Studies on the etiology of the runting syndrome in mice
by John William Safford

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
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Abstract:
Although runt disease in neonatal mice injected with allogeneic, adult spleen cells has been extensively studied the pathogenesis and cause of death is incompletely understood. The possibility that the developing flora in the gut of the neonatal mouse plays a role in the pathogenesis of runt disease was investigated.

Female C3H and A/Jax mice were immunized against endotoxins or bact-erins prepared from Escherichia coli and Salmonella species. Neonatal mice born to immunized females received 40 X 10^6 C57B1/Ks spleen cells intraperitoneally and the course of runt disease in these passively immunized mice was compared to that in mice born to nonimmunized mothers.

It was found that the bacterin-immune and endotoxin-immune C3H runts gained significantly more weight than did the nonimmune controls. Although the endotoxin-immune and nonimmune suffered the same mortality (94%), the bacterin-immune runts suffered a markedly lower mortality of 70%. The bacterin-immune and nonimmune A/Jax runts showed a similar weight-gain and high mortality (92.8% and 88.5% respectively), whereas the endotoxin-immune runts gained weight nearly as rapidly as the normally developing mice and suffered a markedly lower mortality of 69.7%. The mean survival times of the nonimmune and immune C3H and A/Jax were essentially the same.

Bacteriological examination of the heart blood, liver and spleen of 34 A/Jax runts yielded 20 positive cultures. The principle organism isolated was an extremely fastidious, unidentified gram-negative rod.

Hematological studies showed that runted A/Jax mice suffered a severe polymorphonuclear leucocytosis and that the leucocytosis was reduced by intraperitoneal injections of Chloromycetin. The antibiotic did not prevent the diarrhea and liver lesions from developing in the runted mouse.

The gross appearance of antibiotic treated runts and untreated runts was identical.

These data are interpreted as supporting the concept that the normal flora of the gut and their toxic products, such as endotoxin, contribute to the pathogenesis of the runting syndrome.

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ABSTRACT

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Female C3H and A/Jax mice were immunized against endotoxins or bacterins prepared from Escherichia coli and Salmonella species. Neonatal mice born to immunized females received 40 X 10^6 C57Bl/Ks spleen cells intraperitoneally and the course of runt disease in these passively immunized mice was compared to that in mice born to nonimmunized mothers.

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INTRODUCTION

In 1953 Snell (cited by Eichwald, 1963) formulated a number of tissue transplantation laws based on the genetic disparity between donor and recipient animals which describe the immune reactions of the recipients against tissue grafts from related or unrelated donor animals. These "laws of tissue transplantation" may be briefly stated as follows: (1) grafts exchanged between genetically identical individuals will be accepted and retained indefinitely, (2) grafts exchanged between genetically disparate individuals will be rejected, (3) an F_1 hybrid will accept a graft from either parent, and (4) neither inbred parent will accept a graft from the F_1 hybrid. In the second and fourth situation the graft contains antigens not recognized as "self" by the recipient animal and subsequently, the host mounts an immunological assault on the graft and destroys it. The principle cellular antigens of the mouse are determined by alleles of the H-2 locus which segregate according to simple Mendelian laws (reviewed by Snell, 1957; Eichwald, 1963). These antigens are termed histocompatibility antigens.

The laws of tissue transplantation describe the reactions of the host against the graft, but Snell's laws also apply to the phenomenon of a graft's raction against the host. In this situation the grafted tissue reacts more effectively against the host than vice versa, i.e. a graft-versus-host reaction (hereafter referred to as GVH reaction). This event occurs when a graft rich in immunologically competent cells is injected into a foreign recipient which, for reasons discussed below, is unable to reject that graft. When this occurs, the grafted cells mount an immunological attack against the host which is usually detrimental to the host.

Three experimental situations have been described in which a GVH reaction leads to a fatal outcome. These are (1) runt disease (reviewed by Nisbet and Heslop, 1962; Simonsen, 1962), (2) F_1 wasting disease (reviewed by Simonsen, 1962) and (3) secondary disease (reviewed by Koller, Davies and Doak, 1962).

The work of Danchakoff (1916) is probably the earliest mention in the literature of a GVH reaction. She found that splenomegaly resulted after grafting a "tissue mesh" onto the allantoic membrane of a 7-day chick embryo. It was postulated that the splenomegaly was the result of donor cell proliferation within the host spleen. In 1953 Dempster, and independently, Simonsen proposed that kidneys transplanted from one dog to another may react immunologically against the recipient (cited by Simonsen, 1962). However the concept of the GVH reaction remained on purely theoretical grounds until 1957.

