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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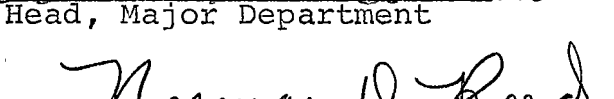
Approved:


Head, Major Department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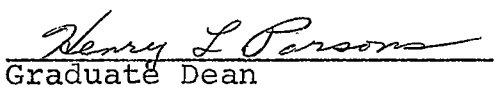
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## ABSTRACT

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

TABLE II

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

| | Number of Mice | Mean PFC/Spleen | Mean PFC/$10^6$ Spleen cells |
|---|---|---|---|
| Nudes | 7 | 4137 | 40 |
| Normals | 9 | 6800 | 30 |

humoral factor of maternal or littermate origin, similar to the effect observed by Osoba (58). Therefore, in a separate experiment, four nude mice (six weeks old) from a mating between a nude female and a nude male and subsequently foster-nursed by a normal female were immunized, IV, with 10 μg LPS and assayed four days later. The responses of the individual mice are shown in Table III. The responses of these nudes were essentially the same as shown previously for nudes derived from heterozygous parents.

Effect of Varying Doses of LPS on the Primary Immune Response. Because it has been observed that high doses of SE could partially overcome the depressing effects of thymectomy on the response to that antigen (59), the response of nudes to different doses of LPS was tested. The results are shown in Table IV. A ten-fold increase or decrease in the initial 10 μg dose of LPS did not markedly affect the response. A hundred-fold decrease in the initial 10 μg dose produced a more marked change in the immune response. However, nudes and normals appeared to be affected equally. A 1000 μg dose was lethal for nudes.

Response to a Single Injection of LPS Assayed at Various Times After Immunization. In order to determine the

TABLE III

The primary immune response of nudes derived from nude parents to 10 μg *E. coli* lipopolysaccharide[a] assayed four days post-immunization

| | Mean PFC/Spleen | Mean PFC/$10^6$ Spleen cells | Mean HA Titer |
|---|---|---|---|
| Nude 1 | 4080 | 37 | 40 |
| Nude 2 | 3560 | 57 | 80 |
| Nude 3 | 5320 | 54 | 80 |
| Nude 4 | 3440 | 34 | 80 |
| Means | 4100 | 45 | 70 |

[a] injected IV

TABLE IV

The effect of varying doses of E. coli lipopolysaccharide on the primary immune response

| | Number of Mice | Dose of LPS (IV) | Mean PFC/Spleen | Mean PFC/$10^6$ Spleen cells |
|---|---|---|---|---|
| Nudes | 3 | 0.1 µg | 880 | 6 |
| | 4 | 1 µg | 3495 | 36 |
| | 14 | 10 µg | 5753[a] | 48[a] |
| | 2 | 100 µg | 4100 | 48 |
| Normals | 3 | 0.1 µg | 1413 | 8 |
| | 4 | 1 µg | 2050 | 25 |
| | 13 | 10 µg | 5548[a] | 27[a] |
| | 1 | 100 µg | 5300 | 41 |

[a] These results are taken from Table I

time after immunization at which the PFC response is greatest and to study the kinetics of the PFC response to LPS in nudes and normals, mice were assayed at varying times after a single IV injection of 10 µg LPS. The results are shown in Table V. The optimum time for the PFC response to LPS was four days post-immunization for both nudes and normals. In addition, there was no apparent difference in the responses of nudes and normals either before or after the peak PFC response. No unequivocal evidence of a cyclical nature of the response as observed by Britton (43) was detected. The somewhat higher PFC response of nudes at 20 days post-immunization was due to an extremely high response (9400 PFC/Spleen) of one nude. Further studies are needed to determine whether this is a significant difference.

Primary Response to Horse Erythrocytes and Lipopolysaccharide when Administered to the Same Animal. In order to confirm that nude mice which gave a normal response to LPS would show an impaired response to heterologous erythrocytes, nude and normal mice were given 0.25 ml of a 10% suspension of HE, IP, on day 0 and 10 µg LPS, either IP or IV, on day 1. The spleens of these animals were assayed

TABLE V

Mean plaque forming cell per spleen response to 10 μg *E. coli* lipopolysaccharide assayed at various times after immunization.

| Number of days post-immunization | Nudes | Normals |
|---|---|---|
| 3 | 1092 (5)[a] | 2356 (5) |
| 4 | 5753[b] (14) | 5848[b] (13) |
| 5 | 1840 (5) | 1460 (5) |
| 6 | 1054 (7) | 1000 (6) |
| 10 | 427 (3) | 740 (2) |
| 14 | 1547 (3) | 1440 (2) |
| 16 | 920 (3) | 2187 (3) |
| 18 | 1572 (5) | 2376 (5) |
| 20 | 4313 (3) | 1167 (3) |

[a] number in parentheses = number of mice

[b] these results are taken from Table I

on day 5 for the PFC response to LPS sensitized SE and non-sensitized HE.

