



Bacterin-induced wasting in mice
by Bruce Albert Braaten

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

Eksted and Nishimura (1964) reported that a wasting syndrome was induced in neonatal mice by the repeated injection of heat-killed bacterins containing either streptococci or staphylococci. The experiments reported in this paper were designed to establish an etiology for the syndrome.

The bacterin preparations were made according to the procedure of Ekstedt and Nishimura (1964). Generally, Swiss mice were given intra-peritoneal injections of a particular bacterin every other day for 30 days, and the first injection was given 24 hours after birth. Failure to gain weight normally during an observation period of 30 days was the major criterion of wasting.

Mice injected with a total dose of 3×10^{10} Group A, type 30, streptococcus bacterin were observed to waste and dosages of two and four times greater caused an increase in the severity of the wasting process and death was commonly observed. A systemic bacterial infection was present in mice receiving the higher doses of Group A, streptococcus bacterin.

Mice injected with *Streptococcus faecalis* or *Bacillus cereus* bacterins showed only very slight wasting.

Mice injected with a bacterin made of *Escherichia coli* had an average weight of only 10.18 grams at 30 days whereas normal and stress control mice had an average weight of 18.91 grams and 16.32 grams respectively. Cultural studies revealed no *E. coli* in tissues and organs. Wasting in mice was observed to be less severe when the first injection of *E. coli* bacterin was given 96 hours and 72 hours after birth than when given at 48 hours.

Mice receiving a total dose of 3×10^8 heat-killed *Salmonella* (0 somatic group B) failed to waste, however, mice receiving a total dose of 2×10^9 showed signs of wasting. *Salmonella* (0 somatic group B) was not isolated from the intestinal contents of these mice.

In conclusion, the above results support the concept that neonatal mice receiving repeated injections of bacterins become immunologically unresponsive to the antigens found in the -bacterins. The reduced immunologic capacity on the part of the host in turn leads to a wasting syndrome caused by the host's normal flora which contain the same antigens as the bacterin.

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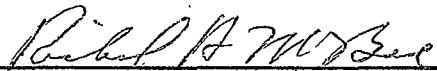
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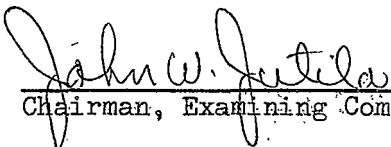
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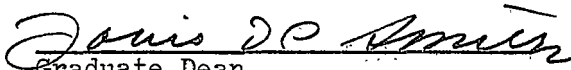
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Microbiology

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Bozeman, Montana

June, 1966

ACKNOWLEDGEMENT

The author wishes to thank Dr. John W. Jutila for his guidance, encouragement, patience, and, most of all, for his infectious enthusiasm for the science of immunology. ,

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ABSTRACT

Ekstedt and Nishimura (1964) reported that a wasting syndrome was induced in neonatal mice by the repeated injection of heat-killed bacterins containing either streptococci or staphylococci. The experiments reported in this paper were designed to establish an etiology for the syndrome.

The bacterin preparations were made according to the procedure of Ekstedt and Nishimura (1964). Generally, Swiss mice were given intraperitoneal injections of a particular bacterin every other day for 30 days, and the first injection was given 24 hours after birth. Failure to gain weight normally during an observation period of 30 days was the major criterion of wasting.

Mice injected with a total dose of 3×10^{10} Group A, type 30, streptococcus bacterin were observed to waste and dosages of two and four times greater caused an increase in the severity of the wasting process and death was commonly observed. A systemic bacterial infection was present in mice receiving the higher doses of Group A, streptococcus bacterin.

Mice injected with Streptococcus faecalis or Bacillus cereus bacterins showed only very slight wasting.

Mice injected with a bacterin made of Escherichia coli had an average weight of only 10.18 grams at 30 days whereas normal and stress control mice had an average weight of 18.91 grams and 16.32 grams respectively. Cultural studies revealed no E. coli in tissues and organs. Wasting in mice was observed to be less severe when the first injection of E. coli bacterin was given 96 hours and 72 hours after birth than when given at 48 hours.

Mice receiving a total dose of 3×10^8 heat-killed Salmonella (O somatic group B) failed to waste, however, mice receiving a total dose of 2×10^9 showed signs of wasting. Salmonella (O somatic group B) was not isolated from the intestinal contents of these mice.

In conclusion, the above results support the concept that neonatal mice receiving repeated injections of bacterins become immunologically unresponsive to the antigens found in the bacterins. The reduced immunologic capacity on the part of the host in turn leads to a wasting syndrome caused by the host's normal flora which contain the same antigens as the bacterin.

INTRODUCTION

Wasting syndromes can be produced by thymectomizing newborn animals (post-neonatal thymectomy syndrome) (Miller, 1962), or by the injection of newborn mice with allogenic lymphoid cells (runt disease) (Billingham and Brent, 1959), cortisol (Schlesinger and Mark, 1964), estradiol (Thompson and Russe, 1965), or multiple doses of certain bacterins (Ekstedt and Nishimura, 1964). A similar disease can be produced in F₁ hybrid mice by the injection of parental lymphoid cells (wasting disease) (Schwartz, Upton, and Congdon, 1957) and by the establishment of allogeneic radiation chimeras in adult inbred mice (secondary disease) (White, 1958). The diseased animals exhibit a wasted appearance, ruffled fur, diarrhea and, in many cases, die from the disease. A striking pathological feature of these syndromes is a diminution or loss of lymphoid tissue and immunological competence.

Trentin (1957) proposed the name "homologous disease" to cover all the complications arising from a graft-versus-host reaction (GVH-reaction) occurring as the result of a successful transplantation of homologous lymphoid cells to immunologically unresponsive hosts. "Runt disease", "secondary disease", and "wasting disease" all fall under the general category of "homologous disease".

The ultimate cause of death in mice affected with "homologous disease" has not been entirely elucidated. It has been generally concluded that cells vital to the life of the animal are destroyed by the grafted lymphoid cells. The capacity of macrophages and lymphocytes to kill a variety of target cells has been conclusively demonstrated both in vivo (Weiser, Granger, Brown, Baker, Jutila, and Holmes, 1965) and in vitro (Granger and

Weiser, 1964).

The possibility that infection representing a complication of the GVH-reaction as contributing to the death of wasted animals has been investigated by numerous workers. Denko and co-workers (1959) made a histologic study of irradiated CF 1 and DBA/2 mice given bone marrow cells of the other strain in order to investigate the nature of late deaths that occurred after irradiation recovery. They found that in many instances the mice died of pneumonia, necrotizing and infected livers, and other infections. Nowell and Cole (1959) attributed the death of non-irradiated adult hybrid mice injected with parental spleen cells to an acute infection present in all injected animals. Van Bekkum, Vos, and Weyzen (1959) found that the prevalent pathological condition in irradiated animals transplanted with allogeneic lymphoid cells was colitis associated with other infections. Simonsen (1957) suspected an infectious process as the basis for death in chickens experiencing a GVH-reaction. However, he reported that exhaustive bacteriologic studies gave negative results. Safford and Jutila (1965) immunized female C3H mice against endotoxins of Escherichia coli and Salmonella typhi or formalized cells of E. coli, S. typhi, S. paratyphi A, and S. typhimurium. They found that whereas all of the offspring of non-immune mice died of the runting syndrome, many offspring of females immunized with bacterins or with endotoxins failed to die of runt disease.

Howard (1961a) found that the GVH-reaction was associated with a considerable rise in phagocytic activity towards intravenously injected India ink. The activity was greatest two weeks after injection of the particles. Cooper and Howard (1961) found a similar increase in the phagocytosis of live

Diplococcus pneumoniae and Salmonella typhimurium injected intravenously 12 days after the onset of the GVH-reaction. Significantly, Howard and Woodruff (1962) found that the formation of agglutinins to Salmonella typhi H antigen was depressed when the bacterial antigen was administered 19 days after the injection of the GVH-reaction. Howard (1961b) observed that resistance to Shigella dysenteriae type I endotoxin is markedly decreased in runt disease although the resistance to the live bacteria was increased. Other investigators (Howard, Biozzi, Halpern, Stiffle, and Mouton, 1959) have reported that stimulation of the reticuloendothelial system in mice by Mycobacterium tuberculosis (BCG) infection coincides with a profound lowering in resistance to the lethal effect of endotoxin from gram-negative bacteria. These results suggest that in some forms of "homologous disease" endotoxins may contribute to the pathologic picture and death of the diseased animals.

Research has led to the discovery of other syndromes which share a common pathological picture with "homologous disease" but which differ in that no GVH-reaction is involved. Miller (1962) found that the thymectomy of infant mice was associated with significant mortality between one and four months of age. The syndrome is known as the "post-neonatal thymectomy wasting syndrome" (McIntire, Sell, and Miller, 1964). The thymectomized mice developed a syndrome characterized by wasting, lethargy, ruffled fur, hunched posture, diarrhea, and death. A marked involution of the lymphoid tissue is also characteristic of this syndrome. Evidence favors the concept that the thymus is responsible in the development of immunological maturity in mice and that early extirpation of the organ

severely impairs that maturity (Miller, Marshall, and White, 1962). Wilson, Sjodin, and Bealmeier (1964) demonstrated that mice did not waste after neonatal thymectomy if kept in a germfree state. They suggest that the primary factor in the pathogenesis of the "post-neonatal thymectomy wasting syndrome" may be an infectious agent or its product to which neonatal thymectomized mice are susceptible because of their reduced immunological capacity. Azar (1964) found that there was a significantly higher incidence of bacterial infections and wasting in neonatally thymectomized rats than in sham-operated controls. In all instances of wasting he found evidence of chronic infection and that the incidence of sepsis and wasting was considerably reduced in rats treated with oxytetracycline.

