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Effects of Purified Salmonella enteritidis Endotoxin on the Immune Response of Balb/c Mice

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THE EFFECTS OF PURIFIED *SALMONELLA ENTERITIDIS* ENDOTOXIN ON THE IMMUNE RESPONSE OF BALB/C MICE

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ABSTRACT

Wasting disease was produced in neonatal Balb/c mice with purified *Salmonella enteritidis* endotoxins. The endotoxins were chemically characterized to provide the study with a potent endotoxin with constant biological activity. Neonatal mice received an initial injection of 35 μg/g body weight of endotoxin and a dose of 75 μg every third day until day 18.

The degree of wasting was estimated by the runting (weight loss) index. Mortality was calculated to be 46%, with a mean survival time of approximately 15 days. The hematologic studies conducted at days 10, 20, and 30 showed an absolute neutrophilia and leukocytosis by day 10 which persisted to day 30. Immunologic data indicated that the responsiveness to sheep erythrocytes was depressed to day 20 and continued to persist at day 30. It was postulated that endotoxin either directly or indirectly affects the immune apparatus which allows the normal flora of the animal to establish an infection leading to wasting and death.

INTRODUCTION

Wasting disease is a syndrome characterized by ruffled fur, diarrhea, wrinkled and dry skin, a high stepping gait, failure to gain weight, and, in some instances, death. Wasting diseases have been produced in newborn mice by neonatal thymectomy (Miller, 1961) or by treatment with cortisol acetate (Schlesinger, 1964; Reed and Jutila, 1965) or estradiol (Reilly et al., 1967). The similarities of these diseases have caused many workers to postulate that common pathological or physiological events are responsible for the wasting and death of diseased animals.

There is considerable evidence that the microbial flora of the animal may play a major role in the development of symptoms of many forms of the disease. It was shown that germ-free mice were highly resistant to cortisol acetate-induced wasting disease (Reed and Jutila, 1965). Similarly, Wilson et al. (1964). Independently, McIntire et al. (1964) showed that thymectomized germ-free mice failed to waste in contrast to severe wasting exhibited by conventionally-reared mice thymectomized at birth.

The disease's course often has been mitigated by treatment with antibiotics. Hence, neonatally thymectomized mice treated with oxytetracycline (Azar, 1964) displayed less severe symptoms and a lower mortality than their untreated counterparts. Similarly, the antibiotic treatment of mice injected neonatally with cortisol acetate improved the clinical course and reduced the incidence of death. Thus, these observations tend to incriminate microorganisms and/or their products in the development of pathologic events that contribute to symptoms of wasting disease.

Among the normal flora isolated from tissues and organs of mice suffering from various forms of wasting diseases are several species of the *Enterobacteraceae* (Jutila and Reed, 1967; Jutila and Cantrell, 1970). A common component of gram-negative enteric bacteria is endotoxin, known to adversely influence the immune mechanism and produce pathologic changes similar to those observed in many of the wasting diseases. Since the normal microbial flora has been implicated in the pathogenesis of many forms of wasting disease, endotoxins, as products of the normal flora, were used to induce wasting in neonatal Balb/c mice.

METHODS AND MATERIALS

Inbred Balb/c mice were used throughout the study. The mice were originally obtained from the National Cancer Institute (Bethesda, Maryland) in the germ-free state and were conventionalized six months later. The mice have since been maintained by successive brother-sister matings in the Montana State University Rodent Breeding Colony. All animals received Purina Laboratory Chow and water *ad libitum*.

Endotoxin extracts were prepared from *Salmonella enteritidis*, strain S-795, (kindly supplied by Dr. E. Ribi) grown in trypticase soy broth (BBL). The endotoxin was prepared by either the dioxane or aqueous ether methods of Ribi (1958) and Fukushi et al. (1964).

The dioxane method involved the addition of 1,4 dioxane to an equal volume of 0.15 M saline containing 10 mg per ml of washed cells. The suspension was stirred at room temperature for 12 hrs and the resulting extract clarified by centrifugation at 2500 g for 70 min. The supernatant fluid was dialyzed against several changes of distilled water for six days and then lyophilized.

