THE USE OF BACTERIAL LIPOPOLYSACCHARIDES TO SHOW THAT TWO SIGNALS ARE REQUIRED FOR THE INDUCTION OF ANTIBODY SYNTHESIS*

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We assume that antigen-sensitive cells are committed to the expression of a unique antibody and respond to antigen either by differentiation to antibody-forming cells (induction) or by inactivation in that they are no longer inducible (paralysis). The interaction of antigen with the receptor on the antigen-sensitive cell is common to both the inductive and paralytic pathways. The question arises as to how antigen-sensitive cells distinguish between inductive and paralytic signals. In this paper we consider the use of bacterial lipopolysaccharides (LPS)[1] in studying the nature of the signals required for the induction of antibody synthesis. There in a vast literature dealing with the activation of lymphocytes by plant lectins and LPS (1–9). LPS stimulates DNA synthesis and cell division in B lymphocytes and stimulates immune responses to various antigens in the absence of thymus-derived cells (5, 10, 11). LPS are high molecular weight polymeric molecules that contain both a polysaccharide and lipid region (12–16). The experimental work in this paper shows that LPS can be used to stimulate immune responses to monovalent haptens in nude spleen cultures. We consider the mitogenic effect of LPS is derived from its ability to complete an inductive signal to B lymphocytes. This interpretation implies that two signals are required for the induction of antibody synthesis. The first signal is delivered to antigen-sensitive cells via the antigen-receptor interaction, and the second is delivered either directly as a consequence of LPS binding to antigen-sensitive cells, or indirectly via the interaction of LPS with an accessory cell type. We suggest that the results of other experiments performed with LPS can be interpreted within the frame of this signal system.

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[1] *Abbreviations used in this paper:* FBS, fetal bovine serum; HRBC, horse erythrocytes; LPS, lipopolysaccharides; MSA, mouse serum albumin; PFC, plaque-forming cells; PVP, polyvinylpyrrolidone; SRBC, sheep erythrocytes; TdR, thymidine; TNP, trinitrophenyl; T_{SRBC}, activated thymus cells.

Materials and Methods

Mice.—Congenitally athymic (nude) mice obtained from Dr. C. Friis, Rye, Denmark, were bred by us following a schedule outlined elsewhere (5). Nude mice between 3 and 4 wk of age were used for experiments. C57BL/6 mice were purchased from Jackson Laboratory, Bar Harbor, Maine. BALB/c mice were obtained from a Salk Institute breeding colony.

Spleen Cultures.—In vitro immune responses were studied in spleen cell suspensions prepared from C57BL/6 and nude mice exactly as described elsewhere (17–19). Spleen cultures contained 1×10^7 spleen cells in 1 ml of Eagle's minimal essential medium supplemented with 5% fetal bovine serum (FBS) and antigen as specified in the text. Cultures were fed daily with a nutritional mixture supplemented with the FBS used in preparing the culture (19).

Elsewhere we have described the effects of different batches of FBS on in vitro immune responses (17, 19). Mouse cells cultured in medium supplemented with deficient FBS do not support primary immune responses to heterologous erythrocyte antigens. However, the immune responses that are elicited in the presence of LPS are very similar in normal and deficient sera. Therefore, we make no reference in the text to the batch of FBS used in each experiment.

Antigens.—Sheep (SRBC) and horse (HRBC) erythrocytes were supplied by Colorado Serum Co., Denver, Colo. 2,3,4-trinitrophenyl-glycine (TNP-gly), TNP-glycylglycine (TNP-gly-cyl), TNP-leucyl-tyrosine (TNP-leu-tyr), and TNP_5-mouse serum albumin (TNP_5-MSA) were prepared following the procedures of Okuyama and Satake (20). Since the nude gene is being bred onto a BALB/c background (5), the MSA was obtained from BALB/c serum and was prepared by fractionation through a column of Sephadex G-100. The fraction containing albumin was pooled and TNP conjugated to it (20).

Polyvinylpyrrolidone (PVP), with an average mol wt of 360,000, was obtained from Sigma Chemical Co., St. Louis, Mo. Dextran 1355 (α 1:3 linked) was obtained from Dr. Allene Jeanes, pneumococcal C carbohydrate prepared as described elsewhere (21), and Type III carbohydrate obtained from Dr. E. Kabat.

Specificity of Antibody-Forming Cells.—At various days after culturing the number of direct hemolytic plaque-forming cells (PFC) in spleen cultures were determined (18) using a microscope slide assay. When SRBC or HRBC were used as antigens in culture, the same erythrocytes were used in the slide assay to determine the PFC directed against SRBC or HRBC. When TNP-conjugates were used as antigens in culture, lightly coupled TNP-SRBC or TNP-HRBC were prepared according to the procedure of Rittenberg and Pratt (22) and used for plaque formation in the slide assay.

Since the addition of LPS to spleen cultures lacking SRBC or HRBC as antigens caused increases in the background PFC (see text), the specificity of PFC directed against TNP was always checked by inhibition procedures. To analyze the specificity of the plaques TNP_{15}-MSA was incorporated into the slide assay at a final concentration of 10^{-6} M. At this concentration there was no suppression of the formation of PFC directed against non-crossing-reacting determinants (SRBC) but greater than 95% inhibition of TNP-specific plaques. Cell recoveries varied depending upon whether LPS was present in the cultures. Therefore in all experiments we have expressed our data as the number of PFC per culture as averaged from duplicate cultures. Unless stated otherwise in the text, *all* TNP-specific PFC presented are the number of PFC that were inhibited from developing in the slide assay in the presence of 10^{-6} M TNP_{15}-MSA. The presence of up to 100 μg of LPS or lipid A per slide in the plaque assays did not affect the appearance of hapten-specific or erythrocyte-specific plaques.

