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Relationship of the Structure of Bacterial Lipopolysaccharides to Its Function in Mitogenesis and Adjuvanticity

(lipid A/B lymphocytes)

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ABSTRACT **The ability of bacterial lipopolysaccharides to induce lymphocyte mitogenesis and to act as an adjuvant of antibody formation was attributable to the lipid-A region of the molecule. Measured by induction of DNA synthesis, lipid A was mitogenic for bone marrow-derived lymphocytes obtained from spleens of congenitally athymic mice but not for thymocytes obtained from thymuses of normal mice. Adjuvanticity was demonstrated by the ability of lipid A to convert a tolerogenic regimen of antigen into one eliciting an immune response and by its ability to markedly enhance the antibody response to a weak antigen.**

Bacterial lipopolysaccharides (LPS) exhibit several biological effects on the immune system. They are mitogenic for lymphocytes, a phenomenon restricted to the bone marrow-derived (B) lymphocyte population (1, 2), powerful adjuvants of antibody formation (3, 4), and highly immunogenic in that extremely low concentrations will trigger a specific antibody response in experimental animals (5–7). These three immunologic properties of LPS may be related if the molecule is viewed as a composite of two distinct functional moieties, a site carrying antigenic activity and another site carrying adjuvant activity. The adjuvant activity may in turn derive from the ability of LPS to induce B-cell mitogenesis, an effect whose net result might be to lower the threshold of antigen concentration necessary to stimulate a specific B lymphocyte. The ability of LPS to circumvent thymus-derived (T)-cell helper function otherwise required for the initiation of antibody response to T-cell-dependent antigens (8–11) is compatible with such an interpretation.

The model of LPS as an antigen that carries its own adjuvant was tested by experiments that attempted to correlate the immunologic activities of LPS with its chemical structure. This molecule has been chemically characterized as consisting of three principal regions (12): (*1*) the *O* polysaccharide which is linked to (*2*) the core polysaccharide which is in turn linked to (*3*) lipid A by a trisaccharide of 2-keto-3-deoxyoctanoic acid. With LPS obtained from different bacterial mutants or components isolated from chemical degradation, it was found that both mitogenicity and adjuvanticity were activities that could be attributed to the lipid region of the molecule, whereas, as described (12), the major antigenic specificity was correlated with the polysaccharide region.

Abbreviations: LPS, lipopolysaccharide; B cell, bone marrow-derived lymphocyte; T cell, thymus-derived cell; PFC, plaque-forming cells.

MATERIALS AND METHODS

Mice. A/J male mice, 5 weeks of age, were purchased from the Roscoe B. Jackson Laboratory (Bar Harbor, Me.). Congenitally athymic (nude) mice were generously provided by Dr. Norman D. Reed at Montana State University and were subsequently bred and raised at Scripps Clinic. All mice were maintained on Purina Chow pellets and chlorinated acidified water (13) at liberty.

Bacterial Cultures. Escherichia coli 0111:B4 (ATCC 12015) and the galactose-epimeraseless strain from this organism, mutant J5, were obtained from Dr. Loretta Leive. The former was grown in a Tris-based minimal medium as described (14), whereas mutant J5 was grown in 3% trypticase soy broth (BBL, Cockeysville, Md.). *Salmonella minnesota* strain Re 595 obtained from Dr. John Ryan was also grown in 3% trypticase soy broth.

