ACTIVATION OF T AND B LYMPHOCYTES IN VITRO

I. Regulatory Influence of Bacterial Lipopolysaccharide (LPS) on Specific T-Cell Helper Function*

By DIETER ARMERDING‡ and DAVID H. KATZ

(From the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115)

The elicitation of antibody responses to all but a selected class of antigens involves a series of apparently complex intra- and inter-cellular events (reviewed in reference 1) whose nature has remained largely obscure despite intensive analysis in recent years. Attempts to circumvent much of the obscurity concerning intercellular reactions (i.e. between T and B lymphocytes) have recently involved detailed experimental assessment of the action of various agents on the biological activation events of one or the other lymphocyte class. Most of these studies have focused on the cellular responses to a variety of mitogenic substances utilizing in vitro systems and several sources of *relatively* pure T or B lymphocyte populations (for review, see reference 2). Furthermore, in experiments on in vitro antibody production, the predominant antigen employed has been the intact heterologous erythrocyte from one or more species.

Bacterial endotoxin or lipopolysaccharide (LPS) has been a recent focal point of great interest because of its striking biological effects on lymphocytes. This material has been shown to be highly mitogenic for B lymphocytes (3–8) and to have rather potent adjuvant properties both in vivo (9–14) and in vitro (5, 8, 15–17). The target cell(s) of its adjuvant effects has not been conclusively established. Indeed, there are compelling data indicating that adjuvanticity of LPS reflects its action on B lymphocytes selectively (8, 11–13, 15, 16) and comparably compelling data that LPS has biological effects on T cells as well (9, 14). The present studies were undertaken to determine the nature of the effect of LPS on antibody production. We have done this by making comparative studies of the effects of LPS on in vitro primary and secondary antibody responses to soluble hapten-protein conjugates and to particulate and soluble sheep erythrocyte antigens. The results obtained demonstrate that a major biological effect of LPS on antibody production is mediated through its influence on antigen-specific helper T-cell function.

Materials and Methods

Proteins and Hapten-Carrier Conjugates.—Keyhole limpet hemocyanin (KLH) was purchased from Pacific Bio-Marine Supply Co., Venice, Calif. Ascaris proteins (ASC), extracted

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from *Ascaris suum* as described in reference 18, were kindly supplied by Dr. Kurt J. Bloch. Hen ovalbumin (OVA), 5X recrystallized, was obtained from Pentex Biochemical, Kankakee, Ill. Fowl γ-globulin (FγG) was precipitated from normal chicken serum with 33% saturated ammonium sulfate. Random copolymers of L-glutamic acid and L-lysine (L-GL) and of D-glutamic acid and D-lysine (D-GL) were purchased from Pilot Chemicals, Inc., Watertown, Mass. Both isomers had an average molecular weight of 55,000 and a ratio of G:L of 60:40.

The following 2,4-dinitrophenyl (DNP) conjugates were prepared as previously described (in references 19 and 20): DNP$_{14}$-KLH; DNP$_{2.1}$-ASC; DNP$_8$-OVA; DNP$_{20}$-FγG; DNP$_{50}$-L-GL; and DNP$_{37}$-D-GL. Subscripts refer to the number of moles of DNP per 100,000 mol wt units of KLH, moles of DNP $\times$ 10^{-7} per milligram of ASC protein, and moles of DNP per mole of carrier for the remainder.

Heterologous Erythrocyte Antigens.—Sheep red blood cells (SRBC) were obtained from Colorado Serum Company, Denver, Colo. For preparation of soluble red cell antigens, washed packed red cells were diluted 1:2 with fetal bovine serum and incubated 1 h at 37°C. The red cells were spun down and the supernatant filtered through a 0.45 μ Millipore filter (Millipore Corp., Bedford, Mass.).

Endotoxin.—Lipopolysaccharide B from *Escherichia coli* 026:B6 was obtained from Difco Laboratories, Detroit, Mich., and was stored frozen at a concentration of 1.0 mg/ml in sterile Hanks's balanced salt solution.

