands.

Homograft immunity and anti-SRBC response of nude females which had given birth to thymus bearing young.

The results of attempts to reconstitute nude animals using Osoba's pregnancy system (37) involving the transplacental effect of a fetal thymic hormone on the mother are summarized in Tables III and IV. Nude females bred to either Balb/c males or littermates were immunized with 0.25 ml of a 10% suspension of SRBC within one month after delivering their wild type young. Table III shows that these animals failed to produce higher numbers of PFC/10^6 spleen cells or PFC/spleen than did similar nude mice which had never delivered thymus bearing young. Likewise, these animals did not produce higher humoral antibody titers of either the hemagglutinating or the hemolytic type than did the virgin nudes. Neither group of nudes produced PFC or humoral antibody responses comparable to those produced by littermate females immunized with similar numbers of SRBC. Nude mothers which had delivered wild type young were also incapable of rejecting CBA skin homografts when challenged within one month of delivery, as shown in Table IV. Similarly, Table IV shows that virgin nudes were unable to reject CBA skin grafts. Normal littermates of nudes, however, were able to reject

TABLE III

ANTI-SRBC RESPONSE OF VIRGIN NUDES, VIRGIN LITTER MATES AND NUDE FEMALES WHICH HAD DELIVERED WILD TYPE YOUNG[a]

GROUP	No. OF ANIMALS	DIRECT PFC/10^5	DIRECT PFC/SPLEEN	SERUM ANTIBODY HA	SERUM ANTIBODY HL
NUDES (preg.)[a]	11	18	2187	176	146
NUDES (virgin)[a]	9	16	1218	149	142
LMC (virgin)	8	170	22,323	1280	4000

a. Anti-SRBC responses of nude females delivering wild type young compared to similar responses of virgin nudes and virgin LMC. Within three weeks of delivery, experimental and control animals were immunized with .25 ml of a 10% suspension of SRBC and were assayed five days later.