



Cortisol acetate-induced wasting disease in germfree and conventionally reared mice
by Norman Duane Reed

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY in Microbiology
Montana State University
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Abstract:

A single subcutaneous injection of cortisol acetate into newborn mice induced a wasting syndrome similar to that observed in the post-neonatal thymectomy syndrome. The course of the disease was less severe if the dose of the drug was decreased or if the mice were older at the time of injection. The administration of maintenance doses of cortisol acetate, after the large neonatal dose, did not prevent the wasting syndrome.

Bacteria, predominantly those considered part of the normal flora, were isolated from the liver, lungs, spleen and heart blood of wasting mice. *Escherichia coli* and *Streptococcus fecalis* were the organisms most commonly isolated.

The course of the wasting disease was not altered by the intra-peritoneal administration of rabbit immune globulin prepared against Bacto-Lipopolysaccharide *Salmonella typhosa* 0901 (LPS) or by allowing the wasting young mice to nurse on females immunized against LPS, an *Escherichia coli* bacterin, or both LPS and a *Streptococcus fecalis* bacterin.

Oral administration of terramycin or terramycin and penicillin reduced the incidence of death following neonatal injection of cortisol acetate.

Symptoms characteristic of the wasting syndromes were observed in cortisol acetate treated germfree mice. However, the incidence of death in germfree mice treated with cortisol acetate was markedly lower than in conventionally reared mice given a comparable dose. Mortality in germ-free mice treated with large doses of cortisol acetate was greatly increased by monocontamination with *Escherichia coli*.

Germfree mice, due to the low incidence of death in these animals, were used in studies concerning the primary effects of neonatal cortisol acetate administration. The neonatal injection of cortisol acetate caused a rapid and severe involution of the thymus and other lymphoid tissue, and prevented the development of normal immunological competence. The lymphoid involution was followed, within 30 days, by apparent recovery of these tissues. However, the ratio of small lymphocytes/large lymphocytes in the peripheral blood of apparently recovered mice was decreased for at least 30 days, and the homograft reaction was impaired for at least 50 days, following neonatal cortisol acetate treatment.

The results support the concept that the wasting mice, being immunologically deficient due to the primary effects of cortisol acetate on the thymus and other lymphoid tissues, are invaded and killed by microorganisms present in their environment.

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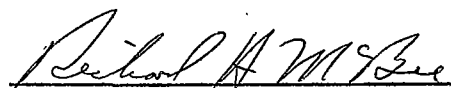
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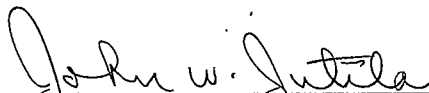
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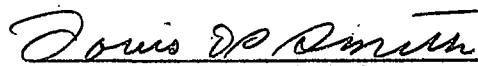
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ACKNOWLEDGMENT

The author wishes to express his gratitude to Dr. John W. Jutila for his support and advice in this investigation. The frequent help of Mr. James T. Hunter, Mr. John W. Safford, Jr., Mr. John C. Potter, and Miss Janet Mattson is appreciated. The aid and suggestions of Dr. R. I. Hamilton, Dr. R. H. McBee, and Mrs. Sharon Reed in the preparation of the manuscript are appreciated.

The author is grateful to the National Institutes of Health for financial support through the training program in microbiology 5T1 Al 131-05 and predoctoral fellowship 1-Fl-GM-24,866-01A1.

The author takes special pride in acknowledging the support given, and sacrifices made, by his wife and children.

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ABSTRACT

A single subcutaneous injection of cortisol acetate into newborn mice induced a wasting syndrome similar to that observed in the post-neonatal thymectomy syndrome. The course of the disease was less severe if the dose of the drug was decreased or if the mice were older at the time of injection. The administration of maintenance doses of cortisol acetate, after the large neonatal dose, did not prevent the wasting syndrome.

Bacteria, predominantly those considered part of the normal flora, were isolated from the liver, lungs, spleen and heart blood of wasting mice. Escherichia coli and Streptococcus fecalis were the organisms most commonly isolated.

The course of the wasting disease was not altered by the intra-peritoneal administration of rabbit immune globulin prepared against Bacto-Lipopolysaccharide Salmonella typhosa 0901 (LPS) or by allowing the wasting young mice to nurse on females immunized against LPS, an Escherichia coli bacterin, or both LPS and a Streptococcus fecalis bacterin.

Oral administration of terramycin or terramycin and penicillin reduced the incidence of death following neonatal injection of cortisol acetate.

Symptoms characteristic of the wasting syndromes were observed in cortisol acetate treated germfree mice. However, the incidence of death in germfree mice treated with cortisol acetate was markedly lower than in conventionally reared mice given a comparable dose. Mortality in germ-free mice treated with large doses of cortisol acetate was greatly increased by monocontamination with Escherichia coli.

Germfree mice, due to the low incidence of death in these animals, were used in studies concerning the primary effects of neonatal cortisol acetate administration. The neonatal injection of cortisol acetate caused a rapid and severe involution of the thymus and other lymphoid tissue, and prevented the development of normal immunological competence. The lymphoid involution was followed, within 30 days, by apparent recovery of these tissues. However, the ratio of small lymphocytes/large lymphocytes in the peripheral blood of apparently recovered mice was decreased for at least 30 days, and the homograft reaction was impaired for at least 50 days, following neonatal cortisol acetate treatment.

The results support the concept that the wasting mice, being immunologically deficient due to the primary effects of cortisol acetate on the thymus and other lymphoid tissues, are invaded and killed by microorganisms present in their environment.

INTRODUCTION TO THE WASTING SYNDROMES

In addition to the neonatal administration of cortisol acetate, wasting syndromes can be produced by several other methods. Although these wasting syndromes may vary in certain details, they are all characterized by progressive weight loss, failure to develop a normal coat, diarrhea, and in many cases death. A pathological finding that is common to all of these wasting syndromes is a profound atrophy of the lymphoid organs (Reed and Jutila, 1965). In view of the similarity of cortisol acetate-induced wasting to other wasting syndromes, a consideration of the various wasting syndromes is in order. A brief description and classification of the wasting syndromes follows.

Wasting diseases mediated by a graft-versus-host reaction (allogeneic* or homologous diseases)

Graft-versus-host reactions result when the recipient of a graft of immunologically competent cells is incapable of rejecting the graft. This situation can occur in very young individuals, in F_1 hybrids receiving parental cells, and in animals whose immunological capacity has been suppressed by chemical or physical agents. Animals involved in a graft-versus-host reaction show transient enlargement of spleen and liver, grossly visible foci of fibrinoid necrosis in the liver, late lymphoid atrophy, anemia, emaciation, and failure to thrive (Eichwald, 1963). The severity of the reaction is influenced by the age of the recipient, dose of donor cells, and the degree of antigenic disparity between donor and

*Several terms, usually restricted to the fields of immunology and gnotobiology, are defined in the appendix.

host. Graft-versus-host reactions have been demonstrated in many species, but the mouse has been studied in greatest detail. Simonsen (1962) has provided a scholarly review of the graft-versus-host reactions.

Runt Disease

Billingham and Brent (1957, 1959) encountered this disease during their studies on the induction of specific immunological tolerance in newborn mice. In certain strain combinations, when adult spleen cells were injected into newborn mice, the injected animals developed a peculiar wasting disease. The injected newborn mice failed to thrive; they grew slowly, their skin became dry and thin, their hair failed to develop normally, they developed sores around their orifices, and many died. At autopsy, atrophy of lymphoid tissue was observed. Billingham and Brent concluded that this wasting condition, which they named "runt disease", was due to a graft-versus-host reaction. Simonsen (1957) independently reported that adult splenic cells, when injected into newborn or embryo chickens and mice, produced a disease that he interpreted as an outcome of an immunologic response by the grafted splenic cells against the host.

Newborn animals injected with allogeneic immunologically competent cells usually grow normally for the first week after injection. This is followed by a failure to gain weight or loss of weight, severe diarrhea, alopecia, dermatitis and death. Runted animals characteristically have splenomegaly and hepatomegaly at sometime during the course of the disease, however, the lymph nodes, thymus, and Peyer's patches are atrophied. The runting animals may succumb quickly, or the disease may take a chronic

form in which the animals linger for many weeks before death.

Wasting Disease (F₁ Hybrid Disease)

F₁ hybrid mice injected with immunologically competent cells of parental strain donors may develop a wasting disease (Kaplan and Rosston, 1959). Since parental strain antigens are present in F₁ hybrids, hybrids do not react immunologically against parental strain antigens. However, part of the antigenic make up of an F₁ hybrid is foreign to both parental strains, and both parental strains can react immunologically to F₁ hybrid cells and tissues. Thus, when F₁ hybrids are injected with immunologically competent cells of parental strain donors, circumstances favor a graft-versus-host reaction.

F₁ hybrid disease has been described in irradiated and unirradiated F₁ hybrid recipients, in both young and adult animals. The dominant features of the disease are lymphoid depletion, atrophy of body fat with edema of the connective tissues, adrenal cortical hypertrophy, severe anemia, leukopenia, and decreased serum protein concentrations (Kaplan and Rosston, 1959).

Secondary Disease.

Allogeneic (homologous) and xenogeneic (heterologous) spleen and marrow cells injected into lethally irradiated mice survive and repopulate the blood-forming tissues of the irradiated recipient animals (Koller, Davies and Doak, 1961). This repopulation permits survival beyond the 30 day period during which acute radiation deaths are usually manifest.

Later, however, the animals frequently die of a disease termed "delayed", "homologous", or "secondary" disease. The animals lose weight, often develop diarrhea, and die with profound atrophy of lymphoid tissues as the most striking finding (Congdon and Urso, 1957). There is general agreement that secondary disease is dependent upon an immunologic reaction, since it occurs only in the presence of antigenic differences between donor and host. Most evidence suggests that the immunologic reaction is a graft-versus-host reaction (Russell and Monaco, 1965). Since the immune system of the host is suppressed by irradiation, immunologically competent donor cells may attack the host without encountering normal host defenses.

Parabiosis Intoxication

Surgical parabiosis of experimental animals is followed by massive cross-circulation and exchange of cellular elements. In genetically distinct parabionts a strange disease may occur in which one animal remains apparently well, while the other loses weight rapidly and dies with profound atrophy of the lymphoid organs and body fat (Finerty, 1952). Van Bekkum and coworkers (1959) have provided evidence for a graft-versus-host reaction in parabiosis intoxication. When F_1 hybrids and parental strain mice were placed in parabiotic union, the F_1 hybrid partners were the first to die. Additional evidence for a graft-versus-host reaction in parabiotic parabiotic partners has been given (Nakič, Nakič, and Silobrčić, 1960).

The Post-Neonatal Thymectomy Syndrome

Mice thymectomized soon after birth fail to develop a normal

lymphocyte population and have serious immunological defects (Miller, 1961). Delayed-hypersensitivity reactions, the homograft reaction, and the humoral antibody response are impaired (Miller, 1961, 1962; Martinez et al., 1962; Jankovic et al., 1962). Neonatal thymectomy in many strains of mice is followed by the development of a wasting syndrome (Miller, 1962; Parrott, 1962). This wasting syndrome, which usually develops at one to three months of age, is characterized by wasting, lethargy, ruffled hair, hunched back, diarrhea and death. There is a marked and generalized involution of lymphoid tissue. If thymectomy is delayed until a few days after birth, the severity and incidence of this wasting syndrome decreases (Parrott, 1962).

The Bacterin-Induced Wasting Syndrome

Ekstedt and Nishimura (1964) observed that if newborn mice were injected repeatedly with large amounts of a washed, autoclaved suspension of staphylococci or streptococci, some of the animals failed to develop normally. Weight gain was retarded in the treated animals. Cortical and medullary zones in the thymus could not be delineated. Lymph nodes could not be found in runted animals and there was atrophy in other lymphoid tissues. The wasting disease was mitigated in animals over 24-hours old at the time of initial injection.

Braaten (1966) confirmed and extended the observations of Ekstedt and Nishimura. Braaten produced wasting in mice by repeated injections of heat-killed bacterins of staphylococci, group A streptococci, Streptococcus faecalis, Bacillus cereus, Salmonella (O somatic group B) and

Escherichia coli. Braaten (1966) suggested that the bacterin injected mice became immunologically unresponsive to the antigens of the bacterins, allowing antigenically related organisms of the hosts normal flora to cause wasting.

Wasting Syndromes Induced With Pharmacologic Compounds

The wasting disease induced with cortisol acetate (Schlesinger and Mark, 1964) will be discussed in the next section Introduction to the Problem.

A wasting syndrome, similar to that induced with cortisol acetate, has been induced by injecting newborn mice with estradiol (Thompson and Russe, 1965). When 0.05 mg of estradiol was injected subcutaneously, three times a week, into newborn mice a syndrome reminiscent of "runt disease" regularly developed within two to three weeks. The mice failed to gain weight, had a hunched posture, scant hair growth, occasional diarrhea, and frequently an oily appearance. Approximately 78% of the treated mice died within six weeks. Autopsy revealed atrophy of peripheral lymph nodes and a four to five fold reduction in thymic weight. Cortical atrophy in the thymus was observed. Lymphocyte counts in estradiol treated mice were greatly reduced.

Wasting Syndromes Caused by Infectious Agents

Viruses may cause wasting syndromes which resemble classic runt disease. Polyoma virus, mouse leukemia virus, mouse reovirus, lymphocytic choriomeningitis virus and unidentified viruses have been

incriminated in wasting syndromes (Sinkovics and Howe, 1964).

Brooke (1964) has recently produced a disease in newborn mice, which resembles "runt disease", using pure cultures of Salmonella typhimurium var. copenhagen. However, it is important to note that this disease differs from the other wasting syndromes in that the mice did not have lymphoid atrophy. Thus, this disease should not be considered a typical wasting syndrome.

Considering the severe lesions of the lymphoid system found in the wasting syndromes it seems likely that infection might play an important role in the pathogenesis of these diseases. There are several reports which indicate that infection or intoxication occurs in animals whose immunologic capacity has been suppressed. It has been shown that antibiotics significantly prolonged the life and reduced the wasting of young rats treated daily with massive doses of cortisone (Stoerk, 1953). Miller, Hammond, and Tompkins (1950, 1951) found that bacteremia, caused by microorganisms normally present in the lower intestinal tract, was common in mice during the second and third weeks after exposure to 450 roentgens (r) or 600 r total body x-irradiation. This dose of irradiation suppresses the capacity to give an immunological response. This group (Miller et al., 1952) found that bacteremia could be controlled by the administration of antibiotics. Other workers have reported that the administration of suitable antibiotics has a beneficial effect in irradiated animals (Miller et al., 1952; Rosoff, 1963). Rosoff (1963) found that oral non-

absorbable antibiotics, neomycin sulfate or polymyxin B, prevented death in rats which had received a lethal dose of whole body radiation. The antibiotics suppressed the gram-negative bacterial flora of the intestinal tract. It is interesting that the protective effect was observed only when cultural data demonstrated the successful elimination of the coliform flora in the gut (Rosoff, 1963). Rosoff (1963) also observed that systemic antibiotic therapy did not improve the survival rate even when it prevented bacteremia, and suggested that products of bacterial activity in the gut might cause death following irradiation. This concept is supported by the observation that there is a reduction in the lethal effect of x-rays in Swiss mice when these animals are raised under pathogen-free conditions, with a fecal flora containing chiefly lactobacilli (Dubos and Schaedler, 1962).