Animals and Immunizations.—Mice of the inbred lines BALB/c and (BALB/c $\times$ A/J)F$_1$ hybrids (CAF$_1$), DBA/2, and C$_{57}$BL/6 were obtained from the Jackson Laboratory, Bar Harbor, Maine. Mice were immunized at 2–3 mo of age intraperitoneally with 100 μg of either unconjugated proteins or DNP-protein conjugates emulsified in complete Freund's adjuvant (CFA, Difco) and were used as primed spleen cell donors 3–6 mo thereafter. Mice primed with SRBC received a single intraperitoneal injection of 10^8 SRBC 2 mo prior to culture.

Depletion of T Lymphocytes.—The preparation of anti-θ-serum, determination of anti-θ. serum cytotoxicity, and anti-θ-treatment of spleen cells have been previously described (21)-

Spleen Cell Cultures.—The Mishell-Dutton system (22) was used with the following modifications: spleen cells were cultured for 3–5 days in Micro-Test II tissue culture plates (Falcon Plastics, Division of BioQuest, Oxnard, Calif). Preliminary studies in our laboratory showed that cells could be cultured without rocking and without supplemental daily feeding. Daily feeding of cultures in this microsystem with fetal calf serum and nutritional cocktail did not alter the magnitude of antibody responses obtained in comparative cultures provided with supplemental nutrients. By pilot experiments we also established optimal conditions of cell density, total volume, and antigen concentration. Cultures were set up in a total volume of 0.3 ml in three steps; antigen, endotoxin (or plain medium in appropriate controls), and cells were added to the culture wells in this order. The pipetted volume of each was 0.1 ml. All concentrations referred to in the figures and tables presented herein represent concentrations of the respective components dispensed into the wells (i.e. final concentrations in the actual cultures are 3-fold lower). Each culture well contained 0.5–1.0 $\times$ 10^6 spleen cells (i.e. final cell density of 1.67–3.33 $\times$ 10^6 cells/ml).

Determination of In Vitro Antibody Responses.—After 3–5 days of incubation, cells from triplicate culture wells were harvested, pooled, and washed in Hanks's balanced salt solution. Plaque-forming cells (PFC) were assayed by a modification of the hemolytic plaque technique (23, 24) for immunoglobulin M (IgM) and immunoglobulin G (IgG) anti-DNP and anti-SRBC antibody-producing cells. SRBC lightly conjugated with 2,4,6-trinitrobenzene-sulfonic acid (TNP) (25) and unconjugated SRBC were used as indicator cells. IgG PFC were developed using a rabbit antimouse immunoglobulin-facilitating serum. The numbers of DNP-specific PFC reported are corrected for background SRBC antibody-forming cells. To permit comparison with results obtained in cell cultures set up in conventional Petri dishes (10 $\times$ 10^6 cells/ml) all data presented here is calculated on the basis of 10^7 cultured cells.

RESULTS

Induction of Spontaneous In Vitro IgM and IgG Anti-DNP Antibody Production by LPS.—Unprimed mouse spleen cells develop primary in vitro anti-DNP antibody responses to soluble DNP-protein conjugates of variable magnitude in our hands. The first series of experiments were designed to determine the effect of exposing unprimed spleen cells to LPS or to LPS plus DNP-KLH. A representative pattern of DNP-specific PFC responses in five separate experiments is presented in Table I. Cells were cultured either in the presence or absence of an optimal dose of DNP-KLH (100 µg/ml) with or without LPS (200 µg/ml). Note that: (*a*) the development of primary anti-DNP responses to DNP-KLH (without LPS) is variable, ranging from no detectable response (exp. 2) to very high responses (exp. 5); (*b*) the responses obtained occur in both IgM and IgG classes; (*c*) in cultures devoid of added DNP-KLH, the presence of LPS at this dose induced appreciable numbers of IgM and IgG anti-DNP plaques; and (*d*) the addition of DNP-KLH to cultures also containing LPS either suppressed or had no effect on the LPS-induced antibody response (but clearly did not further increase the antigen-independent response). In other experiments not shown, comparable results were obtained with other DNP-carrier conjugates (DNP-FγG, DNP-D-GL).

