Bacteriological studies of post-thymectomy wasting disease
by John Leonard Cantrell

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:
Neonatal BALB/c mice thymectomized 12-24 hours after birth and receiving rabbit anti-mouse thymocyte serum (RAMTS) 3 and 48 hours post-thymectomy showed an increased severity, incidence and early development of wasting disease compared to neonatally thymectomized mice. Livers, spleens, lungs and blood from RAMTS-treated thymeatomized mice demonstrated bacterial infection as early as day 8 post-thymectomy and increased in severity to day 28. Sham-thyme atomized mice received either 2 injections of sterile distilled water or 2 injections of RAMTS. The control mice demonstrated no symptoms of wasting disease. Organisms isolated from the tissues and organs of test mice were also isolated from the gut of normal young BALB/c mice. Erysipelothrix sp. was the predominate organism isolated from the tissues or organs up to day 20, thereafter, Streptococcus faecalis was the predominate organism isolated. The tissues and organs of test mice showing severe symptoms of terminal wasting disease (day 42-45) yielded large numbers of Citrobacter sp. and S. faecalis. Thymectomized RAMTS-treated mice displayed a severe leucopenia (3200 white blood cells/mm3) and an inflammation of the small intestine. The results show that RAMTS-treated thymectomized mice develop a chronic infection beginning between day 8-10 post-thymectomy and persisting until death. The results also show that the organisms isolated from the tissues or organs of RAMTS-treated thymectomized mice are the inhabitants of intestinal flora in normal BALB/c mice.

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ABSTRACT

Neonatal BALB/c mice thymectomized 12-24 hours after birth and receiving rabbit anti-mouse thymocyte serum (RAMTS) 3 and 48 hours post-thymectomy showed an increased severity, incidence and early development of wasting disease compared to neonatally thymectomized mice. Livers, spleens, lungs and blood from RAMTS-treated thymectomized mice demonstrated bacterial infection as early as day 8 post-thymectomy and increased in severity to day 28. Sham-thymectomized mice received either 2 injections of sterile distilled water or 2 injections of RAMTS. The control mice demonstrated no symptoms of wasting disease. Organisms isolated from the tissues and organs of test mice were also isolated from the gut of normal young BALB/c mice. Erysipelothrix sp. was the predominate organism isolated from the tissues or organs up to day 20, thereafter, Streptococcus faecalis was the predominate organism isolated. The tissues and organs of test mice showing severe symptoms of terminal wasting disease (day 42-45) yielded large numbers of Citrobacter sp. and S. faecalis. Thymectomized RAMTS-treated mice displayed a severe leucopenia (3200 white blood cells/mm^3) and an inflammation of the small intestine. The results show that RAMTS-treated thymectomized mice develop a chronic infection beginning between day 8-10 post-thymectomy and persisting until death. The results also show that the organisms isolated from the tissues or organs of RAMTS-treated thymectomized mice are the inhabitants of intestinal flora in normal BALB/c mice.

INTRODUCTION

The physiological function of the thymus gland was uncertain prior to 1961, although early investigators Beard (1900) and Hammer (1921) postulated that the thymus played a role in the physiology of the lymphoid system and influenced lymphopoiesis. In 1961, the investigation of Miller (1961, 1962a), Good (1962) and Martinez et al., (1962) emphasized the importance of the thymus in the development of immunological functions. Miller demonstrated that neonatal thymectomy led to severe lymphopenia and an impaired ability to reject homografts in adulthood (Miller 1961, 1962a). Martinez (1962) demonstrated that the longevity of skin homografts in neonatally thymectomized mice was prolonged in certain donor-host combinations. Later work, employing thymectomized and thymus-grafted newborn mice, led to the hypothesis that the most important function of the thymus is immunologic and related to the development of immunologically competent cells early in life (Miller 1962a). These studies showed that lymphopenia and loss of immunological function following thymectomy can be avoided by the grafting of either syngenic or allogenic thymic tissue into the thymectomized animals.