Activated Thymus Cells.—BALB/c mice were irradiated (850 R) and injected with 10^8 thymus cells from nonirradiated donors and 2×10^8 SRBC as described elsewhere (20). After 8 days spleen cells were harvested and used as the source of activated thymus cells (T_{SRBC}).

DNA Synthesis.—Spleen cultures were prepared containing 5×10^6 cells in 1 ml of medium containing 5% FBS. Each day duplicate cultures were incubated for 4 h with 10^{-7} M fluorodeoxyuridine, 10^{-5} M thymidine, and 0.5 μCi of [^{3}H]thymidine (52 Ci/mmol; New England Nuclear, Boston, Mass.). Cells were collected by centrifugation, the DNA was precipitated using 5% trichloroacetic acid, and the precipitate collected by membrane filtration. These filters were incubated with 0.2 ml of NCS solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.) at 70°C for 60 min and then radioactive measurements were made after the addition of a toluene-Liquifluor scintillation fluid.

Mitogens.—Salmonella typhosa (W0901) lipopolysaccharide was obtained from Difco Laboratories, Inc., Detroit, Mich. LPS was dissolved in 0.01 M phosphate buffer (pH 8.0) at a concentration of 1 mg/ml, boiled for 60 min, and stored frozen until use.

Lipid A was also prepared from *S. typhosa* LPS by mild acid hydrolysis (14). For experimental use lipid A was resuspended in saline at 100 μg/ml using triethylamine as described elsewhere (15).

RESULTS

Effect of Antigen on LPS-Induced Immune Responses.—The data presented in Table I describe the effect of LPS on background and induced immune responses to SRBC or HRBC in nude spleen cultures. The following is consistently observed: (*a*) In the absence of LPS, no response to SRBC or HRBC is observed. When different concentrations of LPS are added to nude spleen cultures lacking SRBC or HRBC, an increase in PFC is observed when the cultures are assayed for PFC directed against SRBC or HRBC. The increase in PFC directed against SRBC is always larger than that observed for HRBC, but in the presence of optimal concentrations of LPS the number of PFC is always less than 200 per culture. We stress that in normal spleen cultures

TABLE I

Effect of LPS on Immune Responses to Erythrocyte Antigens and Haptens (TNP) in Nude Spleen Cultures

Concentration of LPS	PFC/culture					
	Minus antigen* assayed on				Plus antigen‡ assayed on	
	SRBC	HRBC	TNP-HRBC	TNP-HRBC + 10^{-6} M TNP_{16}-MSA	SRBC	HRBC
$\mu g/ml$						
0	<10	<10	<10	<10	<10	<10
0.1	20	<10	82	10	320	180
0.5	30	10	190	46	1,050	680
1.0	100	40	320	84	1,950	1,350
2.5	150	70	310	96	3,540	1,620
5.0	200	60	304	98	2,250	1,590

* Nude spleen cultures contained 10^7 nude spleen cells without added SRBC or HRBC.

‡ Identical cultures contained either 3×10^6 SRBC or 3×10^6 HRBC. All cultures were assayed on day 4 using the erythrocytes indicated in the plaque-forming assay and each figure represents the mean of duplicate cultures.

(23–25) and in adult mice (26) this increase in background immune responses in the presence of LPS is much greater. (*b*) The addition of SRBC or HRBC with different concentrations of LPS results in a marked stimulation in the immune response to each of these antigens. The stimulation varies between 5- and 50-fold depending upon the concentration of LPS in cultures. (*c*) Maximum stimulation of in vitro immune responses to SRBC or HRBC in nude spleen cultures is elicited by LPS in the range of 1–5 μg per ml of culture. (*d*) A background immune response to TNP is also found. When nude spleen cells are cultured with different concentrations of LPS and assayed at various times using HRBC or TNP-HRBC as the indicator in the plaque-forming assay, PFC specific for both HRBC and TNP can be distinguished. The number of PFC detected using TNP-HRBC is approximately three to five times the number of PFC detected using HRBC alone. The PFC detected using TNP-HRBC appear to be specific for TNP since they can be suppressed by the addition of 10^{-6} M TNP_{15}-MSA to the assay mixture. Similar background immune responses were detected with SRBC and TNP-SRBC, but since the background responses assayed on these erythrocytes were consistently higher they were judged less suitable for use.

The kinetics of in vitro immune responses in nude spleen cultures elicited by optimal amounts of LPS differed significantly from those observed in identical cultures stimulated with activated thymus cells. The experiment of Fig. 1 compares the kinetics of the immune response to SRBC in nude spleen cultures containing 5 μg of LPS with those containing 5×10^6 BALB/c T_{SRBC} cells. In the presence of LPS the response increases rapidly during the initial 3 days of culture and then declines. By contrast, in nude spleen cultures containing BALB/c T_{SRBC} cells, the immune response to SRBC is initiated more slowly, but increases until day 5 of culture, where the number of PFC is often two to four times the number observed at the peak of the LPS-induced response (Fig. 1).

When nude spleen cultures were incubated with 5 μg of LPS for increasing periods of time before SRBC were added, the immune response subsequently elicited against SRBC decreased. Exposure of cells to LPS for between 20 and 30 h before the addition of SRBC decreased the in vitro immune response to less than 10% of control immune response (Fig. 2). Control immune responses were determined by incubating nude spleen cells for the same time period in the absence of LPS and SRBC, and then adding both simultaneously to cultures. Thus by the time DNA synthesis is induced in spleen cells treated with LPS in the absence of antigen, the cells have lost the ability to respond to antigen (Fig. 2).