Preparation of LPS. LPS from *E. coli* 0111:B4 was extracted from whole cells in aqueous butanol (15) and subsequently fractionated on Sepharose (manuscript in preparation). Briefly, aqueous suspensions of bacteria were treated with butanol-1 at 4°. The aqueous extract of the biphasic system formed by centrifugation at 35,000 × *g* for 20 min was incubated with Pronase (Calbiochem., La Jolla, Calif.) overnight at 37° and centrifuged (17,000 × *g* for 10 min), and the supernatant was concentrated by Amicon ultrafiltration. This extract was then fractionated by gel filtration on Sepharose 4B (Pharmacia, Rathway, N.J.) into two well-resolved fractions BI and BII, containing by weight about 1% and 10% lipid A, respectively. These functions are similar in both physical and chemical properties to those obtained when LPS is extracted by the phenol–water procedure of Westphal (16) and fractionated by gel filtration (in preparation). LPS from the mutant strains *E. coli* 0111:B4, J5, and *S. minnesota* Re 595 were prepared by the phenol–chloroform–petroleum ether procedure (17). Commercial preparations of LPS extracted from phenol–water from *E. coli* 0111:B4 (Lot no. 573564) were obtained from Difco Laboratories (Detroit, Mich.).

Preparation of Lipid A. LPS from *E. coli* 0111:B4 obtained by the phenol–water procedure (17) was hydrolyzed for 30–40 min at 100° in 0.1 N HCl. Lipid A was centrifuged at 2000 × *g* for 10 min, washed three times in distilled water, resuspended in water at a concentration of about 1 mg/ml,

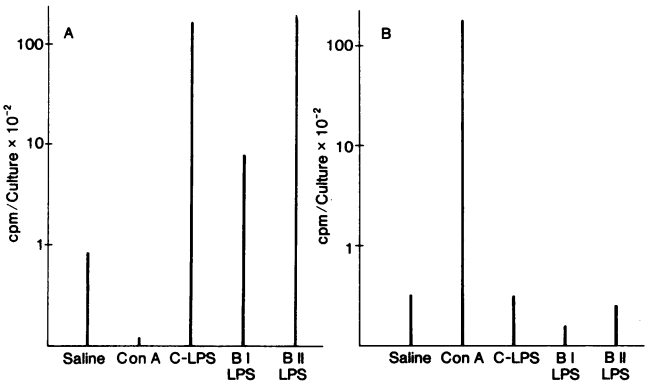

FIG. 1. Mitogenic effect of C-LPS, BI-LPS, BII-LPS, and concanavalin A on spleen cells from congenitally athymic mice (*A*) or on thymocytes from normal A/J mice (*B*). 2×10^6 spleen cells or 12×10^6 thymus cells in 1.0 ml of culture medium were incubated for 72 hr with 10 μg of the LPS preparations or 5 μg of concanavalin A. DNA synthesis was measured for the final 4 hr and is expressed as the arithmetic mean count per min of triplicate cultures. Con A, concanavalin A.

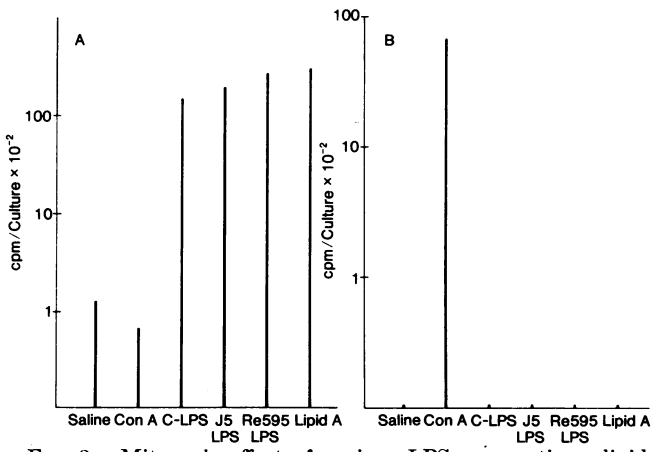

FIG. 3. Mitogenic effect of various LPS preparations, lipid A, and concanavalin A on spleen cells from congenitally athymic mice (*A*) or on thymocytes from normal A/J mice (*B*). Experimental conditions were identical to those described in the legend to Fig. 1.

and solubilized by addition of triethylamine (18) to a final concentration of 1 g/100 ml. These preparations were shown to be free of detectable 2-keto-3-deoxyoctanoic acid. This criterion has been used by other laboratories to assess the purity of lipid A preparations (19, 20).