An abundant literature indicates that neonatal thymectomy impairs cell-mediated as well as humoral immune responses. There is a marked impairment of ability to reject homograft and heterografts in the neonatally thymectomized mice (Martinez 1962, Miller 1962b). This situation has also been reported in other species such as rats (Azar 1964a, 1964b) and hamsters (Sherman 1964).

Evidence for a long range immunosuppressive effect of neonatal thymectomy can be inferred from the development of a wasting disease several weeks after thymectomy. Neonatal thymectomy has been associated with wasting disease in rats (Azar 1964a, 1964b), hamsters (Sherman 1964, 1969), and mice (Miller 1962b, 1964; Hunter 1968). The wasting disease is characterized by diarrhea, weight loss, ruffled fur, lethargy, hunched posture and ultimately death (Azar 1964; Miller 1962b). Significantly, symptoms of this form of wasting disease are reminiscent of those obtained for wasting disease induced with cortisol acetate (Reed 1967; Jutila 1968), estradiol (Reilly 1966), anti-lymphocyte serum (Denman 1967, 1968; Gray 1966; Monaco 1966; Van der Werf 1967), and anti-thymocyte serum (Russe 1967; Barth 1968a, 1968b; Agnew 1968). The syndrome does not occur in all strains of mice and a 4 week to 4 month time interval before the onset may occur depending on the species and strain of animal involved. Experimentation with different animal species and strains, variations in techniques, seasonal factors or bacterial flora may all have contributed to the marked differences in results of neonatal thymectomy obtained in various laboratories.

The etiology of the post-thymectomy wasting disease is not completely understood but it has been postulated (Azar 1964b) that the immunological deficiency of neonatally thymectomized rats is, on the one hand, an expression of pre-occupation with sepsis and contributes to sepsis on the other. The incidence of sepsis and

wasting in thymectomized rats, usually in the form of upper-mediastinal abscesses or bronchopulmonic infection, was significantly higher in neonatally thymectomized rats than in sham-operated or oxytetracycline-treated thymectomized animals (Azar 1964a). The implantation of thymus autografts markedly reduced the incidence of sepsis and wasting in neonatally thymectomized rats (Azar 1964b) and mice (Law 1964).

Miller demonstrated that neonatally thymectomized germ-free mice had identical growth rates as the nonthymectomized germ-free controls and failed to manifest symptoms of wasting disease. On the other hand, thymectomy of conventionally-reared counterparts displayed symptoms typical of wasting and died within 2-3 weeks of the onset of symptoms (Miller 1967). The blood lymphocyte levels of germ-free thymectomized mice did not differ from the control germ-free mice but the mice exhibited a depressed response to sheep red blood cells (SRBC) similar to that found in conventionally-reared, neonatal, thymectomized mice. Similar results were obtained by Wilson (1964), who demonstrated that germ-free thymectomized mice were kept free of wasting symptoms up to 8 months while conventionally-reared thymectomized mice died from wasting disease between 4-8 weeks. Wilson also showed that when an isolator containing neonatal thymectomized germ-free mice became contaminated with environmental bacteria, the mice died from wasting disease within two months.

The purpose of this investigation was (1) to detect an infectious process in thymectomized mice, (2) to perform a sequence study to determine the time of onset of infection and (3) to determine the nature of the infectious agents in the tissues and organs of wasting mice.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

The inbred BALB/c mice used in these experiments were originally obtained in 1966 from the National Cancer Institute, Bethesda, Maryland in the germ-free state. The mice were conventionalized 6 months later and maintained by frequent brother-sister matings.

All stock animals were fed Purina Laboratory Chow while the breeding mice were maintained on Purina Mouse Breeder Chow. Experimental animals were fed Purina Mouse Breeder Chow and water ad libitum.

SURGICAL PROCEDURES

ANESTHESIA. Twelve to 24-hour-old BALB/c mice were anesthetized by a cooling technique modified from the procedures described by East and Parrott (1962) and Hunter (1968b). The newborn mice were seperated from their mothers and placed in a domestic refrigerator freezing compartment and held at -10°C for 6-7 minutes. The mice, at the end of 6 minutes, demonstrated a decrease in respiratory function, a cyanotic coloration, and a lack of reaction to tactile stimuli. The mice were then placed on an aluminum foil, wrapped ice block to maintain the cool temperature and operated on within the next 8-10 minutes. Post-operative recovery was accomplished by warming under a 100 watt lamp. Upon recovery, the coloration,

respirations and general activity returned to normal.