The aqueous ether extraction was performed by resuspending freshly harvested and washed cells in saline on the basis of turbidity (scale reading of 770 in a Klett-Summerson colorimeter, filter number 540). Two volumes of precooled (6-12° C) diethyl ether were added to the cell suspension at the same temperature and the mixture shaken for six consecutive ten-minute intervals with precaution taken to release the pressure after each interval. The suspension was left overnight at 6-12°C, after which the aqueous phase was drawn off and the residual ether removed by bubbling air through the suspension. The remaining steps were performed at 4-6°C. The residual cells were removed from the supernate containing soluble endotoxin by centrifugation at 2500 g for 70 min. The supernate was dialyzed against daily changes of distilled water for five days. NaCl was added to a final concentration of 0.15 M and the endotoxin precipitated by slowly adding absolute ethanol until a final concentration of 68% by volume was reached. After the suspension was allowed to stand at 6°C overnight, the precipitate was collected by centrifugation at 2000 g for 45 min, dissolved in the same volume of 0.15 M NaCl, and reprecipitated with ethanol. Then, the precipitate was lyophilized.

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The lyophilized preparation, after rehydration, was autoclaved at 18 pounds pressure for 15 min.

Total protein content was determined by Lowry's technique (Lowry, 1951) using bovine serum albumin to establish a standard curve. The carbohydrate determination was done by the anthrone reagent method and glucose to establish the standard curve (Morris, 1948). Lipid content was determined by a modification of the gravimetric technique by Entenman (1957).

The LD_{50} was determined by injecting one of four groups of mice intravenously (i.v.) with a given dose of endotoxin. Each group contained five adult Balb/c mice and were given the following doses: Group 1 received 100 μg per gram (μg/g) body weight; Group 2, 50 μg/g body weight; Group 3, 25 μg/g body weight; and Group 4, 12.5 μg/g body weight. The LD_{50} was determined by a modification of the Reed-Muench technique described by Carpenter (1956).

Experimental litters were sized to contain between five and eight neonatal mice of mixed sex. The average weight of these mice at the time of the initial injection was between 1.0 and 2.0 g. The initial injection was given intraperitoneally (i.p.) to animals less than 24 hrs old. The initial dose of endotoxin was 35 μg/g body weight. Subsequent doses of 75 μg of endotoxin were given i.p. every third day until day 18. The time of onset and severity of wasting was approximated by a runting index (RI) described by Keast (1968).

Blood for hematological studies was drawn from the tail or retro-orbital sinus. Differential leukocyte counts were performed on Wright's stained smears and recorded as lymphocytes, polymorphonuclear leukocytes (PMN) and monocytes. Total leukocyte counts were performed on a Model B Coulter Counter (Coulter Electronics, Hialeah, Florida). Hematocrit values were obtained by drawing blood into heparinized capillary tubes and centrifuging in an Adams Autocrit centrifuge (Clay-Adams Inc., New York).

Following sacrifice, the spleen, liver, and thymus were removed and bathed in phosphate buffered saline (PBS) until weighed. An organ index (O.I.) was calculated by a modification of the Simonsen method as follows (Simonsen, 1958):

$$\text{O.I.} = (\text{organ weight} \div \text{body weight}) \times 100$$

All animals were injected with 0.1 ml of 10% suspension of thrice-washed sheep red blood cells (SRBC) at either 10, 20, or 30 days of age. At days 5 and 10 after immunization, ten drops of tail or retro-orbital blood was collected in 0.5 ml saline and the serum removed for titering. The peak of IgM hemolysin production was presumed to occur at day 5, whereas the 10-day period approximated the peak of IgG hemagglutinating antibody (Ab) production.

Hemagglutinin titers were determined by serial two-fold dilutions of individual sera in a volume of 0.1 ml saline. To each serum dilution, 0.1 ml of a 1% SRBC suspension was added. The tubes were incubated for 30 min at 37°C and stored at 4°C overnight. The tubes were centrifuged at 3500 rpm for 1 min and the degree of agglutination scored. If agglutination occurred, the cells came up as one large clump (scored as 4), several large clumps (scored as 3), small clumps and loose cells (scored as 2), or mainly loose cells with some persistant clumps (scored as 1). In the absence of agglutination, there was a cloud of loose cell with no clumping (scored as negative). The titer was taken as the last serum dilution in the series to be scored as 1 or more.