Culture Reagents. Supplemented Minimal Eagle's Medium was prepared as follows: 100 ml contained 89.0 ml of medium, 1.0 ml of 100 × Na pyruvate, 1.0 ml of 100 × nonessential amino acids, 0.1 ml of 100 × glutamine (all from Microbiological Assoc., Albany, Calif.), 3.0 ml of 5.6% $NaHCO_3$ (Flow Laboratories, Inglewood, Calif.), 5.0 ml of fetal-calf serum (Reheis, Chicago, Ill., Lot No. JF6006), and 1.0 ml of 100 × penicillin and streptomycin (final concentration of 100 units/ml and 100 μg/ml, respectively). Agents used in mitogenesis studies, including LPS and concanavalin A (Miles–Yeda Ltd., Rehovet, Israel), were sterilized by exposure to ultraviolet light.

Lymphocyte Suspensions. The methods used are based on those developed by Mishell and Dutton (21). Spleens and/or thymuses were removed asceptically from exsanguinated mice and placed in sterile balanced salt solution (22) that contained 100 units of penicillin and 100 μg of streptomycin per ml. Spleen-cell suspensions were prepared by teasing the tissue with mouse-tooth forceps. Thymus-cell suspensions were obtained by grating the glands against a sterile stainless steel screen. Cell clumps and debris were removed by gravity sedimentation for 3 min, after which the supernatants were centrifuged at 225 × *g* for 10 min. Cell pellets were suspended in 10 ml of supplemented Minimal Eagle's Medium, and the number of viable cells was determined by trypan blue exclusion. Spleen and thymus cells were diluted with supplemented medium to final densities of 2×10^6 and 12×10^6 viable cells per ml, respectively.

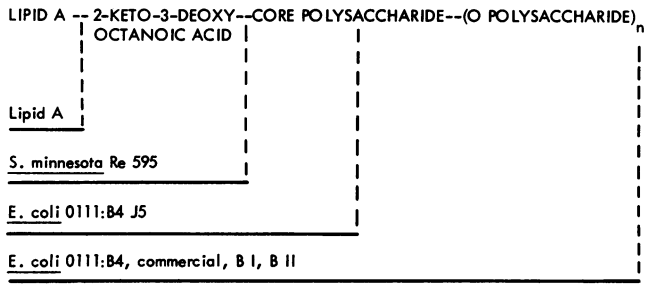

FIG. 2. General structure of LPS and its relationship to the various LPS preparations used in the present studies. BI-LPS, $n \cong 10$; BII-LPS, $n \cong 1$.

Lymphocyte Cultures. Tissue culture dishes (Falcon Plastics, Oxnard, Calif.) were seeded with 1 ml of the appropriate cell suspension and placed in a Lucite box that maintained an atmosphere of 10% CO_2–7% O_2–83% N_2. The box was incubated at 37° on a rocker platform (Bellco Glass, Vineland, N.J.) oscillating at 3–4 complete cycles/min. The cultures were each fed daily with four drops of nutritional cocktail (21).

Measurement of DNA Synthesis. After 68 hr of culture, cells were incubated for 4 hr with 1.0 μCi/ml of [^{3}H]thymidine (5.0 Ci/mmol; Amersham/Searle, Arlington Heights, Ill.). The cells were then scraped from the dish with a rubber policeman, washed once in cold normal saline, and precipitated with cold 5% trichloroacetic acid. The precipitates were collected on 0.45-μm Millipore filters (Bedford, Mass.) which were then dried in scintillation vials. Acquosol scintillation fluid (New England Nuclear Corp., Boston, Mass.) was added, and radioactive measurements were made in a Beckman LS 230 liquid scintillation counter (Beckman Instruments, Fullerton, Calif.). Results are expressed as the arithmetic mean of triplicate cultures.