Recent studies using germfree rats (Reyniers et al., 1956), chicks (McLaughlin et al., 1958), and mice (Wilson and Piacsek, 1961; McLaughlin et al., 1964; Wilson, 1963; Wilson et al., 1964) indicate that the irradiated germfree animal has a better chance for survival than its conventionally reared counterpart. The germfree mouse can tolerate more radiation than either a conventionally reared mouse or an Escherichia coli monocontaminated mouse (McLaughlin et al., 1964). The radiation effect after doses of 550 r to 950 r was observed and after all doses, the germ-free mice survived longer than conventionally reared or E. coli monocontaminated mice. Germfree mice are also more resistant than normal mice to the delayed lethal effect of nitrogen mustard (White and Claflin, 1963).

The possibility that infection or intoxication may contribute to the pathogenesis of the various wasting syndromes has been considered. Investigations of the wasting syndromes mediated by a graft-versus-host reaction have given equivocal results in this respect. In studies on "runt disease", attempts to isolate infectious agents from wasting mice have been negative (Simonsen, 1962). However, Safford and Jutila (1965) immunized female mice against various endotoxins and bacterins and found that whereas all of the offspring of non-immune mice died of runt disease, many offspring of the immune females failed to die. Previous reports (Howard, 1961a, 1961b; Cooper and Howard, 1961) stated that resistance to endotoxin was markedly decreased in mice undergoing a graft-versus-host reaction although phagocytic activity and resistance to live bacteria was increased. Stimulation of the reticuloendothelial system in mice by Mycobacterium tuberculosis (BCG) infection caused a lowering in resistance to the lethal effect of endotoxin from gram-negative bacteria (Howard et al., 1959). In contrast to "runt disease", infectious agents have frequently been incriminated in the pathogenesis of "secondary disease" and "wasting disease" (Denko, Simmons and Wissler, 1959; van Bekkum, Vos and Weyzen, 1959; Nowell and Cole, 1959; Koller, Davies and Doak, 1961; van Bekkum, van Putten and de Vries, 1962).

These observations on "runt disease" and "secondary disease" have recently been supported by experiments utilizing germfree mice. McIntire, Sell and Miller (1964) and Salomon (1965) have observed that the mortality of newborn mice injected with allogeneic spleen cells (runt disease) was similar in conventionally reared and germfree mice, whereas workers at the

Lobund Laboratory (Connell and Wilson, 1965a, 1965b) report that "secondary disease" is absent in x-irradiated germfree mice treated with allogeneic bone marrow.

Although infectious agents have been suspected in the post-neonatal thymectomy syndrome, early attempts to demonstrate such a factor failed (Good, Peterson and Gabrielsen, 1965). East and coworkers (East et al., 1963) demonstrated a hepatotropic viral agent, but their findings do not suggest that the virus has a significant role in the runting process or the early death of the thymectomized mice. Azar (1964) found that chronic bacterial infections played an important role in the development of wasting in neonatally thymectomized rats. Administration of oxytetracycline or implantation of thymic autografts immediately after thymectomy markedly reduced the incidence of bacterial infection and wasting in thymectomized rats. Mortality in neonatally thymectomized mice was significantly reduced when they were "kept under nearly pathogen-free conditions" (Miller, 1961) and did not occur in "specific pathogen-free" mice (Hess, Cottier and Stoner, 1963). McIntire, Sell and Miller (1964), and independently Wilson, Sjodin and Bealmear (1964) found that germfree mice thymectomized at birth do not develop the post-neonatal thymectomy syndrome. Upon contamination the ex-germfree mice, thymectomized at birth, develop the disease within four to eight weeks. This suggests that the primary factor in the pathogenesis of the post-neonatal thymectomy wasting syndrome appears to be an environmental factor, presumably an infectious agent (or its product) to which only neonatally thymectomized mice, because of their diminished immunological capacity, are susceptible (McIntire

et al., 1964). Similarly, Ekstedt and Nishimura (1964) found that germfree mice were much more resistant to the bacterin-induced wasting syndrome than conventional mice of the same strain.

Significantly, the wasting syndromes induced with pharmacologic compounds have not been previously studied in germfree animals.

INTRODUCTION TO THE PROBLEM

Schlesinger and Mark (1964) have described a wasting syndrome induced in young mice by the administration of cortisol acetate. When one-day old mice were given a single subcutaneous injection of 0.25 mg of cortisol acetate, growth of the treated animals was noticeably altered by the third day after drug administration. The skin was thinned and wrinkled and hair growth was markedly impaired. Diarrhea, often of the hemorrhagic type, appeared about a week after injection and the mice died within six to fifteen days after the injection. The course of the disease was prolonged and less severe when smaller doses or older animals were used. With a dose of 0.25 mg cortisol acetate per gram of body weight, all mice 10 days of age or younger at the time of injection died of a fatal wasting disease. The same dose had an irregular effect on mice 14 to 21 days old, and failed to produce a fatal disease in mice that were 10 weeks old at the time of injection. Surviving mice were emaciated, exhibited muscular atrophy, walked with a characteristic high stepping gait and recovered after a variable period of stunted growth. Duhig (1965) has also produced wasting disease in young mice by a single injection of cortisol acetate.

Pathological changes observed (Schlesinger and Mark, 1964) in wasting mice included marked reductions in the weights of the thymus and spleen, accompanied by increases in the weights of the liver, kidneys, and heart. Hemorrhage was often present in the small intestine and foci of calcification were occasionally seen in the myocardium. Overt signs of infection that may have led to the death of the animals were never

observed by these investigators (Schlesinger and Mark, 1964).

Schapiro (1965), apparently unaware of the work of Schlesinger and Mark, described a similar wasting condition in rats. Two-day old rats injected intraperitoneally with a single large dose (roughly equivalent to 14 mg per 100 grams of body weight) of cortisol acetate developed a wasted condition similar to that produced in rats following neonatal thymectomy (Azar, 1964). Schapiro (1965) referred to these animals as "corticoid runts".

Fachet and coworkers (1966) studied the effect of a single large dose of four different glycocorticoids, including cortisol acetate, injected into newborn rats. These workers reported that such treatment produced a fatal cachectic condition very similar to the wasting syndrome which follows the neonatal thymectomy of rats. Total body weights and weights of thymus, spleen, and adrenals were lower in treated rats than in non-treated littermate controls. Also, the number of small lymphocytes in the peripheral blood and in the lymphoid organs was decreased. The wasting rats exhibited ruffled fur, thin skin, absence of subcutaneous fat, and diarrhea occurred in many animals. When smaller doses of glyco-corticoid were used survival time was prolonged and the mortality rate decreased (Fachet et al., 1966).

The pathogenesis of cortisol acetate-induced wasting disease is not well understood. The administration of large doses of cortisol acetate produces severe metabolic derangements (White, Handler and Smith, 1964). Cortisol acetate influences carbohydrate metabolism by altering the rates of three processes: (1) increasing glucose release from the

liver; (2) accentuating gluconeogenesis from amino acids; and (3) decreasing the peripheral utilization of glucose. Cortisol inhibits protein synthesis in muscle and other tissues, resulting in a negative nitrogen balance. Cortisol also influences lipid metabolism and to a limited extent electrolyte and water metabolism. The numbers of blood lymphocytes, erythrocytes, and eosinophiles are influenced by cortisol, as is the structure and function of lymphoid tissue. In the latter tissue, cortisol produces a decrease in the numbers of lymphocytes and a decrease in the size of lymphoid organs (Dougherty, 1952). Cortisol increases the secretory activity of the gastrointestinal tract, possibly precipitating the formation of ulcerative lesions in the gastrointestinal tract during prolonged therapy (White, Handler and Smith, 1964).

The physiological effects of cortisol have to a large extent been studied in adult animals. It is evident that the effect of cortisol acetate is accentuated in newborn and young animals, since in these animals a single injection is sufficient to inhibit growth for a prolonged period and leads to a fatal wasting disease. Thus, the effect of cortisol in neonates requires special consideration.

Although the similarities between the various wasting syndromes have been stressed by many investigators, it is becoming increasingly apparent that these syndromes differ in important pathological processes. Thus, the post-neonatal thymectomy syndrome, bacterin-induced wasting disease, and "secondary disease", do not occur in germfree mice, whereas the wasting syndrome which follows the injection of neonates with allogeneic cells (runt disease) is very severe in germfree mice. In view of

the differing response to the germfree state in these wasting syndromes, it would be of interest to study other wasting diseases in germfree animals.

The purpose of the following work was, therefore, to compare the pathogenesis of the cortisol acetate-induced wasting syndrome in conventionally reared and germfree mice.

MATERIALS AND METHODS

Experimental Animals

The Swiss mice used in this study were originally obtained in 1964 from the germfree stock of Manor Farms, Staatsburg, New York. Randomly mated colonies of both conventionally reared and germfree Manor Swiss mice have since been maintained in the Department of Botany and Microbiology, Montana State University.

Inbred mice of the A/jax strain were used to maintain the Sarcoma I tumor. Breeding stock of the A/jax strain and the Sarcoma I tumor were obtained from the Department of Microbiology, University of Washington, Seattle.

Conventionally reared mice were fed ad libitum Purina Laboratory Chow and water. Germfree mice were fed ad libitum sterile Purina Laboratory Chow (Special Formula) 5010 C and sterile water.

Pharmacological Compounds and Antibiotics

Cortisol acetate (hydrocortisone acetate) was obtained in sterile aqueous suspension, 25 mg per cc, from Wolins Pharmacal Corporation, Mineola, New York.

Antibiotics used were: potassium penicillin G (Pentids "400"-Squibb) and oxytetracycline hydrochloride (terramycin animal formula-Pfizer).

Biological Materials

Vaccines

A streptococcus bacterin was prepared from a pure culture of

Streptococcus fecalis isolated from a wasting Swiss mouse. A pure culture of the organism was grown in brain-heart infusion broth (Difco Laboratories, Inc., Detroit) at 37 C for 24 hours. Formalin was added to a concentration of 1.5% and the culture placed at room temperature for 24 hours. Then the cells were harvested by centrifugation, washed twice in 0.85% NaCl and resuspended as a 1.0% cell suspension in 0.85% NaCl.

An Escherichia coli bacterin was prepared from a culture of E. coli isolated from a wasting Swiss mouse. A pure culture of the organism was grown in brain-heart infusion broth for 12 hours at 37 C. The culture was autoclaved (121 C) for 2.5 hours, the cells harvested by centrifugation, washed twice in 0.85% NaCl and resuspended as a 1.0% cell suspension in 0.85% NaCl.

Lipopolysaccharide antigen (LPS) was prepared from Bacto-Lipopolysaccharide Salmonella typhosa 0901 (control 474995, Difco Laboratories, Inc., Detroit). Bacto-Lipopolysaccharide was dissolved in 0.85% NaCl at a concentration of 500 μ g per ml with 1:10,000 Merthiolate.

Antisera

Rabbit anti-Bacto-Lipopolysaccharide Salmonella typhosa 0901 gamma globulin (anti-LPS gamma globulin) was kindly supplied by Mr. John Safford. The anti-LPS gamma globulin was adjusted to a concentration of 25 mg protein per ml of solution using 0.85% NaCl as diluent. Protein determinations were made by the biuret reaction (Campbell et al., 1964) using bovine gamma globulin as a standard. The anti-LPS gamma globulin had a titer of 1:800 as determined by the bentonite flocculation test (Wolf

et al., 1963).

Normal bovine gamma globulin (NBGG) was prepared by dissolving Armour BGG (Fraction II from bovine plasma, Lot No. W30512) in 0.85% NaCl at a concentration of 25 mg per ml. The NBGG preparation had no anti-LPS activity detectable by the bentonite flocculation test.

Sarcoma I Tumor

Sarcoma I tumor was maintained by intraperitoneal passage in adult A/jax mice. One-tenth ml of ascites fluid was removed from the peritoneal cavity of an A/jax mouse bearing a 10-day old tumor and injected into the peritoneal cavity of another A/jax mouse. In experiments requiring quantitation, tumor cells were counted with a Hausser improved Neubauer hemocytometer using 4% glacial acetic acid colored slightly with methylene blue as diluent. Tumor cells, obtained from the peritoneal fluid of an A/jax mouse bearing a 10-day old tumor, were washed once in Hanks balanced salt solution (Hanks and Wallace, 1949) and enumerated prior to use in experiments.

Method of Producing Wasting Disease and Criteria of Wasting

Experimental litters were sized to contain between seven and ten mice with an average weight, at 36 hours of age, between 1.70 and 2.1 grams. Unless otherwise indicated, all mice in a given litter received the same treatment.

To produce wasting disease, newborn mice were given a single subcutaneous injection of cortisol acetate in the dorsal neck region. The varying doses of cortisol acetate injected were always contained in a

volume of 0.05 ml. Cortisol acetate was diluted with sterile distilled water to obtain the concentration desired. Dilutions were made immediately before use. Mice in control litters received a single subcutaneous injection of 0.05 ml sterile distilled water. With the exception of an experiment designed to determine the effect of age on the response to cortisol acetate, mice were always injected at 36 ± 6 hours of age. Only mice dying later than day three after injection were considered as dying of wasting disease. All experimental mice were observed daily and deaths recorded. All experimental mice were weighed every other day for the first 20 days after injection, and then every five or ten days until the experiment was terminated.

Symptoms used to evaluate the severity of wasting included: failure to gain weight normally; loose, wrinkled, and thin skin; impaired hair growth; hunched posture and a high-stepping gait; gross lymphoid depletion; decreased peripheral circulation; absence of subcutaneous fat; encrustation of eyes with an exudate and failure of eyes to open at a normal age; presence of black discoloration in the abdominal cavity; diarrhea; and death.

Hematologic Methods

Blood and organs for various hematologic and histologic procedures were collected as follows: (1) The mice were weighed. (2) The tail was clipped and a blood smear made from the first drop of tail blood. (3) The mice were anesthetized with ether, the thoracic cavity opened, and the heart severed. Heart blood was immediately collected for absolute

leukocyte counts and hematocrit determinations. (4) The thymus, spleen, inguinal lymph nodes, small intestine, cecum, and large intestine were removed and placed in 10% neutral formalin. When germfree mice were involved, this sequence of events was performed within 30 minutes following the removal of the mice from the germfree environment.

