The antibody response of congenitally thymusless (nude) mice to thymus-independent antigens
by Judith Klein Manning

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

Abstract:
The immune response, as assessed by the number of plaque forming cells (PFC) in the spleen and the serum antibody titers, of congenitally thymusless (nude) mice to two alleged thymus-independent antigens, E. coli lipopolysaccharide (LPS) and type III pneumococcal polysaccharide (SIII) , was studied. The primary response to both antigens was determined. In addition, the response to LPS was studied over a wide dose range, at various times after immunization, when the antigen was presented as part of whole E. coli cells or attached to erythrocytes and when treated with a surfactant. A toleriz-ing dose of SIII was also tested. In all of these studies the response of nudes was, in magnitude, equal to or greater than that of normals. These findings indicate that these antigens are truly thymus-independent.

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Date Aug 3, 1972

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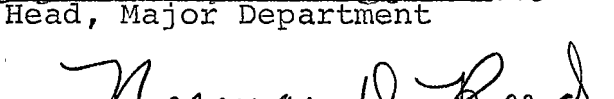
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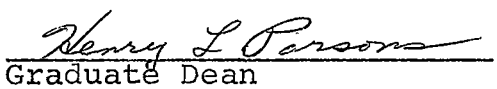
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ABSTRACT

The immune response, as assessed by the number of plaque forming cells (PFC) in the spleen and the serum antibody titers, of congenitally thymusless (nude) mice to two alleged thymus-independent antigens, E. coli lipopolysaccharide (LPS) and type III pneumococcal polysaccharide (SIII), was studied. The primary response to both antigens was determined. In addition, the response to LPS was studied over a wide dose range, at various times after immunization, when the antigen was presented as part of whole E. coli cells or attached to erythrocytes and when treated with a surfactant. A tolerizing dose of SIII was also tested. In all of these studies the response of nudes was, in magnitude, equal to or greater than that of normals. These findings indicate that these antigens are truly thymus-independent.

INTRODUCTION

Studies involving neonatally thymectomized animals and adult thymectomized, irradiated and bone marrow reconstituted animals have demonstrated that the thymus gland plays a definite role in many immune responses. In addition to an impaired cellular response, thymus-deprived animals exhibit a depressed humoral response to some antigens such as sheep erythrocytes, T-2 bacteriophage and certain serum proteins but produce a normal humoral response to other antigens, such as ferritin, Salmonella flagellar antigen and MS-2 bacteriophage (1). These results have led to a classification of antigens as thymus-dependent or thymus-independent.

There is some controversy as to the validity of the concept of thymus-independent immune responses which is based on the responses of thymectomized animals. First, it is possible that the immune response to some antigens matures relatively early in ontogeny and appropriate thymus-derived antigen reactive cells are seeded out from the thymus prior to its removal at birth (2). Second, it has been shown that some thymus-derived cells can survive irradiation and that bone marrow preparations used in reconstitution may contain thymus-derived cells (3). Therefore, alleged thymus-independent antigens may merely require fewer thymus-derived cells to produce an immune response. Alternatively, thymus-

independent antigens may be able to evoke an immune response by some mechanism which does not require the participation of thymus-derived cells.

The nude mouse, described by Flanagan (4) and discovered to be thymusless (5), is the ideal system to study thymus-independent antigens because it avoids the objections stated above. The nude mouse has a detectable abnormal thymic rudiment at the 14-15th day of embryonic life but it never becomes populated with lymphoid cells (6). Wortis (7) has shown that these mice do not lack the precursors for thymocytes but rather that they suffer from a defect of the thymic epithelium. It has been shown that nude mice fail to reject skin homografts and heterografts (8-10). In addition, many studies have confirmed that they do not respond normally to sheep erythrocytes (SE) (9-12). However, the response of nudes to alleged thymus-independent antigens has not been reported prior to this study (13). It was the purpose of this thesis to investigate the humoral response, as assessed by the number of plaque forming cells (PFC) and serum antibody levels, of nudes to two antigens, Escherichia coli lipopolysaccharide (LPS) and type III pneumococcal polysaccharide (SIII).

LPS was classified as a thymus-independent antigen when it was found that addition of thymus cells did not increase

the PFC response to LPS in adult, irradiated and bone marrow reconstituted mice (14-16). The thymus-independency of this antigen was exhibited over a large dose range and in a secondary response (16). SIII was classified as thymus-independent when Humphrey et al. (2) found normal or above normal levels of serum antibody to SIII in neonatally thymectomized animals after immunization. Later, it was found that thymus cell reconstitution of adult, thymectomized, irradiated and bone marrow reconstituted mice had no effect on the PFC response to SIII (17).