Thompson and Russe (1965) induced a wasting syndrome in mice by injecting newborns with estradiol. These workers described the disease as reminiscent of "runt disease". They found that the mice which died had an absence of peripheral lymph nodes and a five fold reduction in thymic weight. The surviving mice had only half the lymphocyte count as the surviving litter mate controls.

Schlesinger and Mark (1964) reported that a single injection of cortisol acetate into young mice induced a wasting syndrome similar to that observed in "runt disease". Adrenal corticosteroids are known to cause lymphoid depletion and severely affect immunologic functions. 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). Reed and Jutila (1965) found death in mice following the injection of cortisol acetate was markedly reduced in a germ-

free environment.

Recently, Ekstedt and Nishimura (1964) produced a form of runt or wasting disease in neonatal mice by the repeated injection of heat-killed bacterins containing either streptococci or staphylococci. The wasting was characterized by failure to gain weight normally and the animals were hyperirritable and assumed a characteristic huddled posture. A few animals demonstrated a deficiency of hair growth and a scaly dermatitis. Histological studies of the wasted animals showed an epidermal hyperplasia of the pinna and that the cortical and medullary zones of the thymus could not be delineated. There was also an obvious decrease of lymphoid tissue in the wasted mice.

Ekstedt and Nishimura (1964) observed that germfree mice treated with staphylococcal bacterin were much more resistant to wasting than conventional animals. They suggested that in conventionally raised mice, natural antibody to staphylococcal antigens passively transferred through the placenta to the newborn animals might play some role in the wasting phenomenon observed in mice treated with staphylococcus bacterin. They further suggested that bacterin-induced wasting was not observed in their germfree control mice because germfree animals might be deficient in natural antibodies. In support of their hypothesis they observed wasting in 10 germfree mice injected with a killed staphylococcal vaccine suspended in a 1:100 saline dilution of staphylococcal rabbit antiserum.

The above workers postulated an anaphylactic mechanism for staphylococcal bacterin-induced wasting. However, it is possible that following the development of immunologic unresponsiveness to staphylococci much or

all the pathology and subsequent wasting of treated mice results from either the invasive or toxigenic properties of staphylococci or other organisms that possess cross reactivity with staphylococci and which are found in the normal flora of mice. The purpose of the present investigation was three fold: to reproduce Ekstedt's and Nishimura's findings on the development of wasting in neonatal mice given repeated injections of Group A, type 30 streptococcus; to extend these observations to other bacterins prepared from organisms isolated from the intestinal tract of suckling mice; and, finally to attempt to establish an etiology for the disease.

MATERIALS AND METHODS

A. Experimental Animals

Newborn of conventionally reared and germfree Swiss mice were used in most of the studies. The mice were originally obtained from the Manor Farms, Staatsburg, New York in 1964 and were maintained by random colony breeding. In one experiment, inbred mice of the A/jax strain were used. Conventionally reared mice were fed Purina Laboratory Chow and water ad libitum. Autoclaved Purina Laboratory Chow (Special Formula) 5010C was fed to the germfree mice. The experimental litters were appropriately toe-marked and observations were made on them every other day.

B. Preparation of Bacterins

Bacterins were prepared from broth cultures of a strain of Group A, type 30 streptococcus, Salmonella (O somatic group B), Escherichia coli, Streptococcus faecalis, and Bacillus cereus. The type 30 streptococcus was obtained from the American Type Culture Collection (D-24). Bacillus cereus was obtained from a stock culture maintained at Montana State University. Cultures of Salmonella (O somatic group B), E. coli, and S. faecalis were isolated from the intestinal contents of 15-day old Swiss mice.

Bacterins were made of each of the above bacteria by the procedure described by Ekstedt and Nishimura (1964). Pure cultures of the organisms were inoculated in brain-heart infusion broth (Difco Laboratories, Inc., Detroit) and grown at 37 C for 18 to 24 hours. The cells were harvested by centrifugation in a model HR-1 International Centrifuge at 5000 r.p.m. using a number 856 head. The cells were washed twice and resuspended in

25 ml of saline. The number of cells in the suspension was estimated by the use of a hemacytometer. The suspension was adjusted to contain approximately 10 billion organisms per ml of saline. The final suspension was poured into 30 ml vaccine bottles, corked with rubber stoppers, and autoclaved at 121 C for 20 to 25 minutes. Prior to use, all vaccine preparations were checked for sterility by inoculating one ml of the preparations into brain-heart infusion broth and incubating at 37 C for four or five days.

C. Method of Producing "Wasting Disease" in Newborn Mice

Most of the experimental litters were standardized to contain eight mice. In most instances, the mice were given intraperitoneal injections of a particular bacterin every other day for 30 days, and the first injection was given within 24 hours after birth. In some experiments the injection schedule and dose of bacterin were varied. In all experiments, 0.1 ml of a given concentration of bacterin was given for the first five injections. Thereafter, the mice were large enough to receive 0.2 ml of a particular concentration of bacterin. A few litters were injected with brain-heart infusion broth.

All mice were weighed every other day during the course of the experiment and their weights were compared with untreated control animals of the same age. All deaths occurring in experimental litters within 10 days after birth were considered due to trauma or maternal cannibalism and were not included in the results. Mice were selected from a few of the experimental groups and were sacrificed for bacteriologic studies.

D. Criteria of Wasting

Failure to gain weight normally during an observation period of 30 days was the major symptom of wasting. The wasted animals assumed a characteristic hunched posture at rest and walked with a high stepping gait. Occasionally the mice exhibited diarrhea, lethargy, suppurative lesions, and, in some instance, died.

E. Bacteriology

Mice experiencing symptoms of wasting disease were sacrificed and the liver, heart blood, spleen, and intestinal contents were cultured on blood agar, eosin methylene blue agar, thiogel medium, phenylethyl alcohol medium, and S S agar. The cultures were incubated aerobically at 37 C and observations for growth were made at 24 and 48 hours.

F. Germfree Techniques

Stock germfree Swiss mice were maintained in Trexler flexible film isolator chambers (Snyder Laboratories, New Philadelphia, Ohio). The units were housed in a clean room equipped with an entry room and a temperature-regulating device. Surgical gowns, masks, and caps were donned for entry and work in the germfree room.

A stock solution of 40% peracetic acid (F.M.C. Corporation, Inorganic Chemicals Division, New York 17, New York) diluted to 2% with double distilled water containing sodium alkylarylsulfonate (1g/liter) was used for sterilizing Trexler germfree units. The units received 1.5 liters of 2% peracetic acid which was introduced with a Trigger Teejet Spraying System (5870) powered by a General Electric pump at 15 pounds pressure.

The units were operated for 24 hours to free them of peracetic acid before admitting germfree mice.

A feed with a high vitamin content was used (Purina 5010C) for maintaining germfree mice. Feed pellets were coated with talcum powder to prevent them from sticking together during sterilization. The feed was placed in double small paper sacks and sealed with autoclave tape. The feed was subjected to a vacuum for a half hour, sterilized at 121 C for a half hour, and again vacuumed for twenty minutes. It was presumed that the first vacuuming served to evacuate deep seated air pockets which then increased the efficiency of steam sterilization within the pellet. The second vacuuming simply served to dry the pellets and the paper wrapping. Prolonged sterilization apparently lowers the vitamin content of the feed and renders the feed unpalatable to the mice. Because of the short time of sterilization the feed was always assayed for sterility before it was used by placing entire feed pellets into thiogel, brain-liver-heart semi-solid agar, and cooked meat phytone media.

The cage bottoms, cage tops, and animal drinking vessels were doubly wrapped with paper and autoclaved. For these items, the autoclave was vacuumed a half hour, brought to 121 C for three hours, and finally vacuumed for a half hour. Sawdust bedding was placed in double paper sacks and autoclaved as above except that it was sterilized again a week later. Water was sterilized in 800 ml glass bottles by autoclaving for three hours at 121 C.

Packages or bottles containing sterile materials were placed in the germfree unit and sprayed with 200 ml of 2% peracetic acid. After

spraying, the items were allowed to stand in the entry port over night. This procedure served to sterilize the outside surface of the packages or bottles. After 12 hours the inside cap was removed and the sterile materials were brought into the main chamber. At the same time, animal waste material, empty bottles, or empty packages were placed in the entry port for removal from the unit.

Experimental germfree work was carried out in a smaller tetrahedron unit recently developed by Snyder Laboratories. The experimental unit differed from those used to maintain stock mice in that it was smaller and equipped with very thin gloves so that more delicate work could be performed. The experimental unit was supplied with a scale for weighing mice, forceps, syringes, needles, and a vaccine in addition to the basic materials found in the stock unit.