THYMECTOMY. Mice were thymectomized within 24 hours after birth using a modification of the techniques described by Miller (1961), East and Parrott (1962), and Hunter (1968b). The mice were anesthetized, placed in a supine position on the ice block and taped with Scotch tape in such a fashion that the head was extended back, thus protruding the thoracic cavity upward toward the operator. The hind limbs were also taped to the block to totally immobilize the animal.

An incision over the sternal region was made by utilizing a Bard-Parker #11 blade. A pair of irrisectomy scissors was used to split the sternum by placing one point at the manubrial notch and pushing to the level of the third or fourth rib. With the point held high, the sternum was split with a single stroke of the scissors. The incision was spread with a pair of fine point forceps to expose the thymus. The thymus was removed by gentle aspiration using a Pasteur pipette attached to a vacuum pump by a segment of rubber tubing. The incision was closed by using two 6-0 sutures with no attempt to align the sternum edges. A drop of collodion was applied to the wound and the mouse was placed under the warming lamp. Two mice in each litter were sham-thymectomized and underwent the above treatment with the exception of the thymic

aspiration.

The first attempts at thymectomy yielded a high incidence of death due to poor surgical technique, however, with practice the mortality was reduced to less than 5%.

To insure the completeness of thymectomy, rabbit anti-mouse-thymocyte serum (RAMTS) (see below), was administered in a volume of 0.05 ml subcutaneously into the neck region 2-3 hours post-thymectomy and again 24 hours after the first injection. One of the sham-thymectomized litter mates received RAMTS as above and the remaining sham operated mouse received an injection of 0.05 ml sterile distilled water.

The animals were checked for completeness of thymectomy at the end of the experimental procedures macroscopically and any suspicious tissue was removed and a histological examination performed. Those animals that demonstrated thymic remnants were discarded.

RABBIT ANTI-THYMOCYTE SERUM

Rabbit anti-mouse-thymocyte serum (RAMTS) was prepared by using a modification of the technique described by Billingham (1961). Thymus glands from adult BALB/c mice were aseptically removed and placed in 1 ml of sterile Alsever's solution. The thymus was

minced using sterile scissors and forceps and washed 3 times in sterile Alsever's solution. Thymocytes were re-suspended in a small volume of Alsever's and emulsified with an equal volume of Freund's adjuvant (Difco). Rabbits were prepared for inoculation by shaving their back and washing with 70% alcohol. Rabbits received a total of 0.50 ml of the thymocyte emulsion subcutaneously at multiple sites to give a total of approximately 10×10^6 cells per rabbit. Booster injections of cell suspensions (10×10^6 cells) without adjuvant were given intervenously on 3 successive days 4 weeks later and the rabbits were bled from the ear vein 7 days after the last injection. The blood was allowed to clot at room temperature and then the serum was separated, pooled, and stored at $-20^{\circ}C$ after addition of Merthiolate (1:10,000, E. Lilly & Co.). The RAMTS was decomplemented at $56^{\circ}C$ for 30 minutes. Prior to use, the serum was absorbed with washed (three times in saline), packed BALB/c mouse erythrocytes using a ratio of 5 volumes of serum to 1 of erythrocytes. This absorption step was repeated until no hemagglutinins to the mouse red cells could be detected.