Bacterial LPS, also called endotoxins, are complex macromolecules consisting essentially of a phospholipid covalently linked to a core polysaccharide to which are attached serologically specific, repeating oligosaccharide chains, the number and length of which are not known (18). Estimates of the molecular weight of LPS particles vary from 1 to 20 x 10^6 (19). LPS has been shown to be associated with the outer layer of the cell wall of gram-negative bacteria (20).

LPS is a good immunogen in both rabbits (21) and mice (22). It is generally believed that the polysaccharide side chain portion is responsible for the O-antigenic specificity of the Enterobacteriaceae (19). LPS also produces a variety of other physiological and pathological effects including

lethal shock, fever, hemorrhagic necrosis of tumors, the Shwartzmann reactions, disturbances in sugar metabolism, increased non-specific resistance to disease and changes in the numbers of circulating polymorphonuclear leukocytes (23).

LPS can be extracted from gram-negative bacteria by a variety of procedures. The three more commonly used methods are the trichloracetic (TCA) extraction (24), the phenol-water extraction of Westphal (25), and the aqueous-ether extraction of Ribi (26). The TCA extract, also called Boivin antigen or complete antigen, contains protein (19) whereas the other extractions do not. Phenol and ether extracts differ in that ether extracts contain much less lipid (19, 27). All of these methods yield products which are both toxic and antigenic, but some differences exist, especially among strains of bacteria (28).

Two models have been proposed for the physical conformation of LPS. Ribi and others (29) suggested that it is an aggregation of linear units which form a micellar structure. Other workers (30-33) have proposed that LPS has an ordered bileaflet, membrane-like structure consisting of a bilayer of polysaccharide and lipid with the non-polar lipids occupying the interior of the bilayer.

Much research has been directed toward the relationship between biological activity and chemical composition and/or physical conformation. Only those studies concerned with the immunogenicity of LPS will be discussed.

Landy (34) has shown that Boivin antigen freed of protein retains its immunogenicity. Therefore, protein is not necessary for immunogenicity. However, it has been shown that Boivin antigen, when compared with other LPS preparations, is the only extract to remove all antibodies formed against whole bacteria (35).

Acetic acid extraction of whole bacteria yields a product which is pure polysaccharide (degraded polysaccharide) (36). Although it has O-antigenic specificity, it is not immunogenic unless attached to protein (19). The molecular weight of degraded polysaccharide has been estimated at 20,000 - 30,000 (19). Milner (37) has shown that acid hydrolysis of extracted LPS also results in a reduction in immunogenicity.

Similarly, treatment of whole bacteria with alkali results in a product (alkali polysaccharide) which is O-specific and non-antigenic but which contains O-deacylated lipid (19). Likewise, the same substance can be obtained by aklaline hydrolysis of extracted LPS (38). Alkali polysaccharide has a molecular weight of 200,000 and, when affixed to the surface of erythrocytes, can elicit an antibody response (38).

Treatment of phenol-extracted LPS from Salmonella enteriditis with sodium deoxycholate (NaD) resulted in a reduced bacterial agglutination titer and PFC response in rabbits (39). This effect could be reversed by removal of NaD by dialysis. However, Jackson (40) has found that, in mice, NaD treated LPS produced a reduction in bactericidal titer but not in passive hemagglutination (HA) titer. In fact, one type of LPS showed an enhancement in the HA titer.

Previous work (41) has indicated that upon treatment with NaD, LPS dissociates into subunits of approximately 20,000 daltons. Removal of NaD by dialysis results in reaggregation of the subunits into particles with a molecular weight of 500,000 - 1,000,000 (41).

From the foregoing it is difficult to evaluate the effect of chemical composition and physical conformation on the immunogenicity of LPS. Studies with alkali and degraded polysaccharides may imply a role for lipids. However, these substances have had covalent bonds broken and have a low molecular weight (19). It is therefore difficult to pinpoint the factor responsible for reduced immunogenicity. Studies with NaD indicate that the requirements for immunogenicity may be different in rabbits and mice.