A summary of the results is presented in Table VI. This experiment confirmed that nudes which responded normally to LPS did show a depressed response to HE whereas normal mice showed a high response to HE. The somewhat lower LPS response exhibited by normals was probably due to antigenic competition. Perhaps this was not observed in nudes because HE are thymus-dependent.

It should be noted that in the above experiments a difference in the responses of nudes dependent on the injection route of LPS was observed. Thus, nudes showed a somewhat higher response to HE and a somewhat lower response to LPS when the two antigens were administered IP. When the LPS was administered IV the HE response was somewhat lower while the LPS response was somewhat higher. The difference was not clear-cut in normal mice. These observations are probably due to complex interactions involving antigenic competition, the adjuvant action of LPS and thymus-dependent and thymus-independent antigens. Further studies are needed to clarify these different responses but were not within the scope of this investigation. Since the differences in responses did not affect

TABLE VI

The primary response to horse erythrocytes (HE)[a] and E. coli lipopolysaccharide (LPS)[b] when both antigens are administered to the same animal

| | Mean PFC/Spleen[c] | | Mean PFC/$10^6$ Spleen[c] cells | |
|---|---|---|---|---|
| | HE | LPS | HE | LPS |
| Nudes (4)[d] | 2530 | 4710 | 16 | 35 |
| Normals (6) | 105,333 | 2023 | 321 | 5 |

[a] 0.25 ml of a 10% suspension of IP on day 0

[b] 10 µg on day 1

[c] assayed day 5

[d] number of mice in parentheses

the conclusions drawn from the experiments, they were treated similarly.

Secondary Response to LPS. A secondary response to LPS as manifested by a heightened response to a second injection of antigen, has been reported in normal mice (22). Since it has been reported that the secondary response to SE is more thymus-dependent than the primary (1), it was of interest to know whether the secondary response could be elicited in nudes.

A group of mice were primed, IP, with 1 µg LPS on day 0. On day 21 some of these mice were given an additional IV injection of 10 µg LPS while the rest were untreated. In addition, several unprimed mice were given a primary dose of 10 µg LPS, IV, on day 21. All mice were then assayed four days later, day 25. The results are shown in Table VII.

From these results it is apparent that nudes were able to produce a substantial secondary response to LPS which was greater than the secondary response of normals.

In another experiment, the same design was followed except that the priming dose was an IV injection of 10 µg LPS and the interval between primary and secondary injections

TABLE VII

The secondary response to *E. coli* lipopolysaccharide

| | Number of Mice | Day 0 | Day 21 | Day 25 | |
|---|---|---|---|---|---|
| | | LPS Injections | | Mean PFC/Spleen | Mean PFC/$10^6$ Spleen cells |
| Nudes | 8 | 1 µg (IP) | 10 µg (IV) | 54,515 | 265 |
| | 2 | 1 µg (IP) | none | 400 | 4 |
| | 1 | none | 10 µg (IV) | 5,400 | 37 |
| Normals | 6 | 1 µg (IP) | 10 µg (IV) | 23,287 | 158 |
| | 3 | 1 µg (IP) | none | 500 | 3 |
| | 1 | none | 10 µg (IV) | 3,180 | 27 |

was fourteen or sixteen days instead of twenty-one days. Mice were assayed four days after the last injection. Similar results were obtained. The data are not shown.

Immune Response to LPS-Coated Horse Erythrocytes.

The physical form in which an antigen is presented may be important in determining whether or not it is thymus-independent (60). One way of presenting LPS in a different form is to attach it to the thymus-dependent antigen, horse erythrocytes.

It was determined, by $^{51}Cr$ labeling, that 0.5 ml of a 33% suspension of LPS-sensitized horse erythrocytes (HE) contained approximately 1.75 µg LPS.

One group of mice was immunized with 0.5 ml of the diluted, sensitized HE. Another group was immunized with 0.5 ml of a 33% suspension of washed, non-sensitized HE followed, one hour later, by an injection of 1.75 µg LPS. A third group was immunized with 1.75 µg LPS only and a fourth group with 0.5 ml of a 33% suspension of washed, non-sensitized HE only. All injections were IP. Four days later the spleens of these mice were assayed for PFC to both LPS (using sensitized SE) and HE. The results are shown in Table VIII.