The following procedure was used to transfer germfree mice from the stock unit to the experimental unit. A sterile two liter Erlenmeyer flask was brought into the stock germfree unit. Two pregnant mice were placed in the flask and the flask was then corked tightly. The flask was brought out of the stock unit and placed into the entry port of the experimental unit. Since the oxygen in the flask is limited, the mice had to be brought into the main chamber within 35 minutes. To compensate for the lack of sterilization time in the entry port, 4% peracetic acid was used instead of 2%.

RESULTS

A. The effect of litter size on weight-gain of untreated mice.

The weights of untreated newborn Swiss litters containing seven, eight, or nine mice were followed for 30 days in order to determine the effect of litter size on growth rate. The results shown in Figure 1 indicate that mice in larger litters gain weight less rapidly than young mice in small litters. Each curve reflects the average weight of one litter of Swiss mice. Because of the effect of litter size on weight-gain, it was decided to standardize the number of mice in all experimental litters to eight. An average weight curve of 48 untreated mice was prepared for comparative purposes.

B. The effect of injection-stress on weight-gain of newborn Swiss mice.

The injection of protein-rich media into mice produces a severe stress reaction presumably through the agency of an anaphylactoid reaction. To test the effect of stress on weight-gain, newborn Swiss mice were repeatedly injected with a volume of brain-heart infusion broth comparable to that used for mice injected with bacterins. It can be seen in Figure 2 that the injection of media initially retards the growth rate of young mice. Thereafter the mice gain weight at the same rate as untreated mice and appear normal at day 30. The differences in average weight between untreated and stress control mice will be referred to as the stress factor hereafter. These results differ from those of Ekstedt and Nishimura (1964) who reported no effect by treatment with broth.

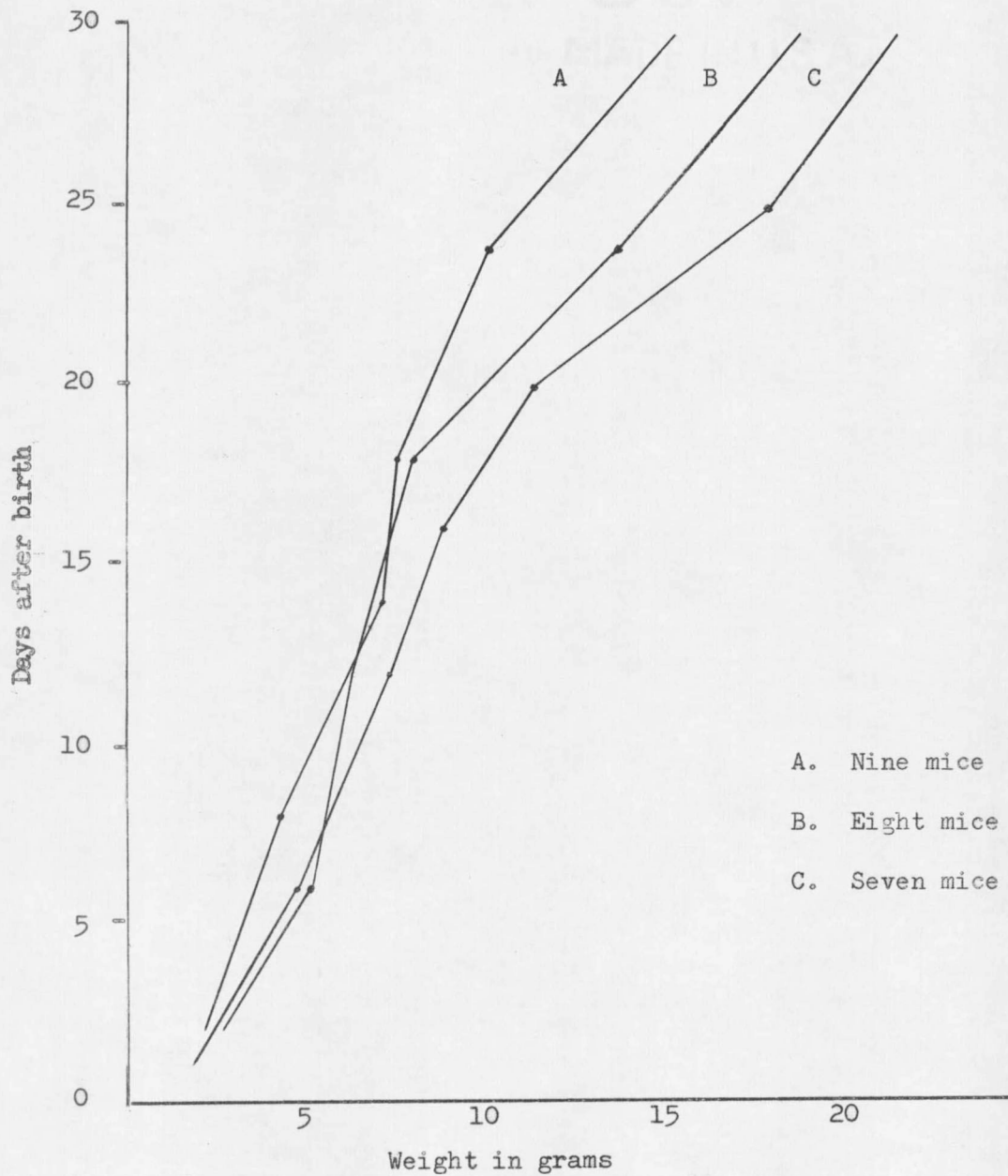


Figure 1. Average weight-gain of mice of varying litter sizes.

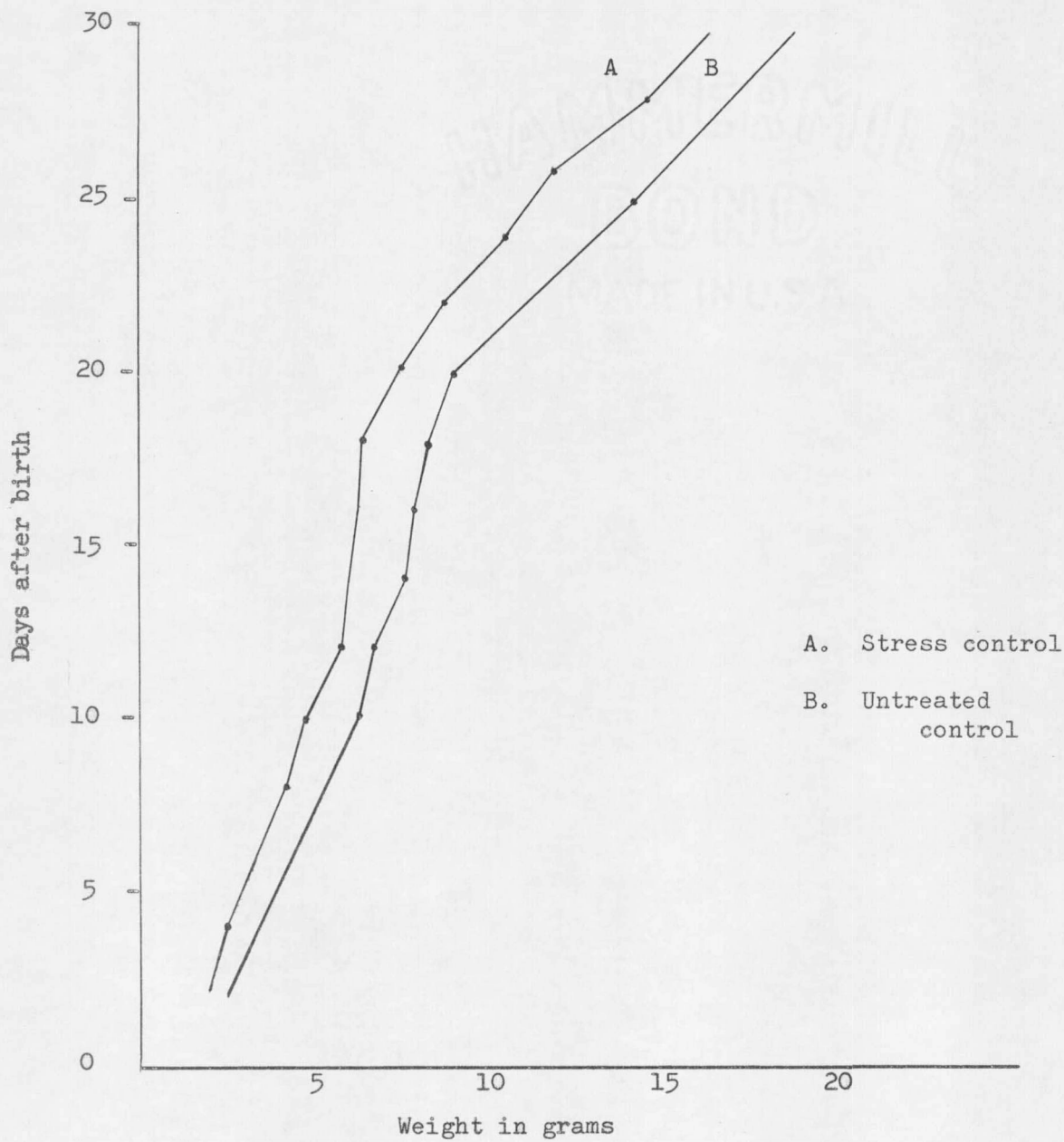


Figure 2. Average weight-gain of three litters of Swiss mice injected with brain-heart infusion broth.