Effect of the Antigen Valency.—Since LPS stimulates immune responses in spleen cultures lacking thymus-derived cells, experiments were carried out to determine whether LPS could be used to study the way antigen delivers a signal to B lymphocytes. Both TNP conjugated to SRBC or to mouse serum

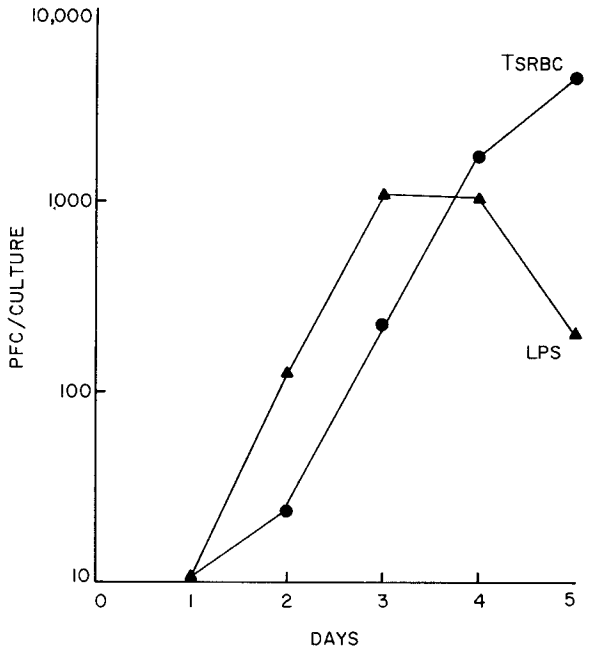

FIG. 1. Comparison of the kinetics of immune responses to SRBC in nude spleen cultures supplemented with 5 μg of LPS or with 5 $\times$ 10^6 activated BALB/c thymus cells (T_{SRBC}). Background responses to SRBC in nude cultures containing 5 μg of LPS but no added SRBC have been subtracted from the data shown for the LPS stimulation.

albumin (MSA) can be used as antigens in nude spleen cultures containing LPS. The experiment of Fig. 3 compares the use of TNP_5-MSA and lightly conjugated TNP-SRBC (22) as antigens. In both cases, the TNP-specific PFC were determined using TNP-HRBC as indicator cells. The data plotted in Fig. 3 represent the PFC observed after the background number of PFC in cultures lacking antigen but containing 5 μg of LPS was subtracted. It was found that the presence of TNP_{15}-MSA in the plaque assay suppressed more than 90% of the PFC that were detected. Thus when 10^{-7} M TNP_5-MSA or 3 $\times$ 10^6 TNP-SRBC are used as antigen in nude spleen cultures, LPS stimulates very marked TNP-specific immune responses above the background responses.

The effect of decreasing the size of the antigen upon the induction of immune responses was analyzed by using small TNP-derivatives. The data presented in Fig. 4 show that TNP-amino acid derivatives, judged too small to interact with more than on receptor-combining site at a time, stimulate TNP-specific immune responses in nude spleen cultures containing LPS. TNP-glyclglycine, TNP-leucyl-tyrosine, and TNP-glycine all support TNP-specific responses in nude spleen cultures containing 5 μg of LPS. Maximum stimulation of the

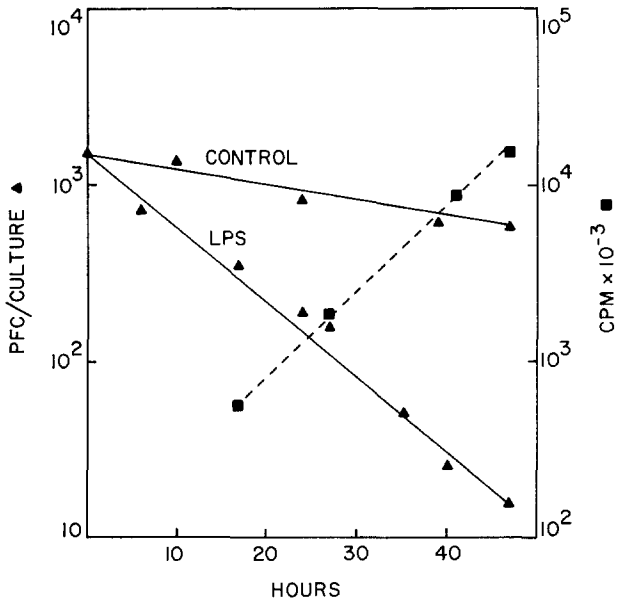

FIG. 2. Effect of preincubating nude spleen cells with LPS on the subsequent induction of immune responses to SRBC. Nude spleen cultures were set up containing 5 μg of LPS and after incubation for the times indicated (0–48 h), 3×10^6 SRBC were added. 4 days after the addition of SRBC, the number PFC directed against SRBC present were assayed. In control cultures, cells were preincubated without LPS and then 5 μg of LPS and 3×10^6 SRBC added also at the times indicated. These cultures were also assayed for PFC directed against SRBC after 4 days. The kinetics of induction of DNA synthesis were followed. Nude spleen cultures containing 5 μg of LPS were incubated with 0.5 μCi of [^{3}H]TdR for 4 h as described in Methods and total radioactivity incorporated into DNA was determined. The points plotted represent the radioactive incorporation at the end of the 4-h labeling periods. Radioactive incorporation into control cultures lacking LPS was determined also, and these background data were subtracted from the figures that have been plotted.

TNP response was observed in the presence of 10^{-6} M TNP-amino acid derivative.

Other polymeric antigens besides LPS considered to be "thymus-independent" antigens were examined for their ability to stimulate TNP responses. PVP-360 (27), pneumococcal carbohydrates (Type III and C) (28), and dextran[2] when added to 10^{-6} M TNP-leu-tyr in nude spleen cultures did not stimulate TNP-specific responses (Table II).

Effect of Lipid A.—The following experiments demonstrate that lipid A contains both the mitogenic and inductive activity described for LPS.