Hemolysin titers were determined by adding 0.05 ml of guinea pig complement (BBL) diluted 1:5 to the standard hemagglutination system described above. The tubes were incubated at 37°C for 30 min and stored at 4°C overnight before reading. The last tube showing complete hemolysis was taken as the end point or titer.

RESULTS

Comparative Chemical Analysis of the Endotoxins.

Chemical analyses were performed on *S. enteritidis* endotoxin prepared by the dioxane method (designated Se-dioxane) and the aqueous ether method (Se-ether), described by Ribi (1958). The chemical composition of these endotoxins and the aqueous ether-extracted endotoxin obtained from Dr. Ribi is described in Table 1.

Table 1. Chemical composition of endotoxin prepared by the aqueous ether or dioxane method.

Endotoxin	Percent Protein	Percent Carbohydrate	Percent Lipid
Se-dioxane	42.0	7.7	N.A.[A]
Se-ether	21.0	38.7	18.0
Se-Ribi[B]	21.5	46.0	13.3

[A] Not available

[B] J. Bact. 87:391

The results show that the chemical composition of endotoxins prepared by the aqueous ether method, both Dr. Ribi's preparation and that prepared for this study, was essentially similar. A protein content of 21 and 21.5%, a hexose content of 38.7 and 47% and a lipid content of 18 and 13.3%, respectively, were determined for Barnett and Ribi endotoxin.

These results are contrasted with those obtained for Se-dioxane where protein estimates were increased to 42% and hexose content decreased to 7.7%. A lipid determination was not performed on Se-dioxane.

Characteristics of Wasting Disease.

The LD_{50} of Se-dioxane for adult Balb/c mice was 22.9 μg/g body weight. Since newborn mice tolerated this dose with few toxic symptoms, the study employed an initial dose of 35 μg/g body weight followed by one of 75 μg/g in the subsequent injections.

The time of onset and severity of wasting disease was estimated by the runting (weight loss) index calculated at various times during treatment with endotoxin. The data shown in Figures 1, 2, and 3 both describe and compare the onset and severity to the disease induced with the three endotoxin preparations. The onset of wasting was evident by the third day in every instance, and marked failure to gain weight was observed over a 12-day period. In the case of Se-dioxane (Fig. 1), weight gains were in a plateau phase between 12 and 20

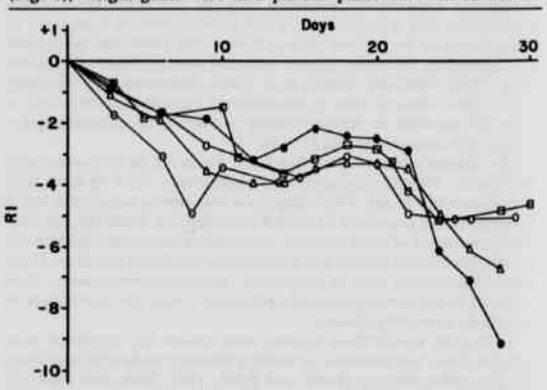

Figure 1. Runting index of Balb/c mice treated with SE-dioxane endotoxin. Newborn mice were injected with 35 μg/g body weight before 24 hours of birth and 75 μg/g of SE-dioxane every third day thereafter for 18 days. The runting index was determined by subtracting the mean weight of the control mice from the mean weight of the treated mice. Each RI unit is equal to 1 gram. Each line represents one litter.

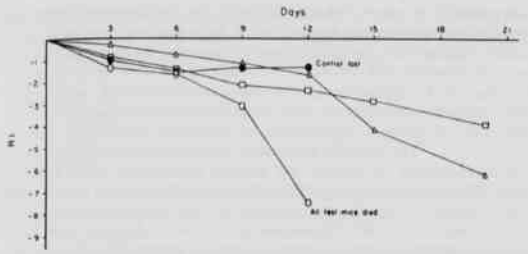

Figure 2. Runting index of Balb/c mice treated with SE-ether. Newborn mice were injected with 35 μg/g body weight before 24 hours of birth and 75 μg/g of SE-ether every third day thereafter for 18 days. The runting index was determined by subtracting the mean weight of the control mice from the mean weight of the treated miced. Each RI unit is equal to 1 gram. Each line represents one litter.