C. The production of wasting disease with bacterins prepared from gram-positive organisms.

1. Wasting disease induced with Group A, type 30 streptococcus bacterins.

The experiment was designed to repeat the observation that Group A, type 30 streptococcus bacterin could produce wasting disease in mice as originally described by Ekstedt and Nishimura (1964). The experiment differed from that performed by these workers in that the concentration of bacterin used was increased two fold. Newborn Swiss mice were injected every other day beginning 24 hours after birth with 2 billion heat-killed streptococci. As can be seen in Figure 3, the average weight-gain of mice receiving the bacterin was considerably below that of the stress and untreated control mice. At day 30, bacterin-treated mice had an average weight of 11.75 grams while the untreated mice had an average weight of 18.91 grams and the stress control mice had an average weight of 16.32 grams. Several experimental mice weighed as little as 9.00 grams. These results essentially confirm those of Ekstedt and Nishimura.

When mice were treated with increasing numbers of heat-killed streptococci the severity of the wasting process was greatly enhanced and death was commonly observed. Table 1 shows that 47% of the mice injected with a total dose of six billion streptococci and 64% of the mice injected with a total dose of 12 billion streptococci died of wasting disease.

The heart blood, liver, and spleen of mice injected with the streptococcal bacterin were cultured on phenylethyl alcohol medium and showed an abundance of streptococci and, unexpectedly, staphylococci. Cultures of tissues and organs from untreated control animals were negative. A striking

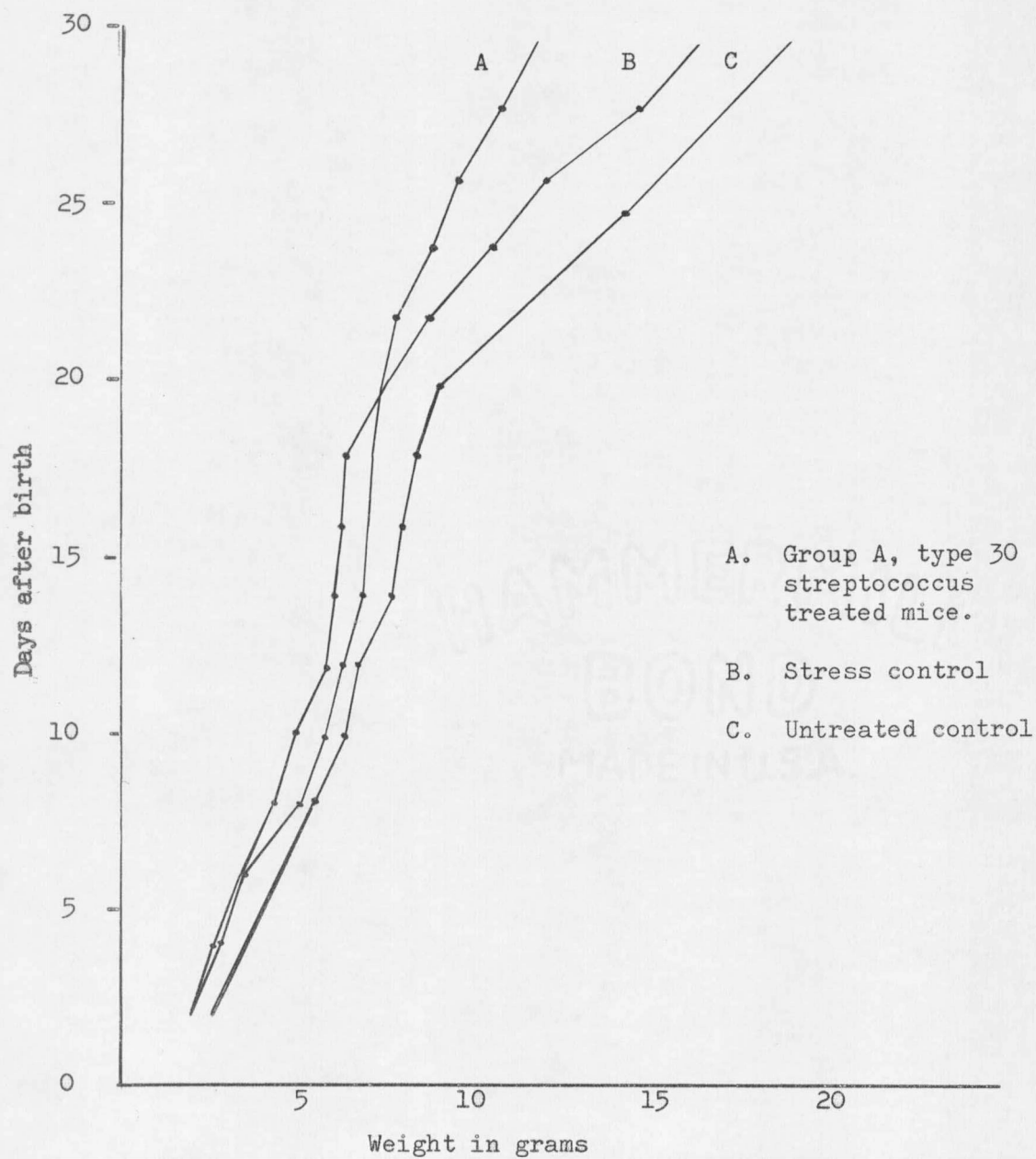


Figure 3. Average weight-gain of two litters of mice treated every other day with 2 billion Group A, type 30 streptococcus bacterin.

finding was that many mice injected with either six billion or 12 billion streptococci developed abscesses at the site of injection. The abscesses were not observed in the mice receiving two billion autoclaved Group A, type 30 streptococci.

Several litters of A/jax and C57Bl/6Ks inbred mice were treated with two billion autoclaved Group A, type 30 streptococcal organisms. Maternal cannibalism was extremely high in the inbred groups after the newborn mice had been handled. Only one experimental litter of A/jax escaped cannibalism after the onset of the experiment. The average weight at day 30 for the mice in this experimental litter was 7.28 grams compared to 12.29 grams in untreated control A/jax mice. It was decided not to use inbred mice in further experiments because of maternal cannibalism.

2. Wasting disease induced with Streptococcus faecalis bacterins.

It is known that 10 to 15 day old mice have an abundance of enterococci (Schaedler and Dubos, 1964) in the gastrointestinal tract which make up a large part of the normal flora. Hence, it was of interest to see if mice given Streptococcus faecalis bacterin would show signs of wasting. One group consisting of five litters of Swiss mice were given 200 million autoclaved S. faecalis organisms and another group of three litters received two billion heat-killed organisms. In one litter of the latter group (S-99), the mother developed an ascites tumor on day 20 so the litter was not included in the results. Figure 4 shows the weight-gain of the two groups as compared to the untreated and stress control groups. It can be seen that the failure to gain weight in both groups was not as

