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 Salmonsella 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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Approved:

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Head, Major Department

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Chairman, Examining Committee

_____
Dean, Graduate Division

MONTANA STATE UNIVERSITY
Bozeman, Montana

June, 1966

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iii

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 A1 131-05 and predoctoral fellowship 1-F1-GM-24,866-01A1.

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

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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.

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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 consider-
ation 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 receiv-
ing 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

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*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 (F_1 Hybrid Disease)

F_1 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 F_1 hybrids, hybrids do not react immunologically against parental strain antigens. However, part of the antigenic make up of an F_1 hybrid is foreign to both parental strains, and both parental strains can react immunologically to F_1 hybrid cells and tissues. Thus, when F_1 hybrids are injected with immunologically competent cells of parental strain donors, circumstances favor a graft-versus-host reaction.

F_1 hybrid disease has been described in irradiated and unirradiated F_1 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 (Nakić, Nakić, 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, Strepto-coccus 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 peripher-
al 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, lympho-
cytic 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 typhi-murium 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 anti-biotics 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 micro-organisms 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 prevent-
ed 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 con-
ventionally 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 mono-
contaminated 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 "second-ary 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 hepatotrophic 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 oxytetra-cycline 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 glycocorticoid 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) decreas-
ing 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 lympho-
cytes, 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 allo-
geneic 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 conven-
tionally 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. Ran-
domly 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 Labora-
tory 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 floculation 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 floculation 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 Newbauer 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 treatement.

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 follow-
ing 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 baso-
phils. 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 hemocy-
tometer. 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 mintues 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 mascerated 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 auto-clave 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 Erlen-
meyer 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 compen-
sate 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).