Differential Leukocyte Counts

Smears of tail blood were stained with Wright's blood stain (Brook-Aloe Scientific) using a pH 6.7 buffer. Differential counts were made on 100 leukocytes using the oil immersion objective. Cells were recorded as lymphocytes, monocytes, neutrophils, eosinophils, and basophils. No attempt was made to distinguish between small, medium and large lymphocytes. Absolute counts of various cell types were calculated by multiplying differential counts by the total leukocyte counts.

Absolute Leukocyte Counts

Absolute leukocyte counts on heart blood were done using standard white cell diluting pipettes and the Hausser improved Newbauer hemocytometer. The diluent used was 4% glacial acetic acid colored slightly with methylene blue. Counts were made using the high dry objective and were corrected for nucleated red blood cells according to Bayliss (1962).

Hematocrit Determinations

Heart blood was collected in heparinized capillary tubes (75 mm length x 1.4-1.6 mm diameter). The tubes were sealed and centrifuged for seven minutes in an Adams Autocrit Centrifuge (Clay-Adams, Inc., New York).

Thymus and Spleen Weights

Thymi and spleens were placed in 10% neutral formalin immediately upon removal from the etherized animals. Between 30 and 90 minutes following removal from the animals, the organs were briefly removed from the formalin, blotted surface dry, and organ weights determined using a Mettler H 16 analytical balance. Weights were determined to the nearest 0.1 mg.

Bacteriologic Methods

Attempts were made to isolate microorganisms from various organs of wasting mice using the following procedure. Mice were killed by ether inhalation and the ventral surface flooded with 70% ethyl alcohol. The abdominal cavity was opened, using care not to cut into the intestines, and the spleen and approximately one-third of the liver removed and placed individually in small petri dishes containing one ml of nutrient broth (Difco). The thoracic cavity was opened next and one lung and approximately 0.2 ml of heart blood were placed individually in small petri dishes containing one ml of nutrient broth. The organs were macerated with a blunt glass rod. Sterile cotton swabs were soaked in the organ homogenates and the swabs were then used to inoculate the surface of bacteriological media. The media employed were: MacConkey agar (Difco); SF agar (BBL); phenylethyl alcohol agar (BBL); brain-liver-heart semi-solid medium (Difco); and blood agar, 5% rabbit or sheep blood in blood agar base (Difco).

After inoculation all of the media were incubated aerobically at

37 C. In addition, a set of inoculated blood agar plates were incubated anaerobically at 37 C. Colonies were counted and recorded at one, three and seven days following inoculation.

Gnotobiotic Methods

Stock germfree Swiss mice were maintained in Trexler flexible film isolators (Trexler and Reynolds, 1957). The isolators were housed in a clean room equipped with an entry air lock, filtered air supply and a temperature regulating device. Surgical gowns, masks and caps were donned for entry and work in the germfree stock room.

Experimental germfree Swiss mice were reared in either Trexler-type isolators or in smaller tetrahedron isolators recently developed by Snyder Laboratories, New Philadelphia, Ohio. Experimental isolators were housed in a clean conventional animal room in an area enclosed by sheet plastic. The air intakes on the blowers of experimental isolators were covered with "EZ KLEEN" filters (Research Products Corporation, Madison 1, Wisconsin) to decrease the demands on the fiber glass bacteriological filters.

Sterilization of Germfree Isolators

Fiber glass filters (Standard Safety Equipment Co., GF Supply Division, Palatine, Illinois) were steam sterilized at 121 C for three hours, vacuum dried, and attached to isolators. Isolators were assembled following the directions of Trexler (Snyder Contamination Control Apparatus, Bulletin No. 1, Snyder Laboratories, New Philadelphia, Ohio).

A stock solution of 40% peracetic acid (Becco Chemical Division,

FMC Corporation, New York, New York) diluted to 2% with double distilled water containing 1 g/l sodium alkylarylsulfonate (Fisher Scientific Company) was used for sterilizing the germfree isolators. The isolators were sprayed with 1.5 liters of 2% peracetic acid, delivered as a fine mist with a Trigger Teejet spraying system (5870-Standard Safety Equipment Co.). Excess liquid peracetic acid was removed with a sponge two hours after spraying. After 24 hours contact with peracetic acid, blowers were started and sterile air passed through the units for at least 72 hours before germfree mice were taken into the isolators.

Sterilization and Entry of Feed and Water

Feed pellets (Purina Laboratory Chow, Special Formula 5010 C) were lightly coated with talc (Merck) to prevent them from sticking together during sterilization. Talc coated pellets then were placed in double small paper sacks, about 700 grams per sack, and sealed with autoclave tape. One sack containing about 1,400 grams of pellets was prepared each time feed was sterilized to use in sterility assays. The sacks of feed were placed in an autoclave, subjected to a vacuum for 30 minutes, heated at 121 C for 30 minutes, and vacuumed again for 20 minutes. The first vacuuming served to evacuate air pockets in the feed sacks and the second vacuuming dried the pellets and paper sacks. The sterilized feed was stored at 4 C in sterilized large paper sacks. Prolonged sterilization lowers the nutrient value of the feed to the extent that it will not support the growth of mice.

Because of the sterilization time limitation the sterilized feed

was always assayed for sterility before it was introduced into a germfree isolator. Feed pellets, taken from the center of a sack containing about 1,400 grams of pellets, were placed in flasks of thiogel, brain-liver-heart semi-solid, and cooked meat phytone media. Sterilized feed was considered suitable for use if all cultures were negative after 10 days of incubation at 37 C.

Distilled water was autoclaved in 400-800 ml screw cap glass bottles for two hours at 121 C and stored in clean paper sacks. Because of the extended sterilization time it was not considered necessary to assay water for sterility. Sterilized water was considered suitable for use if the bottles had a tight seal.

Packages of sterile feed and bottles of sterile water were placed in the entry port of germfree isolators and sprayed with 200 ml of 2% peracetic acid for surface sterilization. After spraying, the items were left in the entry port for at least 12 hours and were then taken into the main chamber of the isolator. At the same time, empty bottles and waste materials were placed in the entry port for removal from the isolator.

Sterilization and Entry of Bedding and Supplies

Corn cob bedding ("Sanicell"-Paxton Processing Company, Paxton, Illinois) and wood shavings from a local lumber mill were used for bedding. Bedding was placed in double paper sacks and the sacks sealed with autoclave tape.

Sacks of bedding, cages, cage tops, water bottles, pencils, tapes, paper and other heat resistant materials were autoclaved for two hours at

121 C and vacuum dried. These pre-sterilized materials were then placed in a sterile supply drum (Figure 1) and the face of the sterile supply drum sealed with a sheet of Mylar plastic (Standard Safety Equipment Co.). The sealed sterile supply drum was placed in an autoclave, the autoclave was vacuumed for 30 minutes, brought to 121 C for three hours, and finally vacuumed for one hour. The sterile supply drum was then connected to the entry port of a germfree isolator (Figure 2) and the area between the Mylar cover on the supply drum and the inside cap of the isolator sprayed with 200 ml of 2% peracetic acid. Twelve hours or more after spraying, the inside cap was removed and the Mylar cover broken. The sterile supplies were then taken into the isolator, empty bottles and waste materials placed in the supply drum, and the inside cap replaced on the entry port.

Transfer of Germfree Mice

The following procedure was used to transfer germfree mice from a stock isolator to an experimental isolator. A sterile two-liter Erlenmeyer flask was delivered into a stock germfree isolator. One or two mice were placed in the flask and the flask tightly sealed with a rubber stopper. The flask was removed from the stock isolator, placed in the entry port of an experimental isolator and sprayed with peracetic acid. Due to the limited amount of air in the flask, the mice had to be taken out of the flask within 35 minutes after the flask was sealed. To compensate for the lack of sterilization time in the entry port, 4% peracetic acid was used instead of the usual 2% preparation.

Figure 1. A Sterile Supply Drum

Left. The Framework of a Sterile Supply Drum.

To prepare for use the stainless steel mesh was covered with fiber glass filter material (Standard Safety Equipment Co., GF Supply Division, Palatine, Illinois) and loaded with pre-sterilized supplies. The face of the drum was then sealed with heat resistant Mylar plastic (Standard Safety Equipment Co.).

Right. A Loaded Sterile Supply Drum.

Loaded sterile supply drums were autoclaved and then connected to germfree isolators (see Figure 2).

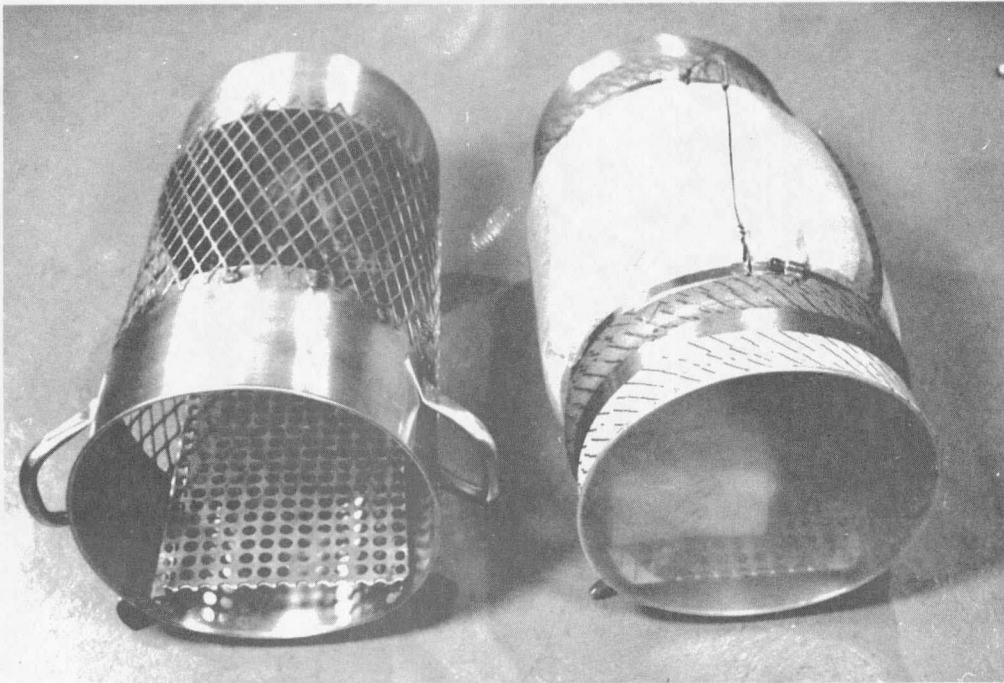


Figure 2. A Loaded Sterile Supply Drum Connected to a Germfree Isolator.

The area between the Mylar plastic cover on the sterile supply drum and the inside cap of the germfree isolator was sprayed with 200 ml of 2% peracetic acid. Twelve hours or more after spraying, the inside cap was removed and the Mylar cover broken. The sterile supplies were then taken into the germfree isolator, empty bottles and waste materials placed in the supply drum, and the inside cap replaced on the entry port.



Determination of Gnotobiotic Status

Fresh feces, cecal contents and various organs were taken from germfree mice and placed in culture media. Culture media employed included: brain-liver-heart semi-solid (Difco); thiogel (Baltimore Biological Laboratories); blood agar, 5% rabbit or sheep blood in blood agar base (Difco); fluid thioglycolate broth (Difco); PPLO medium (Difco); and a freshly prepared cooked meat-liver infusion medium. Cultures were incubated for 21 days under aerobic and anaerobic conditions at 25 C, 37 C and 45 C. The germfree status of stock mice was checked every three weeks. Experimental mice were extensively assayed every two weeks, in addition, "spot checks" using two or three different media were made every time an entry was made, usually twice a week. Examinations for protozoa and parasite ova were made every three or four months in wet mounts prepared from cecum and intestinal contents.

Virus assays were not routinely done. Thus, the virological status of the Montana State University germfree mouse colony is undetermined. Grossly, there was no evidence of viral infection in these mice. In one virus assay using primary mouse fibroblasts and two assays using embryonating chick eggs, no viral or rickettsial agents were detected. However, it is possible that these mice carry latent viruses or viruses not detected by the inadequate assays performed.

When germfree mice were intentionally placed in association with bacteria, cultural procedures were used to demonstrate that only those bacterial species intentionally introduced were present in the mice or in the gnotobiotic environment.

Production of Dibiotic Mice

To facilitate the production of dibiotes (Luckey, 1963) the litter-size isolator shown in Figure 3 was developed (Reed and Jutila, 1965). The litter-size isolator was sterilized and taken into a Trexler-type germfree isolator using a sterile supply drum. A germfree female, her litter (treated in any manner required by experimental design), bedding, and enough feed to supply the experiment were placed in the small isolator and the entry sealed with sterile autoclave tape. After the small isolator was removed from the Trexler-type isolator a changable, sterile water supply was attached to the isolator.

The sterile water supply consisted of a conventional watering bottle attached to the isolator by a short piece of rubber tubing (Figure 3). Water bottles were filled and rubber stoppers, fitted with glass tubing, were taped tightly in place. The glass tubing was covered with a test tube and the bottles autoclaved at 121 C for 40 minutes. To change the sterile water supply the empty bottle was detached and the rubber tubing dipped in 8% peracetic acid for one minute. Then the test tube was removed from over the glass tubing of a full water bottle and the bottle was quickly attached to the isolator by the rubber tubing.

Bacteria used to render germfree mice dibiotic were introduced into the isolator through the sterile water supply. A measured amount of a broth culture of the desired organism was placed in a water bottle, before connecting the bottle to the isolator, by introducing a long 13-gauge needle connected to a 10 ml syringe into the bore of the glass tubing. After injecting the culture into the water bottle, the bottle

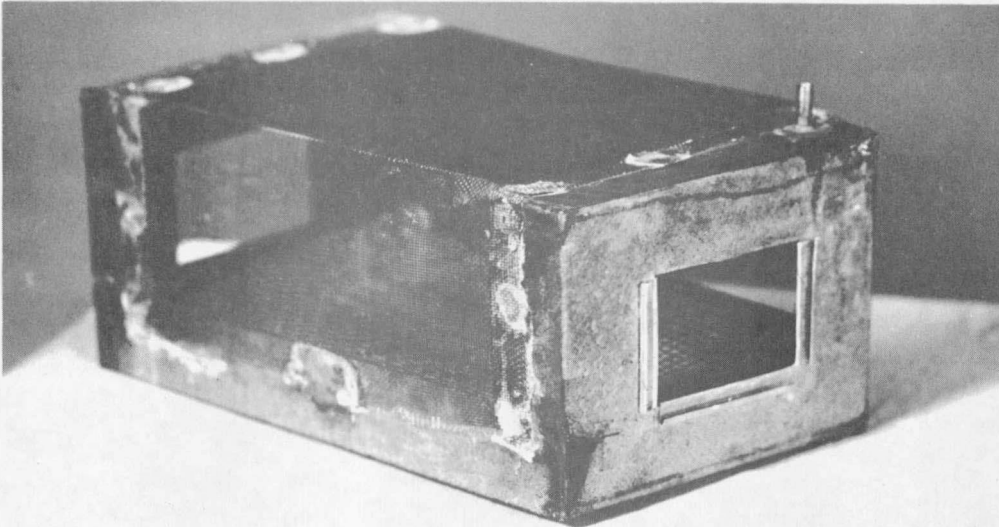
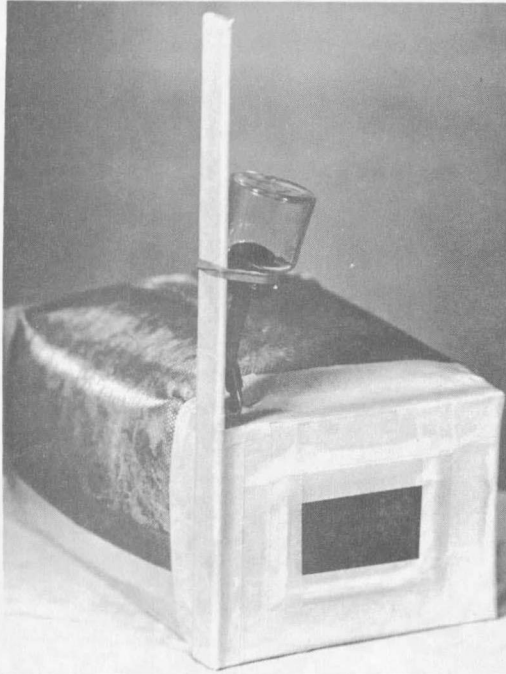
Figure 3. A Litter-Size Isolator.