TABLE VIII

The response to *E. coli* lipopolysaccharide (LPS) coated horse erythrocytes (HE).

| Antigen | Type of mouse | Number of Mice | LPS | HE |
|---|---|---|---|---|
| | | | Mean PFC/Spleen | Mean PFC/Spleen |
| LPS-coated HE[a] | Nudes | 3 | 2000 | 2,293 |
| | Normals | 2 | 1680 | 25,800 |
| HE followed by LPS one hour later[b] | Nudes | 3 | 3480 | 693 |
| | Normals | 3 | 813 | 58,733 |
| LPS only[c] | Nudes | 3 | 2946 | 14 |
| | Normals | 3 | 1693 | 14 |
| HE only[d] | Nudes | 3 | 107 | 253 |
| | Normals | 3 | 50 | 7,667 |

[a] 0.5 ml of a 33% suspension of sensitized HE contains 1.75 µg LPS

[b] doses were equivalent to those in a above

[c] 1.75 µg in 0.5 ml PBS

[d] 0.5 ml of a 33% suspension

The response to LPS of nudes was at a level expected for that dose. The somewhat decreased response to LPS by normals when HE were given also was probably due to competition with thymus-dependent antigens. This was not observed when LPS-coated HE were given. The low response of nudes to HE compared with that of normals was predictable from the SE response shown by other workers. It is noteworthy that the HE response of nudes was enhanced by LPS and that the enhancement was greatest when LPS was bound to the cells. This was not the case with normals. The low response to HE given alone by normals may be due to the large dose administered. LPS enhanced this response but the enhancement was greatest when LPS was not bound to HE.

Immune Response to Heat-Killed E. coli Cells. Immunization with heat-killed E. coli cells is another means of changing the form in which LPS is presented.

The heat-killed E. coli 0113 cells were washed 2x in PBS and then diluted to a concentration of 5-6 x $10^9$ cells/ml. Mice were injected with 0.2 ml of this preparation (approximately $10^9$ cells per mouse). In one experiment the cells were given IV and in another they were given IP. The spleens were assayed four days after immunization.

The PFC response for the individual mice in both experiments are presented in Table IX. These results indicate that the response of nudes, but not normals, may be dependent upon the route of immunization. Thus, nudes appeared to be able to respond as well as normals to the LPS of intact cells when the cells are given IV but showed a decreased response when they are given IP. The normals appeared to respond similarly in both cases.

Primary Response to Sodium Deoxycholate-Treated Lipopolysaccharide. Sodium deoxycholate has been shown to dissociate LPS into subunits of approximately 20,000 daltons (41). It was of interest to determine whether the dissociated LPS was thymus-independent. Therefore, nudes and normals were injected, IV, with 10 ug LPS in 0.05 ml of 1% NaD. The spleens were assayed four days later for the PFC response to LPS. The results are presented in Table X. Except for one nude mouse, all mice showed an apparently normal response to LPS. It appears, then, that dissociated LPS is thymus-independent.

Primary Immune Response to SIII. Mice were immunized IP with 0.5 ug SIII and they were assayed five days later.

TABLE IX

The primary immune response to $10^9$ heat-killed E. coli cells (individual responses)

| | Route of Injection | PFC/Spleen | PFC/$10^6$ Spleen cells |
|---|---|---|---|
| Nudes | IV | 33,400 | 172 |
| | | 39,000 | 342 |
| | IP | 7,240 | 34 |
| | | 5,800 | 28 |
| | | 3,400 | 11 |
| Normals | IV | 24,520 | 108 |
| | | 20,120 | 109 |
| | IP | 7,960 | 22 |
| | | 22,360 | 54 |
| | | 27,920 | 76 |

TABLE X

The primary response to sodium deoxycholate treated E. coli lipopolysaccharide (individual responses)

| | | PFC/Spleen | PFC/$10^6$ Spleen cells |
|---|---|---|---|
| Nude | 1 | 13,680 | 33 |
| | 2 | 5,160 | 27 |
| | 3 | 40 | <1 |
| Normal | 1 | 2,800 | 13 |
| | 2 | 2,400 | 13 |
| | 3 | 3,280 | 22 |
| | 4 | 10,120 | 57 |

The results are shown in Table XI. The PFC response represents total anti-IgM facilitated plaques only. Direct plaques were very difficult to count macroscopically and the equipment for microscopic readings was not available. If the sensitized SE suspension was diluted 1:15 instead of 1:5 the direct plaques were a little easier to see. They represented about one-half to two thirds of the total facilitated response if they could be counted. Since the determinations of direct plaques could not be done accurately they are not reported here. Serum hemagglutinin titers were done also. The results of the hemolysin titers were sporadic and must be evaluated in a different manner. Hence, the hemolysin titers are not reported.