Effects of LPS on Antigen-Induced Secondary In Vitro Anti-DNP Antibody

TABLE I

Induction of Spontaneous In Vitro IgM And IgG Anti-DNP Antibody Production by LPS*

Exp.	Strain	In vitro antigen	Anti-DNP Antibody Response			
			IgM PFC		IgG PFC	
			No LPS	LPS	No LPS	LPS
				200 µg/ml		*200 µg/ml*
1	BALB/c	None	0	1,782	0	1,848
		DNP-KLH	222	820	120	1,000
2	BALB/c	None	260	2,580	210	1,910
		DNP-KLH	280	2,820	250	2,240
3	CAF₁	None	106	625	93	559
		DNP-KLH	300	520	240	480
4	BALB/c	None	120	660	40	510
		DNP-KLH	580	390	240	210
5	BALB/c	None	0	4,020	0	2,840
		DNP-KLH	1,420	3,020	1,160	3,040

* Unprimed BALB/c or CAF₁ spleen cells were cultured at a density of 3.33×10^6 cells/ml for 4 days with or without antigen (DNP-KLH, 100 µg/ml). Anti-DNP PFC per 10^7 cultured cells are given.

Responses.—The failure of LPS to enhance the primary anti-DNP antibody response could be explained on the basis that LPS acts directly on B cells and that receptors for it and immunoglobulin receptors for antigen are located in close proximity on the cell surface (16). This situation could result in steric hindrance of binding of one of each components. Alternatively, this result could reflect a requirement for a minimum amount of specific helper T-cell function, in order to observe an enhancing effect of LPS on antigen-induced responses. This possibility should be demonstrable and dependent upon the presence and quantity of specific helper T cells for the relevant carrier determinants being studied.

The following series of experiments were designed to delineate the role of T cells in the enhancing action of LPS on in vitro anti-DNP antibody responses.

Enhancement of Secondary Anti-DNP Responses to the Homologous DNP-Carrier Conjugate by LPS.—Spleen cells from DNP-KLH-primed BALB/c or CAF$_1$ mice were cultured with or without antigen, either the homologous DNP-KLH conjugate or a heterologous conjugate such as DNP-ASC or DNP-OVA, in the presence or absence of varying concentrations of LPS. The results of several such experiments concentrating on several different parameters are illustrated in Figs. 1–3.
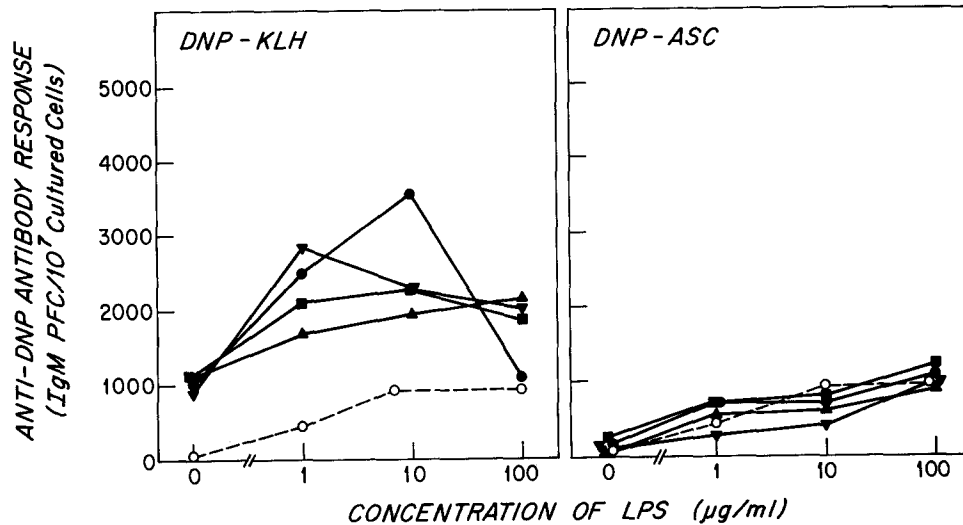

FIG. 1. Effect of LPS on secondary in vitro anti-DNP antibody response. Spleen cells from BALB/c mice primed with DNP-KLH 6 mo previously were cultured for 4 days in the presence of varying doses (0.1–100.0 μg/ml) of DNP-KLH (left panel) or DNP-ASC (right panel) or no antigen (dashed line: the same data are plotted in the left and right panel). The concentration of LPS was varied from 0–100 μg/ml. The final cell concentration was 3.33 × 10^6 cells/ml. Only the IgM anti-DNP-PFC responses are shown. Antigen concentration: O, none; ●, 0.1 μg/ml; ▲, 1.0 μg/ml; ■, 10.0 μg/ml; and ▼, 100.0 μg/ml.