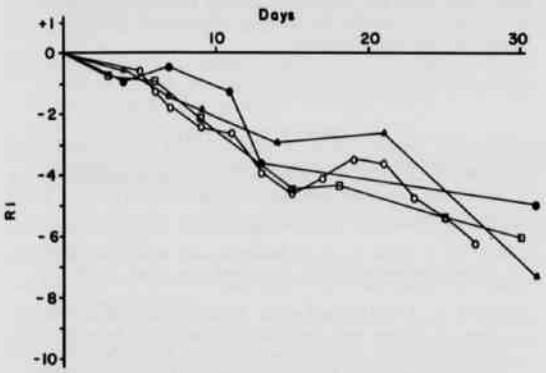

Figure 3. Runting index of Balb/c mice treated with SE-ribi. Newborn mice were injected with 35 μg/g body weight before 24 hours of birth and 75 μg/g of SE-ribi every third day thereafter for 18 days. The runting index was determined by subtracting the mean weight of the control mice from the mean weight of the treated mice. Each RI unit is equal to 1 gram. Each line represents one litter.

days, and then, following weaning, a marked failure to gain weight was again observed. Aqueous ether endotoxins generally produced a more pronounced effect on weight gain, and they were considered the more potent inducers of wasting disease. A regular finding was that a commercial endotoxin preparation, *Escherichia coli* lipopolysaccharide (Difco L.P.S.-B; *E. coli* 0111:B4 #3922), failed to induce symptoms of wasting despite the injection of comparable doses of endotoxin.

The incidence of wasting disease was found to be 100% regardless of the endotoxin employed as determined by the defined criteria (Table 2). Although, the symptoms of diarrhea and high stepping gait were not as pronounced as those seen in other forms of the disease, the failure to gain weight and other symptoms were severe. The mortality was calculated at 38% with Se-dioxane treated mice, 60% in Se-ether, and 42% in Se-Ribi treated mice. Only those mice dying later than 48 hrs after the initial injection were considered as dying from endotoxemia. Most deaths occurred during the interval from day 2 to day 25. Many mice appeared to be recovering by day 40.

Table 2. The incidence of wasting among Balb/c mice treated with endotoxin.

Endotoxin	Percent Wasting	Mean Mortality	Mean Survival Time (Days)
Se-dioxane	100	38	17.8
Se-ether	100	60	12.7
Se-Ribi	100	42	14.8
E. Coli (Difco)	0	0	--

The Effect of Endotoxin on the Hematology of Balb/c Mice.

The hematological changes during and following treatment with endotoxin are shown in Table 3. The results indicate that a pronounced leukocytosis had developed as early as day 10 and represented an increase in neutrophils. The neutrophils persisted to day 30 but the degree of shift to the left, i.e. increase in immature cell forms, could not be ascertained because of the failure to obtain total WBC's at this time. No significant differences (t-test) in the hematocrit could be detected between the control and the experimental groups for each test period. The weights of thymus, spleen, and liver of mice treated with endotoxin were nearly identical to organ weights of control mice (data not shown). The failure to obtain atrophy of thymus and spleen following treatment with endotoxin was correlated with little or no change in absolute lymphocyte counts.

Table 3. The hematology of Balb/c mice treated with multiple doses of SE-dioxane.

Treatment[a]	Day of Test	Total WBC[b]	Hematocrit	Lymphocytes	PMNs	Monocytes
Endotoxin	10	6514	52.2 0[c,d]	46.1±0.0	5±1.0	1.8±0.8
Control	10	4050	52±2.2	84±7.1	15±7.0	1
Endotoxin	20	9200	36±1.0[d]	49±0.0	51±0.0	0
Control	20	7319	43±4.24	64±8.5	37±2.0	1
Endotoxin	30	N.A.[e]	45±2.75	55±8.4	44±8.2	3±1.5
Control	30	N.A.	50±1.26	81±5.5	16±2.3	5±0.25

[a]Test animals received injections of endotoxin every third day from birth to day 18.
[b]All values expressed as means.
[c]Standard deviation
[d]Not available
[e]Not significant by the student t-test

The Effect of Endotoxin on the Immunological Response of Balb/c Mice.