Antigens. Human gammaglobulin was obtained through the courtesy of the American Red Cross and prepared as Cohn Fraction II by E. R. Squibb and Sons (New York, N.Y.). Before use, IgG from this fraction was purified on DEAE-cellulose chromatography by elution with 0.01 M phosphate buffer (pH 8.0). A monomeric preparation of DEAE-purified IgG was obtained by ultracentrifugation at 116,000 × *g* for 150 min in a swinging bucket rotor. This deaggragated IgG will induce a total state of specific unresponsiveness to human gammaglobulin when injected in A/J

TABLE 1. *Immunogenicity of various LPS preparations*

Source of LPS injected*	Response† PFC‡ per spleen
E. coli 0111:B4 (C-LPS)	1790
E. coli 0111:B4 (BII-LPS)	2180
E. coli 0111:B4 (BI-LPS)	2550
E. coli 055:B5	0

* Mice were injected intravenously with 10 μg of each preparation in 0.2 ml.

† Arithmetic mean of the response of 8 mice for each group injected 5 days previously.

‡ Direct plaque-forming cells specific to LPS of *E. coli* 0111:B4 (C-LPS).

mice (23). Bovine-serum albumin (Lot no. F71703) was purchased from Reheis Laboratories, Chicago, Ill.

Enumeration of Antibody-Forming Cells. Cells forming antibody specific to human gammaglobulin, bovine-serum albumin, or lipopolysaccharide were assayed by use of modifications of the Jerne hemolytic plaque assay (24) in which either protein antigens (22) or LPS (6) were coupled to goat erythrocytes (Colorado Serum, Denver, Colo.). Indirect plaque-forming cells (PFC) were developed with antibodies to mouse IgG from squirrel monkey used at a concentration determined to be optimal in the assay.

RESULTS

Mitogenic effect of different LPS preparations

Mitogenic stimulation of spleen cells from nude mice (B cells) was assessed after incubation with LPS of *E. coli* 0111:B4 either obtained commercially (C-LPS) or extracted by the butanol method and fractionated into two forms, BI-LPS (1% lipid A by weight) and BII-LPS (10% lipid A by weight). The results obtained are seen in Fig. 1*A* and reveal that each of the three preparations of LPS tested was mitogenic for B cells. Of the two preparations obtained by butanol extraction and Sepharose fractionation, the one with the lower lipid A concentration (BI-LPS) was markedly less stimulatory. The mitogenic effect of each of the three preparations on thymocytes was negligible, although these cells were readily activated by concanavalin A (Fig. 1*B*), a protein previously described to be mitogenic for T cells but not for B cells (2). Each of the LPS preparations in contrast was equally immunogenic as tested by the ability to induce a specific antibody response when injected into mice (Table 1).

Mitogenic effect of LPS with different chemical structures

The above experiment suggested that the mitogenic effect of LPS could be related to a particular structural region of the molecule, namely, the lipid A region. In order to further test this hypothesis, LPS preparations from the following bacteria were tested: (*a*) from *E. coli* 0111:B4 (C-LPS), (*b*) from a galactose epimeraseless mutant of *E. coli* 0111:B4, J5 (J5-LPS), and (*c*) from the heptoseless mutant of *S. minnesota*, Re 595 (Re 595-LPS). The chemical nature of each of these preparations is depicted in Fig. 2. In addition, lipid A obtained by mild acid hydrolysis of the LPS from *E. coli* 0111:B4 was also tested. It was observed that mitogenesis of B cells was induced by each of the preparations of LPS tested as well as by lipid A (Fig. 3*A*). The activity of the various LPS fractions and of lipid A was restricted to B cells since thymocytes were not stimulated (Fig. 3*B*). In contrast, concanavalin A was stimulatory to thymocytes (Fig. 3*B*) but not to B cells (Fig. 3*A*). Not shown are results that demonstrate that spleen cells obtained from normal mice are mitogenically activated by either LPS or concanavalin A as would be predicted, since a normal spleen contains both B and T cells.

Therefore, the results shown in Figs. 1*A* and 3*A* demonstrate that the mitogenic activity of LPS resides in the lipid A region of the molecule.