Fig. 1 demonstrates that the secondary response to DNP-KLH (left panel) can be markedly enhanced by the addition of LPS and that the degree of enhancement is related to concentration employed of both antigen and LPS. Note that the optimal LPS concentration is again 1–10 μg/ml and that higher doses tend to diminish the maximal response obtained. It is also noteworthy that the peak PFC response elicited by LPS plus antigen occurred with the lowest dose (0.1 μg/ml) of DNP-KLH used. In contrast, these DNP-KLH-primed cells could not be triggered to develop antibody responses by DNP-ASC plus LPS in any of the dose combinations employed (right panel). Comparable data have been obtained using DNP-ASC-primed spleen cells exposed to DNP-ASC or DNP-KLH as in vitro antigens.

Fig. 2 reiterates these findings in DNP-KLH-primed CAF$_1$ spleen cell cultures
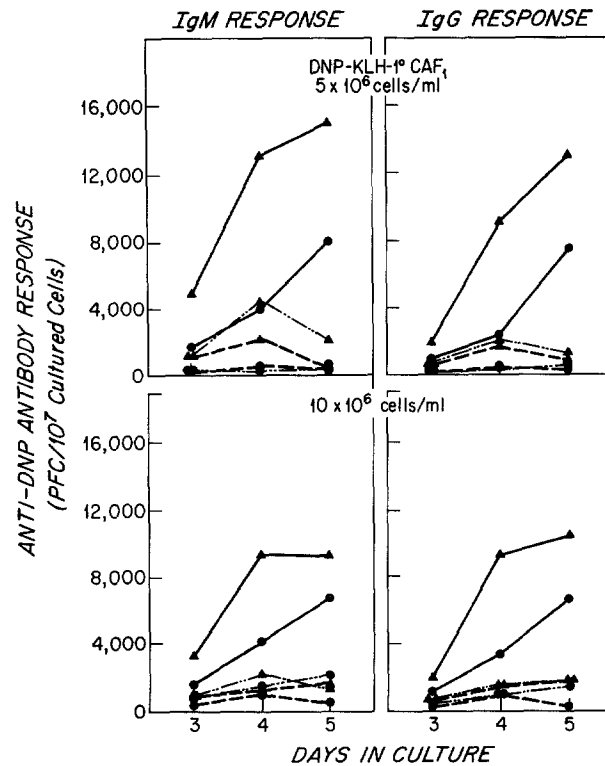

FIG. 2. Kinetics of enhancement of secondary anti-DNP antibody response by LPS. Spleen cells from CAF$_1$ mice primed with DNP-KLH 7 mo previously were cultured for 3, 4, or 5 days without antigen or in the presence of 1.0 μg/ml DNP-KLH either with or without LPS (10 μg/ml). Cell density was 1.67 × 10^6 cells/ml (upper panel) or 3.33 × 10^6 cells/ml (lower panel). The IgM (left panel) and the IgG (right panel) anti-DNP-PFC responses are shown. ●, no LPS; ▲, LPS (10 μg/ml); ——, DNP-KLH; ———, DNP-OVA, and —·—·—, no antigen.

with the additional exploration of parameters of kinetics and cell density ef-
fects. Thus, LPS enhancement of the DNP-KLH-induced response clearly
follows a similar kinetic course as that of the response to DNP-KLH alone,
namely rising sharply from day 3 to day 4 and continuing to increase on day 5.
This pattern contrasts distinctly from the kinetics of development of PFC
induced by LPS in the absence of added antigen which peaks by day 4 and falls
off by day 5. It is interesting that the maximal augmentation by LPS of the
DNP-KLH-induced response was obtained at the lower cell density (5×10^6
cells/ml, top panel) although the kinetic pattern did not differ appreciably from
that observed at a density of 10×10^6 cells/ml (lower panel). The effects on
IgM and IgG antibody synthesis tended to parallel one another. Finally, note
again the failure of LPS to stimulate responses to a heterologous carrier con-
jugate, DNP-OVA.