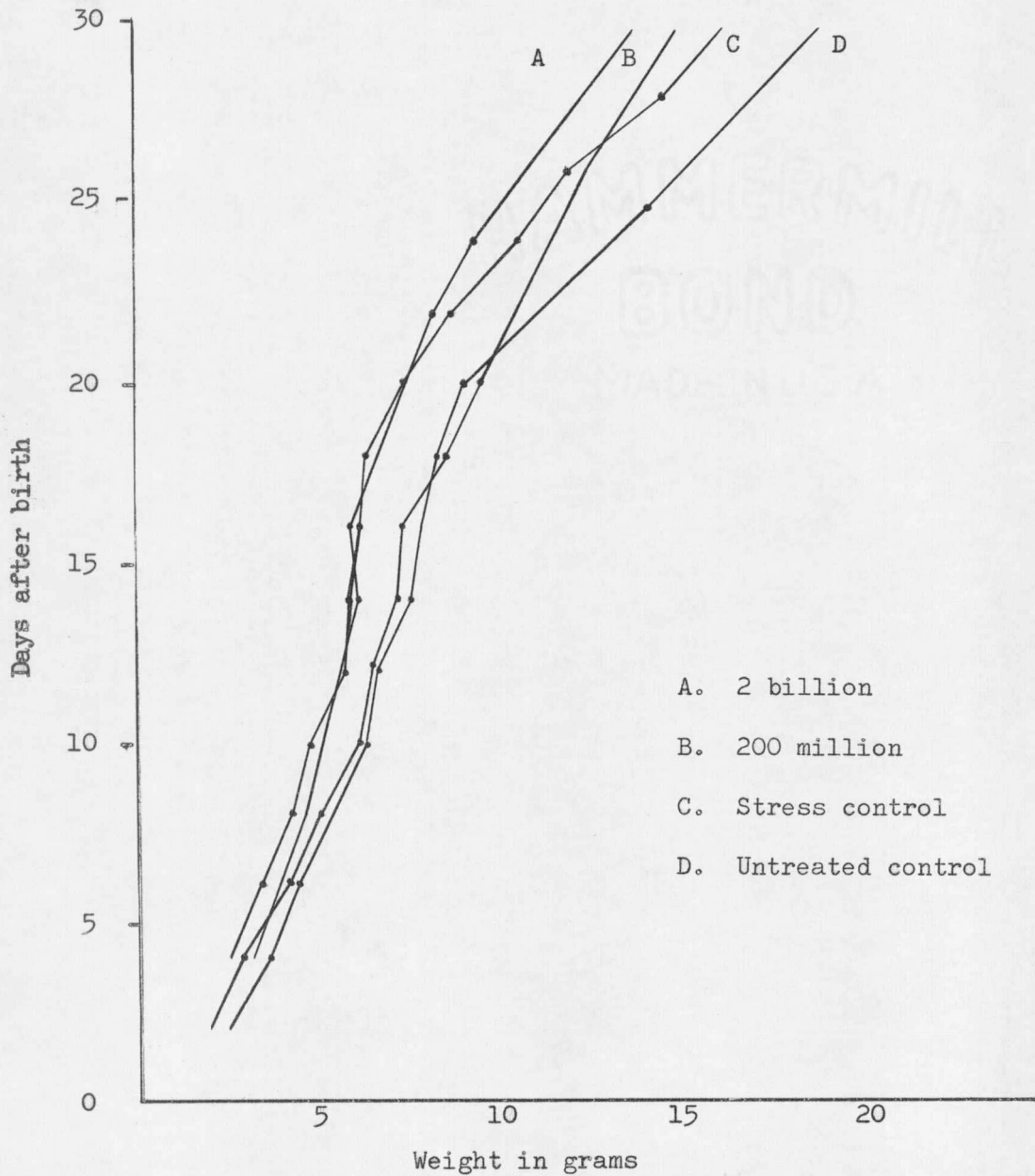


Figure 4. Average weight-gain of five litters of Swiss mice injected every other day with 200 million Streptococcus faecalis bacterin and of two litters injected every other day with 2 billion.

severe as in experiments employing Group A streptococcal bacterins. Indeed, when these data are compared to the weight curve of stressed control animals only a slight difference in weight existed between control and test groups at day 30. Only one mortality that might be attributed to a bacterin induced wasting was observed, and this mouse was gaining weight normally when it died from an unknown cause. Cultures of liver and spleen from experimental mice were made on blood agar, eosin methylene blue agar, and in thiogel medium. All cultures were negative.

3. Wasting disease induced with Bacillus cereus bacterins.

Three litters of Swiss mice were injected with 200 million autoclaved Bacillus cereus organisms. Figure 5 shows the average weight-gain of each of the three litters compared with the control groups. The results were extremely varied. One litter of seven (S-72) almost paralleled the normal control in weight-gain. Another litter of eight (S-81) showed severe wasting, and a third litter of eight (S-76) gained weight normally until day 26 when they suffered a weight loss and two mice died. Cultures of liver and spleen from diseased mice on blood agar and eosin methylene blue agar were uniformly negative.

D. The production of wasting disease with bacterins prepared from gram-negative organisms.

1. Wasting disease induced with Escherichia coli bacterins.

Since Escherichia coli is found in abundance as part of the normal intestinal flora of the 15 to 30 day old Swiss mouse, a bacterin prepared from an intestinal isolate of E. coli was used to induce wasting in

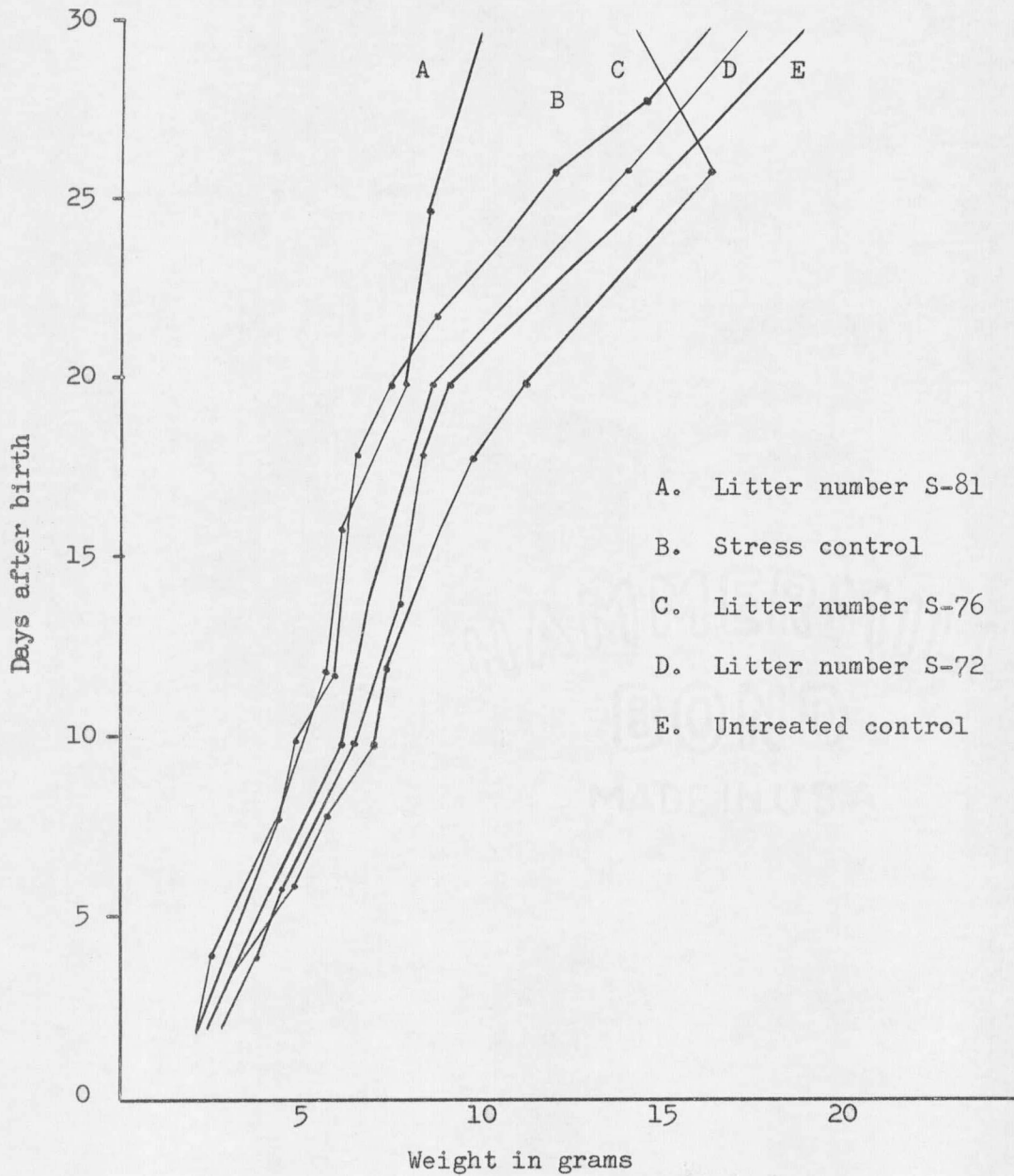


Figure 5. Average weight-gain of each of three litters of mice treated every other day with 200 million Bacillus cereus bacterin.

Table I. Mortality in Swiss mice injected with bacterins of Streptococcus (Group A), Streptococcus faecalis, and Bacillus cereus.

Bacterin	Total dose of organ- isms	No. dying of wasting No. injected	Percent Mortality	Mean Survival Time (days)
<u>Strepto- coccus</u> (Group A)	3×10^{10}	1/15	6.7	29
	6×10^{10}	8/17	47.0	22
	12×10^{10}	9/14	64.2	19
<u>Strepto- coccus</u>	3×10^9	* 1/38	2.6	30
<u>faecalis</u>	3×10^{10}	0/16	0	--
<u>Bacillus</u> <u>cereus</u>	3×10^9	2/23	8.7	30

* Showed no weight loss during observation period

newborn Swiss mice. It was observed that an initial injection of one billion or 100 million heat-killed E. coli organisms was lethal to newborn mice presumably through the agency of endotoxin induced shock. When given an initial injection of 10 million heat-killed E. coli, the mice lived and gained weight normally until day 10 at which time they began to show symptoms of wasting disease. By day 30 the mice showed severe symptoms of wasting (Figure 6) and several mice weighed 8.00 grams or less. Three of the 24 mice receiving injections of bacterin died. Although fecal cultures showed an abundance of E. coli, cultures of liver, spleen, and heart blood from diseased mice were uniformly negative for bacterial growth.

In another experiment, a second E. coli bacterin was administered to three litters of Swiss mice in the same concentration as above. One litter showed severe signs of wasting but only mild wasting was observed in the other two litters. Only one mortality among 24 treated mice was observed. Cultural studies were not done.

