Suppression of Antibody-Forming Cells to *Serratia Marcescens* Lipopolysaccharide After a Large Dose of Antigen[1] (34498)

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Immunoplaque procedures in semisolid gel permit rapid detection and enumeration of individual antibody-forming cells. Many studies have utilized sheep erythrocytes as the antigen both for immunization and as the indicator target cell (1–6). More recent studies have shown that various antigens may be passively "coated" onto carrier red blood cells and used for similar antibody plaque assays (7–20). Such studies have permitted rapid expansion of knowledge concerning cellular events which occur during the immune response to a variety of complex macromolecular antigens, as well as to simpler haptenic determinants conjugated onto larger proteins (7–20).

Most studies to date have been concerned with the response of normal individuals to optimum immunizing dosages of antigen. However, several reports have been concerned with enumeration of antibody-forming cells in experimental animals injected with relatively large tolerogenic doses of antigen at birth (21–24). Sheep erythrocytes, *Shigella paradysenteriae* lipopolysaccharide, and a chemical hapten have been used for such studies, employing mice, rats, or rabbits. On the other extreme, it has been shown that a bacterial antigen, in nanogram quantities or less, may readily stimulate detectable antibody-forming cells in regional lymph nodes or the spleen (25–26).

In the present study, an indirect immunoplaque assay was used to study the cytokinetics of the immune response of mice to either small or large doses of a somatic lipopolysaccharide antigen derived from *Serratia marcescens*. The dose response-effect was studied in detail in terms of the number of antibody plaque-forming cells in the spleen and per million nucleated spleen cells. The relatively larger doses of the lipopolysaccharide antigen resulted in markedly fewer antibody-forming cells than did smaller doses. The suppressive effect did not seem to be related to the endotoxic properties of the antigen. Furthermore, the larger dose of antigen stimulated the appearance of large numbers of "nonspecific" antibody-forming cells to sheep erythrocytes, as compared to nontreated controls.

Methods and Materials. Animals. Noninbred NIH albino A mice and Swiss Webster mice were used. They were generally 6-8 weeks old at the start of an experiment and were obtained from a local dealer in the Philadelphia area (3, 12). The mice were housed in groups of five to six in plastic cages and fed Purina mouse pellets and water *ad libitum*.

Antigens. A lipopolysaccharide (LPS) somatic antigen derived from *Serratia marcescens* was used. The antigen was extracted by the TCA precipitation method, using overnight broth cultures of the microorganism, and was prepared at Difco Laboratories, Detroit, Michigan. The lot number used in this study was 487261.

Immunization. Mice were injected with various concentrations of the LPS prepared in sterile saline and stored at 4° as a stock solution of 1.0 mg per ml. For injection, 0.1 ml of a predetermined concentration of LPS was injected intraperitoneally (ip) into test

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mice. Control animals were injected with saline only.

Preparation of sensitized RBC's. LPS, at a concentration of 1.0 mg per ml saline, was boiled for 1 hr. Washed, packed sheep erythrocytes (RBC) were treated with this LPS, at a ratio of one part RBC's to nine parts LPS in 0.15 *M* PO_4 buffered saline, pH 7.2. This mixture was incubated for 1 hr at 37° and then washed three times by serial centrifugation in the cold. The cells were resuspended to a 10% concentration with buffered saline for use as the indicator in the antibody plaque procedure. Appropriate serologic tests indicated that these LPS-treated RBC's could be specifically hemolyzed by anti-*Serratia* sera, plus complement, but not by normal or heterologous sera.

Immunoplaque assay. Five or more mice from each experimental or control group were sacrificed on a given day and their spleens excised. Dispersed cell suspensions were prepared, essentially as described previously, using the "teasing" procedure with needles and forceps (3, 23). The cells were washed several times in the cold, using sterile Hanks' solution. For determining the number of antibody plaque-forming cells (PFC), an 0.1-ml aliquot of a cell suspension from an individual spleen was added to 2.0 ml of warm, melted 0.7% Noble agar containing 0.1 ml of a 10% suspension of LPS-sensitized sheep red blood cells. The agar also contained 1 mg DEAE–dextran as an inhibitor of the anticomplement activity of the agar. The agar-leukocyte-sensitized RBC mixture was carefully poured onto a previously prepared base layer of 10–15 ml 1.4% Noble agar in a 100-mm diameter petri plate. When the upper layer had solidified, the plates were incubated for 1 hr at 37°, and then treated with approximately 5 ml of a 1:15 dilution of sterile guinea pig serum, as a source of complement. After further incubation for 30 min to 1 hr, discrete zones of hemolysis (plaques) appearing against the background of unlysed red cells were considered to be due to individual PFC's. The number of plaques on three or more plates containing similar concentrations of leukocytes was determined and the number of PFC's per whole spleen and per million leukocytes plated was calculated. In addition, spleen cells were tested exactly as above except that nonsensitized control RBC's were used instead of the LPS-treated RBCs. The plaques appearing on such plates were considered to be due to either "background" PFC's or nonspecifically stimulated anti-sheep RBC antibody.