The experiment depicted in Fig. 3 was designed to study in detail the difficulty
of eliciting an anti-DNP response with a heterologous DNP carrier in the pres-
ence of LPS. One possible reason for this might be that the presence of free
DNP carrier in relative excess could compete with LPS indirectly for accessi-
bility to respective cell surface receptor sites. In order to minimize this pos-
sibility, DNP-KLH-primed BALB/c spleen cells were either cultured as usual
with antigen for 4 days ($0.1 \mu g$/ml of DNP-KLH, DNP-OVA, or DNP-D-GL)
or preincubated with one of the latter conjugates ($1 \mu g/10^6$ cells at 4°C for 30
min) and then washed thoroughly before dispensing into the culture wells.
Hence, the only antigen incorporated into the latter cultures would be that
which is cell-bound (i.e. predominantly to lymphocytes or macrophages). All
cells were then cultured in the absence or presence of LPS in one of three doses.

As shown in Fig. 3, LPS at a concentration of $1.0 \mu g$/ml again markedly
enhanced the secondary response to DNP-KLH. This was considerably greater
in cultures containing antigen throughout than in cultures of preincubated cells,
but was demonstrable in the latter case to some extent as well. Note that in the
absence of LPS, antigen-preincubated cells developed somewhat higher re-
sponses than those elicited by constantly present antigen. As shown previously,
DNP conjugated to unrelated carriers is unable to act synergistically with LPS
in inducing anti-DNP antibody responses. In Fig. 3, only the results obtained
with OVA and D-GL are depicted, but comparable data have been obtained
using ASC, L-GL, or FγG as heterologous carriers.

*Enhancement by LPS of Primary In Vitro Anti-DNP Antibody Responses of
Carrier-Primed Spleen Cells.*—The preceding results are most likely explained
by the presence of carrier-reactive T cells in sufficient frequency in primed
spleen cells to manifest LPS-related effects on antigen-induced responses. The
present experiment investigates this question directly by examining the effects
of LPS on the magnitude of augmented primary in vitro anti-DNP responses of
various carrier-primed spleen cells. Spleen cells from unprimed CAF$_1$ mice and
from CAF$_1$ mice primed with either unconjugated ASC, KLH, or FγG were

cultured either alone or in the presence of DNP-ASC, DNP-KLH, or DNP-FγG with or without LPS.