Bottom. The Framework of a Litter-Size Isolator.

The metal framework was covered with fiber glass filter material (Standard Safety Equipment Co.), sterilized, and delivered into a large germ-free isolator using a sterile supply drum (Figures 1 and 2). A germfree female, her litter, bedding, and enough feed to supply the experiment were placed in the small isolator and the entry sealed with sterile autoclave tape. After the small isolator was removed from the large germfree isolator, a changable sterile water supply was attached (Figure 3, top).

Top. A Litter-Size Isolator with Sterile Water Supply Attached.

Bacteria used to render germfree mice ,dibiotic were introduced into the isolator through the water supply.



was connected to the isolator. It was presumed that soon after the mother mice licked their nipples, after drinking from the water supply, the young contacted the bacteria.

In some experiments, mice in tetrahedron isolators were rendered dibiotic by adding broth cultures of the desired bacteria to the water supply.

Statistical Methods

Mean survival times and standard deviations were calculated according to Huntsberger (1961) and are designated as $MST \pm SD$.

RESULTS

The Effect of Age at Injection and Dosage on Cortisol Acetate Induced Wasting Disease

When Manor Swiss mice were given a single subcutaneous injection of 0.20 mg cortisol acetate per gram body weight, the mice developed a fatal wasting disease (Table I). Growth of the treated animals was noticeably impaired by the third day after cortisol administration. The skin was loose, wrinkled and thin, hair growth was impaired, and the mice exhibited a hunched posture and a characteristic high stepping gait. The eyes of the wasting mice failed to open at a normal age and were encrusted with an exudate. A gross depletion of lymphoid organs and an absence of subcutaneous fat were observed. The feet and tails of the mice had an ischemic appearance presumably due to decreased peripheral blood circulation. A black discoloration in the abdominal cavity, which appeared to be due to hemorrhage into the lumen of the gut, was frequently observed. Diarrhea was common in the wasting mice and the terminal event, death, occurred in 95% of the mice (Table I). The mean survival time of mice which died was 8.6 ± 1.8 days. The few mice which survived this treatment developed a normal appearance after a variable period of time, usually by day 50 after injection.

When mice were injected at 10 days of age with 0.20 mg cortisol acetate per gram of body weight, the disease was less acute than in 36-hour old mice. Although these mice developed a nearly normal coat, they were emaciated and suffered muscular atrophy. Diarrhea was common and in most mice the eyes failed to open at the normal age and were encrusted with an exudate. The mice lost weight or failed to gain weight for the first

10 days after injection with cortisol, and weight gain was less than normal for the next 15 to 20 days. As shown in Table I, mortality in this group was decreased and mean survival time increased, as compared to mice injected with an equal weight adjusted dose at 36 hours of age.

Table I. The effect of age at injection and dosage on the mortality of mice given a single dose of cortisol acetate.

Cortisol Acetate Dose	Age at injec- tion	No.* of Mice	Cumulative Mortality (%) at 30 days after injection	MST $\pm$ SD (days)
0.25 mg/mouse	36 hrs	4/25	16.0	9.0 $\pm$ 2.6
0.50 mg/mouse	36 hrs	58/64	90.6	9.9 $\pm$ 3.8
0.15 mg/g body weight	36 hrs	45/49	91.8	9.1 $\pm$ 1.6
0.20 mg/g body weight	36 hrs	57/60	95.0	8.6 $\pm$ 1.8
0.15 mg/g body weight	6 days	3/8	37.5	14.0
0.20 mg/g body weight	6 days	8/8	100.0	10.8 $\pm$ 4.4
0.20 mg/g body weight	10 days	14/17	82.4	18.8 $\pm$ 5.6
0.20 mg/g body weight	20 days	2/13	15.4	8.0

*Numerators are the numbers of mice dying of wasting disease; denominators are the numbers of mice treated.

Mice 20 days old at the time of cortisol injection (0.20 mg/g body weight) did not develop a fatal wasting disease. Mortality following this treatment was low (Table I) and the gross appearance of the mice was normal.

The mice failed to gain weight normally for the first 10 days after injection, but weight gain thereafter returned to normal.

Thus, a single injection of cortisol acetate into young mice induced a wasting syndrome similar to that observed in runt disease and in the post-neonatal thymectomy syndrome. The course of the disease was less severe if the dose of the drug was decreased (Table I) or if the mice were older at the time of injection. Any attempt to explain the pathogenesis of cortisol acetate induced wasting disease should consider the effect of age on the susceptibility to cortisol acetate.

The Effect of Maintenance Doses of Cortisol Acetate
on Cortisol Acetate-Induced Wasting Disease

Early in the course of this work it was suggested by a visiting physician that the wasting disease which follows the neonatal administration of cortisol in mice might be the result of adrenal insufficiency. A form of acute adrenal insufficiency in humans occurs in patients who have previously been treated for long periods of time with either corticotropin or an adrenal corticosteroid (Thorn, 1963). In such cases, supplementary hydrocortisone or cortisone must be administered. Also, it has been observed that the administration of 11-oxysteroids leads to atrophy of the zona fasciculata, a histologically defined zone of the adrenal gland believed to be responsible for cortisol production (Bloom and Fawcett, 1962). It seemed possible that adrenal function might be suppressed in young mice by an encounter with a large dose of cortisol early in life, resulting in a fatal wasting disease.

To test this possibility, Manor Swiss mice were injected subcutan-

eously with a large dose of cortisol acetate at 36 hours of age and were later given subcutaneous maintenance injections of acetate. Maintenance doses were calculated from the data of Kaplan and Rosston (1959), who maintained adrenalectomized mice by the injection of desoxycorticosterone. Cortisol was considered as being 25% as active in life maintenance as desoxycorticosterone (White, Handler and Smith, 1964). The results of this treatment are presented in Table II. The administration of maintenance levels of cortisol acetate did not lessen the severity of cortisol acetate-induced wasting disease.

Table II. The effect of maintenance doses of cortisol acetate on cortisol acetate-induced wasting disease.

Cortisol Acetate : Wasting Dose : (mg/g. body wt) :	Age at : injec- : tion :	Cortisol Acetate : (maintenance dose) : per injection :	Days (after : injection of : wasting dose) : of maintenance : doses :	No.* : of : Mice :
0.20	36 hrs	0.05 mg/mouse	5	10/10
0.20	36 hrs	None		57/60
0.15	36 hrs	0.025 mg/mouse	6, 9, 12	7/7
0.15	36 hrs	None		5/7
0.15	60 hrs	0.025 mg/mouse	6, 9, 12	4/8
0.15	60 hrs	None		2/8

*Numerators are the numbers of mice dying between 0 and 30 days after injection; denominators are the numbers of mice treated.

Attempts to Isolate Bacteria from Wasting Mice

In their original report of cortisol acetate-induced wasting disease, Schlesinger and Mark (1964) state, "In no case did we observe overt signs of infection that may have led to the death of the animals." Since these workers did not indicate what methods were used to look for infections, several attempts were made to isolate bacteria from wasting mice in the present study. Cultural methods are detailed in the Materials and Methods section.

Manor Swiss mice were injected with cortisol acetate at 36 hours of age. Wasting mice were sacrificed at various times after injection and the liver, lung, spleen, and heart blood were cultured. Moderate to large numbers of microorganisms were isolated from the tissues and organs of 24 of 30 wasting mice (Table III). Mixed infections of the liver were commonly encountered. Escherichia coli and Streptococcus fecalis were the organisms most commonly isolated from the wasting mice. However, E. coli was never isolated from the lungs of diseased mice. In three mice, Staphylococcus aureus and Bacillus cereus were involved in mixed infections of the liver, lung and spleen.

Since E. coli and S. fecalis were the organism most commonly isolated, bacterins of these organisms were used in experiments on passive immunity, reported in the next section of this thesis.

The Effect of Maternal Immunization on Mice Injected with Cortisol Acetate at 36 Hours of Age

Because coliform group organisms and enterococci were frequently isolated from wasting mice, attempts were made to alter the course of

Table III. Bacteria isolated from wasting mice.

Cortisol	:	:	:	:	:	:	:	:	:
Acetate Dose:	Time of	:	No. of	No. of	Distribution of				:
(mg/g body	Culture	:	Mice	Mice	Bacteria*				:
weight) at	(days after	:	Cultured	Infected					Bacteria Isolated
36 hrs of	injection):	:	:	:	Liver	Lung	Spleen	HB	:
age	:	:	:	:	:	:	:	:	:
None	7		3	0					
None	9		2	0					
None	11		4	0					
0.15	7		2	0					
0.15	11		2	2	2/2	1/2	1/2	1/2	<u>Streptococcus sp.</u> (alpha hemolytic) Coliform group organism
0.20	7, 8 and 9		17	15	11/17	5/17	9/17	7/17	Gram negative rod (unidentified); <u>Escherichia coli</u> ; <u>Streptococcus fecalis</u> ; <u>Staphylococcus aureus</u> ; <u>Bacillus cereus</u> .
0.20	11 and 12 days		9	7	4/9	5/9	2/8	3/9	<u>Escherichia coli</u> ; <u>Streptococcus fecalis</u> ; <u>Staphylococcus aureus</u> ; <u>Bacillus cereus</u> .

*Numerators are the number of organs infected; denominators are the number of organs cultured.

cortisol acetate-induced wasting disease by passively immunizing wasting mice against these organisms or related antigens. To avoid the trauma of gavage, the experiments were designed so that cortisol treated suckling mice could be expected to receive gamma globulin passively through their mother's milk.

One group of Manor Swiss females was immunized against an Escherichia coli bacterin (see Materials and Methods). These mice received five weekly intraperitoneal injections of the E. coli bacterin in successive doses of 0.1 ml, 0.1 ml, 0.25 ml, 0.25 ml and 0.50 ml. The females were placed in cages with males on the day of the last injection. When obviously pregnant, they were separated from the males and placed individually in cages. The females were given a single 0.05 ml subcutaneous booster injection about 36 hours after parturition.

A second group of Manor Swiss females was immunized against Bactolipopolysaccharide Salmonella typhosa 0901 antigen (LPS--see Materials and Methods). Five weekly subcutaneous injections of 50 μ g each were administered to these females. The females were placed in cages with males on the day of the last injection and removed to individual cages when they became pregnant. A single intraperitoneal booster injection of 100 μ g was given about 36 hours postpartum.

The third experimental group was composed of female Manor Swiss mice which were immunized against both LPS and a Streptococcus fecalis bacterin (see Materials and Methods). These mice first received four weekly subcutaneous injections of 50 μ g LPS. Two weeks after the last LPS injection these females received three weekly intraperitoneal injections

tions of Streptococcus fecalis bacterin in successive doses of 0.25 ml, 0.25 ml, and 0.50 ml. On the day of the last injection the females were placed with males. Subcutaneous booster injections of LPS (25 μ g) and Streptococcus fecalis bacterin (0.05 ml) were given about 36 hours post-partum.

If the immune females were used for more than one litter, the booster injections described above were repeated after the birth of each litter. The subcutaneous injections were never administered near the mammary glands, since the females would not lactate normally. The pregnancy rate in the immune females was lower than normal, but litter size and general condition of the young born were normal. The serum or lacteal secretions of the immunized females were not titrated to determine antibody levels. However, Safford and Jutila (1965) have reported that runt disease is mitigated in young mice suckled on females immunized by a similar procedure.

Mice born to the immune females were given a single subcutaneous injection of cortisol acetate at 36 hours of age. The observations made on these mice are summarized in Table IV. The gross appearance of wasting mice suckled on immune females did not differ from that of wasting mice suckled on non-immune females. No protective effect was observed when females were immunized against LPS or E. coli bacterin. A moderate decrease in mortality and increase in weight gain was observed in the cortisol treated young of females immunized against both LPS and Streptococcus fecalis bacterin (Table IV).

Table IV. The effect of maternal immunization on mice injected with cortisol acetate at 36 hours of age.

Cortisol Acetate Dose (mg/g body wt)	Antigen*	No. # of Mice	Average weight [#] at days after injection				Cumulative Mortality (%) at 30 days after injection	MST $\pm$ SD (days)
			6	10	20	30		
0.20	<u>E. coli</u>	18/18	2.59(16)	3.00(3)			100.0	9.1 $\pm$ 2.0
0.15	<u>E. coli</u>	6/7	2.70(6)	3.50(3)			85.7	9.8 $\pm$ 1.1
0.20	LPS	16/17	2.00(19)	2.84(12)			94.1	7.13 $\pm$ 1.7
0.15	LPS-Strep.	31/39	3.64(37)	4.02(20)	7.56(9)	11.85(8)	79.5	10.5 $\pm$ 3.5
0.20	None	57/60	2.76(53)	3.60(14)	6.80(3)	10.30(3)	95.0	8.6 $\pm$ 1.8
0.15	None	45/49	2.69(48)	3.64(10)	6.93(4)	9.89(4)	91.8	9.1 $\pm$ 1.6

*E. coli = Female was immunized with Escherichia coli bacterin.

LPS = Female was immunized with Bacto-Lipopolysaccharide Salmonella typhosa 0901 antigen.

LPS-Strep. = Female was immunized with both Bacto-Lipopolysaccharide Salmonella typhosa 0901 antigen and Streptococcus fecalis bacterins.

[#] Numerators are the numbers of mice dying of wasting disease; denominators are the numbers of mice treated. Numbers in parentheses indicate the number of animals observed.