Inhibition experiments. Various quantities of LPS were incorporated directly into the 2-ml agar tubes prior to plating in order to specifically inhibit plaque formation to this antigen (22). In addition, rabbit anti-mouse gamma globulin serum was used in the agar plates as an inhibitor to demonstrate the globulin nature of the antibody plaques (23).

Results. As can be seen from Fig. 1, there was a rapid appearance of specific PFC's to the *Serratia* antigen in spleens of immunized mice. The number of PFC's at the peak of the response, which generally occurred 4–5 days after immunization, was directly related to the dose of antigen injected into the animal. Mice inoculated with 50 μg LPS had the largest number of PFC's on Day 4, as calculated either per spleen or per million leukocytes. Animals injected with 5 μg had markedly fewer PFC's, and the peak response generally occurred on Day 4 when calculated per spleen or on Day 5 when calculated per million leukocytes. Animals injected with 0.5 μg of antigen had even fewer PFC's, with the peak occurring usually on Day 4. In general, there were about 10 times fewer PFC's in spleens of mice receiving this dose than those receiving 100 times more antigen. Mice injected with 500 μg LPS also had markedly fewer PFC's than those inoculated with the 50- or 5.0-μg dose. Animals injected with 100 μg of LPS had somewhat more PFC's, but this was still below the maximum level obtained with a dose one-half smaller.

The rate of increase of PFC's was relatively similar in the spleens of mice injected with any of the antigen concentrations. However, mice injected with the 0.5- and 500-μg doses appeared to have the slowest PFC rise. There

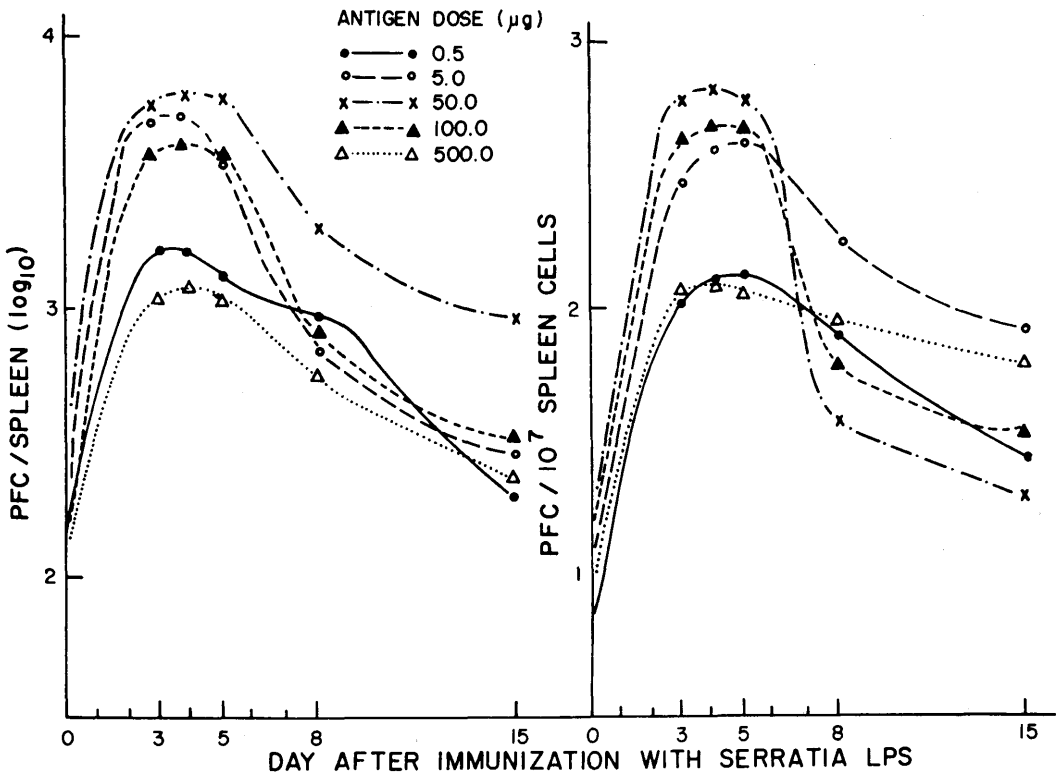

FIG. 1. The cytokinetics of appearance of antibody plaque-forming cells to *Serratia marcescens* lipopolysaccharide in mice injected ip with graded doses of antigen. Each point represents the average number of PFC in spleens of five or more mice tested on the indicated day after immunization. The "background" range of PFC's of nonimmune mice is indicated on Day "0" and was not subtracted from the number of PFC's detected after immunization.

was no significant late appearance of PFC's in spleens of mice receiving the highest dose of antigen. Such mice had as few splenic PFC's 8 and 15 days after immunization as did animals receiving the other antigen doses.

