

IMMUNOLOGICAL RESPONSES OF MICE TO NATIVE
PROTOPLASMIC POLYSACCHARIDE AND
LIPOPOLYSACCHARIDE

Functional Separation of the Two Signals Required to Stimulate a
Secondary Antibody Response*

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There have been several recent hypotheses about the nature of the signals which cause activation of bursa-equivalent cells (B cells) during an immunological response (cf. 1). In one theory it was proposed that a single, antigen-specific signal induced B cells to produce antibodies (2). On the other hand, Coutinho et al. (3) suggested that activation of B cells was the result of one nonspecific signal which was mitogenic for B cells. In a third theory it was postulated that two signals were required to activate B cells to produce antibodies (4-8). These two signals were thought to be (a) the antigenic signal, or the binding of a specific immunodeterminant group to immunoglobulin-like receptors on the surface of a B cell and (b) a second, nonspecific signal. The second signal might have been some product of antigen-activated thymus-derived cells (T cells) in the case of T-cell dependent antigens (9-22) or, as postulated for T-cell independent antigens, the second signal could have been associated with the antigen itself (8, 23-25). With respect to lipopolysaccharide (LPS),¹ a T-cell independent antigen (26-28), the second signal was reported to reside in the Lipid A portion of the LPS molecule (8, 29-32).

Work in our laboratory has been directed toward investigating the immunological responses of mice injected with LPS (33, 34) or a related material, native protoplasmic polysaccharide (NPP) (35). NPP, extracted from the protoplasm of *Escherichia coli* (36, 37), was a simple polysaccharide with an average mol wt of 163,000 daltons (38). Once thought to be a protoplasmic precursor to cell wall

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¹Abbreviations used in this paper: LPS, lipopolysaccharide; NPP, native protoplasmic polysaccharide; RML mice, outbred mice from the Rocky Mountain Laboratory stock; nude mice, congenitally athymic nude mice; LPS-0113, LPS from *E. coli* 0113; LPS-0111, LPS from *E. coli* 0111; NPP-0113, NPP from *E. coli* 0113; ReGL-G30/C21, glycolipid from the Re mutant G30/C21 of *S. typhimurium*; PBS, phosphate-buffered saline; PFC, direct plaque-forming cells; HGG, human gamma globulin.

LPS (36, 37), NPP was more recently described as a product of aberrant metabolism (38). Immunochemically, the O-antigenic determinants of NPP cross-reacted completely with the O-antigenic determinants of homologous LPS (37, 39). However, NPP did not contain Lipid A, 2-keto-3-deoxyoctulosonate, or heptose, and failed to elicit some of the host reactive properties associated with LPS (37, 39). NPP was nontoxic for chicken embryos, nonpyrogenic for rabbits, and nonimmunogenic for rabbits (37, 39) and guinea pigs.² Also, NPP was found to be nonmitogenic for murine spleen cells (31) and, in the presence of homologous antiserum, unable to fix complement (37, footnote 3).

With the availability of the model system of immunochemically homologous NPP and LPS, experiments were designed to demonstrate a separation of immunological phenomena which might be attributed to a separation of immunological signals. This was achieved by showing that NPP (antigenic signal only) would stimulate mice to give a primary response and would sensitize mice for a secondary response, whereas LPS (antigenic signal and second signal) was necessary to trigger a secondary response.

Materials and Methods

Mice. Mice of both sexes were obtained from the Rocky Mountain Laboratory (RML), Hamilton, Mont. Congenitally athymic nude mice were bred in our laboratory and were the offspring of heterozygous animals obtained by crossing nude males with RML females. In order to improve their general health, nude mice received water containing oxytetracycline (OTC, Pharmaceutical Co., Karkov, Poland) and metronidazole (Flagyl, Searle & Co., Columbus, Ohio). Postmortem examinations confirmed that the nude mice used in the experiments were athymic.