TABLE 2. *Adjuvant effect on the immune response of A/J mice to bovine-serum albumin*

Material injected*	Response† PFC‡ per spleen
Saline	10
Concanavalin A	160
Pneumococcal polysaccharide	110
C-LPS	130,800
J5-LPS	1047,600
Re 595-LPS	559,900

* Mice were injected intravenously on day 0 with 500 μg of bovine-serum albumin in 0.2 ml, and 3 hr later with 50 μg of one of the preparations or saline. On day 14, the mice were challenged again with 200 μg of bovine-serum albumin, given intraperitoneally in 0.2 ml.

† Response obtained 4 days after the second challenge with bovine-serum albumin. Although the entire kinetic curve of the secondary response was obtained, this day represents the peak of the response.

‡ Indirect plaque-forming cells specific to bovine-serum albumin.

Adjuvanticity of LPS preparations

Two procedures were used to determine whether the various LPS preparations and lipid A had *in vivo* activity as adjuvants of antibody formation.

(*a*) *Response to bovine-serum albumin:* A/J male mice (9 weeks of age) were injected intravenously with 500 μg of bovine-serum albumin. 3 Hr later, they received another intra-

TABLE 3. *The ability of different LPS preparations to convert a tolerogenic injection of deaggregated human gammaglobulin into an immune stimulus*

LPS injected	Response* PFC† per spleen
None (saline)	70
C-LPS	7,730
BII-LPS	8,010
J5-LPS	10,470
Re 595 LPS	11,740
Lipid A	12,270

* Mice were injected with 2.5 mg of deaggregated IgG intraperitoneally, and 3 hr later with one of the listed preparations (50 μg given intravenously). Their spleens were assayed 10 days after this treatment.

† Indirect plaque-forming cells specific to human gammaglobulin.

venous injection of saline, C-LPS, J5-LPS, Re 595-LPS, concanavalin A, or Type III pneumococcal polysaccharide. 14 Days later, all groups were injected with 200 μg of soluble bovine-serum albumin given intraperitoneally, and the number of plaque-forming cells specific to bovine-serum albumin was quantitated in the spleens of these animals at various times after the last antigenic challenge. The results of this experiment are shown in Table 2 and clearly reveal that the potent adjuvant effect of LPS can be attributed to those preparations of LPS that contain lipid A. Thus, LPS obtained from the *S. minnesota* mutant Re 595, which structurally contains only lipid A and a trisaccharide of 2-beta-3-deoxy-octanoic acid, displays adjuvanticity. In contrast, neither the capsular polysaccharide of pneumococcus nor concanavalin A markedly enhance the response to bovine-serum albumin.

(*b*) *Conversion of tolerance to immunity:* It has been recently observed that LPS treatment after injection of deaggregated IgG can convert this usually tolerogenic regimen to an immune response specific to human gammaglobulin (manuscript in preparation). Since this phenomenon may be regarded as a stringent definition of an adjuvant effect, it was used as a system to provide another index of the adjuvant activity of the various LPS preparations and of lipid A. A/J male mice, 6 weeks of age, were injected intraperitoneally with 2.5 mg of deaggregated IgG given in a 1-ml volume. 3 Hr later, they were given an intravenous injection of saline or 50 μg of C-LPS, BII-LPS, J5-LPS, Re 595-LPS, or lipid A. From the data presented in Table 3, we conclude that the effect of converting a tolerogenic injection of deaggregated IgG to a specific antibody response to that antigen is a function that can also be attributed to the lipid A portion of the LPS molecule.