Lipid A stimulates DNA synthesis in nude spleen cultures (Fig. 5). At optimal

[2] Blomberg, B., and M. Cohn. 1973. The stimulation of immune responses to dextran in nude mice. Manuscript in preparation.

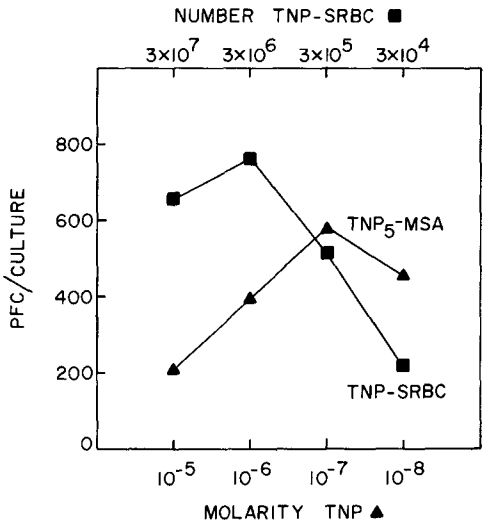

FIG. 3. The stimulation of immune responses to TNP in nude spleen cultures. Each culture contained 10^7 cells with 5 μg of LPS and the number of lightly coupled TNP-SRBC (22) or the concentration of TNP_5-MSA as indicated. Cultures were assayed on day 4 using TNP-HRBC as the indicator cells in the plaque assay. The data expressed are the number of PFC observed after the background response to TNP in cultures lacking antigen but containing 5 μg of LPS was subtracted. When TNP_{15}-MSA was added to the plaque assay more than 90% of the PFC were suppressed from developing. Nude spleen cultures containing different concentrations of TNP-SRBC or TNP_5-MSA but no LPS showed no response to TNP (<10 PFC/culture).

concentrations of lipid A the stimulation of DNA synthesis was more than 50-fold above the detectable background. At the same concentration LPS was more mitogenic than lipid A; however, lipid A did not suppress DNA synthesis in the range of 5–20 μg as was observed for LPS.

Lipid A was found to stimulate an immune response to SRBC in nude spleen cultures (Fig. 6 *A*). Also, when different concentrations of lipid A are added to nude spleen cultures containing TNP-amino acid derivatives, there is a stimulation in the TNP-specific immune response. The data presented in Fig. 6 *B* describe the effects of different concentrations of lipid A in nude spleen cultures containing 10^{-6} M TNP-leu-tyr. When control cultures containing different concentrations of lipid A but no hapten were assayed using HRBC and TNP-HRBC a background response as described for LPS was observed. The addition of 10^{-6} M TNP-leu-tyr resulted in an increase of the stimulation of the TNP-specific immune response in cultures containing between 0.5 and 5 μg lipid A.

DISCUSSION

A monovalent hapten, too small to extend beyond the antibody-combining site of the receptor on an antigen-sensitive cell, is not itself immunogenic. It

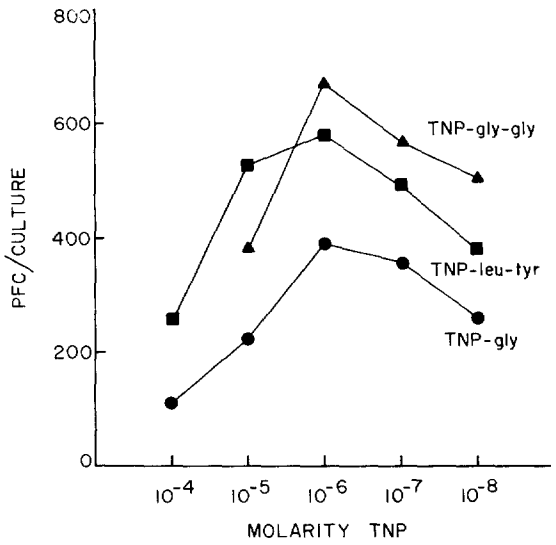

FIG. 4. The stimulation of immune responses using TNP-amino acid derivatives in nude spleen cultures. All cultures contained 10^7 nude cells. In cultures containing the different concentrations of TNP-amino acid derivatives indicated, but no LPS, no immune response to TNP was detected (10 PFC/culture). Background immune responses to TNP determined in cultures stimulated with 5 μg of LPS, but no antigen, did not exceed 80 PFC/culture. The cultures were all assayed on day 4 using TNP-HRBC as the indicator cells in the plaque assay. The data plotted represent the number of PFC that were detected and suppressed by the addition of 10^{-6} M TNP_{15}-MSA to the plaque assay mixture.

cannot participate in the normal cooperative interaction required for the induction of antibody synthesis. The unique finding in this study is that such a monovalent hapten can be rendered immunogenic by the addition of LPS in nude spleen cultures. These data extend reports that T cell-depleted spleen cultures (anti-θ-treated or derived from nude mice) can be rendered responsive to erythrocytes by the addition of LPS (5, 9). Also in thymectomized mice LPS stimulates an immune response to TNP when the TNP is coupled to isologous mouse red cells (MRBC), a carrier which is nonimmunogenic because of a lack of T cells recognizing the self-antigens of MRBC (11).

There are two ways an inductive stimulus may be completed by monovalent haptens and LPS, namely by delivering one or two signals to antigen-sensitive cells. A one-signal hypothesis might be invoked if the TNP-amino acid derivatives bind nonspecifically to LPS or to a substance to which both hapten and LPS bind. The hapten-LPS complex would activate cells by the presentation of a particular density of TNP units to antigen-binding receptors (29, 30). The addition of any assumption involving the delivery of a signal to cells via interaction of LPS with the B lymphocyte membrane or the cooperating cell system would make a two-signal hypothesis.