The Effect of Intraperitoneal Gamma Globulin Administration
on Mice Injected with Cortisol Acetate
at 36 Hours of Age

Because endotoxins were thought to be involved in the pathogenesis of wasting syndromes (Safford and Jutila, 1965), attempts were made to alter the course of cortisol acetate induced wasting disease by the passive administration of rabbit anti-Bacto-Lipopolysaccharide Salmonella typhosa 0901 gamma globulin (anti-LPS gamma globulin--see Materials and Methods). Mice were injected with 0.25 mg cortisol acetate per gram body weight at 36 hours of age. Four days after cortisol injection the mice received an intraperitoneal injection of 0.1 ml of either anti-LPS gamma globulin or normal bovine gamma globulin (NBGG). Gamma globulin injections were repeated every third day until a total of five injections had been made, or until all animals in a litter were dead. The design of this experiment varied from all others in that control mice and cortisol treated mice were littermates. Thus, each litter was composed of two control mice and five to eight cortisol treated mice. The results of intraperitoneal gamma globulin administration are shown in Table V. It is apparent that the repeated intraperitoneal administration of anti-LPS gamma globulin did not alter the course of cortisol acetate induced wasting in mice. The weights of control mice in this experiment were higher than control weights observed in other experiments (see Table VI). The observation is explained as due to the control mice competing for milk with wasting littermates rather than healthy littermates.

Table V. The effect of intraperitoneal gamma globulin administration on mice injected with cortisol acetate at 36 hours of age.

Cortisol Acetate Dose (mg/g body wt)	Gamma Globulin*	No. # of Mice	Average Weight [#] at Days After Injection				Cumulative Mortality (%) at 30 Days After Injection	MST $\pm$ SD (days)
			6	10	20	30		
None	NBGG	0/9	5.53(9)	9.20(9)	14.67(7)	21.00(2)	0	
None	anti-LPS	0/9	5.42(9)	9.01(9)	14.45(7)	22.50(2)	0	
0.25	NBGG	27/30	2.93(27)	3.52(9)	6.48(3)	8.00(2)	90.0	9.4 $\pm$ 2.8
0.25	anti-LPS	29/34	3.05(32)	3.64(17)	7.33(4)	10.23(4)	85.4	10.0 $\pm$ 2.4
0.25	None	58/64	2.87(55)	3.80(25)	7.69(7)		90.6	9.9 $\pm$ 3.8

*NBGG = Normal bovine gamma globulin

anti-LPS = Rabbit anti-Bacto-Lipopolysaccharide Salmonella typhosa 0901 gamma globulin

[#]Numerators are the numbers of mice dying of wasting disease; denominators are the numbers of mice treated. Numbers in parentheses indicate the number of animals observed.

The Effect of Antibiotic Administration on Mice
Injected with Cortisol Acetate at
36 Hours of Age

Rosoff (1963) found that oral antibiotic administration prevented death in rats which had received a lethal dose of whole body irradiation, whereas systemic antibiotic administration did not improve the survival rate even when it prevented bacteremia. Thus, the oral route of antibiotic administration was selected. To avoid the trauma which accompanies gavage, antibiotics were added to the water supply of the mother mouse. It was assumed that the suckling experimental mice obtained the antibiotic orally through their mother's milk.

Manor Swiss mice, 36 hours old, were given a single subcutaneous injection of cortisol acetate. Antibiotics were added to the water supply immediately after the young were injected with cortisol and every day for 30 days thereafter. Antibiotic solutions were prepared fresh every day in cool tap water. The results obtained in these experiments appear in Table VI.

When 36-hour old mice were treated with 0.15 mg cortisol acetate per gram body weight and suckled on mothers which received terramycin or terramycin and penicillin in their water supply, the cumulative mortality was less than that observed in cortisol treated mice which did not receive antibiotics (Table VI). The protective effects of terramycin alone and terramycin combined with penicillin were similar; 68.6% and 61.8% cumulative 30 day mortality, respectively, in contrast to 91.8% in cortisol treated mice not receiving antibiotic. However, when a larger dose of cortisol (0.20 mg/g body weight) was used, terramycin given alone had a

Table VI. The effect of antibiotic administration on mice injected with cortisol acetate at 36 hours of age.

Cortisol Acetate Dose (mg/g body wt)	Antibiotic*	No. # of Mice	Average weight [#] at days after injection				Cumulative Mortality (%) at 30 days after injection	MST $\pm$ SD (days)
			6	10	20	30		
0.15	None	45/49	2.69(48)	3.64(10)	6.93(4)	9.89(4)	91.8	9.1 $\pm$ 1.6
0.15	T	24/35	2.68(31)	4.26(14)	8.41(11)	11.55(11)	68.6	8.5 $\pm$ 2.7
0.15	T + P	21/34	3.05(31)	3.83(25)	6.86(13)	7.91(13)	61.8	11.8 $\pm$ 1.5
0.20	None	57/60	2.76(53)	3.60(14)	6.80(3)	10.30(3)	95.0	8.6 $\pm$ 1.8
0.20	T	44/71	2.84(68)	3.92(44)	7.35(27)	11.90(26)	62.0	10.3 $\pm$ 4.3
0.20	T + P	20/23	2.84(23)	3.40(7)	6.28(3)	7.73(3)	87.0	9.3 $\pm$ 2.3
None	T	2/34	4.04(33)	5.58(32)	7.70(32)	15.55(32)	5.9	
None	T + P	10/35	3.19(32)	4.54(29)	6.03(27)	9.61(25)	28.6	13.5 $\pm$ 9.6
None	None	2/37	4.72(24)	6.61(24)	9.73(24)	19.85(24)	5.4	

*T = 20 mg % terramycin in the mother's water supply.

P = 4,000 units/ml penicillin in the mother's water supply.

[#]Numerators are the numbers of mice dying of wasting disease; denominators are the numbers of mice treated. Numbers in parentheses indicate the number of animals observed.

greater protective effect than the combined administration of terramycin and penicillin (Table VI).

Two observations should be considered in any attempt to evaluate the effect of antibiotic administration on cortisol acetate-induced wasting disease. As shown in Table VI, when young mice, not treated with cortisol, were suckled on mothers receiving terramycin, the weight gain of the suckling mice was retarded. When young mice, not treated with cortisol, were suckled on mothers receiving terramycin and penicillin, weight gain was severely retarded and the 30 day cumulative mortality was 23% higher than that of control young mice suckled on mothers not receiving antibiotics (Table VI). Daily inspections were made and no evidence of the antibiotic treated mother mice being off feed or failing to nurse their young was recorded.

The second observation concerns the length of time antibiotics had been used in the conventional animal colony. In the first antibiotic experiment, using young mice injected with 0.20 mg/g body weight of cortisol and suckled on females receiving terramycin, a protective effect was observed (Table VI). This experiment was followed by one using young mice injected with 0.20 mg/g body weight of cortisol and suckled on females which received both terramycin and penicillin. The protection observed in the latter case was unimpressive (Table VI). The experiments using terramycin, and terramycin with penicillin, in mice injected with 0.15 mg/g body weight of cortisol acetate were conducted during the same time period. Antibiotics had not been used in the animal colony for several weeks before these experiments were started. It is possible that

the continued use of terramycin in the mouse colony resulted in the development and selection of terramycin-resistant microorganisms, reducing the effectiveness of terramycin therapy in wasting mice. Terramycin resistant cultures of Proteus have been isolated from wasting mice in this laboratory following prolonged use of terramycin in the mouse colony (Jutila, 1966).

Thus, although the administration of antibiotics reduced the mortality in cortisol acetate induced wasting disease, the usefulness of antibiotic therapy was limited by the detrimental effect of the antibiotics on control mice and the possible selection of antibiotic resistant organisms.

The Effect of the Germfree State on Mice Injected
with Cortisol Acetate at 36 Hours of Age

Germfree Manor Swiss mice, 36 hours old, were injected subcutaneously in the dorsal neck region with varying doses of cortisol acetate. Conventionally reared Manor Swiss mice treated with equal doses of cortisol acetate were included as controls in these experiments. Details of the materials and techniques used in maintaining the germfree animals have been given in the Materials and Methods section.

A single injection of 0.25 mg of cortisol acetate into mice 36-hours old resulted in moderate symptoms characteristic of the wasting syndromes in both conventionally reared and germfree mice. The skin was thin and wrinkled and the growth of hair was impaired. When the coat did develop, it was sparse and ruffled. Most of the mice recovered after a variable period of time and reproduced normally. Mortality with a dose

of 0.25 mg was low in both the conventionally reared and the germfree groups (Table VII). Although the weight gained by germfree mice injected with 0.25 mg cortisol acetate approached that of the normal control groups, the weight gained by conventionally reared mice injected with 0.25 mg cortisol acetate was retarded during a 30 day observation period (Table VII).

When 0.50 mg of cortisol acetate was given to germfree and conventionally reared mice at 36 hours of age, both groups showed severe signs of wasting and a marked failure to gain weight. The skin was extremely loose, wrinkled, and thin, and the growth of hair was markedly impaired. The mice exhibited a hunched posture and characteristic high stepping gait. The ischemic appearance of the feet and tail and black discoloration in the abdominal cavity, previously noted in conventionally reared mice, was also observed in the germfree mice. Diarrhea was observed in both groups. However, with this dose a striking difference in mortality between the germfree and conventionally reared groups was observed (Table VII). These data show a cumulative mortality at 30 days after injection, of 90.6% (58/64) in conventionally reared mice as contrasted with 15.1% (5/33) in germfree mice. By 40 days after injection the germfree survivors had developed a normal appearance and later reproduced. The response of germfree and conventionally reared animals to doses of 0.75 mg per mouse and 0.20 mg per gram of body weight are also summarized in Table VII.

Although many of the symptoms characteristic of the wasting syndromes appeared in germfree neonatal mice treated with cortisol acetate, the incidence of death was greatly reduced and the mean survival time

Table VII. Cortisol acetate-induced wasting disease in germfree (GF) and conventionally reared (CR) mice.

Cortisol Acetate Dose	Status of Mice	No.* of Mice	Average Weight* at Days After Injection				Cumulative Mortality (%) at 30 Days After Injection	MST $\pm$ SD (days)
			4	12	20	30		
None	CR	2/37	3.72(24)	7.33(24)	9.73(24)	19.85(24)	5.4	
0.25 mg/ mouse	CR	4/25	2.72(25)	5.63(21)	7.96(21)	13.86(21)	16.0	9.0 $\pm$ 2.6
0.50 mg/ mouse	CR	58/64	2.59(64)	4.44(14)	7.69(7)		90.6	9.9 $\pm$ 3.8
0.20 mg/g body weight	CR	57/60					95.0	8.6 $\pm$ 1.8
0.75 mg/ mouse	CR	10/10					100.0	5.8 $\pm$ 1.6
None	GF	1/14	3.55(14)	6.95(14)	11.44(12)	20.99(5)	7.1	
0.25 mg/ mouse	GF	3/24	2.94(24)	6.57(23)	10.67(21)	17.95(21)	12.5	15.0 $\pm$ 3.5
0.50 mg/ mouse	GF	5/33	2.55(24)	5.22(20)	8.41(19)		15.1	11.0 $\pm$ 3.1
0.20 mg/g body weight	GF	14/44					31.8	11.5 $\pm$ 2.4
0.75 mg/ mouse	GF	5/10					50.0	10.8 $\pm$ 2.7

*Numerators are the numbers of mice dying of wasting disease; denominators are the numbers of mice treated. Numbers in parentheses indicate the number of animals observed.

increased as compared to conventionally reared neonates given a comparable dose. Thus, the germfree animal provides an opportunity to study the pathogenesis of cortisol acetate-induced wasting disease in the absence of complicating factors introduced by the presence of microorganisms.

Cortisol Acetate-Induced Wasting Disease
in Dibiotic Mice

Because microorganisms that are considered part of the "normal flora" were isolated from wasting mice, and because the mortality in germfree mice treated with cortisol acetate was greatly reduced as compared to conventionally reared mice, experiments were conducted to determine the effect of monocontamination on germfree mice treated with cortisol acetate. The procedure used to render germfree mice dibiotic is detailed in the section on Materials and Methods. To determine that the bacteria used as monocontaminates were successfully established in the mice, the contents of the stomach, small intestine, large intestine, and cecum were cultured and the approximate numbers of bacteria per gram determined by plating serial dilutions on blood agar. The plates were incubated for seven days under aerobic and anaerobic conditions. Table VIII shows the numbers of bacteria found in the organs of five dibiotic mice which were assayed.

It is obvious from these data (Table VIII) that the procedure used was successful in producing dibiotic mice. The numbers of bacteria found in the various parts of the intestinal tract in these studies are, with one exception, very similar to the values reported by Schaedler, Dubos, and Costello (1965). These workers reported 10^8 and 10^9 coliform bacilli

Table VIII. Numbers of bacteria in the intestinal tract of five dibirotic mice.

Bacteria* Used	:Cortisol Acetate: :Dose at 36 Hours:	Approximate Number of Bacteria Per Gram			
		Stomach	Small Intestine	Large Intestine	Cecum
<u>Bacteroides</u> sp.	0.15 mg/g body weight	4×10^3	4×10^2	9×10^6	6×10^8
<u>Bacteroides</u> sp.	0.15 mg/g body weight	3×10^4	4×10^3	3×10^7	1×10^9
<u>Bacteroides</u> sp.	None	2×10^3	5×10^4	2×10^7	8×10^7
<u>Bacteroides</u> sp.	None	4×10^3	4×10^5	3×10^8	8×10^7
<u>Escherichia</u> <u>coli</u>	None	8×10^3	3×10^6	2×10^8	4×10^9

*Bacterial cultures used to render mice dibirotic were added to the mother's water supply when the young were 36 hours old, numbers of bacteria were determined 32 days later.

(a slow lactose fermenter) per gram of tissue in the stomach and small intestine of dibirotic mice, in contrast to about 10^4 and 10^6 in the present study. However, only one mouse was assayed in the present study, and the coliform cultures used were different.

The results obtained when cortisol acetate treated germfree mice were made dibirotic with cultures of Escherichia coli or Bacteroides sp. are given in Table IX. The bacteria used were isolated from mice in the conventional mouse colony. Germfree mice were injected with cortisol acetate and monocontaminated at 36 hours of age.

Dibirotic mice treated with 0.15 mg/g body weight of cortisol acetate had a lower mortality than germfree mice treated with this dose. Why the germfree mice treated with 0.15 mg/g body weight showed such a high mortality in this experiment is not clear.

Table IX. Cortisol acetate-induced wasting disease in monobiotic and dibiotic mice.

Cortisol Acetate Dose	Monocontaminating Bacteria	No.* of Mice	Cumulative Mortality (%) at 30 Days After Injection
None	<u>Escherichia coli</u>	1/16	6.3
0.15 mg/g body weight	<u>Escherichia coli</u>	1/6	16.6
0.50 mg/mouse	<u>Escherichia coli</u>	13/16	81.7
None	<u>Bacteroides sp.</u>	2/10	20.0
0.15 mg/g body weight	<u>Bacteroides sp.</u>	2/10	20.0
0.15 mg/g body weight	None	6/13	46.2
0.50 mg/mouse	None	5/33	15.1

*Numerators are the numbers of mice dying of wasting disease; denominators are the numbers of mice treated.