Antigens and Endotoxic Mitogens. *E. coli* 0113 (Braude strain) and *E. coli* 0111:B4 (Difco strain) were cultivated, fractionated, and extracted as described previously (40). Briefly, the cells were disrupted in a refrigerated cell fractionator and the cell walls were separated from the protoplasmic fraction by differential centrifugation. LPS from *E. coli* 0113 (LPS-0113) and *E. coli* 0111:B4 (LPS-0111) were extracted from the cell walls by the phenol-water procedure (41); NPP was extracted from the protoplasmic fraction of *E. coli* 0113 (NPP-0113) with cold trichloroacetic acid (37). Glycolipid was extracted by the phenol-water procedure (41) from whole cells of *Salmonella typhimurium* G30/C21 (ReGL-G30/C21) which is an Re mutant strain (42, 43).

Bioassays revealed that the maximum possible contamination of NPP-0113 with LPS-0113 was less than 1/5,000 by weight. Results of antigen titration studies indicated that such trace contamination of NPP-0113 by LPS-0113 could not account for the immunogenicity of NPP-0113.

Lyophilized preparations of the materials described above were dissolved in phosphate-buffered saline (PBS-0.15 M NaCl, 0.0033 M PO₄, pH 7.2) and stored at -20°C until used. All materials were injected intravenously (i.v.) via a lateral tail vein.

Immunoassays. Standard procedures employing sheep erythrocytes (SRBC) coated with LPS-0113 as indicator cells were used to determine the numbers of direct plaque-forming cells (PFC) (27) and titers of humoral antibodies (33). Humoral titers are expressed as values of x derived from the equation $x = \log_2 (HD/2)$, where HD is the reciprocal of the highest dilution of serum which produced hemagglutination of sensitized SRBC (34). Thus, the titer is the tube number of the end-point when the first tube contains a 1/4 dilution of antiserum. Sera which gave no hemagglutination at the lowest dilution tested were arbitrarily assigned a titer of 0, i.e., a dilution of 1/2.

²Rudbach, J. A. The role of immunological factors in infectious, allergic, and autoimmune diseases. Immunogenicity of Lipopolysaccharides. Proceedings of the Eighth Miles International Symposium. In press.

³Luoma, M. K., and J. A. Rudbach. Manuscript submitted for publication.

Results

Kinetics of the Appearance of Antibodies in Mice Injected with NPP-0113 or LPS-0113. In order to quantitate the time of appearance of antibodies in mice injected with NPP-0113 or LPS-0113, groups of RML mice were injected with one dose (on day 0) or two doses (on days 0 and 21) of $1 \mu\text{g}$ of antigen and were then bled on succeeding days. The kinetics of appearance of antibodies after both the primary and secondary injections of NPP-0113 or LPS-0113 are presented on Fig. 1. It was observed that the peak titers to primary injections of NPP-0113 and LPS-0113 were similar (4.6 and 5.6, respectively). However, LPS-0113 stimulated a significantly faster rate of increase in humoral antibodies (i.e., the day 4 titers