TABLE II

Effect of Thymus-Independent Antigens on the Induction of Immune Responses in Nude Spleen Culture

Antigen	Concentration	PFC/Culture	
		TNP	SRBC
	μg/ml		
—	—	<10	<10
LPS	0.5	283	960
LPS	1.0	440	1,540
PVP-360	0.5	<10	<10
PVP-360	1.0	<10	<10
Pneu-C	0.5	<10	<10
Pneu-C	1.0	<10	<10
Pneu-Type III	0.5	<10	<10
Pneu-Type III	1.0	<10	<10
Dextran	0.5	<10	<10
Dextran	1.0	<10	<10

TNP cultures contained 10^{-6} M TNP-leu-tyr as antigen; SRBC cultures contained 3×10^6 SRBC. Cultures were assayed on day 4 using TNP-HRBC to determine the TNP response or SRBC for the SRBC response.

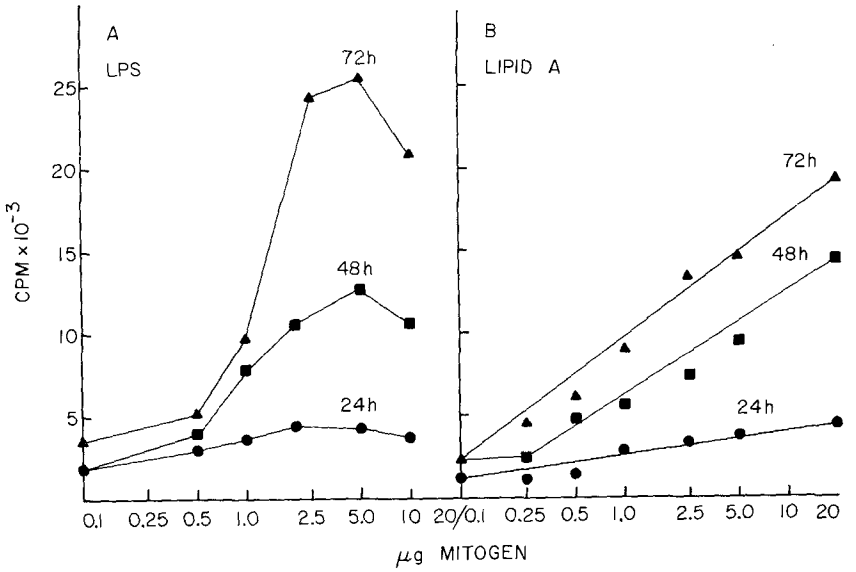

FIG. 5. Effect of LPS and lipid A on DNA synthesis in nude spleen cultures. Each point represents the mean of duplicate cultures. In the absence of LPS or lipid A, the radioactive incorporation into nude spleen cultures after 24, 48, or 72 h did not exceed 600 cpm. After 24, 48, and 72 h duplicate cultures were labeled for 4 h with [^{3}H]TdR as described in Methods.

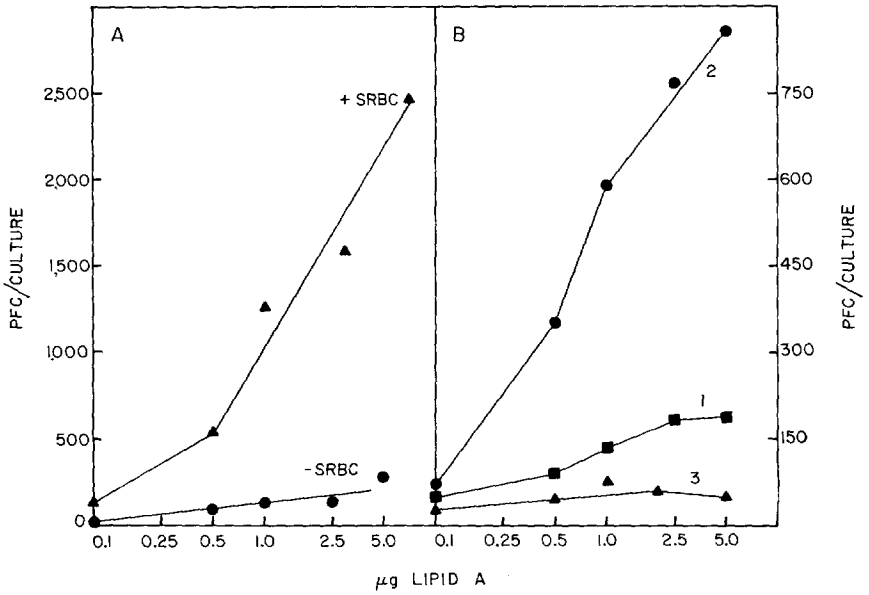

FIG. 6. The stimulation of in vitro immune responses by lipid A. Nude spleen cultures containing 10^7 cells and the concentrations of lipid A indicated were prepared. (*A*) The immune responses to SRBC were assayed on day 4 and compared in cultures containing only lipid A (−SRBC) and cultures to which 3×10^6 SRBC (+SRBC) had been added at the start of cultures. (*B*) The immune responses to TNP were assayed on day 4 in (*1*) cultures containing only lipid A, (*2*) cultures containing lipid A and 10^{-6} M TNP-leu-tyr, and (*3*) cultures containing lipid A and 10^{-6} M TNP-leu-tyr, but were assayed in the presence of 10^{-6} M TNP_{15}-MSA in the plaque-forming mixture.

For the case studied here, the two-signal hypothesis, which we favor, is that the monovalent hapten delivers via the receptor a signal to the cell but the inductive stimulus is not completed until a second signal is delivered via LPS. Normally, the inductive signal is delivered as a consequence of recognition of two determinants coupled together on the antigen, one by the receptor and the other by the cooperating cell system. The experimental system used here reveals the independent delivery of the two signals because the determinants (monovalent hapten and LPS) are uncoupled.