At this time, Billingham (1958), in his attempts to induce tolerance to foreign skin grafts, unexpectedly observed that many of the mice succumbed to a runting syndrome. The disease developed 2 to 3 weeks after the injection of spleen cells from adult mice into neonatal allogeneic recipients (a genetically disparate recipient within the same species). At the same time, independent experiments of Simonsen (1957) demonstrated pathological changes in neonatal mice and embryonic or neonatal chicks injected with allogeneic spleen cells or buffy coat cells. Both Billingham and Simonsen concluded that the pathology of this disease was the direct result of a GVH reaction. They postulated that the immunologically

immature recipients were unable to defend themselves from the immunologically mature lymphoid cells derived from adult allogeneic donors.

Since Billingham's and Simonsen's demonstration of runt disease in mice and chicks, the same syndrome has been induced in the neonatal rat (Billingham, et al., 1960), in the neonatal hamster (Jutila, 1960) and in the fetal rabbit (Porter, 1960a) with essentially the same results.

Billingham's (1958) initial studies of runt disease showed a high degree of variation in mortality from one donor-recipient combination to another. Jutila and Weiser (1962) studied the immunological mechanisms involved in the production of runt disease in a number of donor-recipient combinations and in parental to F_1 hybrid combinations. They found that the incidence of runt disease was highest in those combinations differing at the H-2 locus and that prior sensitization of the donor was required to produce runt disease in combinations identical at the H-2 locus but differing at "weaker" histocompatibility loci (i.e. H-1 and H-3). Studies designed to determine the influence of age of the recipient on the incidence of runting showed that F_1 hybrids remained susceptible to injections of parental cells until about day 10 whereas inbred combinations failed to develop runt disease shortly after 5 days of age. An acute form of runt disease was observed when C57Bl/K peritoneal macrophages sensitized to sarcoma I, a tumor indigenous to the A strain mouse, were injected into 5 day old A/Jax. Runt disease induced with immune cells has been termed acute allogeneic disease (Weiser, et al., 1965). These findings are in direct agreement with and confirm the hypothesis that runt disease is indeed the result of an immunological attack on the host by the allogeneic

lymphoid cells. The importance of disparity of donor and recipient mice at the H-2 locus is stressed.

This concept is further strengthened by the studies of Siskind and Thomas (1959). They showed that the cell inoculum used for inducing runt disease must consist of viable, immunologically competent lymphoid cells. Runt disease was not produced by cells of non-lymphoid origin (liver and kidney) or by frozen-thawed lymphoid cells. The observation that large amounts of specific antiserum administered to neonatal mice failed to produce runt disease shows that the disease is caused by a cellular rather than humoral immunological attack (Jutila and Weiser, 1962).

Billingham and Silvers (1961) carried out an extensive comparative study on the ability of different lymphoid tissues from CBA mice to induce runt disease in A strain mice. They found that lymph node cells were most effective for producing runt disease followed by spleen cells and leucocyte concentrates, marrow cells and thymocytes from young donors, and thymocytes from adult donors. They also suggested that lymph nodes from different anatomical sites may not be equivalent in immunological abilities. This was suggested by the observation that axillary and brachial node cells were more effective for producing runt disease than mesenteric node cells. Cohen, et al. (1963) used the C57B1 to A strain donor-recipient combination to study the relative abilities of newborn and adult thymus cells to initiate a GVH reaction. They found that the same number of cells from either neonatal donors or adult donors were equally effective for initiating the GVH reaction. Sosin, Hilgard and Martinez (1966) confirmed the work of Cohen, et al. (1963) and also found that spleen cells from

neonatal mice were totally ineffective in producing a GVH reaction. Also, adult spleen cells were found to be about ten times more effective than adult or neonatal thymocytes. In contrast to the above observations, Terasaki (1959) found that thymocytes from young donors would not initiate a GVH reaction in the embryonic chick.

Terasaki (1959) extended his studies to the effectiveness of chicken blood cells to induce the GVH reaction. He found that wheras blood lymphocytes were able to induce the GVH reaction monocytes were ineffective. He assumed that the immunologically competent cell in the blood was the large lymphocyte since he considered the small lymphocyte to be a non-dividing end cell. Burnet and Burnet (1960) reported that the lymphocyte was responsible for GVH reactions on the chorioallantoic membrane of chick embryos. Cannon, et al. (1958) concluded that the polymorphonuclear leucocyte was also able to manifest GVH activities in chick embryos. Vredevoe and Hildemann (1963), and Hildemann (1964) demonstrated that purified small lymphocytes from blood were immunologically reactive when introduced into a genetically disparate neonatal mouse. Hildemann (1964) went on to show that the injected cells rapidly spent themselves or were destroyed by "allergic injury." This had been postulated by Kaplan and Rosston (1959) who stated that both donor and recipient lymphoid cells die in a stoichiometric interaction.