There is some controversy as to the type of

immunoglobulin (Ig) produced in response to LPS. Most studies have indicated that the response is predominantly IgM (21, 42-43). Other workers claim that IgG immunoglobulins are produced (44-45). Walters and Jackson (46) have reported that IgA antibodies are produced.

A secondary response to LPS occurs and the magnitude of the response depends on the ratio of priming dose to secondary dose and the interval between immunizations (22). The secondary response is also thought to be predominantly IgM (42).

Pneumococcal polysaccharide, in the form of soluble specific substance, is an extract of the capsular substance of Diplococcus pneumoniae. There are numerous serological types among this species, each having a different chemical composition. Type III pneumococcal polysaccharide (SIII) consists of repeating units of cellobiuronic acid and has a molecular weight of about 60,000 (47).

Most of the early studies on the immune response to SIII have centered on the ability to protect an organism against a challenge of live, virulent bacteria. The discovery that SIII can be linked to erythrocytes (48) has led to more detailed study of the immune response elicited by SIII.

In mice the PFC response is thought to be exclusively IgM and two types of antibody have been identified (49-50). One type is detected by the direct PFC test, whereas the other is only detectable when the plaques are developed with an anti-IgM antiserum.

The treatment of mice with anti-lymphocyte serum (ALS) resulted in an enhancement of the direct PFC response to SIII (51). Baker et al. (50) postulated that thymus cells are able to exert a suppressive, regulatory effect on the direct PFC response to SIII.

Other workers (52-53) have also detected two types of antibody in the serum of mice. However, one antibody identified as a hemagglutinin, is thought to be IgA, whereas the other, identified as a hemolysin, is thought to be IgM (53).

Baker (54) has reported that no short term secondary response to this antigen is produced. However, Kearney and Halliday (52) reported a long term secondary response that is dependent upon the ratio of primary dose to secondary dose.

MATERIALS AND METHODS

Mice. A breeding nucleus of mice, carrying the nude (nu) gene, was obtained from the University of Edinburgh, Scotland. Since nudes are frequently sterile and the females are unable to suckle their young, an experimental stock was obtained by breeding heterozygous animals. The breeding scheme of Rygaard (8) was used. Heterozygous males carrying Rex and nude (in trans position) were bred to specific pathogen free (SPF) Balb/c females. Non-Rex heterozygous animals from these matings were used as breeders (F_1) as well as some F_2 females. Occasionally, nude males proved fertile and were used as breeders. Some heterozygous breeders were obtained by mating Balb/c females to nude males.

At birth the litter size was adjusted so that the number of phenotypically normal animals (+/+ or +/nu), hereafter referred to as normals, was equal to the number of nudes (nu/nu). The genotypes of the normal mice were not determined.

All mice were given sterile Purina 5010C feed and acidified-chlorinated water (55). The mice ranged in age from 5-12 weeks and the sexes were not distinguished in the experimental design.

Antigens. E. coli 0113 lipopolysaccharide (LPS), extracted by the phenol-water method (25) and whole E. coli 0113 cells, boiled for 150 minutes, were supplied by Dr. J. A. Rudbach of the University of Montana. The LPS was dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mg/ml and kept frozen. At the time of immunization it was thawed and diluted to the required concentration.

LPS sensitized horse erythrocytes (HE) were prepared as described below for sensitization of sheep erythrocytes (SE).

Sodium deoxycholate dissociated LPS (NaD-LPS) was prepared by dissolving LPS at a concentration of 1 mg/ml in 1% NaD (dissolved in 0.1 M tris buffer, pH 8). This mixture was further diluted in 1% NaD so that 0.05 ml of the solution contained 10 µg LPS. This preparation was left at room temperature for 30 minutes and then injected.

HE (Colorado Serum Co.) were washed 3x in PBS and then adjusted to the required concentration.

All cell washings were done in the cold.

The type III pneumococcal polysaccharide (SIII), prepared by a modification of the procedure of Felton (56), was supplied by Dr. P. J. Baker of the National Institutes of Health, Bethesda, Maryland. The SIII was dissolved in saline at a concentration of 1 mg/ml, kept refrigerated and diluted

to the required concentration in PBS at the time of immunization. This same preparation was used for sensitizing SE.

Sensitization of SE with LPS. The sensitization of SE with LPS was done according to the method of Neter (38). LPS was dissolved in 0.1 M potassium phosphate buffer (pH 7.3-7.4) at a concentration of 10 mg/10 ml. This preparation was placed in a boiling water bath for 2.5 hours. It was kept frozen and used as needed.