The results of the immunological studies in mice treated with Se-dioxane are presented in Table 4. The endotoxin-treated mice demonstrated a depression in both the hemolysin and the hemagglutination responses at days 20 and 30. It was observed that 68% of the experimental mice responded at day 20 to an immunizing dose of sheep erythrocytes, whereas 100% of the controls responded. Although the antibody titer of the 30-day endotoxin-treated mice had increased well above that of the 20-day old test group, apparently a severe impairment of responsiveness to sheep erythrocytes still persisted.

Table 4. The effect of multiple administration of SE-dioxane on the hemolysin and hemagglutination response in young Balb/c mice.

Treatment[a]	Days of immunization[b]	Days of Titration[c]	Antibody Titers[d]					
			Hemolysin			Hemagglutination		
			No. responded[e]	Mean	Range	No. responded	Mean	Range
Endotoxin (SE-dioxane)	20	5	2/3	88	0-144	2/3	56	0-96
		10	6/9	33	0-80	5/9	52	0-160
Control	20	5	2/2	120	96-144	2/2	87	86-87
		10	2/2	75	70-80	2/2	360	80-640
Endotoxin	30	5	8/12	70	0-320	7/12	52	0-200
		10	8/11	130	0-320	9/11	148	0-320
Control	30	5	5/5	512	320-640	5/5	384	320-640
		10	5/5	240	80-320	5/5	448	320-640

[a] The mice received injections of endotoxin every third day from birth to day 18.

[b] Designates day after birth that mice were immunized with 0.1 ml of 10% sheep red blood cells i.p.

[c] Day mice were bled for antibody titrations.

[d] Titers are expressed as reciprocals of serum dilutions and the means are rounded off to the nearest whole number.

[e] The numerator designates those animals responding to immunization; the denominator the total number of animals immunized.

DISCUSSION

The symptoms of wasting disease induced with endotoxin were reminiscent of those observed with other forms of the disease, most notably, those induced with cortisol acetate (Schlesinger and Mark, 1964; Reed and Jutila, 1965) or neonatal thymectomy (Miller, 1961). Hence, it could be concluded that some of the pathological events associated with wasting induced by these methods were produced, in part, by the effects of endotoxin. The incidence of deaths among mice treated with endotoxin (Table 2) was similar to mortality among thymectomized mice treated with rabbit anti-(mouse) thymocyte serum (Jutila and Cantrell, 1970) but significantly less than the mortality among cortisol acetate treated mice (Reed and Jutila, 1965).

The hematological response of an animal treated with endotoxin was characterized by a neutrophilia which developed by day 10 and persisted through day 30 (Table 3). A similar finding was made in mice treated with cell wall preparations rich in endotoxin (Eksedt and Hayes, 1967). In contrast, the hematologic picture differs in thymectomized mice given anti-lymphocyte serum (Monaco, 1967; Agnew, 1968; Jutila and Cantrell, 1970; Jutila, 1969) and those treated with cortisol acetate (Reed and Jutila, 1967; Jutila, 1969). Thymectomized mice and neonates treated with cortisol acetate characteristically demonstrated a lymphopenia. In addition to this, Reed and Jutila (1967) observed an initial depression in the neutrophil counts after treatment with cortisol acetate; but when infections developed, a neutrophilia occurred. The discrepancy between the data obtained from mice treated with endotoxin and that obtained from thymectomized mice or mice treated with cortisol acetate may be explained by the fact that lymphocyte-producing organs such as thymus and spleen were unaffected by the treatment in one case and severely damaged or abated in the other. It seems especially pertinent that a neutrophilia, as observed in this or other forms of wasting, is commonly associated with infection.