With a dose of 0.50 mg per mouse (about 0.25 mg/g body weight) the mortality in dibiotic mice associated with Escherichia coli (81.7%) was greater than the mortality in germfree mice (15.1%) treated with an equal dose. Using this dose, the mortality in dibiotic mice associated with Escherichia coli (81.7%) was comparable to that observed in conventionally reared mice (90.6% - see Table VII).

Involution and Recovery of the Thymus and Spleen in Mice Injected with Cortisol Acetate

Since the mortality following treatment with cortisol acetate was low in germfree mice, these animals provided information which could not be obtained from conventionally reared mice.

Germfree and conventionally reared Manor Swiss mice were injected with cortisol acetate (0.20 mg/g body weight) at 36 hours of age. At 0, 2, 6, 10, 20 and 30 days after injection mice were weighed and hemato-

logic and lymphoid organ weight studies performed. With the exception of treated conventionally reared groups later than day six post-injection, 8-12 mice were studied in each group. The techniques used are given in the section on Materials and Methods. The mice sacrificed for study at any given day after cortisol injection were obtained from several different litters, so that any variation between litters was evenly distributed throughout the observation periods. Germfree and conventionally reared mice, injected with water at 36 hours of age, served as controls.

Figure 4 is a plot of the ratio, thymus weight (mg) / body weight (g), of the cortisol treated and control mice. The control curves are very similar to those reported for normal germfree and conventionally reared mice by Wilson, Bealmear, and Sobonya (1965).

Thymic involution was marked in both germfree and conventionally reared mice injected with cortisol (Figure 4). Observations on conventionally reared mice were terminated at day 10 after injection due to death of the animals. In contrast, most of the germfree treated mice survived and a rapid recovery of the thymus, starting between six and ten days after injection, was observed. The weight of the thymus, relative to body weight, had recovered to control values by 30 days after injection (Figure 4). Similar involution and recovery was observed in the case of the spleen (Figure 5). The hematologic observations made on these mice are reported in the next section.

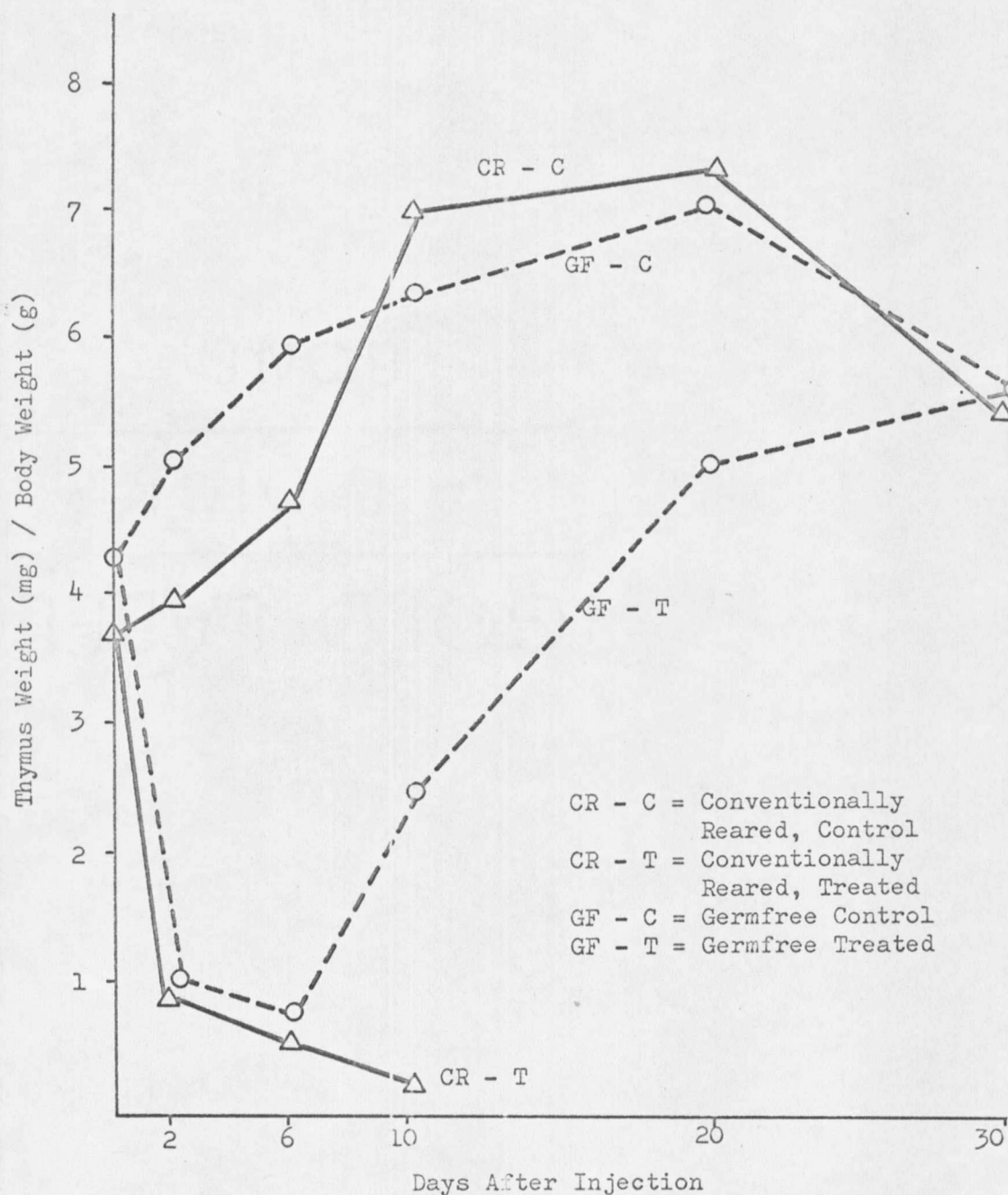


Figure 4. Thymus Weight (mg) / Body Weight (g) Ratio in Germfree and Conventionally Reared Mice Injected with 0.20 mg Cortisol Acetate Per Gram Body Weight at 36 Hours of Age.

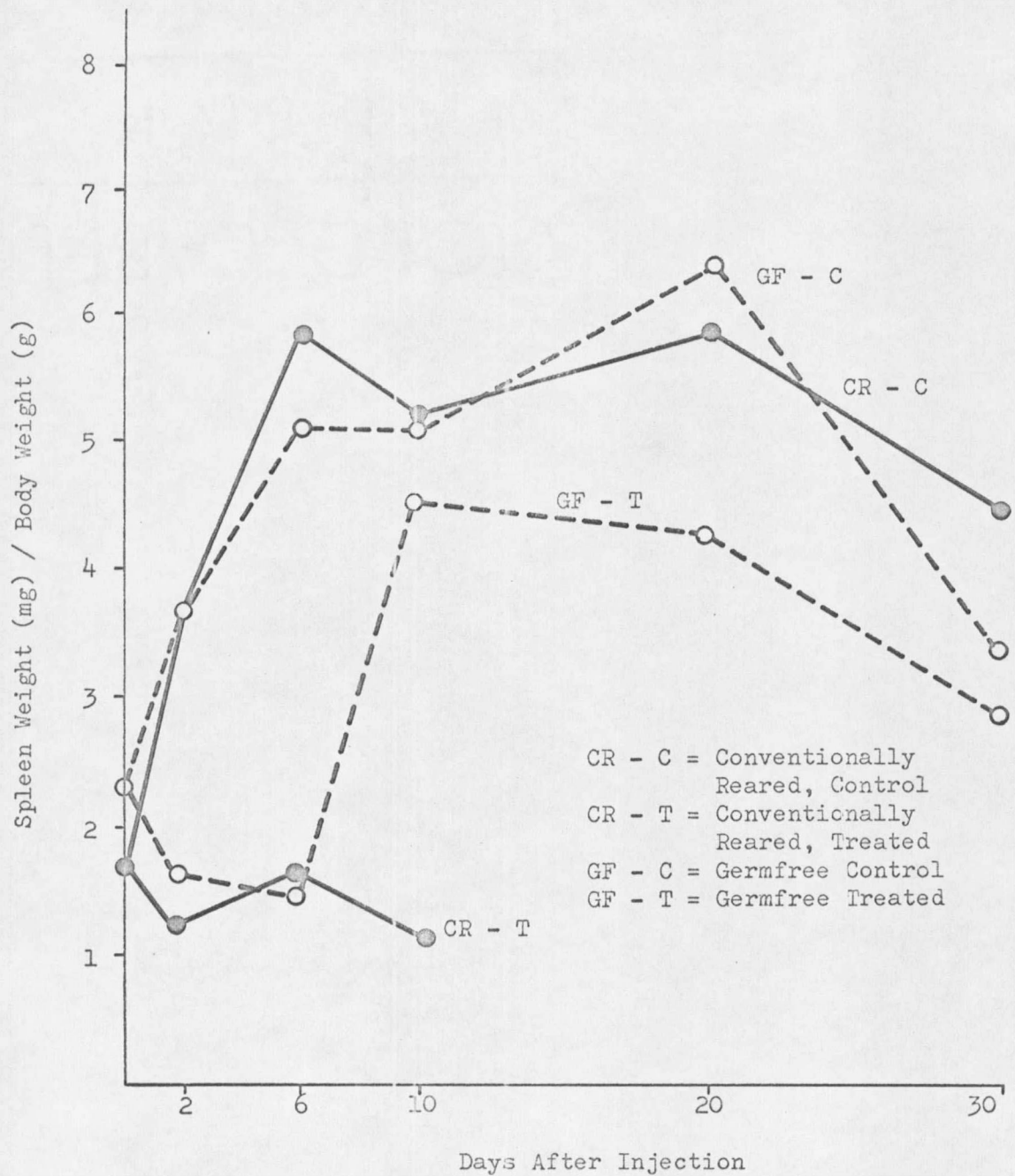


Figure 5. Spleen Weight (mg) / Body Weight (g) Ratio in Germfree and Conventionally Reared Mice Injected with 0.20 mg Cortisol Acetate Per Gram Body Weight at 36 Hours of Age.

Hematologic Observations on Germfree and Conventionally
Reared Mice Injected with Cortisol
Acetate at 36 Hours of Age

Germfree and conventionally reared Manor Swiss mice were injected with 0.20 mg of cortisol acetate per gram of body weight at 36 hours of age. Observations made on the thymus and spleens of these animals are recorded in the previous section. Hematologic studies were done at 0, 2, 6, 10, 20 and 30 days after injection. The hematologic techniques employed are detailed in the section on Materials and Methods.

The results of differential leukocyte counts and total leukocyte counts are listed in Table X. In order to eliminate an element of subjective interpretation and to obviate the distinction between small, medium, and large size lymphocytes and monocytes, these cells are hereafter referred to collectively as mononuclear leukocytes. Gorer (1946) has pointed out that while differentiation between these cell types is generally easy in man, it is difficult in the mouse.

Table XI depicts body weight, hematocrit values, and the ratios of mononuclear/polymorphonuclear leukocytes in tail blood. Absolute counts of mononuclear leukocytes are presented graphically in Figure 6. The absolute counts of polymorphonuclear leukocytes are shown in Figure 7. The observation that normal conventionally reared animals had larger numbers of mononuclear and polymorphonuclear blood cells than normal germfree animals (Figure 6 and 7) has also been made in other species (Luckey, 1963).

Both germfree and conventionally reared neonates responded to cortisol injection with a leukopenia (Table X). In germfree animals the

Table X. Differential leukocyte counts and total leukocyte counts of germfree and conventionally reared mice injected with cortisol acetate at 36 hours of age.*

Cortisol: Acetate : (mg/g body wt):	Status of Mice	# : Day : After : Injec- tion :	Differential Counts ^{1/2} (%)					Total WBC Count/mm ³
			Neut	Eosin	Baso	Lympho	Mono	
None	CR	0	77.1	1.0	0	17.5	4.4	2,321
None	GF	0	75.9	0.9	0	16.8	6.4	2,554
None	CR	2	53.2	0.8	0	42.2	3.8	3,304
0.20	CR	2	91.3	0.3	0	6.2	2.2	1,143
None	GF	2	59.6	1.0	0	34.7	4.7	2,186
0.20	GF	2	94.5	0	0	3.7	1.8	903
None	CR	6	39.2	2.5	0	54.0	4.3	3,651
0.20	CR	6	93.8	0.1	0	3.6	2.5	2,815
None	GF	6	37.5	0.5	0	56.2	5.8	3,200
0.20	GF	6	94.1	0	0	4.7	1.2	708
None	CR	10	26.4	3.2	0	65.4	5.0	4,446
0.20	CR	10	94.0	0	0	6.0	0	1,390
None	GF	10	25.2	0.5	0	68.6	5.7	3,298
0.20	GF	10	81.1	0.7	0	14.3	3.9	1,683
None	CR	20	21.7	1.1	0	74.7	2.5	3,433
0.20	CR	20	all mice dead					
None	GF	20	13.2	1.3	0	82.2	3.3	3,126
0.20	GF	20	39.6	5.1	0	52.1	3.2	2,735
None	CR	30	22.2	1.5	0	71.7	4.6	3,154
0.20	CR	30	all mice dead					
None	GF	30	22.1	2.4	0	73.3	2.2	2,305
0.20	GF	30	23.5	1.1	0	74.1	1.3	4,194

#CR = Conventionally Reared
 GF = Germfree
 Neut = Neutrophil
 Eosin = Eosinophil

Baso = Basophil
 Lympho = Lymphocyte
 Mono = Monocyte

*With the exception of values after day 6 post-injection for treated conventionally reared (CR) groups, all values listed are the average from 8-12 mice.

Table XI. Body weight, hematocrit values, and mononuclear leukocyte/polymorphonuclear leukocyte ratios of germfree and conventionally reared mice injected with cortisol acetate at 36 hours of age.*

Cortisol:	Status#:	Day :	:	:	:	:
Acetate :	of :	After :	Body :	Hematocrit :	M/P Ratio#	
(mg/g :	Mice :	Injec-:	Weight :	(Heart blood) :	(Tail blood)	
body wt):	Mice :	tion :	:	:	:	
None	CR	0	1.85	39.9		0.30
None	GF	0	1.86	38.0		0.32
None	CR	2	2.57	34.7		0.92
0.20	CR	2	2.23	39.0		0.10
None	GF	2	2.52	33.3		0.72
0.20	GF	2	2.48	35.0		0.06
None	CR	6	4.76	35.4		1.49
0.20	CR	6	2.75	34.5		0.07
None	GF	6	4.50	36.8		1.70
0.20	GF	6	3.06	37.7		0.07
None	CR	10	7.12	41.8		2.72
0.20	CR	10	2.85	29.0*		0.07
None	GF	10	6.14	35.1		3.63
0.20	GF	10	4.83	37.0		0.24
None	CR	20	11.24	47.7		4.50
0.20	CR	20		all mice dead		
None	GF	20	13.66	43.5		6.27
0.20	GF	20	8.82	44.2		1.79
None	CR	30	20.12	43.3		4.72
0.20	CR	30		all mice dead		
None	GF	30	22.60	42.4		3.83
0.20	GF	30	12.46	47.8		3.56

#CR = Conventionally Reared

GF = Germfree

M = Mononuclear leukocytes, includes small, medium, and large lymphocytes and the blood monocytes

P = Polymorphonuclear leukocytes, includes neutrophils, eosinophils and basophils

*With the exception of values after day 6 post-injection for treated conventionally reared (CR) groups, all values listed are the average from 8-12 mice.