The general protocol and the data from this experiment are summarized in Fig. 4. Unprimed spleen cells were spontaneously induced to anti-DNP anti-
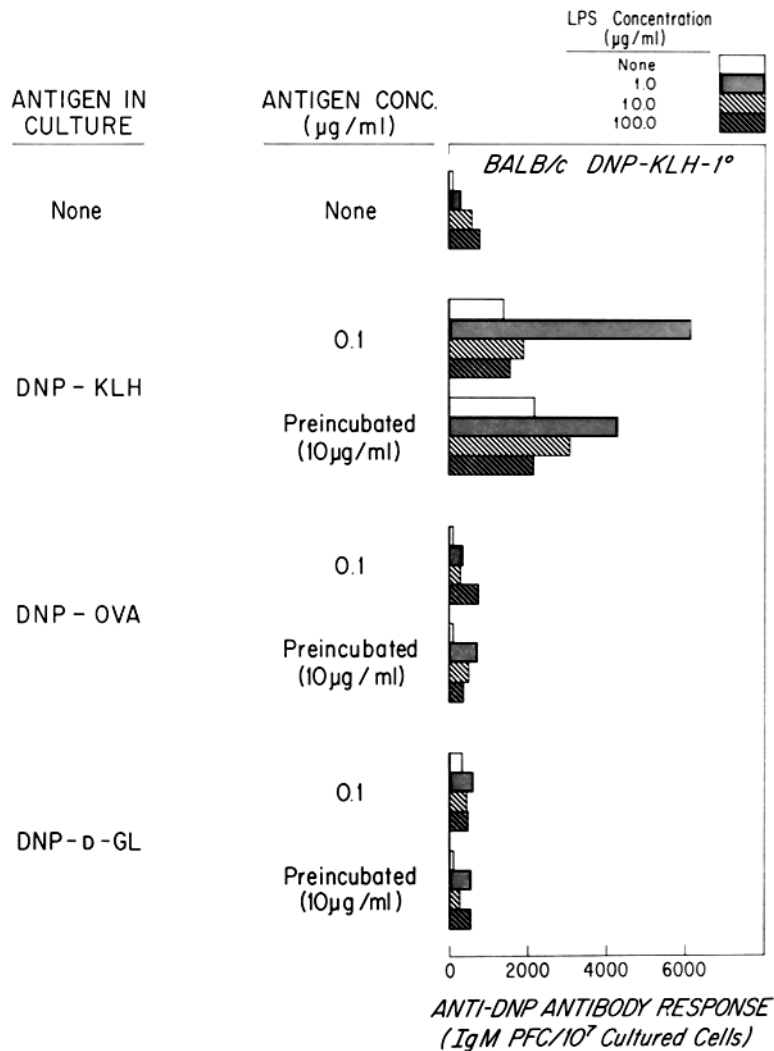
FIG. 3. Effect of LPS on the secondary anti-DNP antibody response to homologous and heterologous DNP-carrier conjugates. Spleen cells from BALB/c mice primed with DNP-KLH 6 mo previously were cultured in the presence of no antigen or one of the following DNP-carrier conjugates: DNP-KLH, DNP-OVA, or DNP-D-GL. Antigen concentration was 0.1 µg/ml or cells were preincubated with antigen (see Results). Final cell concentration was 3.33×10^6 cells/ml. The IgM anti-DNP-PFC responses of cells cultured with or without LPS (1 to 100 µg/ml) are shown.
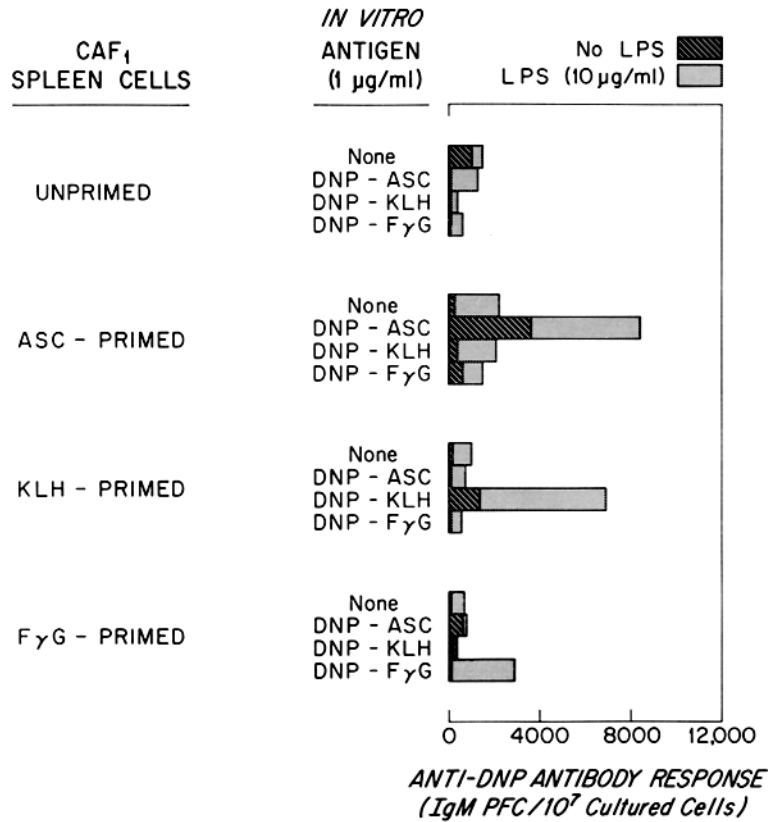
FIG. 4. Enhancement by LPS of primary in vitro anti-DNP antibody responses of carrier-primed spleen cells. Spleen cells from CAF_1 mice either unprimed or primed 4 mo previously with FγG or ASC, or 5 mo previously with KLH were cultured for 5 days in the presence of 1 μg/ml of either DNP-ASC, DNP-KLH, or DNP-FγG or without any antigen. The IgM anti-DNP-PFC responses of cells cultured without (dark hatching) or with (gray hatching) LPS (10 μg/ml) were compared. Final cell concentration was 1.67×10^6 cells/ml.