Does the added hapten interact with receptors as a monomer or aggregate when it delivers one signal while LPS independently is delivering the other? The answer deals with the mechanism by which the first signal is delivered that a receptor-hapten interaction has occurred. The data do not favor that the added monovalent hapten acts as an aggregate. Monovalent haptens added to antisera are known not to polymerize antibody, fix complement, or activate histamine release, reactions which depend upon aggregation of the hapten. Furthermore, sufficiently stable nonspecific interactions with albumins, for example, would be via the hydrophobic TNP moieties making them unavailable

for other interactions. Given this interpretation, the only way that a hapten acting monovalently could deliver a signal to the cell is if a conformation change occurs in the receptor.

The question arises as to how LPS delivers a second signal to the B lymphocytes. The activity of LPS appears to be due to the lipid A structure (Fig. 6). It is likely that LPS and lipid A exert their effects by binding to the lipid bilayer of cell membranes. LPS may provide a second signal to B lymphocytes either by direct interaction with the membrane of these cells, or indirectly by its interaction with the cooperating cell system. A direct interaction of LPS with the membrane of B lymphocytes may lead to a signal that mimics that normally provided by the thymus-derived cooperating system. This is an interesting idea because it gives the term thymus independence a precise meaning. Unfortunately, this hypothesis is not applicable to other so-called thymus-independent antigens (SIII, dextran, PVP, etc.) because they do not stimulate an immune response to monovalent haptens (Table II). The idea that LPS acts via the cooperating cell system has two positions. The first position is that the integration of LPS into the B lymphocyte membrane may provide an array of determinants that are recognized by the thymus-derived cell system and this recognition results in the delivery of second signal to the B lymphocyte. The nude mouse cannot be assumed to be free of any thymus-derived contribution to the inductive stimulus (5). Most breeding programs involve matings between homozygous nude males (nu/nu) and heterozygous females (nu/+), thus permitting maternal (nu/+)-fetal (nu/nu) and fetal (nu/+)-fetal (nu/nu) transfer of cells, hormones, and antibodies that may restore, however minimally, the thymus function of the homozygote (nu/nu). However, the finding that concanavalin A and phytohemagglutinin do not stimulate DNA synthesis in nude spleen cultures (5) suggests that nude spleen cells are devoid of reactive T lymphocytes; therefore, it is unlikely that LPS is being recognized by these cells. A second alternative is that LPS may bind to other cell types found in the accessory cell system, namely macrophages (29, 30), and induce these cells to release humoral factors. Such factors may interact with B lymphocytes and provide a second signal for the completion of the inductive stimulus (31, 32).

The use of LPS to delineate the two signals required in the inductive process will be challenged because LPS has a number of different effects that need resolving:

(*a*) LPS stimulates, in the absence of added erythrocytes or TNP determinants, a low level response that is greatly enhanced by addition of the given antigen (Table I; [26]). This in itself should occasion no surprise since the culture medium containing bovine serum is likely to have in it cross-reacting substances that like monovalent haptens are rendered immunogenic by LPS. In view of the possibility that cross-reactions could be quite extensive (32, 34), the assumption is not unreasonable that the in vitro culture contain a large number of determinants recognizable by B lymphocyte receptors.

(*b*) LPS appears able to stimulate DNA synthesis in B, not T lymphocytes (4, 5, 9). Further, LPS stimulates DNA synthesis in most B lymphocytes (9, 35). This has led a number of workers to propose that a direct mitogenic effect of LPS on B cells only stimulates them to secrete antibody in the absence of a determinant-receptor interaction (4, 9). This is a competing hypothesis only if it is meant that a single signal, i.e. LPS acting as a nonspecific mitogen, is all that is required for induction of B, not T lymphocytes. This hypothesis does not account for the marked synergistic stimulation of a specific immune response by addition of a hapten to LPS-treated cells.

(*c*) LPS, when added to nude spleen cultures before the addition of antigen, inhibits the induction of subsequent immune responses to that antigen (Fig. 2).

These three effects have a common explanation if LPS is considered not to be a mitogen per se but is considered to act only as part of the inductive stimulus. The indications are that most B lymphocytes are stimulated to DNA synthesis by LPS (9, 35). If LPS provides one signal of the inductive stimulus, and, all B lymphocytes that begin DNA synthesis have received a complete inductive stimulus, then a large proportion of B lymphocytes at any given moment in time must be receiving the antigenic (first) signal. The question then arises as to how most B lymphocytes can be continuously receiving a signal via antigenic stimulation. Antibody-combing sites appear to be multifunctional in that they bind a number of different haptenic determinants. It has been reported that various purified myeloma proteins bind specifically a number of haptens (33), and that one mouse myeloma protein binds competitively two structurally dissimilar small haptens, DNP-lysine and 2 methyl-1:4 naptho-quinone thioglycolate, to different regions within the antibody combining site (34). Whether all antibodies have multiple binding function is not known. However if combining sites are capable of binding two or more dissimilar haptens, it is likely that all B lymphocytes bearing surface receptors may be capable of binding with determinants found on self-components. These receptor interactions may provide B lymphocytes with a constant source of antigenic stimulation. As B lymphocytes are generated, antigenic stimulation by cross-reacting self-components may allow cells to proceed part of the way along the differentiation pathway to antibody-forming cells. Completion of the inductive stimulus by providing LPS stimulates these B lymphocytes to divide and begin antibody synthesis. This interpretation explains why LPS, in the, absence of test antigen, stimulates background immune responses (4, 5, 9, 23–26). The synergistic stimulation of the immune response by added antigen and LPS may reflect that, in the presence of a constant amount of the second signal (LPS), the background immune response is limited by the rate at which B lymphocytes receive the first signal.

LPS can also suppress the immune response. When nude spleen cells are treated with LPS in the absence of added antigen, the cells immediately begin to lose the ability to respond to a subsequent challenge with that antigen (Fig.