The immunological studies on endotoxin-treated mice (Table 4) closely parallel those seen in wasting mice following thymectomy or treatment with cortisol acetate (Jutila, 1969). A depression of responsiveness to sheep erythrocytes was evident at day 20 with slight recovery at day 30 after initiation of treatment. These results confirm the findings of several investigators (Eksedt and Nishimura, 1964; Eksedt and Hayes, 1967; Chester et al., 1971; Diamantstein et al., 1976; and Nakano et al., 1976) who also demonstrated an impaired antibody response in endotoxin-treated mice. Of interest is the observation by Eksedt and Hayes (1967) that although no circulating antibody to sheep erythrocytes could be detected, the mechanism for rejecting skin grafts was not affected. This result would indicate that although the humoral immune response was impaired, the cellular response was unaffected. It also emphasized the well-known dichotomy between the two systems. The precise reasons for antibody suppression were not determined; however, this effect probably occurred during the induction phase of the immune response. In support of this postulate it was noted that although the lymphocyte counts and morphology appeared to be unaffected, little antibody was being produced. Apparently, the antigen was prevented from reacting with the antigen sensitive cell. Precisely how this effect may be mediated is not known, but it may occur in one of the following ways: The endotoxin may cause an adrenal hyperreactivity which, in turn, provokes the production and release of abundant steroids (L. Hayes, *pers. comm.*). The steroids, because of their predilection for lymphoid tissue, eliminate or damage the antigen recognition units (Miller, 1967; Mitchell and Miller, 1968). Alternatively, steroids may inhibit the lysosome-mediated transformation of the responsive lymphocyte (Hischhorn et al., 1967; Weissmann et al., 1967). Another possibility is that endotoxin, with its interaction with lymphoid cells, directly rather than indirectly, interferes with or damages the unit for antigen recognition.

Immunological suppression in wasting animals may be a necessary prerequisite to the events that kill the animal. It has been demonstrated that symptoms of and death from wasting disease are caused by an infectious process following impairment of the immune mechanism of the animal (Jutila and Cantrell, 1970; Jutila and Reed, 1968; Jutila, 1969; and Azar, 1964, 1964b). The depression allows the normal flora to invade the body of the animal, causing wasting disease induced with endotoxin.

LITERATURE CITED

AGNEW, H. D. 1968. The effect of heterologous anti-lymphocyte serum on the small lymphocyte population of rats. J. Exptl. Med. 128:111.

AZAR, H. A. 1964. Bacterial infection and wasting in neonatally thymectomized rats. Proc. Soc. Expt. Biol. Med. 116:817.

AZAR, H. A., J. WILLIAMS and K. TAKATSUKI. 1964a. Development of plasma cells and immunoglobulins in neonatally thymectomized rats. p. 75-87. In V. Defendi and D. Metcalf (eds.). The Thymus. Wistar Institute Symposium Monograph Number 2. Wistar Institute Press, Philadelphia.

CARPENTER, P. C. 1956. Immunology and Serology. W. B. Saunders Co., Philadelphia. P. 193.

CHESTER, T. J., F. DECLERCG and T. A. MERIGAN. 1971. Effect of separate and combined injections of Poly rI:Poly rC and endotoxin of reticuloendothelial activity, interferon, and antibody production in the mouse. Infect. Immun. 3:516.

DIAMANTSTEIN, T., W. KEPPLER, E. BLITSTEIN-WILLINGER and S. BENEFRAIM. 1976. Suppression of the primary immune response in vivo to sheep red blood cells by B-cell mitogens. Immunol. 30:401.

EKSEDT, R. D. and E. NISHIMURA. 1964. Runt disease induced in neonatal mice by sterile bacteria vaccines. J. Exptl. Med. 120:798.

EKSEDT, R. D. and L. L. HAYES. 1967. Runt diseases induced by non-living bacterial antigens. J. Immunol. 98:110.

ENTENMAN, C. 1957. General procedures for separating lipid components of tissues. p. 310. *In* Methods of Enzymology (eds.), Vol. III. S. P. Colowick and N. O. Kaplan. Academic Press Inc., New York.