BACTERIOLOGIC STUDIES

Thymectomized and control mice were sacrificed by cervical dislocation at day 4 and every other day thereafter to day 28 post-

thymectomy. Mice demonstrating severe symptoms of wasting disease were also sacrificed before the onset of death. The mice in one litter were cultured at each of the time periods. Each litter contained an average of 6 test thymectomized mice and 2 control sham-thymectomized mice. The mice were sponged with 70% ethyl alcohol before the tissues and organs were aseptically removed. Blood for culture was collected by first sponging the tail with 70% ethyl alcohol and then cutting the tail with a sterile scissors. Four to 5 drops of blood were collected in sterile thioglycollate broth. The spleen, liver, and both lungs were removed, placed into a sterile pre-weighed 60-mm Falcon petri dish and weighed on a Metler balance. After weighing, the organs were flooded with sterile Alsever's solution. The organs were then surfaced sterilized with 70% ethyl alcohol which was burned off immediately. One lung, approximately 0.1 g liver, and the entire spleen was minced in 1 ml M/100 phosphate-buffered saline (PBS) pH 7.2 with sterile iris scissors and crushed with the blunt end of a glass rod. A volume of 0.1 ml of each of the tissues was then inoculated on duplicate blood agar plates, EMB, and into thioglycollate broth. One series of plates was incubated anaerobically using a Brewer Jar and the other was incubated under aerobic conditions. All cultures were incubated at 37^{o}C. All blood agar plates were preincubated for 24 hours prior to use and any plate showing questionable contamination was eliminated from the

experiment. Colony counts were performed on all blood agar cultures after 24 and 48 hours of incubation, and the predominate organism selected and isolated for identification. The identification of each organism was performed by using standard bacteriological and serological methods.

HEMATOLOGY PROCEDURES

All mice, prior to sacrificing, had total leucocyte and differental counts performed. The animal was placed under a heat lamp for 3-5 minutes and then anesthetized with ether. Sufficient blood was obtained by cutting the tail with a sterile scapula. The leucocyte count was obtained by using a Neubauer bright-lined hemocytometer. The differental was performed on thin blood smears stained with Wright's stain.

HISTOLOGY PROCEDURES

After the organs were removed for culture procedures, the thoracic cavity was opened and observed for thymic tissue. Any suspicious tissue was removed and prepared for a histological study. The small intestine of all thymectomized and control mice was removed and a portion of the duodenum, jejunum and ileum was prepared

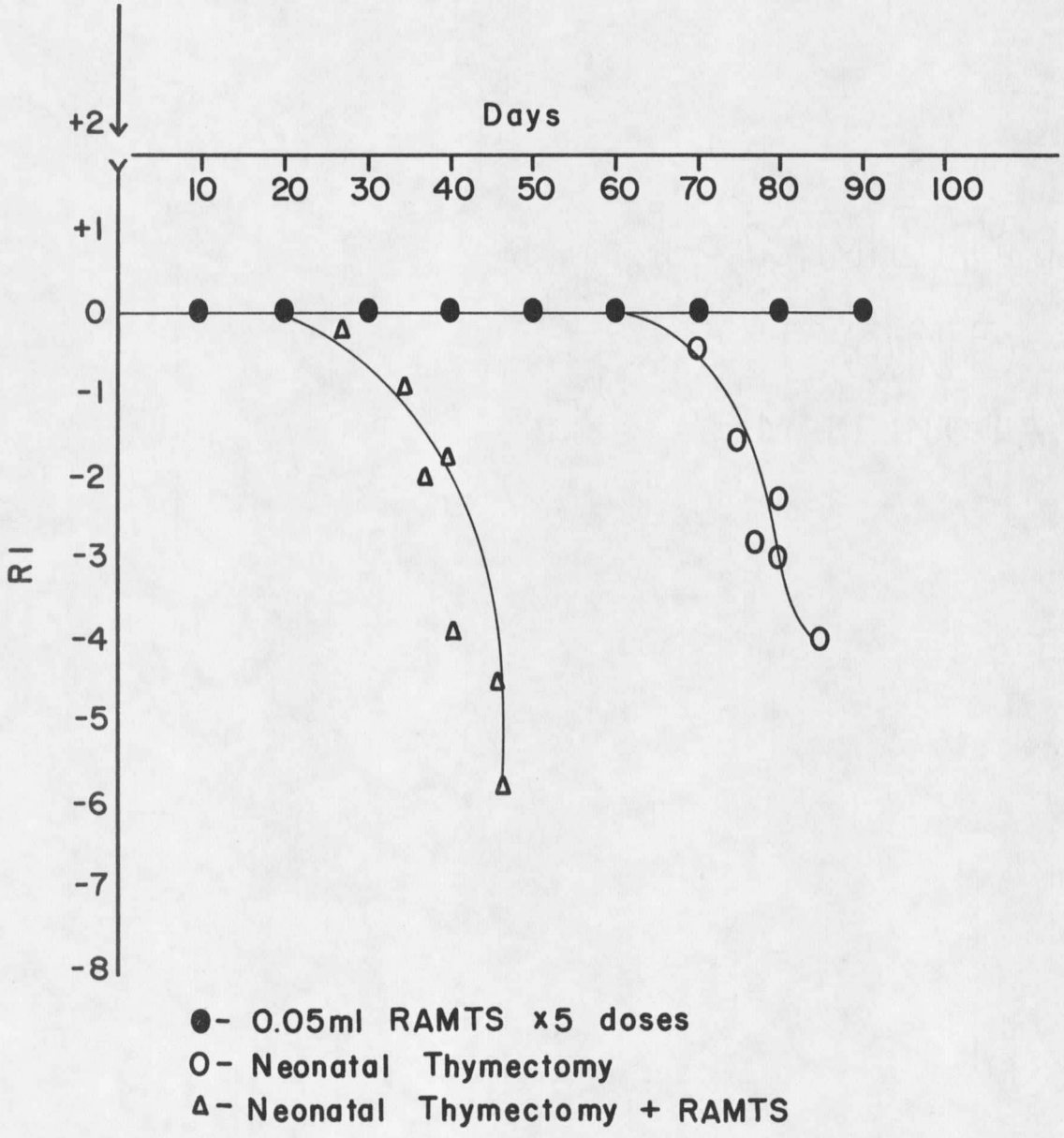
for examination. All tissue for histological examination was fixed in buffered 4% formaldehyde. The fixed tissues were processed in paraffin and thin sections were cut at a thickness of 4 microns. The tissues were stained with hematoxylin and eosin.