It can be seen that the response of nudes to SIII was the same as that of normals. The differences observed between the means were not significant at all levels of probability when tested by Student's t test. Unimmunized animals showed no appreciable background to SIII.

Induction of Immunological Unresponsiveness to SIII. Immunological unresponsiveness to a thymus-dependent antigen has been shown to involve thymus-derived cells and bone marrow cells (61). It was of interest to determine whether

TABLE XI

The primary immune response to 0.5 µg type III pneumococcal polysaccharide[a] assayed five days post-immunization

| | Number of Mice | Mean facilitated PFC/Spleen | Mean facilitated PFC/$10^6$ Spleen cells | Mean HA Titer |
|---|---|---|---|---|
| Nudes | 15 | 8337 | 72 | 87 |
| Normals | 13 | 8829 | 56 | 70 |

[a] administered IP

unresponsiveness to a thymus-independent antigen, such as SIII, could be induced in nudes.

A group of nude and normal mice was injected IP with one dose of 250 µg SIII on day 0. On day 8, this group of mice plus two untreated mice were given a normal immunizing dose of SIII (0.5 µg IP).

The results (Table XII) are clear-cut. Both nudes and normals that were given a large dose of SIII on day 0 showed an inability to respond to a normal immunizing dose of the same antigen whereas the control mice showed a normal response.

TABLE XII

The induction of immunological unresponsiveness to type III pneumococcal polysaccharide (individual responses).

| | Day 0 | Day 8 | Day 13 |
|---|---|---|---|
| | SIII injections (IP) | | Facilitated PFC/Spleen |
| Nude 1 | 250 µg | 0.5 µg | 0 |
| Nude 2 | 250 µg | 0.5 µg | 360 |
| Nude 3 | 250 µg | 0.5 µg | 0 |
| Nude 4 | none | 0.5 µg | 69,280 |
| Normal 1 | 250 µg | 0.5 µg | 0 |
| Normal 2 | 250 µg | 0.5 µg | 40 |
| Normal 3 | 250 µg | 0.5 µg | 0 |
| Normal 4 | none | 0.5 µg | 18,000 |

## DISCUSSION

These results demonstrate that nude mice, which are genetically athymic, are able to produce a primary humoral response to LPS and SIII which is equivalent in magnitude to that produced by their phenotypically normal littermates. The somewhat higher response of nudes may be due to an effect of the thymusless condition on the spleen cell population. The total nucleated spleen cell count of immunized nudes was not significantly different from that of normals even though it has been shown (62) that, histologically, the spleens of unimmunized nudes appear to be deficient in cells in the thymus-dependent areas. It may be that in nudes each clone committed to antibody production to these antigens is able to expand to a greater extent or that the number of precursor cells reactive to these antigens is greater to begin with.

The results obtained with the nude mice derived from nude parents established that in utero, maternal or littermate thymic influence did not detectably affect the subsequent response of nudes to LPS. However, the obscure possibility remains that some undefined influence on the immune system of these nudes has occurred through nursing.

The more comprehensive studies on LPS showed that the

thymic-independency of this antigen extends over a wide dose range and to the kinetics of the response. In addition, it was found to apply to the secondary response to this antigen as well. It is noteworthy that the secondary response to LPS is also an IgM response (42). The higher secondary PFC response of nudes may or may not be significant. It may merely reflect a variation in the responses of individual mice. It also may be due to an effect on the spleen cell population as delineated above. The author considers it unlikely to be an enhancement effect similar to that observed by Baker (51) in ALS treated mice immunized with SIII, since other workers (63) have not been able to detect a similar enhancement to LPS in ALS treated mice.

These two antigens, LPS and SIII, share certain properties. They are both microbial products associated with virulence factors and the antigenic specificity of the molecules resides in repeating polysaccharide chains. In addition, they both elicit mainly IgM antibody production. Perhaps some or all of these properties are responsible for the thymus-independency of these antigens. Pantelouris (64) has shown that, in nudes, IgG antibody production to SE is more severly impaired than IgM antibody production. This

has also been shown in thymectomized animals. It would be of interest therefore, to know whether polymerized flagellin (POL), reported to be thymus-independent (60), which is protein in nature and elicits both IgM and IgG antibody production, can elicit a similar response in nudes (65). Feldman and Basten (60) have suggested that thymic-independency of an antigen depends on the physical form of an antigen and hence its mode of presentation to the antibody forming system. They found that the monomeric form of flagellin (MON) was thymus-dependent whereas the polymerized form was not. In addition, they showed that DNP was thymus-independent when coupled to POL, but was thymus-dependent when coupled to donkey erythrocytes (a thymus-dependent carrier).