An interesting addition to the wide variety of lymphoid cells accused of being responsible for the GVH reaction is provided by the work of Frenkel, Korst and Raccuglia (1962). These workers injected a plasma cell tumor indigenous to C3H mice into the xenogeneic (a different species) neonatal

Sprague-Dawley rat. The tumor failed to develop in the xenogeneic host but 90 days after the tumor injection the rats began to exhibit dermatitis, weight loss, decreased muscle mass and a terminal diarrhea followed by death 106 to 112 days post-tumor injection. The symptoms were the classical picture of runt disease in the rat and the authors belived that they were the result of an immunological reaction of the tumor against the host. To this author's knowledge, this work represents the only case of runt disease induced in a xenogeneic donor-recipient combination.

Although much confusion still exists as to the specific lymphoid cell involved in the GVH reaction much significance has been placed on the ability of the small lymphocyte to differentiate into large pyroninophilic cells when confronted with the tissue antigens of the allogeneic host (Gowans, 1962). This cell appears to be identical with the circulating histiocyte described by Binet and Mathé (1962). Indeed, lymphocytes in general appear capable of converting into many cell types (Robbins, 1964). For this reason it may be prudent to determine the cell type found within the host once the GVH reaction has become established rather than the type of cell injected into the host.

The pathology of the classic runting syndrome has been studied extensively. Pathologic lesions have been observed and described in the skin, intestine, lymphoid tissue, liver and blood. Studies by Billingham and Brent (1959) have shown that the ability to gain weight is markedly reduced in the runted animal. In some strain combinations a chronic form of the disease with a low mortality was characterized by a weight-gain of one-fourth that of a normal animal. Russell (1960) has used the weight-

gain as an index for the severity of runt disease in mice. He observed near normal rates of weight-gain until about day 7, followed by a period of failure to gain weight referred to as the plateau period. This period continues for about 10 days until death occurs. He points out that the abrupt cessation of growth after about day 7 is reminiscent of the beginning of homograft rejection. In strain combinations where runt disease is not always fatal weight-gain is an extremely useful tool to use in following the course of the disease.

Lesions of the skin were not as dramatic in the mouse as those found in the rat. Russell (1960), using the DBA/1 to C57B1/6 combination, reported no skin abnormalities but observed alopecia around the eyes, nose and anus. Other authors who have studied a variety of donor-recipient combinations of mice reported neither alopecia nor skin abnormalities (Billingham, 1958; Siskind and Thomas, 1959). Cole, Nowell and Davis (1964) studied wound healing in mice undergoing a GVH reaction and found that healing of a circular punch wound was delayed for 2 to 3 days in these mice. Admitting that this phenomenon was very poorly understood they postulated that delayed wound healing could be due to a general mitotic inhibition by the GVH reaction.

The rat, on the other hand, exhibits an entirely different skin involvement. The studies of Billingham, et al. (1960) revealed an extremely dramatic succession of skin lesions. Exfoliation of the skin was followed by a thickening of the epidermis with some degree of keratinization. In extreme cases the epidermis became separated from the dermis and intradermal abcesses were frequently observed. Microsopically the dermis

contained large numbers of histiocytes and very few, if any, lymphocytes. Krén, et al. (1962) reported very similar results using different strains of rats. They concluded that the dermatitis was immunological in nature. Stastney, Stembridge and Ziff (1963) also concluded that the skin lesions in rats undergoing a GVH reaction have an immunological basis and suggest that this particular disease may be used as a model for human autoimmune disease (Stastney, et al., 1965).

Unfortunately the intestinal pathology and diarrhea observed in runt disease has been ignored almost entirely until recently. Rielly and Kirsner (1965) examined the intestines of animals suffering from runt disease and found attenuation of villi, decreased goblet cells, crypt enlargement and immature cells above the usual germinative centers. These pathologic changes were more pronounced in the ileum than in the jejunum and no mononuclear cell infiltration was observed. Because of the lack of cellular infiltration they postulated that the pathology was induced by either antigen-antibody reactions or by intestinal crypt cell uptake of foreign nucleic acid precursors which would lead to an altered host cell metabolism.

A possible explanation for the diarrhea of runt disease has been suggested by the studies of Reilly and Kirsner (1966). Analysis of intestinal epithelial cells from the runt intestine for disaccharidases revealed a gross deficiency of lactose. Since lactose is the only carbohydrate available to nursing mice they postulated that the presence of undigested lactose acidic products of bacterial metabolism leads to acid diarrhea. Indeed, they found that the cecal contents of runts are always in the acidic range (pH 5.5 to 6). It was suggested that this could lead to

intestinal irritation which might pose problems for amino acid and fat absorption in addition to the lack of carbohydrate absorption. These findings also offer an attractive explanation for the emaciated appearance of runts in spite of the full stomachs of these animals reported by Jutila (1960).