RESULTS

The Incidence of Wasting Disease Among Mice Thymectomized at Birth. Since previous work on wasting disease in antibiotic-treated or germ-free and monocontaminated thymectomized mice suggested an infectious process as the cause of disease, studies were undertaken to establish the onset of infection and to identify the infecting organisms.

The incidence of wasting disease in thymectomized BALB/c mice was determined by thymectomizing neonatal mice 12-24 hours after birth. Sham-thymectomized litter mates were used as controls. Whereas mortality rate was initially high due to poor surgical technique and maternal cannibalism, the mortality rate was reduced to less then 5% after practice. Also, cannibalism was reduced by using second and third litters of the stock BALB/c females. All mice were weaned at 21 days of age. Wasting disease was determined by the symptoms of diarrhea, ruffled fur, hunched posture, weight loss and finally death. Diarrhea was first detected beginning on day 10 or 12 and persisted for approximately 4 to 6 days. Other symptoms, such as hunched posture and ruffled fur, were observed during the weight loss period (Fig. 1) and persisted until death. Death due to wasting was obtained beginning on day 75 and increased in numbers until day 85. Mice showing no symptoms of wasting disease were sacrificed at day 85 and checked for thymus gland. Six percent of the thymectomized mice showing thymic tissue were excluded from the experiment.

FIGURE I



The runting index (RI) calculated and graphed from data obtained on the runting syndrome produced in the BALB/c strain of mice.