2). Treatment of nude spleen cells for several hours with LPS decreases their ability to initiate immune responses to SRBC, and if this period is extended to between 20 and 30 h, the immune responses then initiated are less than 10% of the inducible response. The remaining immune response reflects the level of the background response to the test antigen. Similar observations have been reported for in vivo experiments. Treatment of mice with various doses of LPS decreases their ability to respond to SRBC when these erythrocytes are injected 1 or 2 days later (36, 37). Both these in vitro (Fig. 2) and in vivo data indicate that when antigen-sensitive cells are stimulated to cell division by LPS in the absence of a test antigen, the cells undergo changes that decrease their subsequent ability to be induced. Since this inhibitory effect of LPS is transient in nature (36, 37) these data suggest that LPS may also suppress the ability of cells to induce immune responses. This may reflect a property of the second induction signal. An excess of this signal may be capable of suppressing the induction pathway.

The interaction of antigen with the antigen-binding receptor on B lymphocytes has been postulated to lead to paralysis (38, 39). It has recently been shown that the paralytic stimulus delivered by deaggregated human immunoglobulin in adult mice can be converted into an inductive stimulus by LPS (40). These data imply that the antigenic signal delivered to cells is common for both inductive and paralytic pathways, therefore the events initiated in cells as a consequence of the antigenic signal are shared by both pathways. Thus the second signal of the inductive stimulus appears to divert cells from a paralytic to an inductive pathway. In order for a large proportion of cells to be induced by LPS in the background response (Table I), a large proportion of cells must be interacting with antigen. These interactions may be of low affinity so that the rate of paralysis of these cells is limited by the first signal. The level of the second signal upon addition of LPS is constant. In the absence of added antigen the rate of induction must be limited by the antigenic signal. It is because LPS reveals these interactions by completion of the inductive stimulus that a large number of B lymphocytes respond to LPS.

SUMMARY

Evidence is presented that antigen-sensitive cells require two signals for induction. Normally these two signals are delivered to the cell via the recognition of two determinants on the immunogen: the first the receptor on the antigen-sensitive cell, and the second by the cooperating cell system. The special experimental situation described here depends upon the observation that bacterial lipopolysaccharides (LPS) render immunogenic a variety of haptens. When monovalent haptens (TNP-amino acids) are added to spleen cultures, specific antihapten responses are induced in the presence of LPS. After analyzing competing interpretations of this phenomenon, we propose that the antigenic signal is delivered as the consequence of a conformational change

in the receptor upon interacting with antigen, and the second signal is delivered directly via the interaction of LPS with the membrane on the antigen-sensitive cell receiving the antigenic signal, or indirectly via the interaction of LPS with the cooperating cell population. These data imply LPS is not itself a mitogen, but merely completes an inductive stimulus to B cells. The experimental results from these and other studies indicate how these two signals may participate in inductive, suppressive, and paralytic stimuli to antigen-sensitive cells.

REFERENCES

1. Janossy, G., and M. F. Greaves. 1971. Lymphocyte activation. I. Response of T and B lymphocytes to phytomitogens. *Clin. Exp. Immunol.* **9**:483.
2. Greaves, M. F., S. Bauminger, and G. Janossy. 1972. Lymphocyte activation. III. Binding sites for phytomitogens on lymphocyte subpopulations. *Clin. Exp. Immunol.* **10**:537.
3. Stobo, J. D., A. S. Rosenthal, and W. E. Paul. 1972. Functional heterogeneity of murine lymphoid cells. *J. Immunol.* **108**:1.
4. Andersson, J., G. Möller, and O. Sjöberg. 1972. Selective induction of DNA synthesis in T and B lymphocytes. *Cell. Immunol.* **4**:381.
5. Watson, J., R. Epstein, I. Nakoinz, and P. Ralph. 1973. The role of humoral factors in the initiation of *in vitro* primary immune responses. II. Effects of lymphocyte mitogens. *J. Immunol.* **110**:43.
6. Holm, G., and P. Perlmann. 1965. Phytohaemagglutinin-induced cytotoxic action of unsensitized immunologically competent cells on allogeneic and xenogeneic tissue culture cells. *Nature (Lond.).* **207**:818.
7. Stavy, L., A. J. Treves, and M. Feldman. 1972. Capacity of thymic cells to effect target cell lysis following treatment with concanavalin A. *Cell. Immunol.* **3**:623.
8. Dutton, R. W. 1972. Inhibitory and stimulatory effects of concanavalin A on the response of mouse spleen cell suspensions to antigen. *J. Exp. Med.* **136**:1445.
9. Andersson, J., O. Sjöberg, and G. Möller. 1972. Mitogens as probes for immunocyte activation and cellular cooperation. *Transplant. Rev.* **11**:131.
10. Jones, J. M., and P. D. Kind. 1972. Enhancing effect of bacterial endotoxins on bone marrow cells in the immune response to SRBC. *J. Immunol.* **108**:1453.
11. Schmitdke, J. R., and F. Dixon. 1972. Immune response to a hapten coupled to a nonimmunogenic carrier. Influence of lipopolysaccharide. *J. Exp. Med.* **136**:392.
12. Lüderitz, O., O. Westphal, A. M. Staub, and H. Nikaido. 1971. Characterization of bacterial lipopolysaccharides. *In* Microbial Toxins. G. Weinbaum, S. Kadis, and S. J. Ajl, editors. Academic Press, New York. 145.
13. Rietschel, E. Th., H. Cottert, O. Lüderitz, and O. Westphal. 1972. Nature and linkages of the fatty acids present in the lipid-A component of *Salmonella* lipopolysaccharides. *Eur J. Biochem.* **28**:166.
14. Galanos, C., E. Th. Rietschel, O. Lüderitz, and O. Westphal. 1971. Interaction of lipopolysaccharides and lipid A with complement. *Eur. J. Biochem.* **19**:143.
15. Galanos, C., E. Th. Rietschel, O. Lüderitz, and O. Westphal. 1972. Biological activities of lipid A complexed with bovine serum albumin. *Eur. J. Biochem.* **31**:230.