An experiment was designed to test the importance of early injection with E. coli bacterin. Three experimental groups were established in which one was comprised of three litters of Swiss mice and the other two groups contained two litters. The initial injection was administered 48 hours after birth in the first group, 72 hours after birth in the second, and 96 hours after birth in the third group. The concentration of bacterin was 10 million heat-killed E. coli for the first two injections, 15 million for the third, 100 million for the fourth and fifth, and 200 million thereafter. All three groups exhibited wasting but the most severe was observed

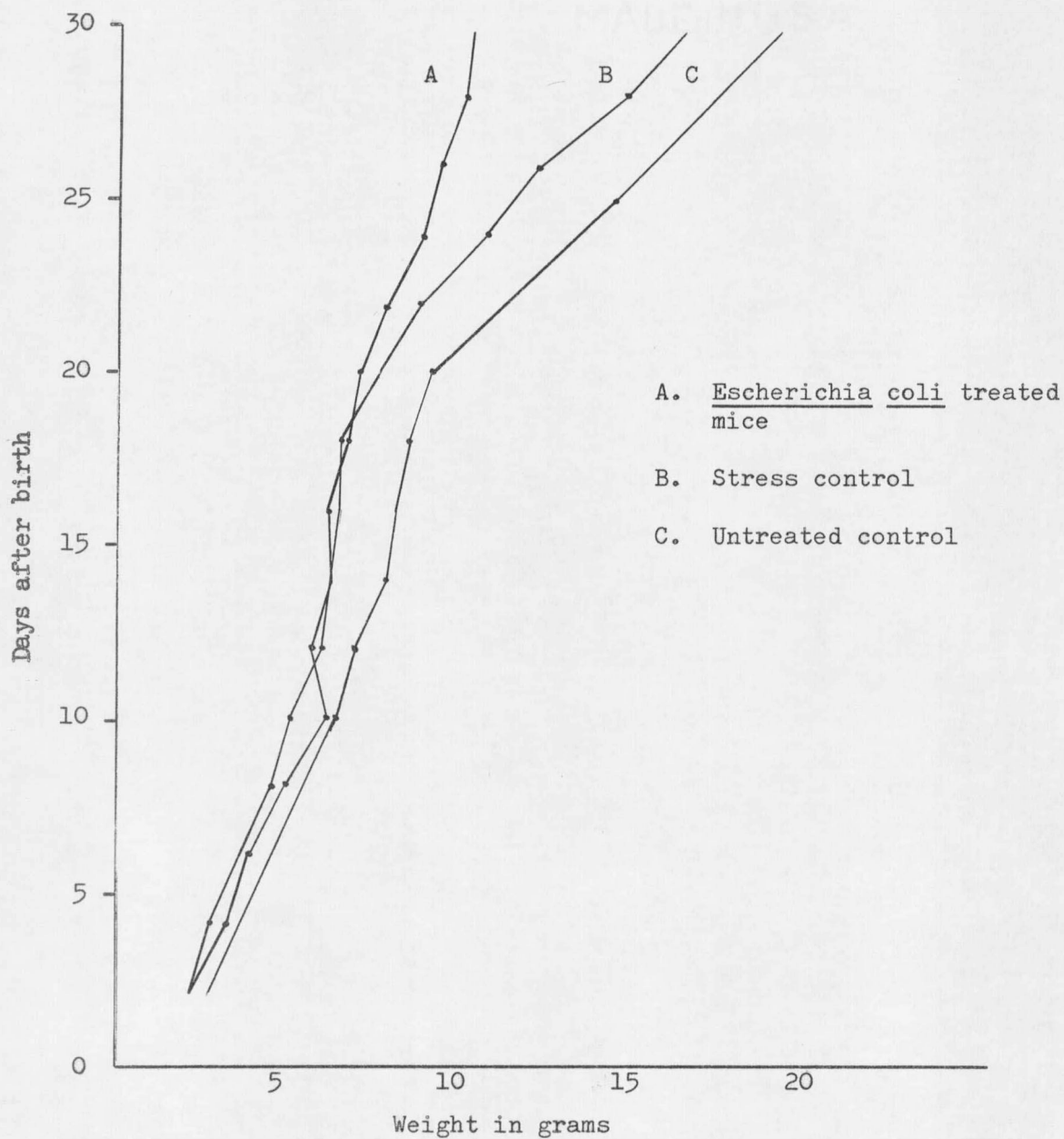


Figure 6. Average weight-gain of three litters of Swiss mice injected every other day with 20 million Escherichia coli bacterin.

in the group receiving the earliest initial injection (Figure 7).

Some of the mice which showed wasting with E. coli bacterin had an unusually small thymus, enlarged adrenal glands, and the intestines appeared mucoid. Histologic studies revealed a very thin walled caecum and in some mice the small intestine showed some signs of necrosis. Numerous mononuclear cells (macrophages) were observed in the mucosa. A striking finding was a severe depletion of lymphocytes in the thymic cortex.

An attempt was made to see if E. coli bacterin could induce wasting in the germfree mouse. For reasons presented in the discussion, nothing definite could be concluded from the results of the germfree work.

2. Wasting disease induced with Salmonella (O somatic group B) bacterin.

It was of interest to see if wasting could be induced in Swiss mice using a gram-negative organism rarely found in the mice. Two litters received neonatal injections of one billion autoclaved Salmonella (O somatic group B) organisms, and all of the mice in both litters died within 48 hours presumably of endotoxin shock. In two other litters the concentration of bacterin was lowered to 100 million for the first injection, and all but five mice died within 48 hours. When the concentration of the initial injection was further lowered to 10 million heat-killed cells, none of the mice died of shock in the three experimental litters receiving this concentration. The average weight-gain of these mice was not markedly below that of the untreated and stress control mice (Figure 8), and only one mortality out of 24 mice was observed. Fecal cultures were

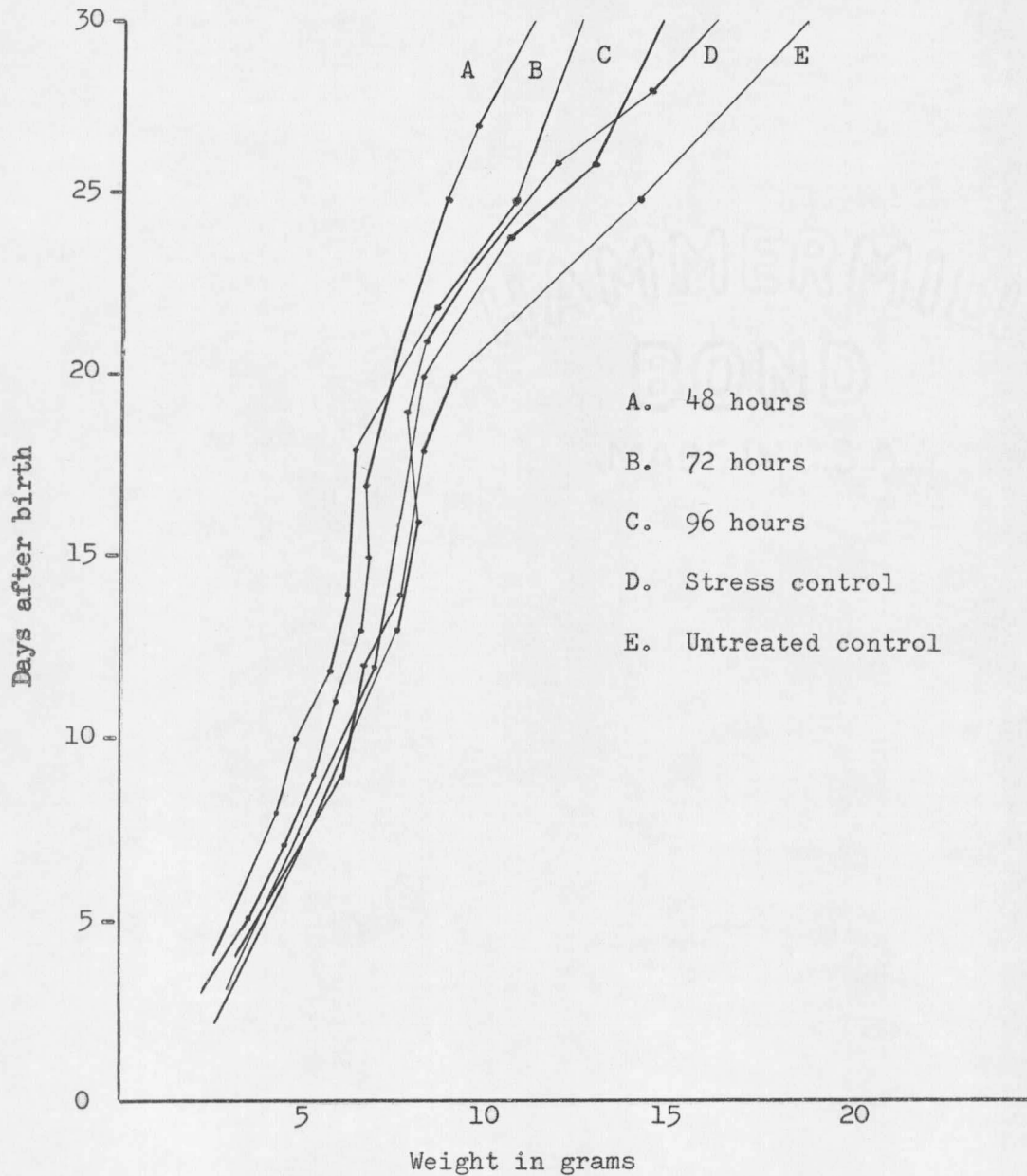


Figure 7. Average weight-gain of three groups of Swiss mice receiving the initial injection of Escherichia coli bacterin in varying times after birth.