Siskind and Thomas (1959) observed focal, coagulative necrotic lesions in the livers of runted mice. These lesions were patchy white areas located along the free edge of the liver and were generally subcapsular with little or no cellular inflammatory reaction. No proof was offered as to the origin of these lesions but the authors did postulate infection, ischemia or possible immunological injury. Sinkovics and Howe (1964) found complement-fixing antibodies in the blood of runted mice which reacted with cytoplasmic liver antigens. Fife, Hook and Muschel (1962), however, were unable to detect complement-fixing anti-liver antibodies in the blood of runted rats.

Simonsen (1957) reported a very severe anemia in chickens undergoing the GVH reaction. The blood cells from these animals gave a positive direct Coombs' test. Siskind and Thomas (1959) reported anemia in mice suffering from runt disease and Jutila (1960) reported that anemia became progressively worse as the disease progressed. He also observed that 30 to 50 per cent of the red blood cells of the runted animal gave a positive direct Coombs' test. Immune hemolysis has also been observed in rats (Krčn, et al., 1962) and in rabbits (Porter, 1960b) suffering from runt disease.

Siskind and Thomas (1959) found that runt disease in mice produce

leucocyte counts varying from severe leucopenia to a marked leucocytosis. Jutila (1960), however, reported a leucocytosis and found no marked leucopenia. Jutila also observed that the leucocytosis was the result of a marked neutrophilia. Kren, et al. (1962) observed that leucocytes increased as the disease progressed in rats and he attributed this to host reactivity. Porter (1960b) observed a steady decrease of all blood cells in the rabbit undergoing a GVH reaction and observed that the bone marrow was hypoplastic or aplastic at birth.

The most striking and consistent of pathological changes induced by the runting syndrome is found in the lymphoid tissue of the runt. The initial studies of Billingham (1958) revealed involution of lymph nodes to such a degree as to make detecion almost impossible. The spleens of runts were reported to be of normal size but were discolored, fibrotic and grossly deficient in the Malpighian corpuscles.

In contrast to the normal spleen size reported by Billingham (1958), Simonsen (1958)and Jutila (1960) observed a significant increase in spleen size of mice undergoing the GVH reaction; an increase of two to three-fold was noted in some strain combinations. Davies and Doak (1960) studied the fate of allogeneic cells containing the T_6 chromosome marker injected intravenously into neonatal allogeneic mice. They observed that the division rates within the spleens were abnormally high but contained few or no donor cells. Nowell and Defendi (1964) studied the same phenomenon in rats by using the sex chromosome marker with the same results. Thus, it appears that the splenomegaly of runt disease is primarily due to host cell proliferation.

Interesting experiments performed by Haller (1964) have shown that the allogeneic spleen cells, when injected intravenously, settled in the recipient spleen, at least for a short time. He found that runted mice splenectomized within 4 hours post-injection suffered one-half the mortality of non-splenectomized controls. To explain the absence of donor cells within the spleen observed by other investigators he postulates a secondary migration of donor cells to other sites. Unfortunately these studies were not extended to delayed splenectomy or to intraperitoneal injections of allogeneic cells.

Both lymphoid atrophy (Billingham, 1958) and lymph node enlargement (Billingham and Brent, 1959) have been observed in runted mice. This apparent contradiction is explained by the fact that the lymphoid follicles are observed to go through two phases in runting mice. First, the normal lymphocytes are replaced by proliferation of other cell types and secondly, if the animal lives long enough, atrophy and fibrosis of the lymphoid tissue occurs (Russell and Monaco, 1965). Therefore, the gross and microscopic appearance of the lymph node is dependent upon the severity of the GVH reaction and upon the time of examination.

Terasaki (1964) observed proliferation of mononuclear cells in the medulla and cortex of lymph nodes of runted mice. This was followed by a decrease in lymphocytes and proliferation and eventual complete replacement of the node by reticular cells. The terminal stage was characterized by a completely effaced nodal structure and a small number of reticulum cells in the sinuses. The examination of runt lymphatic tissue with the electron microscope has revealed that the major cell types involved were

lymphocytes, plasma cells, and histiocytes (Weiss and Aisenberg, 1965). Most of the plasma cells and lymphocytes were damaged and phagocytized while the reticular cells and other fixed cells of the connective tissue were seldom affected.

The major change in the thymi of runted mice is a depletion of cortical lymphocytes which may become so severe that separation of cortex and medulla is lost (Weiss and Aisenberg, 1965). Hildemann, Gallagher and Walford (1964) observed that spleen and lymph node enlargement were associated with the depression of thymic weight. They postulated that early "immunologic thymectomy" could be the primary cause of acute transplantation disease. The importance of the thymus in the immunological development of neonatal mice has been amply demonstrated by Miller (1964).

These data support the concept that the runting syndrome is induced by the immunological activities of allogeneic lymphoid cells in the neonatal, immunologically immature host. The pathological lesions characteristic of this syndrome include lymphoid atrophy, anemia, necrotic foci in the liver, alopecia, dermatitis, intestinal pathology and diarrhea, weight loss, emaciation and, in most cases, death. The specific cause of death in the runting syndrome has not yet been explained.