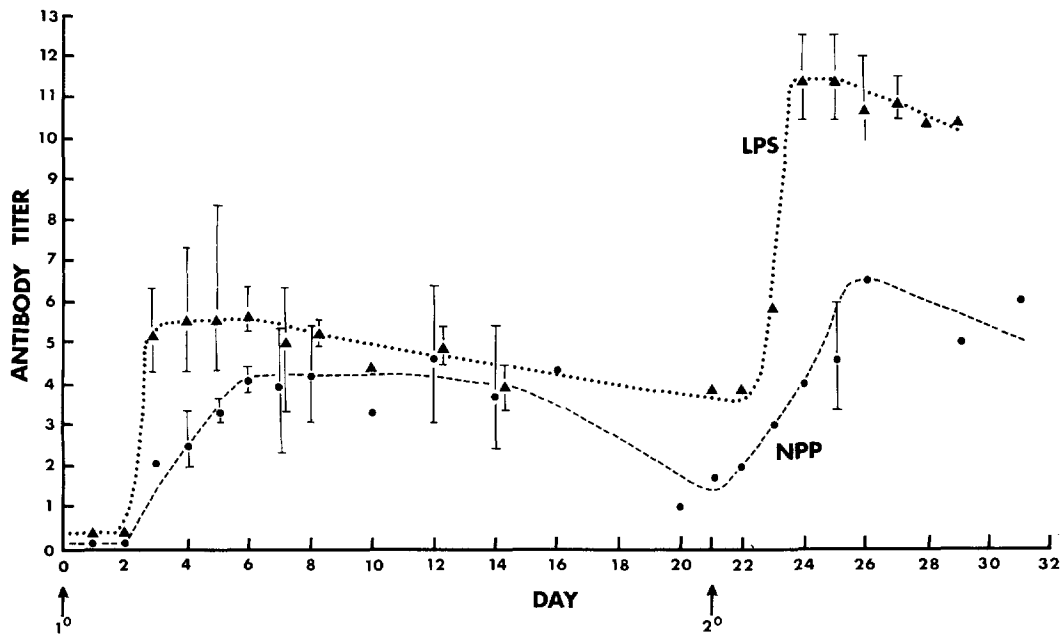


FIG. 1. Kinetics of the antibody responses of RML mice injected with NPP-0113 or LPS-0113. Each point represents the titer of sera pooled from at least five mice. The bars indicate the range of titers observed where the geometric mean titer was calculated from several experiments. (●---●), antibody titers of mice injected i.v. with $1 \mu\text{g}$ of NPP-0113 on days 0 and 21; (▲---▲), antibody titers of mice injected i.v. with $1 \mu\text{g}$ of LPS-0113 on days 0 and 21.

differed at the 99% confidence levels) than did NPP-0113. Other differences in the responses of mice to NPP-0113 and LPS-0113 were also noted after the second injections of antigen. The antibody response after a second dose of NPP-0113 was similar to the primary response to this antigen, whereas two doses of LPS-0113 gave a typical secondary response. These observations stimulated further experimentation as described below.

Requirement for LPS-0113 to Trigger a Secondary Response. Repeated attempts to produce a secondary response with different doses of NPP-0113 failed. Therefore, in an attempt to determine why NPP-0113 would not elicit a

secondary response, the following experiment was performed. Groups of RML mice were injected on day 0 with either 1 μ g of NPP-0113, 1 μ g of LPS-0113, or PBS. After 21 days, some mice from each group were injected with 1 μ g of NPP-0113, 1 μ g of LPS-0113, or with PBS. Sera from mice in each group were collected and examined for antibodies to the 0113 determinant by passive hemagglutination and the splenic cells were assayed for specific anti-0113 PFC. Results of several experiments are compiled in Table I. It was found, again, that two injections of NPP-0113 would not stimulate a secondary response; only another primary response was elicited. However, mice primed with NPP-0113 and challenged with the LPS-0113 responded by producing an antibody and PFC response similar quantitatively to the secondary responses of mice injected twice with LPS-0113. The antibody responses of mice primed with LPS-0113 and subsequently challenged with NPP-0113 were characteristically primary re-

TABLE I
Antibody Responses of Mice to NPP-0113 and LPS-0113

Primary injection (day 0)	Secondary injection (day 21)	Antibody titer (day 25)*	PFC/spleen‡ (day 25)
PBS	PBS	0.4 (0-1.0)	20
1 μ g NPP-0113	PBS	3.0 (2.5-4.0)	40
PBS	1 μ g NPP-0113	3.9 (2.0-5.1)	100
1 μ g NPP-0113	1 μ g NPP-0113	5.4 (4.0-6.5)	800
1 μ g NPP-0113	1 μ g LPS-0113	9.8 (8.0-11.0)	23,000
PBS	1 μ g LPS-0113	5.7 (4.0-7.0)	1,420
1 μ g LPS-0113	1 μ g LPS-0113	10.0 (9.0-11.0)	19,200
1 μ g LPS-0113	1 μ g NPP-0113	5.2 (3.2-8.0)	900

* Geometric mean titer calculated from separate experiments (range in parentheses).