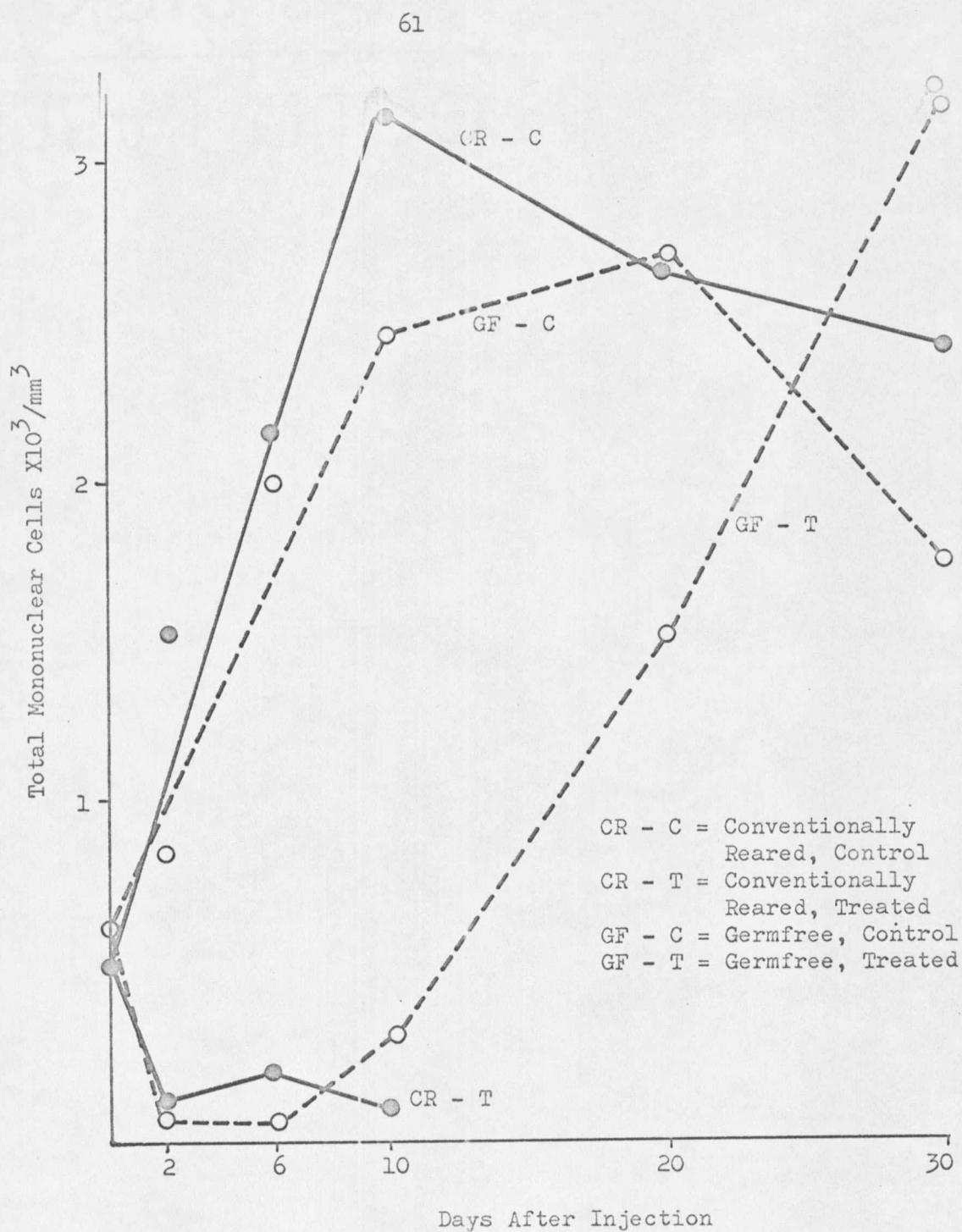


Figure 6. Absolute Numbers of Mononuclear Cells in the Tail Blood of Germfree and Conventionally Reared Mice Injected with 0.20 mg Cortisol Acetate Per Gram Body Weight at 36 Hours of Age.

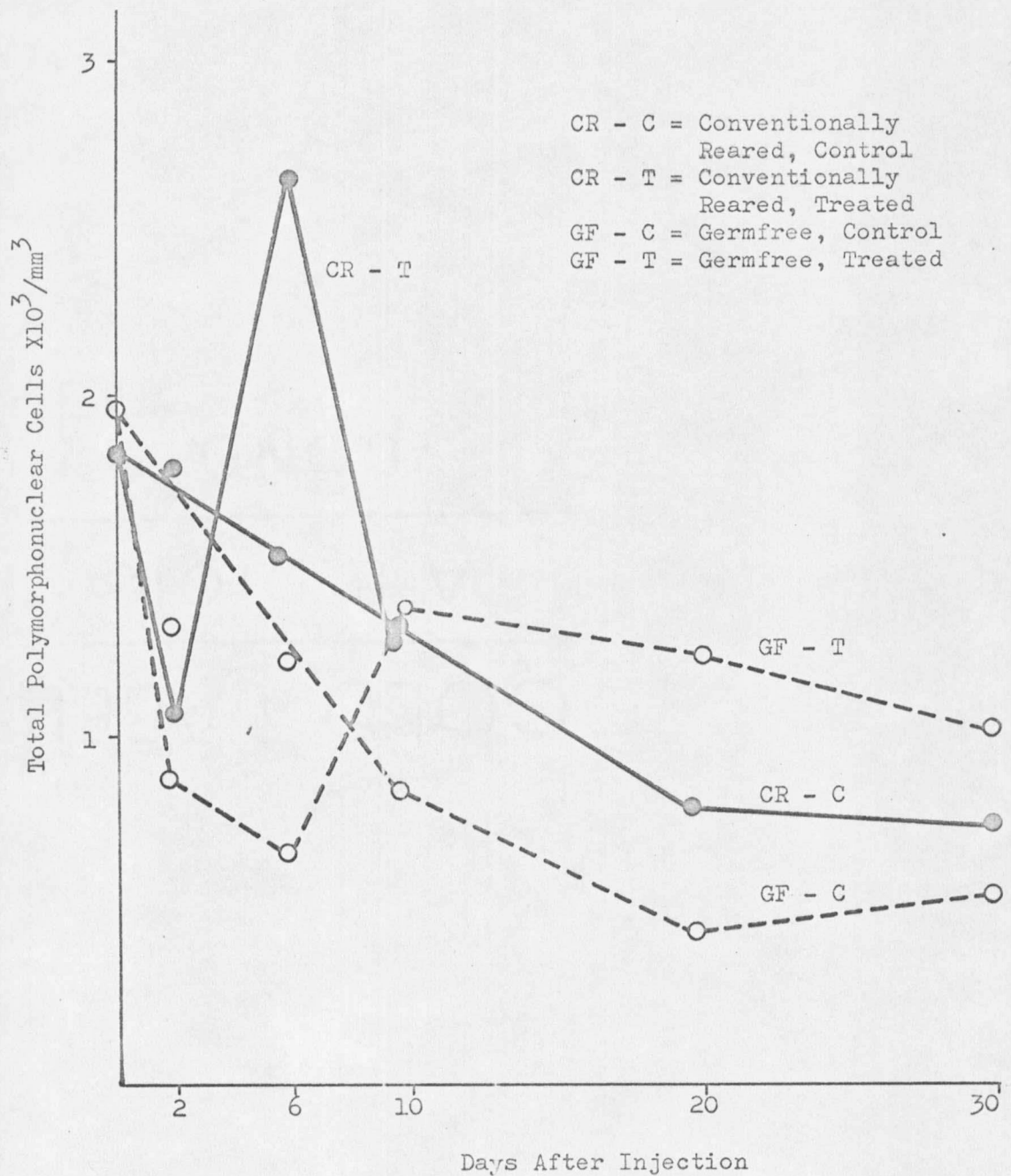


Figure 7. Absolute Numbers of Polymorphonuclear Cells in the Tail Blood of Germfree and Conventionally Reared Mice Injected with 0.20 mg Cortisone Acetate Per Gram Body Weight at 36 Hours of Age.

leukopenia was initially the result of a decrease in the number of both mononuclear and polymorphonuclear leukocytes (Figures 6 and 7). After day 10, however, the decreased leukocyte count was due entirely to a severe lymphopenia (Figure 6), since at this time the number of polymorphonuclear cells in treated germfree animals was higher than in control germfree mice (Figure 7). Between 20 and 30 days after injection the number of mononuclear cells in germfree cortisol treated mice approached control values and at day 30 after injection surpassed control values.

In cortisol treated conventionally reared mice an initial decline in the number of both mononuclear and polymorphonuclear leukocytes was observed (Figures 6 and 7). Lymphopenia persisted until the death of the animals. The number of polymorphonuclear leukocytes, however, increased markedly between two and six days after injection, possibly in response to infection. The decline in the polymorphonuclear count in this group, between six and ten days after injection, should be considered lightly since only two animals were observed. The death of all cortisol treated conventionally reared mice prevented the accumulation of data concerning this group after day 10 post-injection.

In germfree control animals, the mononuclear/polymorphonuclear cell ratio (M/P ratio) increased rapidly during the first six days after injection (Table XI), as the lymphocyte replaced the neutrophil as the predominant blood cell. In contrast, the M/P ratio decreased markedly in cortisol treated mice during this interval (Table XI). A rapid increase in the number of mononuclear cells and a stabilization in the polymorphonuclear count (Figures 5 and 6) in treated mice, between six and 30

days after injection, resulted in a M/P ratio at day 30 which was very close to the control value (Table XI).

With the exception of observations on conventionally reared mice at 10 days after injection, which included only two test animals, hematocrit values in cortisol treated animals were similar to control values.

Immunological Competence of Mice Injected
with Cortisol Acetate at 36 Hours of Age

Previous experiments have shown a morphologic involution and recovery of lymphoid tissues in cortisol acetate treated neonates (Figures 4, 5, 6, and 7). In studying the pathogenesis of this wasting disease it was clearly of interest to determine the immunological capacity of the cortisol treated animals at various times during the recovery of the lymphoid system. Although these studies are still in an early stage, the early results obtained have been of sufficient significance to warrant description and discussion.

The experiments to be described employed the ascites form of Sarcoma I tumor. Sarcoma I is a dibenzanthracene induced tumor indigenous to A-strain mice. In A-strain mice the tumor is not "foreign", thus it does not incite a homograft rejection response; instead, it grows to the death of it's host. This tumor normally starts to grow in mice not of the A-strain, but is then rapidly rejected by a homograft response. Thus, challenge of Manor Swiss mice with the Sarcoma I tumor should serve as a convenient test of the homograft response.

Manor Swiss mice, treated as neonates with cortisol acetate and protected by antibiotics, immune mothers, or the germfree state, were

challenged at various times by the intraperitoneal inoculation of Sarcoma I tumor. Since some of the mice used were under 30 days of age and might have died from wasting disease, all mice dying after tumor challenge were autopsied. Only mice showing massive metastatic tumor growth were recorded as dying of tumor invasion. The details of the experiments, and the results, are recorded in Table XII.

Table XII. The response of normal and cortisol acetate-treated Manor Swiss mice to Sarcoma I tumor.

Cortisol:	Status*	Day(after : cortisol : injection):	No. of Tumor : Cells Injected:	No. : of : Mice :	Result
Acetate : Dose at : 36 Hrs. : (mg/g : body wt):	of Mice	of Tumor : Challenge :	(intraperiton- : eally)		
0.15	T	13	1.3×10^6	2	Died of tumor 23 and 26 days after challenge
None	CR	13	1.3×10^6	8	Rejected tumor 15-18 days after challenge
0.20	GF	50	1.0×10^7	1	Died of tumor 29 days after challenge
None	GF	50	1.0×10^7	1	Rejected tumor in 12 days
0.15	LPS-S immune	52	1.0×10^7	2	Died of tumor 25 and 27 days after challenge
0.15	LPS-S immune	51	1.0×10^7	1	Rejected tumor in 22 days
0.15	<u>E. coli</u> immune	53	1.0×10^7	1	Died of tumor 29 days after challenge
None	CR	42	1.0×10^7	2	Rejected tumor in 12 days

*T = Mother received 20 mg % terramycin in drinking water
 CR = Conventionally reared
 GF = Germfree
 LPS-S immune = Mother immunized against Bacto-Lipopolysaccharide and Streptococcus fecalis bacterin.
E. coli immune = Mother immunized against Escherichia coli bacterin.

All of 11 control mice challenged with Sarcoma I tumor rejected the tumor. In contrast, six of seven cortisol treated mice challenged with Sarcoma I tumor died of tumor growth. Rejection time in the single survivor was longer than in the control groups.

Surprisingly 50 days after cortisol injection, when by appearance and blood studies the mice appeared fully recovered, the homograft reaction was seriously impaired. It appeared that the morphologic recovery of the lymphoid tissues was not accompanied by a functional recovery.

In an effort to determine why these apparently recovered mice could not reject a tumor homograft, many of the blood smears which provided the data for Figures 4, 5 and 6, and Tables X and XI, were re-examined.

Lymphocytes 9μ or less in diameter were considered small lymphocytes, those larger than 9μ were considered large lymphocytes. The re-examination indicated that the lymphoid recovery was not morphologically normal (Table XIII). The proportion of small lymphocytes, the cell type generally considered to be intimately involved in immune responses (Gowans and McGregor, 1965), was reduced in the cortisol treated mice (Table XIII).

Table XIII. Small lymphocyte/large lymphocyte ratios in control and cortisol acetate-treated germfree mice.

Cortisol Acetate Dose (mg/g body wt)	Day After Injection	S/L Ratio*
None	10	0.736 (53/72)
0.20	10	0.022 (2/89)
None	30	0.712 (41/59)
0.20	30	0.428 (45/105)

*S = Small lymphocyte, $\leq 9\mu$.

L = Large lymphocyte, $> 9\mu$.

Numbers in parentheses indicate the number of cells observed.