Rudbach (21) has suggested that the LPS-LPS subunit system is analogous to the polymerized flagellin-monomeric flagellin system. However, some of our findings are not consistent with this analogy. Thus, NaD-treated LPS which should be analogous to MON, was found to be thymus-independent in our system. The possibility remains that in this particular experiment the NaD did not successfully dissociate the LPS. However, it is also reasonable to think that NaD treated LPS is not analogous to MON.

Immunization with whole E. coli cells of LPS-sensitized HE (a thymus-dependent "carrier") possibly represents a different mode of presentation of the antigen. Both of these forms of LPS are fully immunogenic in nudes. Thus, these results appear to be inconsistent with those observed with DNP by Feldmann and Basten (60). However, their system is different in that DNP is a hapten whereas LPS is not. The response to haptens is known to be very much dependent on their carriers. In addition, it is possible that, when injected into the animal, the LPS becomes detached from the erythrocytes and therefore is really not in a different form when presented to immunocompetent cells. However, it appears very unlikely that this could occur with whole E. coli cells. These results suggest that perhaps the mode of presentation of an antigen is not the only factor that determines whether an antigen is thymus-independent. Alternatively, it may be that LPS attached to erythrocytes or as part of whole cells does not represent a truly different mode of presentation of the antigen.

The differences in response to LPS, observed in nudes when immunized with whole E. coli cells by different injection routes is difficult to account for. Since the number of animals in each experiment is small, the differences may

just be random variation. It is also possible that the depressed response of nudes when the E. coli cells were injected IP represents some abnormality in the peritoneal macrophages of nudes. Further studies are required to test these possibilities.

The attachment of LPS to erythrocytes appeared to enhance the respose of nudes to the erythrocytes to a greater extent than when the two antigens were administered separately one hour apart. Similar findings have been reported by other workers (16) in thymectomized mice and they suggested that this enhancement was due to more rapid and efficient uptake of the sensitized cells by the reticuloendothelial system and related to the adjuvant action of LPS. Another possibility is that LPS attaches directly to antibody-producing cells to stimulate antibody production and in so doing the erythrocytes, which are attached to the LPS, may also stimulate the antibody forming cells resulting in cells which produce antibodies to both LPS and erythrocytes.

The response by nudes to a single dose of SIII appears not to be contradictory to Baker's theory (50) that two types of thymic-derived cells (a suppressor and an amplifier cell) act in an opposing manner to regulate the immune

response to SIII, and that ALS affected the suppressor cell, thereby allowing the amplifier cell to function alone. Thus nudes, which presumably lack both types of cells would show neither a suppressed nor an enhanced response to SIII, as when only one of the cell types is depleted. It is possible that the amplifier cell is not thymus-derived and may be present in nudes. Accordingly then, the response to SIII by nudes may represent an amplified response.

The inability to do direct plaque counts probably does not affect the conclusions of these experiments since, presumably, a total PFC response developed with an anti-IgM antiserum would reflect an enhancement of the direct PFC response. Preliminary studies done in this laboratory in cooperation with Dr. P. J. Baker indicate that nudes do not show an enhancement of the direct PFC response.

The induction of immunological paralysis, by nudes, to SIII indicates that, with this particular antigen, this phenomenon can be thymus-independent.

Although the results presented here confirm and extend the findings in thymus-deprived animals with respect to these two antigens, it would be imprudent to extrapolate

these results to other alleged thymus-independent antigens. An antigen should not be considered truly thymus-independent unless it is able to elicit a normal response in nudes.

## SUMMARY

The congenitally thymusless (nude) mouse is an ideal system for studying the thymus-independency of antigens. The objections raised in the use of conventionally thymus-deprived animals namely, that cell seeding may occur before birth or that thymus-deprivation may not be complete, are avoided.

It was found that nudes were able to respond fully as well as their phenotypically normal littermates to a single immunizing dose of either E. coli lipopolysaccharide (LPS) or type III pneumococcal polysaccharide (SIII). More comprehensive studies with LPS showed that these findings extend over a wide dose range, to various times of assay after immunization, to the secondary response and to the presentation of this antigen as either a part of whole E. coli cells or attached to erythrocytes. In addition, nudes showed a normal response to sodium deoxycholate (NaD) treated LPS. Immunological unresponsiveness to SIII could be induced. These findings establish that these antigens are truly thymus-independent.

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