There were approximately 100–250 PFC's to the *Serratia* antigen in spleens of control, nonimmunized animals. There was also a "background" of about 100 PFC's to nonsensitized red cells in the spleens of the nonimmune mice. However, the PFC's to the RBC's increased rapidly after injection with LPS. As can be seen from Table I, the largest increase occurred in mice injected with 500 μg of LPS. There was a lesser stimulation in mice injected with the smaller antigen doses. The peak of this "nonspecific" hemolytic response to sheep erythrocytes occurred 5 days after LPS injection (Table I).

Appearance of antibody-forming cells to the *Serratia* antigen was inhibited by incorporation of 10–100 μg of specific LPS into the agar plates prior to incubation (Table

TABLE I. Effect of Various Concentrations of *Serratia* LPS on the Average Number of "Nonspecific" PFC's to Unsensitized Sheep RBC's in Spleens of LPS-Immunized Mice.

Antigen dose (in μg)	Day after LPS injection[a]			
	3	5	8	15
None	93	76	89	98
0.5	214	565	431	193
5.0	463	896	609	281
50.0	319	2651	1953	765
100.0	731	2990	2148	907
500.0	1618	4153	2650	861

[a] Groups of five or more mice tested on day indicated after ip injection of graded concentrations of LPS antigens.

TABLE II. Effect of Incorporation of Either *Serratia* LPS, Rabbit Anti-Mouse Globulin, or Normal Serum on Appearance of Specific PFC's in Agar Plates Containing Spleen Cells from Mice Immunized 5 Days before with Graded Concentrations of Antigen.

Dose of antigen used for immunization (in μg)[a]	Antigen concentration in agar (in μg)				Anti-mouse globulin (0.1 ml)	Normal rabbit serum (0.1 ml)
	None	1	10	100		
5.0	486	480	31	<5	<5	467
50.0	678	692	46	<5	<5	625
500.0	139	130	8	<5	<5	111

[a] Five to eight mice per group injected ip with indicated dose of LPS and sacrificed 5 days later; pooled spleen cells tested in agar with or without inhibitors.

II). Few specific PFC's appeared in such control plates, regardless of the dose of antigen used for immunization of the test animal or the number of leukocytes plated. Less inhibition occurred when 1 or 5 μg of LPS was used. Furthermore, incorporation of 50–100 μg of a similar LPS derived from *Escherichia coli* or *Salmonella typhosa* had no effect on appearance of PFC's to the *Serratia* LPS. However, LPS from either *Serratia* or a control organism did not affect appearance of enhanced numbers of PFC's to unsensitized RBC's in plates containing spleen cells from mice injected with *Serratia* antigen. As an additional control, all PFC's, regardless of specificity or source, were completely inhibited by addition of 0.1 ml of a 1:10 dilution of rabbit polyvalent anti-mouse gamma globulin serum (Table II). Normal rabbit serum had no effect.

Discussion. The results of these experiments indicate that a marked immune response can be elicited, as detected on the cellular level, in mice injected with the lipopolysaccharide somatic antigen derived from *Serratia marcescens*. Incorporation of 10-100 μg of *Serratia* LPS into the agar plates prevented appearance of specific antibody plaques, regardless of the dose of antigen used for immunization of the test animal. Furthermore, incorporation of 0.1 ml of a 1:10 dilution of rabbit anti-mouse globulin serum into the test agar also prevented appearance of plaque-forming cells, indicating the globulin nature of the plaques. Incorporation of heterologous lipopolysaccharide, or anti-globulin serum with specificity to species other than the mouse, failed to significantly inhibit plaque formation.

These experiments show that sheep erythrocytes passively sensitized with the lipopolysaccharide antigen derived from *Serratia* can be readily used to determine the number of specific antibody-forming cells in the spleens of mice immunized with this antigen. There was a satisfactory correlation between the number of antibody-forming cells in the spleens of these animals and their immune status as assessed by serum antibody titrations (unpublished data). Furthermore, the peak number of PFC's generally occurred on Days 4 or 5, when the LPS dose was administered within a 5000-fold range (0.5–500 μg). However, spleens of mice injected with the 500-μg antigen dose had fewer antibody-forming cells than did those from mice injected with 5, 50, or 100 μg of antigen, regardless of the day tested.