A wasting disease very similar to runt disease results when F_1 hybrid adult mice are injected with parental strain lymphoid cells. As in runt disease, the host is unable to react immunologically against the injected cells since parental cells are not foreign to the F_1 hybrid recipient. On the other hand, lymphoid tissue grafts from the parent are able to recognize the foreign antigens inherited by the F_1 hybrid from the other

parent.

As in runt disease, wasting disease of F_1 hybrids is characterized by general wasting, anemia and lymphoid depletion which are common to all GVH reactions (Kaplan and Rosston, 1959). Another facet to the complex problem of theetiology of wasting diseases was provided by the work of Nowell and Cole (1959) who state that the specific cause of death appeared to be a non-specific infection involving one or more organs. This, they proposed, resulted from the impairment of the host's antibody-producing mechanisms by the GVH reaction. Kaplan and Rosston (1959) reported finding normal flora of the mouse gastrointestinal tract in blood, peritoneal contents and organs of some wasting F_1 mice. Apparently they felt that this observation was not at all significant and an infectious etiology was not considered.

Howard and Woodruff (1961) found that the GVH reaction in F_1 mice severely depressed the immunological responsiveness of those mice. Skin grafts were rejected slower than normal and the antibody response to Salmonella typhi H antigens was delayed and enfeebled. A singular observation was the exquisite sensitivity of F_1 hybrids undergoing the GVH reaction to bacterial endotoxins (Howard, 1961). Howard postulates that endotoxin absorbed from the gut may contribute to the pathogenesis of F_1 disease. The increased sensititivity to endotoxin may be due to the increased phagocytic activity of the reticuloendothelial system of mice experiencing a GVH reaction (Cooper and Howard, 1961).

Koltay, et al. (1965) found that the serum immunoglobulin levels dropped dramatically during the GVH reaction produced in F_1 mice. They

noted that the survivors of this disease showed a considerable increase in the IgM class but not in the IgA or IgG classes of immunoglobulins. Since antibodies produced against endotoxin reside entirely in the IgM class (Arnason, Vaux St-Cyr and Relyveld, 1964) they stressed the importance of endotoxin as a probable cause of death.

In summary, wasting disease induced in F_1 hybrid mice can be considered to be an adult form of runt disease which is promoted by a GVH reaction. However, the use of adult recipients has allowed the performance of experiments which have shed light on a new approach to the pathogenesis of GVH reactions. The immunological deficiencies of animals undergoing the GVH reaction have pointed to a possible involvement of the bacteria of the gut and their products, most notably the endotoxins, in the etiology of these diseases.

The third form of wasting disease, secondary disease, can be induced in adult animals which have received allogeneic lymphoid cells following a lethal dose of x-irradiation. The irradiated animal infused with allogeneic bone marrow or spleen cells is protected from the early irradiation death but will die within 3 to 8 weeks of a secondary GVH reaction. If the irradiated animal is infused with isologous (genetically identical) bone marrow, it is protected from the lethal irradiation effects. Irradiation apparently destroys the immunological competence of the recipient allowing the grafted allogeneic bone marrow or spleen cells to mount an immunological attack against the helpless recipient. Trentin (1956) has shown that the closer the genetic relationship between donor and recipient, the greater the protective effect of the injected cells. As with F_1

disease, the pathology of secondary disease closely resembles that of runt disease (Koller, Davies and Doak, 1962).

Denko, Simmons and Wissler (1959) studied secondary disease in mice and found that the cause of death was not always apparent. They did observe, however, that many of the mice died of infection; pneumonia being the most common along with necrotizing, infected liver lesions. Van Bekkum and De Vries (1962) fed mice suffering from secondary disease tetracycline and found that the antibiotic treatment decreased diarrhea and mortality. They stated that the diarrhea and wasting was due to infection of graft anti-host lesions and that infection was possible because lymphoid atrophy resulted in poor antimicrobial defense. Significantly, they postulated that most infectious organisms originated from the normal flora of the animals.

The most definitive experiments linking an infectious process with the mortality of secondary disease are those of Connell and Wilson (1965). They found that germfree mice suffered a dramatically lower mortality from secondary disease than did their conventionally reared counterparts. This was attributed to the absence of microbial flora in germfree animals.

Thus, the most common finding, and perhaps the most important, in all GVH reactions is a depletion of lymphoid tissue which leaves the animal immunologically helpless and allows normally harmless microbial flora to become infectious. Indeed, one would not have to postulate an invasion by the microorganisms but could consider their toxic products, such as endotoxins, leaking from the gut and serving as a contributing factor to the death of the animal.