SE (Colorado Serum Co.) were washed 2x in saline or PBS. The boiled LPS was diluted 1:10 in PBS and 0.25-0.50 ml of the packed SE was added for each milligram of LPS. This mixture was incubated at 37°C for 30-35 minutes and then the cells were washed 3x in PBS.

Sensitization of SE with SIII. The method of Baker (48) was used to sensitize SE with SIII. SE were washed 2x in saline (phosphates may interfere with coupling). A 1% stock saline solution of chromium chloride ($CrCl_3 \cdot 6H_2O$, Fisher Sci. Co.), which had turned a bluish color, was diluted 1:10 in saline. For each milliliter of diluted $CrCl_3$, 0.8-1.0 mg of SIII (diluted in saline, 1 mg/ml) and 0.50 ml of packed SE were added and left at room temperature for 5 minutes. The cells were then washed 4x in saline.

Immunological Assays. The slide test of Mishell and Dutton (57) was employed, using SE sensitized with LPS, to determine the PFC response to LPS. In most of the primary response determinations, centrifuged spleen cells were resuspended in 4 ml of 0.83% ammonium chloride to lyse the erythrocytes. After 5 minutes at room temperature the spleen cells were washed 2x in Dutton's balanced salt solution (DBSS). No significant difference in the PFC response was observed with or without ammonium chloride. A background PFC response to non-sensitized SE was determined for each animal and, except in the case of a very high response to LPS was subtracted from the total PFC response. Sensitized and non-sensitized SE were diluted 1:15 in DBSS and 0.05 ml were added to 0.1 ml of a spleen cell dilution in 0.5 ml agarose (0.5%). The slides were incubated at 37°C for 1-2 hours in a moist chamber. Complement (absorbed with SE) was added and the slides were reincubated for 1-2 hours.

The slide method of Baker (48) was used to determine the PFC response to SIII. Spleen cells were suspended in medium 199 adjusted to pH 7.0-7.2. Agarose (0.5%) and complement were suspended in Hanks balanced salt solution (HBSS) adjusted to the same pH. A 20% solution of sensitized and non-sensitized SE was used in the same volume as for LPS

determinations. Duplicate sets of slides were made and after 1-2 hours incubation at 37°C an anti-IgM facilitating antiserum was added to one set of slides which was re-incubated for 1-2 more hours. The other set of slides (direct plaques) was left in the incubator. After washing the trays and slides that contained facilitating antiserum, complement was then added to both sets of slides and then they were incubated for one more hour. A background response to non-sensitized SE was determined for each animal and subtracted from the total response.

Serum hemagglutination titers for the LPS response were done exactly as described by Rudbach (22). The last tube displaying a 1+ agglutination reaction was used as the end point.

Serum hemagglutinin (HA) and hemolysin titers for the SIII response were done by the microtiter method according to Baker (48).

Titers are reported as the reciprocal of the dilution of the end point tube.

RESULTS

Primary Immune Response to LPS. The primary immune response to LPS of nudes and normals is shown in Table I. Mice received a single injection of 10 µg LPS in the lateral tail vein (IV). Four days later, they were bled, sacrificed and their spleens assayed for PFC. The number of PFC was not increased when the plaques were developed with either anti-IgM or anti-IgG antiserum. Individual responses varied greatly but the range was similar in both groups. Unimmunized animals showed no appreciable response to LPS.

The differences observed between nudes and normals with respect to PFC/Spleen and HA titers were not significant, at all levels of probability, when tested by Student's t test. However, the difference between the means of the PFC/10^6 spleen cells was significant when P=0.05 but not when P=0.02. This may indicate that nudes were able to respond slightly better than normal mice.

Similar results were obtained (Table II) when the same dose of LPS was injected by the intraperitoneal (IP) route.

Because the nudes used in these studies were offspring of heterozygous, normal females, it is possible that the nudes were influenced by thymocytes or a thymus-derived

TABLE I

The primary immune response to intravenous injection of 10 μg *E. coli* lipopolysaccharide assayed four days post-immunization

	Number of Mice	Mean PFC/Spleen	Mean PFC/10^6 Spleen cells	Mean HA Titer
Nudes	14	5753 (720-18,000)[a]	48	92
Normals	13	5848 (1240-18,300)	27	65

[a] number in parentheses is the range