It appears unlikely that the depression of the antibody plaque response in mice treated with the larger dose of LPS was due to appearance of 7S antibody, which is known to be less efficient in hemolytic assays (4, 5). It is known that IgM antibody, which generally appears as the exclusive antibody after immunization with bacterial somatic antigens, is much more efficient in mediating the lysis of target erythrocytes, even when passively sensitized with a specific antigen. However, there was no evidence of appearance of 7S antibody to the LPS, using serologic titration procedures and sucrose-gradient centrifugation methods (unpublished data).

Although the mechanism whereby the rela-

tively large concentration of LPS suppressed appearance of specific PFC's is unknown, it is possible that the toxic nature of the antigen is involved. However, it should be noted that the LD_{50} of this particular preparation was calculated to be approximately 850 μg (unpublished results). The 500-μg dose was well below this toxic level and few, if any, animals showed any symptoms of endotoxic poisoning. Furthermore, deaths attributed to endotoxin generally occur within 1 or 2 days after LPS injection. The marked immunosuppression persisted throughout the 2-week observation period of those experiments. Thus, it seems unlikely that the marked depression of the specific antibody response was due merely to a direct toxic effect of the LPS on the antibody-forming system. However, this possibility cannot be excluded at present until appropriate studies are performed.

It should be noted that the largest number of "nonspecific" PFC's to sheep erythrocytes occurred in spleens of mice injected with the 500-μg dose of antigen. For example, mice injected with this concentration of LPS had approximately 4000 PFCs to the erythrocytes 5 days after injection. Mice treated with 50 μg of LPS responded with 2000–2600 hemolysin PFC's. There was even a lesser stimulation in mice treated with the lower doses of LPS.

A number of investigators have shown that injection of relatively large quantities of an antigen, either into adult or neonatal animals, may "overload" the immune system and induce a state of specific immunologic paralysis or unresponsiveness (26, 27). Recent studies with pneumococcal polysaccharide, using an RBC cytoadherence or rosette antibody-cell technique to enumerate immunocompetent cells, indicated that mice tolerant to this antigen had normal numbers of specific antibody-forming cells in their spleen (28). However, antibody could not be detected in their serum, probably due to rapid binding by "excess" antigen. In contrast, cellular studies with *Shigella* lipopolysaccharide antigen, as well as with other bacterial products, have shown that specific tolerance can be readily induced on both the cellular and humoral level in neonatal or adult animals, as well as *in vitro* with lymphoid cell cultures (22, 23). However, induction of such tolerance is generally demonstrated by subsequent challenge immunization with the same antigen. Tolerant animals fail to respond with normal antibody formation after challenge. Similar challenge experiments are in progress with the *Serratia* system and should provide information as to whether mice injected with 500 μg of LPS are actually tolerant, and cannot respond normally after subsequent injection with a known immunizing dose of the LPS.

The specificity of the immunosuppression occurring in the "high dose"-treated animals is also under further investigation. It is possible that the relatively large concentration of LPS may induce a nonspecific unresponsiveness to a number of antigens. However, this seems unlikely because of the enhanced response of "background" antibody-forming cells to sheep erythrocytes. Regardless of the mechanism of the depressed antibody plaque response in mice injected with the large dose of LPS, it is apparent that the concentration of antigen used for immunization markedly affects the magnitude of the immune response as assessed by the immunoplaque procedure.

Summary. The number of specific antibody plaque-forming cells appearing in spleens of mice immunized with various concentrations of the lipopolysaccharide somatic antigen derived from *Serratia marcescens* was studied in detail. Maximum numbers of antibody-forming cells appeared 4–5 days after intraperitoneal injection of 50 μg of antigen. Fewer plaque-forming cells appeared in spleens of mice treated with a lower concentrations of antigen. Furthermore, mice injected with 500 μg of the lipopolysaccharide also had relatively few plaque-forming cells, indicative of a marked depression of the immune response.

The "nonspecific" response to sheep erythrocytes was enhanced in spleens of mice injected with the highest concentrations of antigen, as compared to animals receiving smaller amounts. The specificity of the antibody-plaque response was readily demonstrated by inhibition experiments using either specific antigen or anti-mouse globulin in

the test agar plates. Plaques were inhibited when 10–100 μg of *Serratia* lipopolysaccharide, but not *Salmonella* or *E. coli* antigen, was incorporated into the agar.

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