The experiments to be reported here were undertaken to detect a possible contribution of the normal flora of the gut and/or their toxic products to the etiology of the runting syndrome. Parenthetically, after these studies had begun two reports were published concerning runt disease in germfree mice. Both papers indicated that the runting syndrome progressed in germfree mice just as it did in conventionally reared mice (McIntire, Sell, and Miller, 1964; Salomon, 1965). This study was designed to determine the effect of passive immunization of newborn mice against various endotoxins and gram-negative bacteria and the effects of antibiotics on the course of the runting syndrome. In addition, the bacteriology and hematology of the runted mice were studied.

MATERIALS AND METHODS

A. Experimental animals.

Inbred mice of the C3H, A/Jax and C57Bl/Ks (previously designated C57Bl/6K) strains were used throughout the study. The mice were obtained from the University of Washington in 1961 and have been maintained in this laboratory by frequent brother-sister matings. Breeding mice were fed Purina Mouse Breeder Chow and water ad libitum; stock animals were maintained on Purina Laboaratory Chow and water ad libitum after weaning at 30 days of age. Cage litter consisted of non-autoclaved wood shavings.

B. Production of runt disease.

C57Bl/Ks male mice from 3 to 9 months of age served as spleen cell donors. The mice were sacrificed by cervical dislocation and the spleens (usually about 4) were removed aseptically and placed in a sterile 10 ml syringe fitted with a fine-mesh steel screen. The spleens were forced through the screen into 1 ml of Medium 199 (Cappel Laboratories). Gentle aspiration with the syringe served to break up the splenic pulp and yielded a homogeneous mixture. A 1:1000 dilution of an aliquot from the spleen preparation was made in 4% acetic acid and counted with a standard hemocytometer. The spleen preparation was then diluted with Medium 199 to contain 8×10^8 cells/ml. Viability tests were not performed but Jutila (1966) has reported about 99% viability of spleen cells prepared in a similar fashion. Random bacteriologic sampling of spleen preparations on blood agar confirmed the sterility of these preparations.

Neonatal C3H and A/Jax mice born to either endotoxin-immune, bacterin-immune or nonimmune females were injected with 40×10^6 spleen cells contained in a volume of 0.05 ml within 24 hours of birth. This dose was

administered with a 1.0 or 0.25 ml tuberculin syringe fitted with a 27-gauge needle. Leakage was minimized by injecting through the thigh muscle into the peritoneal cavity; all mice which leaked excessively were removed from the litter.

C. Criteria of runting.

The criteria used for judging runt disease in mice injected with adult allogeneic cells at birth was a failure to gain weight at a normal rate and death, commonly occurring 2 to 3 weeks post-injection. Animals dying before day 8 usually suffered trauma at the time of injection or were victims of maternal cannibalism and were not included in the data.

Other criteria frequently employed were diarrhea, a hunched posture, high-stepping gait, abdominal alopecia, necrotic foci on the liver and splenomegaly. In severe cases the distal portion of the small intestine was swollen and congested and the peritoneal cavity was filled with a watery, milky fluid.

D. Preparation of bacterins.

Bacterins prepared from Escherichia coli B, Salmonella paratyphi A, S. typhimurium and S. typhosa H-901 were used in some experiments. S. typhosa H-901 was obtained from the American Type Culture Collection (#6229); the other organisms were obtained from the Montana State University stock culture collection.

An inoculum was taken from a stock culture agar slant (Difco) and transferred to 50 ml of trypicase soy broth (TSB)(Baltimore Biological Laboratories). After 24 hours of incubation at 37 C with continuous shaking 10 ml of the TSB culture were transferred to 500 ml of fresh TSB

and incubated at 37 C with continuous shaking for 36 to 48 hours. Sufficient formaldehyde was then added to the culture to obtain a 0.4% concentration. The formalized culture was kept at room temperature. Viability of the culture was checked daily by inoculating 50 ml of TSB with 1 ml of the formalized culture. After viability tests had shown that the culture had been killed the cells were harvested by centrifugation (Servall Refrigerated-Automatic RC-2) at 5000 X g and 0 C. The cells were washed twice in sterile saline and resuspended in sterile saline to a concentration comparable to MacFarland tube #5 (approximately 1.5 X 10^9 cells/ml). Merthiolate was added to a concentration of 1:10,000 and the bacterin was stored in vaccine bottles at 4 C.

E. Immunization procedures.

Pilot experiments employed female C3H mice immunized against aqueous-ether extracted endotoxins of E. coli and Salmonella enteritidis (kindly supplied by Dr. E. Ribi, Rocky Mountain Laboratory, Hamilton, Montana) or against bacterins of E. coli B and S. paratyphi A. Later experiments employed female A/Jax immunized against endotoxins (Bacto Lipopolysaccharide B, Difco) of E. coli 0111:B4 or S. typhosa 0901 or against bacterins of E. coli B, S. paratyphi A, S. typhimurium and S. typhosa H901. The three Salmonella species were combined to yield a single polyvalent vaccine.