Wasting Disease Induced in BALB/c Mice by Thymectomizing and Treatment With RAMTS. Since the wasting syndrome occurred in such a low frequency, it was decided to "enhance" the frequency of wasting disease by treating thymectomized mice with RAMTS. This presumed that residual thymus cells lodged in peripheral lymphoid tissue would be eliminated by the cytotoxic action of specific antiserum. The initial studies were designed to test the effect of RAMTS alone on neonatal BALB/c mice. Hence, 15 neonates were injected with 0.05 ml RAMTS subcutaneously every other day for 10 days. Control litter mates received 0.05 ml sterile distilled water in the same manner described above. The RAMTS treated mice failed to demonstrate any symptoms of wasting disease throughout the testing period of 70 days. No difference in the RI (Keast 1968) was observed (Fig. 1) in the treated animals compared to either the control litter mates or the control sham-thymectomized animals. It was assumed that 5 injections of the anti-serum had little or no effect on the immune response of BALB/c mice. The effect of RAMTS on thymectomized BALB/c mice was determined by first thymectomizing the mouse 12-24 hours after birth and then followed by a 0.05 ml subcutaneous injection of RAMTS 2-3 hours after thymectomy. Another injection of RAMTS was administered 48 hours post-thymectomy. Control mice consisted of 2 sham-thymectomized litter mates. One control received 2 injections of RAMTS and the other received 2 injections of sterile distilled water

as described above. Test and control mice were sacrificed at 2 day intervals starting on day 4 post-operative and on out to day 28 post-operative. The data (Fig. 1) indicates that the wasting disease of mice given the RAMTS and also thymectomized was manifested earlier (day 35 compared to day 75) than mice only thymectomized. The test animals demonstrated the same symptoms of wasting disease and the onset of diarrhea occurred on the same day. Total white blood cell and differential counts were performed on all test and control animals (Table I). There was a marked leucopenia in all test animals ranging from 1700 to 4200 white blood cells/mm^3 and a severe lymphopenia was also evident in the test animals. There was no significant difference in the blood picture between control mice treated with RAMTS or sterile distilled water.

Portions of the duodenum, jejunum and ilium of all test and control mice were sectioned and stained for histology study. The RAMTS-treated thymectomized mice demonstrated lesions in the intestinal crypts which were packed with white blood cells. The control mice demonstrated normal histologic tissue.

The Incidence of Infection on Thymectomized BALB/c Mice Treated With RAMTS. Thymectomized and RAMTS treated BALB/c mice were sacrificed at intervals following treatment and their tissues and organs subjected to bacteriologic study. The results presented in

TABLE I

Hematology of thymectomized neonatal BALB/c mice given RAMTS

Day postoperative	Group[a]	Total WBC	Lymphocytes	PMNs	Monocytes
4	Test	2900	38	60	2
	Control	6000	68	32	0
8	Test	3400	32	65	3
	Control	6500	67	32	1
12	Test	2800	45	52	3
	Control	7100	80	18	2
16	Test	3500	37	62	1
	Control	7000	70	28	2
20	Test	2400	45	55	0
	Control	6800	77	22	1
24	Test	3300	44	54	2
	Control	6300	75	24	1
28	Test	3900	40	57	3
	Control	8000	75	25	0

[a]Test animals were thymectomized and received 0.05ml RAMTS every other day for two doses. Control mice were sham thymectomized, receiving either 0.05ml RAMTS or 0.05ml sterile distilled water.

Table II show that whereas the spleens, livers, lungs and blood of all animals sacrificed on day 4 failed to demonstrate any positive cultures, the incidence of infection was 50% by day 8 post-thymectomy. At day 10, 100% of the animals demonstrated at least one organ or tissue containing microorganisms. The numbers of organisms per tissue homogenate were small at day 8 but increased in number progressively to day 28 depending on the organism isolated. Large numbers of organisms were isolated from tissues and organs of thymectomized mice displaying terminal wasting. The numbers and species of organisms isolated from the tissues or organs varied in mice of the same litter. Blood cultures from mice sacrificed on day 8 and 12 were sterile but the livers, spleens, and lungs cultures were positive. Frequently an organism isolated from one tissue or organ was encountered in another tissue or organ in the same animal. Mixed infections were frequently encountered throughout the study.

Types of Bacterial Flora Isolated. The predominate bacterial species isolated from the tissues and organs of the test mice throughout the sequence study (Table III) were presumed to be derived from the enteric flora. The organisms isolated included Streptococcus faecalis, Proteus mirabilis, Erysipelothrix species, Staphylococcus aureus, Corynebacterium species, and a few Bacillus species. An interesting observation was that Erysipelothrix was the predominate

TABLE II

Incidence of infection in tissue and organs in RAMTS treated thymectomized BALB/c mice.