16. Rietschel, E. Th., C. Galanos, A. Taraka, E. Ruschmann, O. Lüderitz, and O. Westphal. 1971. Biological activities of chemically modified endotoxins. *Eur. J. Biochem.* **22:**218.
17. Watson, J., and R. Epstein. 1973. The role of humoral factors in the initiation of *in vitro* primary immune responses. I. Effects of deficient fetal bovine serum. *J. Immunol.* **110:**31.
18. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* **126:**423.
19. Watson, J., and M. Thoman. 1972. A factor that can be used to regulate an *in vitro* primary immune response. *Proc. Natl. Acad. Sci. U.S.A.* **69:**594.
20. Okuyama, T., and K. Satake. 1960. On the preparation of properties of 2,4,6-trinitrophenyl-amino acids and -peptides. *J. Biochem.* (*Tokyo*). **47:**454.
21. Sher, A., and M. Cohn. 1972. Inheritance of an idiotype associated with the immune response of inbred mice to phosphorylcholine. *Eur. J. Immunol.* **2:**319.
22. Rittenburg, M., and K. Pratt. 1969. A nitrophenyl (TNP) plaque assay. Primary response of BALB/c mice to soluble and particulate immunogen. *Proc. Soc. Exp. Biol. Med.* **132:**575.
23. Pasanea, V. J., and O. Mäkelä. 1969. Effect of the number of haptens coupled to each erythrocyte on haemolytic plaque formation. *Immunology.* **16:**399.
24. Pasanea, V. J., and M. Virolainen. 1971. Anti-hapten response *in vitro*. Affinity differences in precursors of anti-hapten antibody-producing cells. *Eur. J. Immunol.* **1:**406.
25. Bullock, W. W., and E. Möller. 1972. "Spontaneous" B cell activation due to loss of normal mouse serum suppressor. *Eur. J. Immunol.* **2:**514.
26. Rank, W.-R., R. Di Pauli, and U. Flügge-Rank. 1972. The lipid A immunity system. I. Induction of heterophile antibodies by enterobacterial lipopolysaccharides and their lipid A component. *Eur. J. Immunol.* **2:**517.
27. Andersson, B., and H. Blomgren. 1971. Evidence for thymus-independent humoral antibody production in mice against polyvinylpyrrolidone and *E. coli* lipopolysaccharide. *Cell. Immunol.* **2:**411.
28. Davies, A. J. S., R. L. Carter, E. Leuchares, V. Wallis, and F. M. Dietrich. 1970. The morphology of immune reactions in normal, thymectomized and reconstituted mice. III. Response to bacterial antigens: salmonellar flagellar antigen and pneumococcal polysaccharide. *Immunology.* **19:**945.
29. Feldmann, M. 1972. Induction of immunity and tolerance in vitro by hapten protein conjugates. I. The relationship between the degree of hapten conjugation and the immunogenicity of dinitrophenylated polymerized flagellin. *J. Exp. Med.* **135:**735.
30. Feldman, M. 1972. Cell interactions in the immune response in vitro. II. The requirement for macrophages in lymphoid cell collaboration. *J. Exp. Med.* **135:**1049.
31. Dutton, R. W., R. Falkoff, J. A. Hirst, M. Hoffmann, J. W. Kappler, J. R. Kettmann, J. F. Lesley, and D. Venn. 1971. Is there evidence for a non-antigen specific diffusable chemical mediator from the thymus-derived cell in the initiation of the immune response. *In* Progress in Immunology. B. Amos, editor. Academic Press, New York. 355.
32. Gorczynski, R. M., R. G. Miller, and R. A. Phillips. 1973. Reconstitution of T

cell-depleted spleen cell populations by factors derived from T cells. I. Conditions for the production of active T cell supernatants. *J. Immunol.* **110:**968.

33. Schubert, D., A. Jobe, and M. Cohn. 1968. Mouse myelomas producing precipitating antibody to nucleic acid bases and/or nitrophenyl derivatives. *Nature (Lond.).* **220:**882.
34. Rosenstein, R. W., R. A. Musson, H. Armstrong, W. H. Konigsberg, and F. F. Richards. 1972. Contact regions for dinitrophenyl and menadione haptens in an immunoglobulin binding more than one antigen. *Proc. Natl. Acad. Sci. U.S.A.* **69:**877.
35. Shands, J. W., D. L. Peavy, and R. T. Smith. 1973. Differential morphology of mouse spleen cells stimulated *in vitro* by endotoxin, photohaemagglutinin, pokeweed mitogen and staphylococcal enterotoxin B. *Am. J. Pathol.* **70:**1.
36. Franzl, R. E., and P. D. McMaster. 1968. The primary immune response in mice. I. The enhancement and suppression of hemolysin production by a bacteria endotoxin. *J. Exp. Med.* **127:**1087.
37. McMaster, P. D., and R. E. Franzl. 1968. The primary immune response in mice. II. Cellular responses of lymphoid tissue accompanying the enhancement or complete suppression of antibody formation by a bacterial endotoxin. *J. Exp. Med.* **127:**1109.
38. Bretscher, P., and M. Cohn. 1970. A theory of self-nonself discrimination. *Science (Wash. D.C.).* **169:**1042.
39. Bretscher, P. 1972. The control of humoral and associative antibody synthesis. *Transplant. Rev.* **11:**217.
40. Chiller, J., and W. O. Weigle. 1973. Termination of tolerance to human gamma globulin in mice by antigen on bacterial lipopolysaccharide. *J. Exp. Med.* **137:** 740.