‡ Direct PFC/spleen from a single experiment.

sponses, similar quantitatively to the responses of mice injected once with LPS-0113. These results showed that NPP-0113, in addition to eliciting a primary response, sensitized mice for a secondary antibody response which could be triggered by LPS-0113. Therefore, the lack of a secondary response following two doses of NPP-0113 did not result from a lack in a capacity of this polysaccharide to sensitize the mice; NPP-0113 simply could not trigger a secondary response in mice sensitized with either NPP or LPS.

Antibody Responses of Mice Injected with NPP-0113 Plus a Heterologous Endotoxic Mitogen. Data presented above suggested that NPP possessed the attributes for primary sensitization of immunocompetent cells, but it lacked the second signal, which was necessary for triggering a secondary response. Also it was shown that both signals were contained in the LPS. Therefore, an attempt was made to trigger a secondary response to the 0113 determinant by supplying the antigenic signal in the form of NPP-0113 and the second signal in the form of two non-cross-reacting endotoxic mitogens, LPS-0111 and ReGL-G30/C21, both of which contained Lipid A (43, 44). The results of one of these experiments are given in Table II. The combination of NPP-0113 and different amounts of

TABLE II
*Antibody Responses of Mice Injected with NPP-0113 Plus a Heterologous
 Endotoxic Mitogen*

Primary injection (day 0)	Secondary injection (day 21)	Antibody titer* (day 25)
1 μ g NPP-0113	1 μ g NPP-0113	5.0
1 μ g NPP-0113	1 μ g LPS-0113	9.0
1 μ g NPP-0113	1 μ g NPP-0113 + 5 μ g LPS-0111	3.0
1 μ g NPP-0113	1 μ g NPP-0113 + 10 μ g LPS-0111	4.5
1 μ g NPP-0113	1 μ g NPP-0113 + 20 μ g LPS-0111	4.5
1 μ g NPP-0113	1 μ g NPP-0113 + 15 μ g ReGL-G30/C21	4.0
1 μ g NPP-0113	1 μ g NPP-0113 + 30 μ g ReGL-G30/C21	3.0
1 μ g NPP-0113	1 μ g NPP-0113 + 60 μ g ReGL-G30/C21	5.5

* Titer of sera pooled from five mice/group from a representative experiment.

LPS-0111 or ReGL-G30/C21 did not act as effective stimuli for triggering a secondary response in mice which were primed with NPP-0113. Other experiments showed that a secondary response could not be stimulated by NPP-0113 and heterologous sources of Lipid A in mice which were primed with LPS-0113. Thus, to be effective in triggering a secondary response, apparently both signals had to be contained in the same molecule.

Antibody Responses of Nude Mice Injected with NPP-0113. Up to this point, the immunological behavior of NPP appeared similar to that of pneumococcal polysaccharide (45) and polyvinylpyrrolidone (46), both T-cell independent antigens (26, 27). In order to determine whether or not the immunological responses to NPP required or were influenced by T cells, the following experiments were performed. Groups of congenitally athymic nude mice were injected with various combinations of NPP-0113 and LPS-0113, and their humoral antibody and splenic PFC responses were quantitated. The data, presented in Table III, showed that a single injection of NPP-0113 could elicit a weak primary response in nude mice. Slightly higher titers were seen in nude mice injected twice with NPP-0113. Most importantly, a single injection of NPP-0113 sensitized nude mice for a secondary antibody response when challenged with LPS-0113. In general, the quantitative patterns of the antibody responses of nude mice to NPP-0113 and LPS-0113 were similar to the patterns of responses of mice possessing T cells. It was concluded, therefore, that the immunological phenomena observed for NPP-0113 were independent of the influence of T cells.