Day postoperative	Group [a]	Liver	Spleen	Lung	Blood
4	Test (6) [b]	0% [c]	0	0	0
	Control (2)	0	0	0	0
8	Test (6)	50	50	50	0
	Control (2)	0	0	0	0
12	Test (5)	100	20	100	0
	Control (2)	0	0	0	0
16	Test (5)	80	20	80	40
	Control (2)	0	0	0	0
20	Test (6)	85	85	100	67
	Control (2)	0	0	0	0
24	Test (5)	100	100	100	100
	Control (2)	0	0	0	0
28	Test (6)	100	100	100	100
	Control (2)	0	0	0	0

[a] Test animals were thymectomized and received 0.05ml RAMTS every other day for two doses. Sham thymectomized mice received either 0.05ml RAMTS or 0.05ml sterile distilled water.

[b] Numbers in parenthesis indicate the number of animals cultured at that time period.

[c] Percent of animals infected at that time period.

TABLE III

Types of organisms isolated at various time intervals from tissues or organs of RAMTS treated thymectomized neonatal BALB/c mice.

Day postoperative	TISSUES OR ORGANS CULTURED			
	Liver	Spleen	Lung	Blood
4	None	None	None	None
8	[a]3/6 Erysipelothrix sp. (50)[b] 1/6 Corynebacterium sp (35)	3/6 Erysipelothrix sp. (35)	3/6 Erysipelothrix sp. (65)	None
12	4/5 Erysipelothrix sp. (100) 1/5 S. faecalis (100)	1/5 Erysipelothrix sp. (75)	5/5 Erysipelothrix sp. (70) 1/5 S. aureus (20) 1/5 S. faecalis (50)	None
16	2/5 Bacillus sp. #1 (25)[c] 2/5 Bacillus sp. #2 (10)	1/5 Erysipelothrix sp. (40)	2/5 Corynebacterium sp. (40) 1/5 Bacillus sp. #1 (20) 1/5 Bacillus sp #2 (20) 2/5 Erysipelothrix sp. (75)	1/5 Erysipelothrix sp. 1/5 Bacillus sp. #1
20	1/6 Proteus mirabolis (8) 4/6 Erysipelothrix sp. (50) 2/6 Corynebacterium sp. (100)	1/6 Proteus mirabolis (20) 4/6 Erysipelothrix sp. (TNTC)[d] 2/6 Corynebacterium sp. (100)	3/6 Proteus mirabolis (25) 4/6 Erysipelothrix sp. (TNTC) 2/6 Corynebacterium sp. (50)	3/6 Erysipelothrix sp. 1/6 S. faecalis
24	2/5 Bacillus sp. #2 (100) 3/5 S. faecalis (TNTC) 1/5 Proteus sp. (20)	2/5 Bacillus sp. #2 (75) 1/5 Bacillus sp. #1 (TNTC) 2/5 S. faecalis (150) 1/5 S. aureus (75)	2/5 Bacillus sp. #2 (150) 3/5 S. faecalis (150) 1/5 S. aureus (65)	3/5 S. faecalis 1/5 Bacillus sp. #1 1/5 Corynebacterium sp.
28	2/6 Proteus sp (40) 2/6 S. faecalis (TNTC) 1/6 Bacillus sp. #1 (200) 1/6 Bacillus sp. #2 (100)	1/6 Proteus sp (30) 3/6 S. faecalis (200) 2/6 Bacillus sp. #2 (100)	1/6 Proteus sp. (60) 1/6 Bacillus sp. #1 (100) 1/6 Bacillus sp. #2 (100) 3/6 S. faecalis (TNTC)	1/6 Proteus sp. 3/6 Corynebacterium sp. 3/6 Erysipelothrix sp.

[a] Numerator represents number of positive cultures from each tissue or organ; denominator represents number of mice from which the specimens were obtained.

[b] Colony counts on blood agar plates — inoculating approximately 0.1 gm of tissue

[c] See text for discription of Bacillus species

[d] Too numerous to count (TNTC)