One group of C3H females received subcutaneous injections at separate sites of 5 mcg of each endotoxin in sterile saline on days 1, 5, 7 and 35. Another group was injected subcutaneously with 0.1 ml of each bacterin at separate sites on the same days. A third group was not treated and served as nonimmune controls.

A/Jax mice immunized against endotoxins received 5 mc X g of one endotoxin subcutaneously at weekly intervals for 2 months. Another group of A/Jax mice received 0.1 ml of the E. coli B bacterin and 0.1 ml of the Salmonella polyvalent vaccine in the same manner. Both groups were given booster injections about every 30 days following the initial immunization schedule. Again, another group served as nonimmune controls.

F. Titration of anti-endotoxin sera.

The immune status of some of the A/Jax mice given endotoxin was determined by titering the sera from the mice according to the bentonite flocculation technique of Wolff, Ward and Landy (1963). The stock bentonite mixture was prepared according to the method of Bozicevich, et al. (1958).

To adsorb the endotoxin to the bentonite, 10 ml of the stock bentonite was sedimented and the pellet was resuspended in 1 ml of distilled water. To this was added either 200 mcg of S. typhosa 0901 endotoxin or 300 mcg of E. coli 0111:B4 endotoxin in 1 ml of 0.15 M saline. This mixture was then allowed to stand at room temperature for 1 hour after mixing. One ml of 1.0% bovine plasma albumin (BPA) (Armour Pharmaceutical Co.) was added and the mixture was allowed to stand for an additional 15 minutes at room temperature. Fifteen ml of distilled water and 1 ml of 0.1% methylene blue chloride was added. The mixture was washed twice with distilled water and the final volume made up to 5 ml with distilled water and stored at 4 C until used.

Mice were anesthetized with ether and bled from the tail vein. The blood was allowed to clot overnight at 4 C and the serum was collected after 15 minutes centrifugation (International Clinical Centrifuge CL).

All sera were stored at -20 C in tightly closed vials until titered.

Serial two-fold dilutions of the anti-endotoxin sera were made with 0.1% BPA in Kahn tubes. One-tenth ml of the dilutions, including a 0.1% BPA control, were dispensed onto a ceramic ring slide (Clay-Adams #A-1751). To these drops of sera and control BPA were added 0.025 ml of the bentonite-endotoxin complex using a 0.25 syringe and a 25-gauge needle. The ring slide was placed on a rotating machine (100 to 120 rpm) for 30 minutes at room temperature. The reactions were read under 100X magnification and the degree of clumping was scored from 0 to 4+. The titers are expressed as the reciprocal of the last dilution showing a 1+ reaction as compared to the negative BPA control.

G. Antibiotic treatment.

Runted A/Jax and normal controls received daily intraperitoneal injections of 0.2 mg chloromycetin (Parke, Davis & Co.) per gram body weight beginning at 5 days of age and continuing until death or until the animals were sacrificed at 14 to 16 days of age for hematological studies as described below.

Attempts were made to administer polymixin B sulfate (Nutrional Biochemicals Corp.) via gavage tube to 5 day old runts and normal controls. Dihydrostreptomycin sulfate (Nutritional Biochemicals Corp.) in combination with penicillin "G" sodium (Nutritional Biochemicals Corp.) was injected intraperitoneally into runted and normal mice.

H. Bacteriology.

In order to determine if there was an overt bacterial invasion of the organs of runted mice the heart blood, liver and spleen were cultured.

Asepsis was maintained by dipping all instruments in 80% enthanol and flaming them.

The mice were sacrificed by etherization and the thoracic cavity was carefully opened, leaving the diaphragm intact. The heart was slit open and blood was collected in a sterile Pasteur pipet and transferred to the media described below. The liver and spleen were removed after opening the abdominal cavity and placed in separate small petri dishes containing 0.5 ml sterile saline. The organs were maceated with a plunger from a 1 ml syringe. The slurry was then transferred to the media by means of a Pasteur pipet. The inoculum was spread over the agar surface by means of a glass spreader.

MacConkey's agar (Difco) and blood agar (Difco) containing 5% rabbit blood were used. During initial studies the plates were incubated anaerobically in a Brewer jar in addition to aerobic incubation of a duplicate set of plates. Since the anaerobe plates failed to reveal additional organisms this step was eliminated and aerobic incubation only was used.

After 48 hours incubation at 37 C, representative colonies were picked and streaked on blood agar. They were then transferred to stock culture agar slants (Difco) and maintained at 4 C. In most cases no attempt was made to characterize the isolated organisms beyond hemolytic activities, gram reaction and morphology. A few isolates were grown on eosin methylene blue agar (Difco) and subjected to the indol, methyl red, Voges-Proskauer and citrate utilization tests.

I. Hematology.

Differential leucocyte counts, total leucocyte counts and hematocrits

were performed on runted and normal mice by the usual hematologic procedures (Wintrope, 1961).