DISCUSSION

A form of acute adrenal insufficiency in humans occurs in patients who have previously been treated for long periods of time with either corticotropin or an adrenal corticosteroid (Thorn, 1963). In such cases, supplementary hydrocortisone or cortisone must be administered. Also, it has been observed that the administration of 11-oxysteroids leads to atrophy of the zona fasciculata, a histologically defined zone of the adrenal cortex believed to be responsible for cortisol production (Bloom and Fawcett, 1962). Early in the course of this work it was suggested by a visiting physician that the fatal wasting disease which follows the neonatal administration of cortisol acetate in mice might be the result of adrenal insufficiency. This suggestion was not supported by the results obtained in the present study. The administration of maintenance levels of cortisol acetate did not favorably alter the course of the disease (Table II), and, significantly, mortality was greatly reduced in germfree mice (Table VII). If the fatal wasting disease resulted from adrenal insufficiency, one would expect the incidence of death in germ-free and conventionally reared mice to be similar.

The course of the wasting disease decreased in severity as the age of the mice at the time of cortisol injection was increased (Table I). In this respect, cortisol induced wasting is very much like the post-neonatal thymectomy syndrome. The earlier in life thymectomy is performed, the higher the incidence of the associated wasting syndrome (Miller, Marshall and White, 1962). Thymectomy after one week of age is not associated with any significant incidence of this syndrome.

Since the primary factor in the pathogenesis of the post-neonatal thymectomy syndrome appears to be an environmental factor, presumably an infectious agent or its product (McIntire et al., 1964), the possibility of an infectious etiology in the wasting disease induced with cortisol acetate was considered. In contrast to the results of Schlesinger and Mark (1964), evidence of infection was observed. Bacteria were frequently isolated from the organs of wasting mice (Table III). Escherichia coli and Streptococcus fecalis were the organisms most commonly isolated from the wasting mice. Due to the difficulties in effectively sampling organs from such small animals (total body weight 2-4 grams), the moribund condition of the wasting mice, and the isolation of predominantly bacteria which are considered part of the normal flora, the results of the bacteriological studies are difficult to evaluate. More exacting bacteriological studies were not performed; instead, attempts were made to mitigate cortisol-induced wasting in conventionally reared mice by passive immunization against bacteria and bacterial products and treatment with antibiotics.

The experiments on passive immunization (Tables IV and V) were prompted by the successes of Safford and Jutila (1965) in reducing the mortality in "runt disease" by passive immunization against various bacteria and bacterial products. In the present study, the repeated intraperitoneal injection of rabbit anti-Bacto-Lipopolysaccharide Salmonella typhosa 0901 gamma globulin did not alter the course of cortisol acetate-induced wasting (Table V). The failure to protect wasting mice in this experiment may have been due to the limited antigenic spectrum involved, or failure of the immune globulin to react effectively with bacteria and

bacterial products in the gut. Rosoff (1963), for example, found that orally administered antibiotics prevented death in irradiated rats only when cultural data demonstrated the successful elimination of the coliform flora of the gut. Systemic antibiotic administration did not improve the survival rate even when it prevented bacteremia (Rosoff, 1963). These observations suggest that to reduce mortality in lethally irradiated animals it is necessary to control the activities of the coliform flora in the gut. Thus, in other experiments concerned with passive immunization the antigenic spectrum was broadened and the immune globulin was orally presented to the newborn through the lacteal secretions of immune mothers.

In contrast to the observations made in studies on "runt disease" (Safford and Jutila, 1965), protection was not observed when cortisol treated neonates were suckled on females immunized against either Bacto-Lipopolysaccharide Salmonella typhosa 0901 or an Escherichia coli bacterin (Table IV). A very moderate decrease in mortality was observed in the cortisol treated young of females immunized against both Bacto-Lipopolysaccharide and a Streptococcus fecalis bacterin. In addition to the narrow antigenic spectrum involved, other factors may have contributed to the lack of protection observed in the experiments involving maternal immunization. Although variable antibody titers can be demonstrated in the sera of female mice immunized by the procedures employed (Safford, 1966), the quality and quantity of immune globulin which reaches the young through the mothers milk or by crossing the placental membranes was not determined. The passage of antibody from blood to lacteal secretions

and across the placental membranes is a selective process, as is the absorption of antibody from the gut (Humphrey and White, 1964). Gamma-macroglobulins (IgM), which probably constitute the bulk of the immunoglobulins formed against endotoxins, are selected against in the above processes (Humphrey and White, 1964). Thus, the failure of passive immunization against Bacto-Lipopolysaccharide, Escherichia coli, and Streptococcus fecalis to alter the course of cortisol-induced wasting is not proof against an infectious etiology in this disease. In fact, the results of experiments involving oral antibiotic administration support the concept of an infectious etiology.

The oral administration of terramycin or terramycin and penicillin reduced the severity of cortisol acetate-induced wasting disease (Table VI). Duhig (1965), also using terramycin in the mother's water supply, has obtained similar results. Azar (1963) reported that the administration of terramycin reduced the incidence of bacterial infection and wasting in neonatally thymectomized rats.

The usefulness of antibiotic therapy was limited in the present study by the detrimental effect of the antibiotics on control mice (Table VI). Dubos and coworkers (Dubos, Schaedler and Costello, 1963) have also observed a growth-depressing effect of antibacterial drugs in mice, and suggested that this effect was mediated through changes in the gastrointestinal microbiota. That changes in the normal flora, brought about by antibiotic administration, can be detrimental to the health of animals was strikingly demonstrated by Newton and coworkers (Newton, Steinman and Brandriss, 1964). These investigators established that when germfree and

conventionally reared Guinea pigs were injected intraperitoneally with potassium penicillin G (120 mg/kg body weight), no mortality was observed in the germfree group (0/30) whereas 52% (22/42) of the conventionally reared animals died between three and eight days after treatment.

The usefulness of antibiotic therapy was also limited in the present study by the indication that the effectiveness of the antibiotics decreased as the time the animal colony was in contact with the antibiotics increased (see Results). This suggested that prolonged treatment with antibiotics led to the development and selection of antibiotic resistant organisms in the mouse colony. This explanation is supported by observations made during studies on the antibiotic treatment of post-irradiation infection in mice (Miller et al., 1952). Miller's group found that antibiotic therapy (including terramycin) reduced the incidence of post-irradiation deaths in early experiments. The early success was followed by therapeutic failure, caused by epidemics of infection by drug-resistant strains of Pseudomonas. Epidemics of Proteus and Salmonella infections were also observed. These epidemics were controlled by terminating all experiments and sterilizing all equipment. Such a house cleaning was always followed by a series of experiments in which antibiotic therapy was once again effective (Miller et al., 1952). Terramycin resistant cultures of Proteus have been isolated from wasting mice in this laboratory, following prolonged use of terramycin in the colony (Jutila, 1966).

Although the factors discussed above limited the usefulness of antibiotic therapy, the protection observed (Table VI) provides evidence that infectious agents are involved in the pathogenesis of cortisol

acetate-induced wasting disease. Confirming evidence was obtained in experiments utilizing germfree mice.

The germfree technique is a highly refined research tool which avoids the variables inherent in experimental designs involving cultural procedures, passive immunization, or antibiotic therapy. Thus, the observation that the severity of cortisol acetate-induced wasting disease is markedly mitigated in germfree mice, as compared to conventionally reared mice, is of singular importance (Table VII). The most obvious difference between germfree mice and their conventionally reared counterparts is the absence of a microbial flora in the germfree animals. Therefore, it seems probable that the higher mortality in cortisol treated conventionally reared mice was due to their microbial flora. This concept is supported by the results obtained when cortisol treated (0.5 mg/mouse) germfree mice were rendered dibiotic with Escherichia coli. The 30-day cumulative mortality in the dibiotic mice, 81.7%, was much greater than in germfree mice, 15.1% (Table IX). However, it is important to stress that this does not incriminate E. coli as the only microorganism contributing to the death of wasting mice, since with lower doses of cortisol (0.15 mg/g body weight) mortality was low in E. coli associated dibiotic mice (16.6%) even though this dose commonly results in a 30-day cumulative mortality of 91.8% in conventionally reared mice (Tables I and IX). Thus, other microorganisms must contribute to the death of wasting animals.

Because the mortality following treatment with cortisol acetate was low in germfree mice, these animals were well suited for studies, over

extended periods of time, on the primary effects of neonatal cortisol administration. A striking effect of such treatment was a rapid and extreme involution of lymphoid tissue, followed by recovery of this tissue. This involution and recovery was quantitated in the case of the thymus (Figure 4), spleen (Figure 5), and the circulating lymphocytes (Tables X and XI; Figure 6).

It is interesting to speculate that the peaks in the spleen weight/body weight ratios of control animals (Figure 5) were caused by periods of intense antigenic stimulation. The first peak might be related to the antigenic stimulation provided by the developing intestinal microflora, which occurs during this time interval (Schaedler and Dubos, 1964). This explanation is supported by a strong peak in the curve describing conventionally reared mice, and a plateau in the curve describing the unstimulated germfree animals. The second peak might be related to the antigenic stimulation involved in changing from a milk diet to a solid diet, which takes place around 20 days of age. This would explain the higher peak in the curve describing germfree animals, since the change to solid feed represents their first intense antigenic challenge.

The lymphoid tissues studied were poorly developed in the normal neonatal animal, but developed rapidly during the first ten days of life (Figures 4, 5 and 6). The neonatal injection of cortisol acetate caused a severe involution of the poorly developed lymphoid system (Figures 4, 5 and 6), resulting in young animals with extremely low levels of lymphoid tissue and decreased immunological competence. It is significant that

this time period in conventionally reared mice (injection to 20 days post-injection) also encompasses the development of the microbial flora of the gut (Schaedler and Dubos, 1964) and the mean survival time of cortisol treated neonates (Table I).

Since neonatal thymectomy also results in failure to develop normal lymphoid tissue (Miller, 1961) it is probable that cortisol functions at two levels to prevent normal lymphoid development: (1) by impairing thymic function (Figure 4), which is necessary for normal development of the lymphoid system, and (2) by a direct lymphocytolytic effect on existing lymphoid tissue (Figures 5 and 6) (White, Handler and Smith, 1964). This mode of action would explain the many similarities noted between cortisol acetate-induced wasting disease and the post-neonatal thymectomy syndrome. This mode of action would also help explain the effect of age on the susceptibility to cortisol acetate. The development of lymphoid tissue and immunological competence in young animals, and the re-establishment of immunological competence following damage to the immune apparatus in adults, are dependent upon the presence of the thymus (Miller, 1961; 1965). Maintenance of immunological competence in adults, however, does not require the presence of the thymus (Miller, 1965). Thus, the thymic involution which follows neonatal cortisol administration (Figure 4) occurs at an age when the thymus is most essential to the immunological capacity of the animal.

The involuted lymphoid tissues recovered and approached control values by 30 days after cortisol injection (Figures 4, 5 and 6; Tables X and XI). Thus, it was expected that the immunological competence of the

recovered animals would be normal. Surprisingly, preliminary experiments indicated that immunological competence was impaired for as long as 50 days after cortisol injection (Table XII). Re-examination of many of the blood smears which provided the data for Figure 6 and 7 and Tables X and XI indicated that the recovery of the lymphoid system in cortisol treated mice was not morphologically normal. That is, although recovery of the total number of lymphocytes was observed (Figure 6), the proportion of immunologically competent small lymphocytes (Gowans and McGregor, 1965) was less than normal (Table XIII).

It would appear that the lymphoid recovery observed in cortisol treated neonates was morphologically and functionally abnormal. The results of future studies on cortisol treated germfree neonates can be expected to contribute to an understanding of normal lymphopoiesis and the development of immunological competence.

SUMMARY

A single injection of cortisol acetate into young mice induced a wasting syndrome similar to that observed in the post-neonatal thymectomy syndrome. The course of the disease was less severe if the dose of the drug was decreased or if the animals were older at the time of injection.

The course of the wasting disease was not altered by the administration of maintenance doses of cortisol acetate, or by passive administration of various antisera. Oral antibiotic therapy, however, reduced the incidence of death in cortisol acetate treated neonates. This observation, and the isolation of bacteria from various organs of wasting mice, suggested that microorganisms were involved in the pathogenesis of cortisol acetate-induced wasting disease. Escherichia coli and Streptococcus fecalis were the organisms most frequently isolated from wasting mice.

Experiments utilizing germfree and dibirotic mice confirmed the involvement of microorganisms in this wasting disease. The incidence of death in cortisol treated germfree mice was significantly lower than in conventionally reared mice given a comparable dose of cortisol. Mortality in germfree mice treated with large doses of cortisol acetate was markedly increased by rendering the mice dibirotic with Escherichia coli. Using smaller doses of cortisol the mortality in dibirotic mice was not increased, as compared to germfree mice, even though the mortality in conventionally reared mice treated with a comparable dose was 91.8%. Thus, Escherichia coli cannot be considered as the only organism contributing to the death of wasting animals.

Germfree mice, due to the low incidence of death in these animals, were well suited for studies concerning the primary effects of neonatal

cortisol administration. The neonatal injection of cortisol acetate caused a rapid and severe involution of the thymus and spleen, greatly decreased the number of lymphocytes in the peripheral blood, and prevented the development of normal immunological competence. The lymphoid involution was followed by apparent recovery, however, additional hematologic and immunologic studies indicated that the lymphoid system is morphologically abnormal for at least 30 days, and functionally abnormal for at least 50 days, after cortisol injection.

This study supports the concept that wasting mice, being immunologically defunct due to the primary effects of cortisol acetate on the thymus and other lymphoid tissues, are invaded and killed by microorganisms present in their environment.

APPENDIX

APPENDIX

Several terms employed throughout the present investigation are defined below:

Allogeneic homograft - A graft transplanted between animals of the same species, but of different strains. Synonym: homograft.

Autologous graft or autograft - A graft placed upon the same animal from which it was removed.

Dibiotic - The state of an organism which exists in intimate contact with only one species other than its own.

Germfree - The condition of existence in the absence of all demonstrable living microorganisms.

Gnotobiotic - The designation of an organism free from contamination or in association with only organisms known to the investigator.

Heterologous graft or heterograft - A graft transplanted between different species, such as rat to mouse. Synonym: xenogeneic graft.

Homologous graft or homograft - A graft transplanted between animals of the same species, but of different strains. Synonym: allogeneic homograft.

Homograft reaction - The reaction of a host to a graft of normal or tumor tissue from a genetically dissimilar individual of the species.

Isologous graft or isograft - A graft between individuals having the same genetic constitution, such as members of an inbred strain.
Synonym: syngeneic homograft.

Monobiotic - The state of an organism which exists in the complete absence of all demonstrable living organisms of other species. This term is more exacting than "germfree" in the sense that "germfree" is

defined in terms of microorganisms.

Syngeneic homograft - A graft between individuals having the same genetic constitution. Synonym: isologous graft.

Xenogeneic graft - A graft transplanted between different species, such as rat to mouse. Synonym: heterologous graft.

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