body formation to a greater extent by LPS alone than by any of the DNP-carrier conjugates used at this dose. Carrier-primed spleen cells, with the exception of the FγG cells, developed considerably higher primary responses to the appropriate DNP conjugate of the carrier to which they were primed. The most notable finding in this experiment relates, however, to the enhancing effects of LPS on these various responses. Thus, enhancement by LPS was extraordinarily selective in that only responses to the DNP carrier for which helper T cells had been specifically primed were so affected. This is particularly striking in the case of the FγG-primed cells which in the absence of LPS failed to develop an expected augmented primary response to DNP-FγG, whereas with LPS in the culture, DNP-FγG elicited nearly 3000 IgM anti-DNP PFC.

Enhancement by LPS of Specific Helper Cell Function in Cooperative Secondary In Vitro Anti-DNP Antibody Responses.—In the preceding experiment, it was shown that LPS enhanced primary anti-DNP antibody responses of carrier-primed spleen cells presumably by its effects on helper cell function. Another direct approach to this issue is to evaluate the effect of LPS on cooperative secondary in vitro anti-DNP responses elicited from mixtures of independently primed DNP-specific B cells and carrier-specific T cells. The protocol and data from such an experiment are summarized in Fig. 5. The cells employed con-
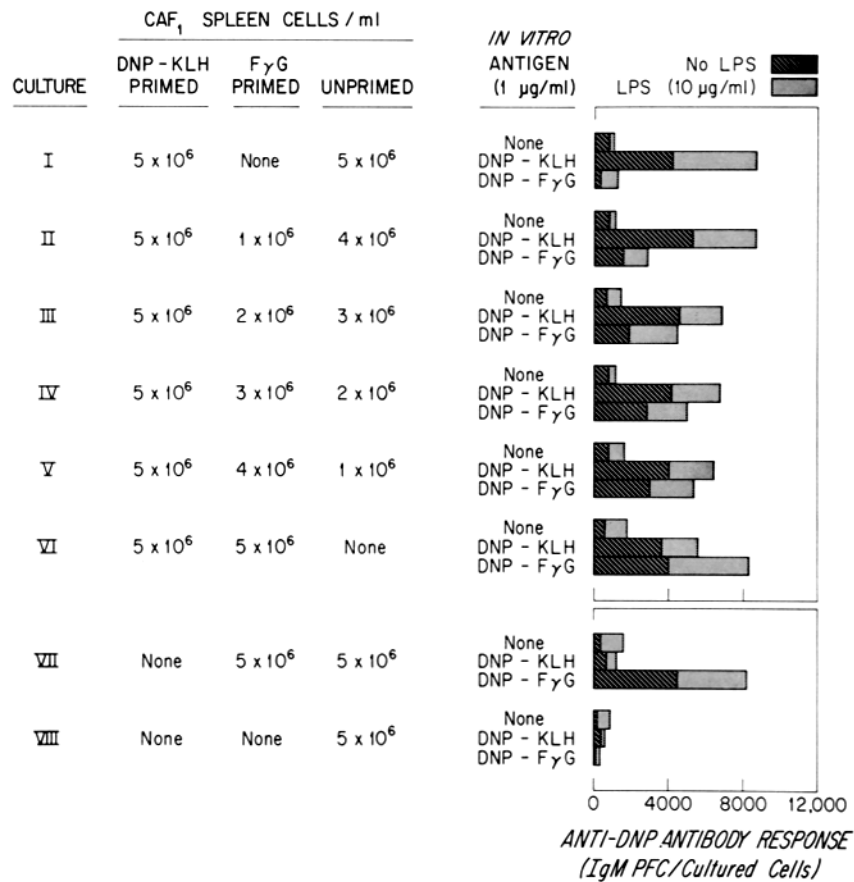

Fig. 5. Enhancement of specific helper cell function in cooperative secondary in vitro anti-DNP antibody responses. Three types of CAF_1 spleen cells were used: (a) unprimed; (b) FγG-primed (3 mo previously); and (c) DNP-KLH-primed (7 mo previously). Cells were cultured in the presence of 1 μg/ml DNP-KLH, DNP-FγG, or no antigen either with (gray hatching) or without (dark hatching) LPS (10 μg/ml). Various cell mixtures were made, as shown. Final cell concentration was 3.33 × 10⁶ cells/ml, except for unprimed cells alone (1.67 × 10⁶ cells/ml). The IgM anti-DNP-PFC responses on day 4 are shown.

sisted of (*a*) DNP-KLH-primed, (*b*) FγG-primed, and (*c*) unprimed CAF$_1$ spleen cells. Various mixtures of these cells were made keeping the final cell density constant at 10^7/ml. The mixtures were then cultured for 4 days in the presence of DNP-KLH, DNP-FγG, or no antigen (for background controls) with or without LPS.