Differential counts were determined from smears of blood taken from the tail vein stained with Wright's stain (Brook, Aloe Scientific) using a buffer of pH 6.7. One hundred cells were counted under oil immersion and scored as either lymphocyte, monocyte, polymorphonuclear leucocyte, eosinophil, basophil or nucleated red blood cell.

Total counts were made on blood taken from the heart. The mouse was sacrificed by etherization, the thoracic cavity opened and the heart was cut open. Blood was drawn into a leucocyte diluting pipet and immediately diluted with 4% acetic acid containing 0.1% methylene blue chloride. After shaking the pipet for 5 minutes (Burton Pippette Shaker #1402) the leucocytes were placed in a standard hemocytometer, allowed to settle for 5 minutes, and counted under 430X magnification. Total counts were corrected for nucleated red blood cells and absolute counts were obtained by multiplying the differential counts by total counts.

Hematocrits were determined for heart blood. The blood was drawn into a heparinized micro-hematocrit tube (75 mm X 0.55 mm) and centrifuged for 10 minutes in an Adam's Autocrit (Clay-Adams). Values were read directly from the scale within the centrifuge.

J. Histology.

Portions of the small intestine were removed from runted and normal mice for histological examination.

Immediately after removal the tissue samples were placed in 10% buffered (pH 7.0) formalin until processed for cutting. The tissues were

then fixed in Zenker's fixative for 24 hours, washed in running tap water for 36 hours and then dehydrated and cleared in a series of alcohols and benzene. After dehydration the tissues were infiltrated with paraffin at 52 C and embedded. Cross-sections were cut at 7 μ and stained with a standard hematoxylin and eosin procedure.

K. Statistical methods.

Average weight-gains, mean survival times, average blood cell counts and standard deviations were calculated according to Huntsberger (1961). The chi-square test was performed as described by Snedecor (1956).

RESULTS

A. Description of gross and microscopic morphology of runt disease in C3H and A/Jax mice.

Mice suffering from the runting syndrome exhibited the classic picture of emaciation, a very fluid diarrhea, varying degrees of abdominal alopecia, a hunched posture and ischemic appendages. The runts did not gain weight at a normal rate and those animals which lost weight invariably died within 24 hours of the weight loss.

Autopsy of C3H mice killed by the runting syndrome revealed varying degrees of splenomegaly, frequent necrosis of the leading edge of the liver and, in severe cases, extreme congestion of the distal portion of the small intestine. The most consistent finding was a thin, dry, parchment-like skin and extreme atrophy of the inguinal and axillary lymph nodes. A finding in disagreement with some authors (Billingham and Brent, 1959; Fiscus, et al., 1962) was the observation that thymic atrophy was extremely rare in the runting syndrome encountered in this study. The stomachs of all animals were full, no matter how severely emaciated.

A/Jax runts were essentially the same in appearance as were the C3H with the exception of abdominal alopecia. While the C3H frequently suffered from a severe form of alopecia the A/Jax seldom exhibited a significant degree of hair loss or failure to develop hair.

Autopsy of the A/Jax revealed the classic picture of lymph node involution, varying degrees of splenomegaly, focal liver necrosis and, very rarely, thymic involution. In a number of terminal runts the liver necrosis had spread throughout all lobes of the liver leaving a white fibrous mass, a phenomenon not seen in the C3H runts. In extreme cases the intestine

was grossly congested and inflamed and the peritoneal cavity was bathed in an odorless milky-colored fluid.

Histological examination of the A/Jax runt intestine showed attenuation of the villi, decreased numbers of goblet cells and congestion of the blood vessels of the submucosa, findings which are in agreement with the studies of Reilly and Kirsner (1965). In addition, two runts examined histologically showed evidence of intestinal hemorrhage.

B. Runt disease in C3H neonates born to immunized and nonimmunized mothers.

Newborn C3H born to females which had been immunized against endotoxins or bacterins were injected with 40 X 10^6 C57Bl/Ks spleen cells (see Materials and Methods). The course of the runting syndrome in mice born to these groups was compared with the runting syndrome in mice born to nonimmunized mothers. Hereafter these three groups will be referred to as endotoxin-immune runts, bacterin-immune runts and nonimmune runts.

Since Russell (1960) showed that weight-gain accurately reflects the course and severity of the runting syndrome this criterion was the first used to determine the severity of runt disease among the treated mice. Figure 1 shows that the weight-gain curves for the immune and nonimmune runts in addition to a curve representing the weight-gain of normally developing mice. Nonimmune runts failed to gain weight at a normal rate, and in many cases, lost weight just prior to death. The weight-gain of runted mice was not plotted beyond day 20 since most animals had died of runt disease. The endotoxin-immune runts and bacterin-immune runts gained less weight than the normal controls, but, significantly, gained more weight than the nonimmune runts. Runting mice feeding on females immunized against