Discussion

Different hypotheses have been presented to suggest mechanisms for the activation of B cells by immunological signals (cf. 1). One current theory stated that two signals were required to activate B cells to secrete antibodies (4-8). However, the individual contribution of each signal to the activation of B cells has not been elucidated. In the present report, functional separation of the two

TABLE III
Antibody Responses of Nude Mice to NPP-0113 and LPS-0113

Primary injection* (day 0)	Secondary injection (day 15)	Antibody titer (day 19)‡	PFC/spleen§ (day 19)
PBS	PBS	0.0 (0-0)	ND
PBS	1 μ g NPP-0113	1.2 (0.0-3.0)	130
1 μ g NPP-0113	1 μ g NPP-0113	4.3 (4.0-5.0)	460
1 μ g NPP-0113	1 μ g LPS-0113	8.2 (7.0-9.0)	20,700
PBS	1 μ g LPS-0113	4.3 (2.0-6.0)	1,600
1 μ g LPS-0113	1 μ g LPS-0113	9.5 (7.0-11.0)	23,400
1 μ g LPS-0113	1 μ g NPP-0113	3.8 (3.0-5.0)	560

* Three or four nude mice were included in each group.

‡ Geometric mean of titer calculated from titers of individual mice from each group (range in parentheses).

§ Spleens from mice of each group were pooled and tested for direct PFC against SRBC coated with LPS-0113.

signals required for induction of immune responses has been accomplished through the use of two immunochemically related, but biologically distinct natural products. NPP-0113 did not contain Lipid A (37-39), was unable to activate complement even in the presence of homologous antibodies, (37, footnote 3), was not mitogenic for splenic cells (31), and did not require T cells to mount an immune response (35 and Table III). Lipid A (8, 29, 30, 32), the third component of complement (6), or some product of antigen-activated T cells (9-22) all have been suggested to act as the second signal during activation of B cells. Therefore, it appeared that NPP-0113 possessed only the antigenic signal. LPS-0113, on the other hand, contained an antigenic signal of the same immunochemical specificity plus the second, nonspecific signal in the form of Lipid A.

A single injection of NPP-0113 elicited a normal primary response in RML mice and a weak primary response in nude mice. This was shown repeatedly by the increase in humoral antibody titers and specific PFC. These results suggested that the antigenic signal alone could stimulate the B cells of a mouse to secrete antibodies. It was believed generally that initial contact between an antigen and the immunocompetent cells of an animal caused this population of cells (*a*) to produce a primary antibody response, and (*b*) to expand in number so that the pool of cells immunologically specific for the sensitizing antigen would be increased (47, 48). Production of a secondary antibody response could be explained by synthesis of antibody by this expanded clone of cells. In this context, a single injection of NPP-0113 efficiently sensitized mice for a secondary response which could be triggered by LPS-0113. Therefore, NPP-0113, which possessed only the antigenic signal and not the mitogenic Lipid A (31), probably caused B cells to proliferate and form an expanded clone of cells specific for the 0113 immunodeterminant group. However, this expanded clone of cells must have been qualitatively different from the progenitor cells in the immunologically virgin animal, because NPP-0113 could not trigger them to produce a secondary response.

What was discussed previously should not be interpreted to imply that a primary injection of NPP-0113 stimulated exactly the same immunological events in an animal as did a primary injection of LPS-0113. In fact, a comparison of the kinetics of the primary responses to these two antigens shows that this probably was not the case. The humoral antibody titer to LPS-0113 increased significantly faster than it did to the same dose of NPP-0113. Ultimately, however, similar peak titers were reached. An explanation for this phenomenon might be that the mitogenicity associated with the Lipid A portion of the LPS-0113 enhanced the rate of cellular division or differentiation. However, the actual numbers of antibody forming cells (reflected by the peak titer) was under the control of the antigenic signal.