DISCUSSION

The ability of LPS to act as a mitogen for B lymphocytes and as an adjuvant for antibody formation are properties that the present data show to be attributable to the lipid A portion of the LPS molecule. The fact that there exists a close parallelism in structure–function relationship between mitogenic stimulation on one hand and adjuvant activity on the other suggests that these two phenomena may be related. The mode of action of LPS as an adjuvant could be viewed as the result of the induction of B-cell mitogenesis mediated by its lipid A, an effect that would lower the amount of antigen necessary for specific B-cell stimulation. A similar mechanism has been proposed to explain the mode of action of the helper function of T cells in the observed T cell–B cell cooperation in antibody formation (25). The ability of LPS to bypass the requirement of T-cell help has been demonstrated in several studies (8–11) and provides an observation compatible with the present interpretation. However, the adjuvant effect of LPS may be mediated other than by its mitogenic action on B cells. Alternatives that could be considered include interference with a feedback mechanism operative in antibody formation (26), toxicity, causing the release of cellular breakdown products such as nucleotides which are themselves adjuvants (27), or the alteration of intracellular processing events that operate in the reticuloendothelial limb of the immune response (28).

The present data offer an insight into a structural relationship between the adjuvant property of LPS and its potent immunogenicity when injected into experimental animals. As first shown by Landy and Baker (5), a specific immune response (PFC) can be observed in the popliteal lymph nodes of rabbits after a footpad injection of a few thousand molecules of LPS from *S. enteritidis*. The observation that a few molecules could immunologically sensitize mice to LPS from *E. coli* also led Rudbach to conclude that this preparation is in fact a "super antigen" (6). Nossal and Ada (7) point out that the strength of the inherent immunogenicity of LPS can be some eleven orders of magnitude greater than that for other antigens. In this regard, the repetitive nature of the antigenic determinants on LPS is not in itself sufficient to explain its immunogenic behavior, inasmuch as the polymerized flagellin of *Salmonella* or the capsular polysaccharide of pneumococcus are five logs less immunogenic than the O antigen of *Salmonella*, although all three are antigens structurally composed of repeating determinants. The hypothesis that LPS is a powerful immunogen because it is an antigen that carries its own adjuvant could be verified if it becomes possible to increase the immunogenicity of weak antigens by attaching to them that part of LPS (lipid A or some of its fatty acids) that presently has been shown to induce mitogenic and adjuvant stimuli.

Lipid A is structurally composed of a phosphorylated D-glucosamine disaccharide of *N*-acetyl glucosamine to which fatty acids (lauric, myristic, palmitic, and 3-hydroxy myristic) are either ester- or amide-linked (29). Although the present data do not provide information on the relative importance of each structural unit to the functional parameters of mitogenicity, adjuvanticity, or immunogenicity, preliminary experiments indicate that the removal of the ester-linked fatty acids by alkaline hydrolysis does abrogate the B-cell mitogenic stimulus obtained with the intact molecule. The possibility that fatty acids may be critical to the immunologic activities of LPS would be compatible with several previous observations that demonstrated that lipids, and even individual fatty acids, are good adjuvants (30–32). More information is necessary before the mechanism by which lipid A triggers lymphocyte stimulation can be understood. This effect may be related to the ability of lipid A to integrate into the lipid bilayer of the plasma membrane inasmuch as LPS is known to insert into membrane structures (33). Why this action should be limited to lymphocytes of the B series is puzzling, unless the initial binding of LPS to the cell membrane is dependent on the presence of LPS receptors such as those reported to exist on human erythrocytes (34), and that quantitative differences of such receptors exist on the surfaces of T and B lymphocytes. Alternatively, qualitative differences in the binding of LPS may account for the observed cellular restriction in mitogenesis in a fashion similar to that observed for concanavalin A, a T-cell mitogen, which nevertheless can just as effectively be bound to B cells (35).