FUKUSHI, K., R. L. ANACKER, W. T. HASKINS, M. LANDY, K. C. MILNER and E. RIBI. 1964. Extraction and purification of endotoxin from Enterobacteriacae: A comparison of selected methods and sources. J. Bact. 87:391.

HIRSCHHORN, R., K. HIRSCHHORN and G. WEISSMANN. 1967. Appearances of hydrolase rich granules in human lymphocytes induced by phytohemagglutinin and antigens. Blood. 30:84.

JOHNSON, A. G. 1964. Adjuvant action of bacterial endotoxins on the primary antibody response. *In* M. Landy and W. Braun (eds.), Bacterial endotoxins. Inst. of Microbiology, Rutgers, The State University, New Brunswick, New Jersey.

JUTILA, J. W. 1969. Wasting disease induced with cortisol acetate III. Immunologic studies. J. Immunol. 102:963.

JUTILA, J. W. and N. REED. 1968. Wasting disease induced with cortisol acetate. II. Bacteriologic studies. J. Immunol. 100:675.

JUTILA, J. W. and J. L. CANTRELL. 1970. Bacteriological studies on wasting disease induced by neonatal thymectomy. J. Immunol. 104:79.

KEAST, D. 1968. A simple index for the measurement of the runting syndrome and its use in the study of the influence of the gut flora in its production. Immunology. 15:237.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:268.

MCINTIRE, K. R., S. SELL and J. F. A. P. MILLER. 1964. Pathogenesis of post-neonatal thymectomy wasting syndrome. Nature. 204:151.

MILLER, J. F. A. P. 1961. Immunological function of the thymus. Lancet. 2:748.

MILLER, J. F. A. P. 1967. The thymus yesterday, today, and tomorrow. Lancet. 2:1299.

MITCHELL, G. F. and J. F. A. P. MILLER. 1968. Immunological activity of thymus and thoracic duct lymphocytes. Proc. Nat. Acad. Sci. 59:296.

MONACO, A. P., M. L. WOOD and P. S. RUSSELL. 1967. Modification of allograft immunity in adult-thymectomized mice given anti-lymphocyte serum. Fed. Proc. 26:418.

MORRIS, D. L. 1948. Quantitative determination of carbohydrates with Dreywood's anthrone agent. Science. 107:254.

NAKANO, M. M., J. TANABE, T. SAITO and T. SHIMIZU. 1976. Immunosuppressive effect of bacterial lipopolysaccharide on antibody response. Jpn. J. Microbiol. 20:53.

REED, N. D. and J. W. JUTILA. 1965. Wasting disease induced with cortisol acetate. Studies in germ-free mice. Science. 150:356.

REED, N. D. and J. W. JUTILA. 1967. Wasting disease induced with cortisol acetate. I. Studies in germ-free and conventionally reared mice. J. Immunol. 99:238.

REILLY, R. S., J. S. THOMPSON, R. K. BIELSKI and C. D. SEVENSON. 1967. Estradiol-induced wasting syndrome in neonatal mice. J. Immunol. 98:321.

RIBI, E., K. C. MILNER and T. D. PERRINE. 1958. Endotoxic and antigenic fractions from the cell wall of *Salmonella enteritidis*. Methods for separation and some biologic activities. J. Immunol. 82:75.

ROWLANDS, T., N. CLAMAN and D. KIND. 1965. The effect of endotoxin on the thymus of young mice. Am. J. Pathol. 46:165.

SCHLESINGER, M. and R. MARK. Wasting disease induced in young mice by administration of cortisol acetate. Science. 143:968.

SIMONSEN, M., J. ENGELBRETH-HOLM, E. JENSEN and P. HEMMING. 1958. A study of the graft-versus-host reaction in transplantation to embryos F1 hybrids, and irradiated animals. Ann. N.Y. Acad. Sci. 73:834.

WEISSMANN, W., W. TROLL, G. BRITTINGER and R. HIRSCHHORN. 1967. Role of hydrolytic enzymes in nuclear activity. J. Cell Biology. 35:140A.

WILSON, R., K. SJODIN and M. BEALMER. 1964. The absence of wasting in thymectomized germ-free (Axenic) mice. Proc. Soc. Exptl. Biol. Med. 117:237.