Data from other studies supported the concept that the antigenic signal alone might result in expansion of a specific clone of cells. For example, Chiller, et al. (49) found that mice which were initially tolerant to human gamma globulin (HGG) responded in a heightened fashion in comparison to mice which had not received the tolerogen, when both groups were challenged with an immunogenic preparation of HGG plus LPS. Apparently, the injection of the tolerogenic form of HGG (which was thought to contain the antigenic signal only) sensitized mice for an enhanced response which was demonstrable when tolerance was broken with LPS. The foregoing results were interpreted from the premise that the tolerogenic and antigenic signals were identical (4, 5). Even more direct evidence in support of the concept that the antigenic signal alone can generate an expanded clone of cells was given by Hünig, et al. (50). They noted that SRBC could stimulate proliferation of specific B cells in cultures of spleen cells derived from nude mice. In the absence of T cells, the SRBC could be regarded as an antigenic signal alone. Thus, the results of experiments with NPP-0113 were in concert with previous evidence indicating that the antigenic signal alone might cause the proliferation of a specific clone of B cells.

Also, the foregoing data introduced a new concept that two signals were required to trigger a specific secondary response in primed animals. This was shown by the requirement for LPS-0113 as the secondary stimulus; NPP-0113 (antigenic signal only) could elicit only another primary response in mice primed with either NPP-0113 or LPS-0113. Other T-cell independent antigens such as Type III pneumococcal polysaccharide (45) and polyvinylpyrrolidone (46) stimulated only primary responses; they were unable to elicit secondary responses. The data presented above would tend to suggest that this occurs not because Type III pneumococcal polysaccharide and polyvinylpyrrolidone could not prime an animal, but rather because they lacked the second signal necessary to trigger a secondary response. That Type III pneumococcal polysaccharide and polyvinylpyrrolidone lack a second signal has been suggested previously by Watson, et al. (8).

The finding that mice primed with NPP-0113 and injected secondarily with NPP-0113 plus a heterologous source of Lipid A (LPS-0111 or ReGL-G30/C21) failed to make a secondary anti-0113 response does not discredit the concept that both signals were required to trigger a secondary response. Possibly, both the antigenic and the second signals were required on the same molecule so that both signals could contact the primed B cells simultaneously. Another explanation for the inability of a mixture of heterologous Lipid A and NPP-0113 to trigger a secondary response in primed mice could be that the binding of NPP-0113 to the immunoglobulin-like receptors on the surface of the B cells might have inhibited subsequent interaction of adjacent membrane sites with the Lipid A

moiety of the heterologous mitogen. A final possibility which should be considered was that the second signal might have been given by something other than Lipid A. Experiments in progress should provide answers to some of these questions.

In conclusion, we would like to make the following suggestions: both the antigenic signal and the second signal play necessary, but distinct roles in the generation of immune responses. For the T cell independent antigens used in this study, the quantity of antibodies produced during a primary response was dependent on only the amount of antigenic signal; the second signal simply accelerated the events leading to the peak primary response. A more important function of the second signal was the clearly demonstrable requirement for it to be associated with the antigenic signal in order to trigger a secondary response. Finally, the observation that the single antigenic signal could stimulate unprimed cells to secrete antibodies, whereas both signals were required to trigger primed cells added to the evidence (33, 48, 51) indicating that qualitative, and not just quantitative differences have occurred at the cellular level during the generation of immunological memory.

Summary

Functional separation of the two signals involved in stimulating immunological responses was achieved through the judicious use of two natural bacterial antigens. Native protoplasmic polysaccharide (NPP) extracted from *Escherichia coli* was immunochemically identical to the lipopolysaccharide (LPS) extracted from the same organism. However, NPP was not endotoxic, not mitogenic, did not fix complement, and was immunologically independent of T cells. The NPP, which appeared to contain only the antigenic signal, could induce a primary antibody response in mice and could sensitize mice for a secondary response. However, the antigenic signal contained in NPP was insufficient to trigger a secondary response in mice primed with either NPP or LPS. LPS, containing both the antigenic and second signals, was required to trigger a secondary response in primed mice.

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