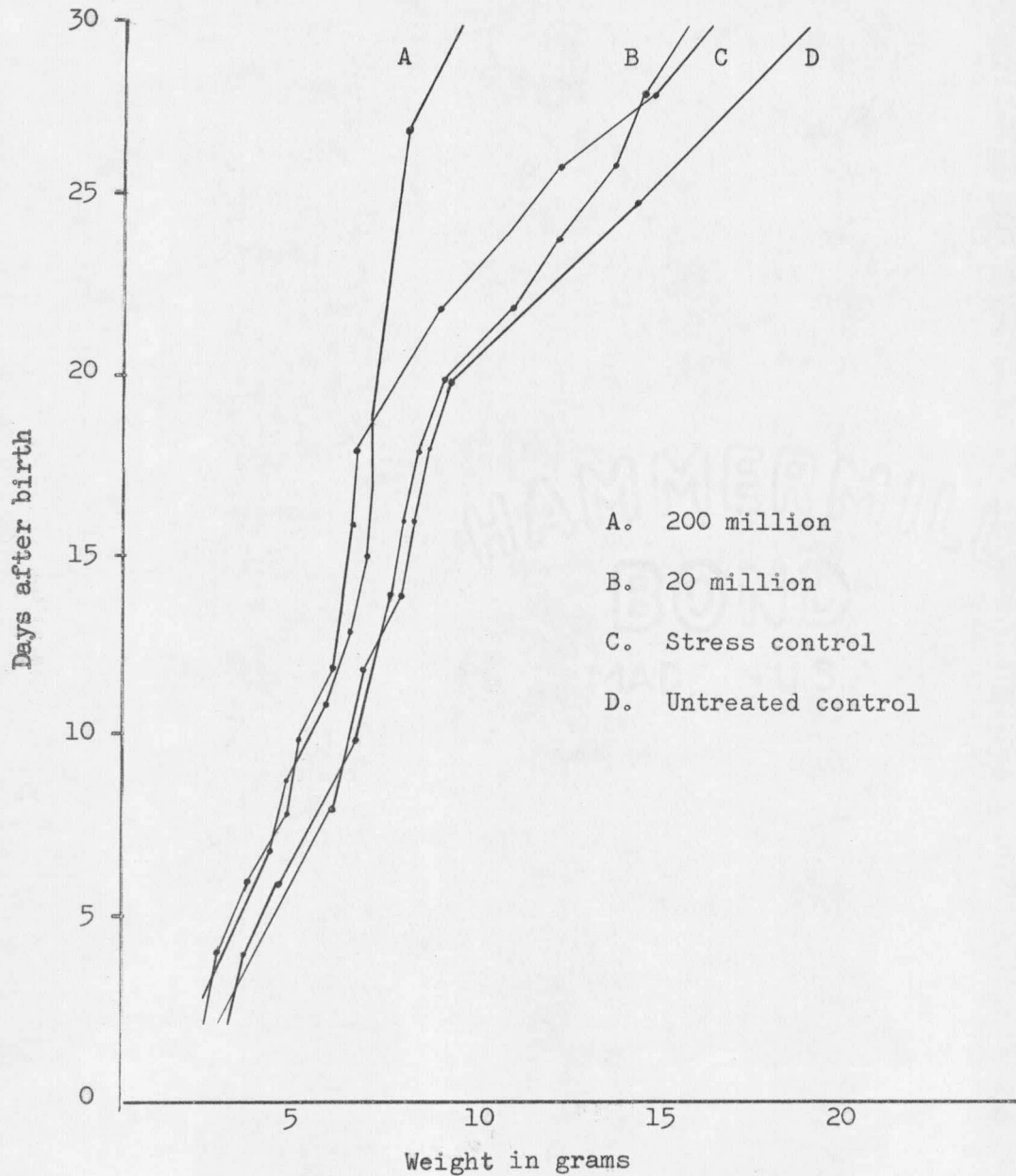


Figure 8. Average weight-gain of three litters of Swiss mice treated every other day with 20 million Salmonella (O somatic group B) bacterin and of two litters receiving 200 million organisms.

made on S S agar and eosin methylene blue agar and all were negative for Salmonella (O somatic group B), but all cultures showed an abundance of Escherichia coli. Cultures of spleen, liver, and heart blood were negative for any growth.

In another experiment, three litters received a neonatal injection of 10 million autoclaved Salmonella. Subsequent doses of bacterin were increased, as in the experiment which received increasing doses of E. coli bacterin, until by day 10 the mice were receiving 200 million cells. Two litters showed severe signs of wasting but the other litter paralleled the weight-gain of the three litters of mice receiving 20 million autoclaved Salmonella organisms. The average weight-gain of the two litters that wasted is shown in Figure 8 and three mortalities were observed. Again, Salmonella (O somatic group B) was not isolated from fecal cultures of these mice.

Table II. Mortality in Swiss mice injected with bacterins of Escherichia coli and Salmonella (O somatic group B).

Bacterin	Total dose of organ- isms	No. dying of wasting No. injected	Percent Mortality	Mean Survival Time (days)
<u>E. coli</u>	3×10^8	3/24	12.5	34
<u>Salmonella</u>	3×10^8	1/24	4.2	22
	2×10^9	3/24	12.5	26

DISCUSSION

The role that the normal flora or their products may play in "homologous disease", and other wasting syndromes with a similar pathological picture, is poorly understood. Most of the evidence against the normal flora being a factor in the etiology of wasting syndromes stems from workers who have failed to observe overt signs of infection. However, the hypothesis is advanced that absence of a systemic infection does not rule out the possibility of bacterial toxic products leaking through the intestinal epithelium and entering the vessels of the mucosa. The toxic products could be excretions or secretions (exotoxins) of the normal flora, or they might come from the breakdown of bacterial cells undergoing lysis (endotoxins).

Much of the data presented in the results points to the concept that the normal flora or their products play an important role in bacterin-induced wasting. It is probable that specific immunologic unresponsiveness may be induced to certain bacteria found in the normal flora when large doses of bacterins are administered to newborn mice. It is proposed that immunologic unresponsiveness to certain bacteria in turn leads to infection of the host or damage to the host by toxic products of bacteria indigenous to the host.

In the case of wasting induced with Group A, type 30 streptococci bacterin, streptococci and large numbers of staphylococci were isolated from the tissues and organs of the wasted mice. The presence of streptococci in tissues and organs of diseased animals is in accord with the above proposal but the presence of staphylococci was an unexpected finding. It seems possible that early streptococcal infection predisposes to a

staphylococcal infection or, alternatively, a certain degree of antigenic similarity exists between staphylococci and streptococci. If the Group A, type 30 streptococcal bacterin possessed any antigenic cross-reactivity with staphylococcus, the staphylococcus which normally inhabit the skin may have gained entrance to the body via the needle puncture caused by injections of bacterin. The fact that lesions were seen only at the site of inoculation supports this notion.

It is known that the higher the concentration of antigen given to a newborn mouse, the more marked will be the immunologic unresponsiveness of that mouse to the antigen as measured by the absence of anaphylaxis and lack of antibody when the mouse is challenged at a later date (Smith, 1961). Wasting in mice injected with heat-killed Group A, type 30 streptococci, as measured by percent mortality and mean survival time, was observed to be increased in mice receiving higher concentrations of the bacterin. This increase in wasting observed in mice receiving the higher concentrations of bacterin correlates with the increase in immunologic unresponsiveness which would be expected to occur when newborn mice receive higher concentrations of antigen.

Wasting was not very severe in newborn mice which received heat-killed Streptococcus faecalis organisms. This observation is not surprising since enterococci are, at best, low grade pathogens and rarely, if ever, invade tissue or organs. In addition, the number of enterococci in the intestine of young mice reaches a maximum between day 10 and 14 and thereafter the number decreases by a factor of five logs (Schaedler and Dubos, 1964). The observation that wasting did occur in newborn mice

receiving larger concentrations of bacterin was somewhat significant and is in accord with the hypothesis that normal flora may contribute to the disease process.

The results observed in mice treated with Bacillus cereus bacterin were ambiguous and no conclusions could be drawn. One litter grew normally and this was expected since the normal flora contains little if any B. cereus. Another litter grew normally until day 26 and then lost weight and two mice died on day 30. The reason for the loss of weight and death observed in these mice is not clear. A third litter was observed to waste. It seems possible that the mother might not have been feeding and caring for the young properly or the bacterin may have possessed some cross-reactivity with some bacteria found in the normal flora.

Wasting induced in newborn mice treated with E. coli bacterin was extremely severe. It is of interest that wasting with E. coli was brought about with the injection of 20 million heat-killed cells every other day while it took the injection, every other day, of two billion heat-killed Group A, type 30 streptococci to produce wasting and the latter wasting was not as severe. Although no signs of a systemic infection were found upon culturing tissues and organs of the treated mice, there was an abundance of E. coli in the intestinal tract. This finding is in accord with the hypothesis that toxic products of the normal flora may play an important role in bacterin-induced wasting as well as other wasting syndromes.