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1. Gery, I., Krüger, J. & Spiessel, S. Z. (1972) *J. Immunol.* **108**, 1088–1091.
2. Andersson, J., Möller, G. & Sjöberg, O. (1972) *Cell. Immunol.* **4**, 381–393.
3. Johnson, A. G., Gaines, S. & Landy, M. (1956) *J. Exp. Med.* **103**, 225–246.
4. Neter, E. (1969) in *Current Topics in Microbiology and Immunology* (Spinger-Verlag, Berlin), Vol. 47, pp. 92–96.
5. Landy, M. & Baker, P. J. (1966) *J. Immunol.* **97**, 670–679.
6. Rudbach, J. A. (1971) *J. Immunol.* **106**, 993–1001.
7. Nossal, G. J. V. & Ada, G. L. (1971) in *Antigens, Lymphoid Cells and the Immune Response*, eds. Dixon, F. J. & Kunkel, H. G. (Academic Press, New York), pp. 245–246.
8. Jones, J. M. & Kind, P. D. (1972) *J. Immunol.* **108**, 1453–1455.
9. Sjöberg, O., Andersson, J. & Möller, G (1972) *Eur. J. Immunol.* **2**, 326–331.
10. Schmidtke, J. & Dixon, F. J. (1972) *J. Exp. Med.* **136**, 392–397.
11. Chiller, J. M. & Weigle, W. O. (1973) *J. Exp. Med.*, **137**, 740–750.
12. Lüderitz, O., Westphal, O., Staub, A. M. & Nikaido, H. (1971) in *Microbial Toxins*, eds. Weinbaum, G., Kadis, S. & Ajl, S. J. (Academic Press, New York), pp. 145–233.
13. McPherson, C. W. (1963) *Lab. Animal Care* **13** (5), 737–742.
14. Leive, L. (1965) *Biochem. Biophys. Res. Commun.* **18**, 13–17.
15. Leive, L. & Morrison, D. C. (1972) in *Methods in Enzymology XXVIII, Complex Carbohydrates*, ed. Ginsburg, V. (Academic Press, New York), pp. 254–262.
16. Westphal, O. & Jann, K. (1965) in *Methods in Carbohydrate Chemistry*, ed. Whistler, R. L. (Academic Press, New York), Vol. 5, 83–91.
17. Galanos, C., Lüderitz, O. & Westphal, O. (1969) *Eur. J. Biochem.* **9**, 245–249.
18. Westphal, O., Gmeiner, J., Lüderitz, O., Tanaka, A. & Eichenberger, E. (1969) *Colloq. Int. Cent. Nat. Rech. Sci.* **174**, 69–78.
19. Galanos, C., Lüderitz, O. & Westphal, O. (1971) *Eur. J. Biochem.* **24**, 116–122.
20. Galanos, C., Rietschel, E. T., Lüderitz, O., Westphal, O., Kim, Y. B. & Watson, D. W. (1972) *Eur. J. Biochem.* **31**, 230–233.
21. Mishell, R. I. & Dutton, R. W. (1967) *J. Exp. Med.* **126**, 423–442.
22. Golub, E. S., Mishell, R. I., Weigle, W. O. & Dutton, R. W. (1968) *J. Immunol.* **100**, 133–137.
23. Chiller, J. M. & Weigle, W. O. (1971) *J. Immunol.* **106**, 1647–1653.
24. Jerne, N. K. & Nordin, A. A. (1963) *Science* **140**, 405.
25. Möller, G. (1970) *Cell. Immunol.* **1**, 573–582.
26. Uhr, J. W. & Möller, G. (1968) *Advan. Immunol.* **8**, 81–127.
27. Braun, W. & Nakado, M. (1967) *Science* **157**, 819–821.
28. Alexander, P. & Evans, R. (1971) *Nature, New Biol.* **232**, 76–78.
29. Rietschel, E. T., Gottert, H., Lüderitz, O. & Westphal, O. (1972) *Eur. J. Biochem.* **28**, 166–173.
30. Dresser, D. W. (1961) *Nature* **191**, 1169–1171.
31. Barrie, J. U. & Cooper, G. N. (1964) *J. Immunol.* **92**, 529–539.
32. Gall, D. (1966) *Immunology* **11**, 369–386.
33. Rothfield, L. & Romeo, D. (1971) *Bacteriol. Rev.* **35**, 14–38.
34. Springer, G. F., Hupriker, S. V. & Neter, E. (1970) *Infect. Immunity* **1**, 98–108.
35. Stobo, J. D., Rosenthal, A. S. & Paul, W. E. (1972) *J. Immunol.* **108**, 1–17.