The earlier the initial injection of E. coli bacterin was administered to newborn mice the more severe was the wasting observed. Since

immunologic unresponsiveness to an antigen is most easily achieved in immunologically immature mice, the above observation correlates with the hypothesis that bacterin-treated mice receiving early treatment exhibit wasting because they are unable to respond to products of the normal flora. The mice receiving the first injection of bacterin 96 hours after birth possibly do not exhibit wasting because they respond to products of the normal flora.

The observation that wasting was not observed in newborn mice treated every other day with 20 million Salmonella (O somatic group B) bacterin is in accord with the hypothesis that treatment of mice with a bacterin of Salmonella (O somatic group B) should fail to waste. Salmonella (O somatic group B) was rarely isolated from the intestinal tract of the bacterin treated mice although E. coli was isolated in abundance. However, wasting was observed in newborn mice receiving this bacterin when the dose was increased to 200 million cells. Although Salmonella were not isolated from the latter mice, it may be that increasing the dose of the bacterin by 10 fold produces a form of protracted endotoxin shock. Significantly, a toxic death was observed when larger doses of both E. coli and Salmonella bacterins were given within 24 hours after birth. Alternatively, a state of immunologic unresponsiveness to endotoxin may have developed by treating mice with large doses of Salmonella bacterin which cross-reacted with the endotoxins of the normal flora.

It could be argued that while immunologic unresponsiveness in newborn mice may be induced to a given bacterin, the subsequent wasting might only be a result of immunologic unresponsiveness to toxic properties of later

injections of bacterin and not to products of the normal flora. No mice that wasted showed signs of a reduced weight-gain before day 10 as compared to the stress control mice. If toxicity to the mice developed solely from the injection of bacterin, newborn mice would be expected to exhibit little or no weight-gain. On the other hand, it is known that the normal flora does not establish in the intestine much before day 10 (Schaedler and Dubos, 1964) and, significantly, symptoms of wasting were not observed earlier than day 10. It appears then, that the establishment of normal flora is important for the development of symptoms of wasting syndromes.

The germfree mouse would serve to elucidate more clearly the role of normal flora in the wasting process. Ekstedt and Nishimura (1964) reported that germfree mice that received large doses of staphylococcus bacterin were much more resistant to wasting than conventionally reared mice. In this study an attempt to produce wasting disease in germfree mice using an E. coli bacterin was unsuccessful. The results with germfree mice were difficult to evaluate because the unit became contaminated accidentally with a gram-positive sporeformer that grew in thiogel medium, brain-liver-heart semisolid agar, and cooked meat phytone medium. It did not grow in nutrient broth or on blood agar. In addition, the three litters were prematurely born and subjected to extreme stress due to some residual peracetic acid and consequently their weight-gain was initially retarded. The premature births were induced possibly by the carbon dioxide build up in the flask in which the pregnant mothers were brought into the experimental unit. Finally, the E. coli bacterin preparation that was to be taken into the germfree unit was autoclaved twice. This precaution proved

to be a mistake since only one conventional litter out of three treated with the twice autoclaved bacterin demonstrated any signs of wasting and in that case the wasting was slight.

As the result of their work in germfree mice, Ekstedt and Nishimura (1964) postulated an anaphylactic mechanism for bacterin induced wasting, i.e. the reaction of antigen contained within the bacterin and antibodies found in the recipient mouse. There are several criticisms that can be made about an anaphylactic mechanism as being the cause of wasting in neonatal mice treated with staphylococcal bacterin. The first, and one that Ekstedt and Nishimura (1964) themselves make, is that germfree animals have been shown to possess some naturally occurring staphylococcal antibody (Cohen, Newton, Cherry, and Updyke, 1963). Hence, it is not entirely valid to say a germfree mouse differs from a conventional one in that it does not possess staphylococcal antibodies. Second, they injected staphylococcal rabbit antiserum into neonatal germfree mice, but they did not treat neonatal germfree mice with normal rabbit serum as a control. This worker has observed death occurring within 12 hours in conventionally reared adult C57Bl/6Ks mice that had received an intraperitoneal injection of one ml of normal rabbit serum. Third, Ekstedt and Nishimura (1964) suggest that natural antibody to staphylococcal antigens passively transferred through the placenta to conventional newborn animals might play some role in bacterin induced wasting. Natural antibodies are macroglobulins (Humphrey and White, 1964), and macroglobulins are not known to pass the placenta (Fahey, 1962). Fourth, macroglobulins are produced soon after

birth in response to antigenic stimulus (Fahey, 1962). Hence, if an anaphylactic mechanism were involved, it would seem that a more pronounced wasting would be observed in mice which received their initial injection of bacterin later than 24 hours after birth rather than earlier, as Ekstedt and Nishimura (1964) found, and as observed by this worker in the case of E. coli-induced wasting. The observations of Ekstedt and Nishimura (1964) on germfree mice appear to lend more support, at this time, to the concept that the normal flora or their products play an important role in bacterin induced wasting.

Lymphoid depletion and a reduced immunologic capacity on the part of the host seem to be common factors in "homologous disease", "post-neonatal thymectomy wasting syndrome", estradiol-, cortisol-, and bacterin-induced wasting. It seems plausible that mice with the above syndromes might in some ways be compared to mice receiving a lethal dose of X-irradiation since the latter are known to have a profound reduced immunologic capacity. Rosoff (1963) found that 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 and the protective effect was observed only when cultural data demonstrated the successful elimination of the coliform flora in the gut. McLaughlin, Dacquoise, Jacobus, and Horowitz (1964) found that the germfree mouse can tolerate more radiation than either a conventional or an E. coli monocontaminated mouse. The radiation effect after doses of 550 to 950 r was observed and after all doses the mice in the germfree state survived longer than the mice monocontaminated with

E. coli. It was also observed that the monocontaminated mice survived longer than the conventional mice.

The importance of the contribution of the normal flora to pathologic changes in lethally irradiated mice seems quite clear. It is not entirely unreasonable to compare the ultimate cause of death in lethally irradiated mice to that in mice suffering from any of the wasting syndromes, including bacterin-induced wasting, since in all cases there is an impairment of the immunological capacity of the host. However, care must be taken not to draw the analogy too far, since lethal whole-body irradiation severely damages the intestinal epithelium.

A damaged intestine is no doubt much more apt to permit the access of the normal intestinal flora and their products to the blood stream than a healthy intestine. An undamaged intestine may admit toxic products of the normal flora but possibly not the bacteria themselves. Indeed, this may explain why in some types of wasting bacteria can be isolated from the tissues and organs while in other types of wasting there is no evidence of a systemic infection. In the former case the immuno-suppressive agent, whatever it may be, also damages the intestinal epithelium while in the latter case the intestine is not damaged.

It might be said that the ultimate cause of death in "homologous disease" cannot be compared to other wasting syndromes with a common pathology since in "homologous disease" immunologically competent cells are transplanted to the host. It could be argued that the normal flora or their products could not contribute to the wasting process observed in the

host since the transplanted cells would protect the host. However, since the transplanted immunologically competent cells will first encounter the numerous antigens of the host, it is possible that most of the cells will be pre-empted to react with only the antigens of the host leaving none or only a few cells to combat the normal flora and their products. One can conjecture that, perhaps, if homologous spleen cells were incubated in a supporting fluid for a few hours in the presence of a complex bacterin made from the normal flora of the recipient, the spleen cells would not be as concerned with the recipients antigens upon injection and would exert a truly protective effect against its normal flora.

In conclusion, something should be said about the value of knowing the etiology of "homologous disease" and other wasting syndromes with a common pathological picture. It is known that many therapeutic drugs used for the treatment of cancer have the unfortunate side effect of reducing the immunologic capacity of the patient and rendering him more susceptible to infection. Knowledge of the etiology of wasting induced with cortisone and other immuno-suppressive drugs could prove of value in reducing the unwanted side effects of these drugs.

If it were not for "secondary disease", leukemia could possibly be cured by giving the patient a lethal dose of whole-body irradiation and transplanting homologous bone marrow. Homologous bone marrow might also be used to save a person who had accidentally received a lethal dose of irradiation. However, it should be kept in mind that even if "secondary disease" could be controlled, it might only let the host live long enough for a chronic graft-versus-host reaction to manifest itself. This disease

might possibly take the form of some of the autoimmune disorders.

SUMMARY

The injection of a large number of heat-killed Group A, type 30 streptococci in neonatal mice produced a wasting syndrome. It was observed that if the dosage was increased two and four times there was a more severe form of wasting coupled with a systemic bacterial infection. Wasting was not observed in mice receiving Streptococcus faecalis bacterin. Only one litter of three litters of mice treated with Bacillus cereus bacterin wasted. Mice exhibited severe symptoms of wasting with Escherichia coli bacterin, and the sooner the initial injection was given after birth the more severe was the wasting. Mice treated with Salmonella (O somatic group B) bacterin failed to waste unless treated with very high doses. The mice receiving bacterins of the gram-negative organisms had an abundance of E. coli in their intestinal contents but no Salmonella (O somatic group B). These observations support the concept that the normal flora or their products contribute in the pathogenesis of bacterin-induced wasting.

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