As shown in Fig. 5, in the absence of added LPS, 5 × 10^6 FγG-primed cells displayed a very good augmented primary response to DNP-FγG but no significant response to DNP-KLH (culture VII). The response to DNP-FγG was markedly enhanced, as before, by addition of LPS. The importance of the presence of carrier-primed cells to obtain these responses is reiterated by the failure of 5 × 10^6 unprimed spleen cells alone to manifest any appreciable response (group VIII).

The particularly relevant data is shown by culture groups I-VI in Fig. 5. DNP-KLH-primed spleen cells (5 × 10^6) mixed with an equal number of unprimed spleen cells responded very well to DNP-KLH—a response enhanced by the addition of LPS—but failed to respond to DNP-FγG irrespective of whether or not LPS was added (culture I). On the other hand, addition of FγG-primed cells, even at the lowest number used (1 × 10^6/ml) resulted in development of secondary anti-DNP responses to DNP-FγG in the absence of LPS, the magnitude of which were clearly related in an increasing linear fashion to the increasing concentration of FγG-primed cells added (cultures II-VI). The addition of LPS to these mixed cell cultures resulted in significant increases in the responses to both DNP-KLH and to DNP-FγG. It is the effect on the latter response that is especially relevant since it clearly requires the presence of the FγG-primed helper cells to be observed. This interpretation follows from the 2 to 3-fold increase in responses to DNP-FγG obtained by adding LPS to cultures II-VII, the absolute magnitude of which always exceeded spontaneous induction by LPS (in absence of added antigen) in these cultures as well as the undetectable effect of LPS on the response to DNP-FγG in culture I. Although only IgM PFC data are illustrated in Fig. 5, parallel results were obtained for the IgG antibody response.

Finally, note that when equal numbers of DNP-KLH and FγG-primed cells are mixed, the magnitudes of responses induced by DNP-KLH and DNP-FγG are nearly the same (culture VII). Moreover, the addition of carrier-primed cells tends to diminish somewhat the magnitude of the antibody response to the homologous conjugate, DNP-KLH (cf. culture groups II and VI). This has been our consistent observation in many such cultures and lacks a definitive explanation. This tendency of inhibition of the DNP-KLH response in such cell mixtures has a bearing on the present experiment since it reflects an undefined influence that apparently prevents DNP-specific memory cells from developing the full height of the secondary response. This probably explains why a mixture of FγG-primed and unprimed cells (culture VII) developed even higher anti-DNP PFC responses than that induced in mixtures of DNP-KLH- and FγG-

primed cells (culture VI). Nevertheless, it is clear from the data that the en-
hancement of antigen-dependent secondary responses by LPS is strictly cor-
related to the presence and frequency of the appropriate helper T cells.

*Enhancement of Primary In Vitro IgM and IgG Anti-SRBC Antibody Re-
sponses by LPS.*—The results of the preceding experiments demonstrate clearly
that LPS exerts a substantial biologic effect on T-cell-helper function. Since
these conclusions are somewhat at variance with other reports of the effects of
LPS on in vitro antibody responses to SRBC we felt it essential to elucidate
the nature of the possible differences. Hence, we evaluated the effect of LPS
on spontaneous and antigen-induced anti-SRBC antibody responses in vitro.
The results of representative experiments in four different mouse strains are
summarized in Table II. The addition of LPS alone to unprimed cells induces a
significant increase in numbers of "background" anti-SRBC plaques. In con-
trast to the results of anti-DNP responses, the incorporation of particulate
SRBC into cultures also containing LPS resulted in a marked enhancement
of the antigen-induced primary response in both IgM and IgG antibody classes.
The magnitude of this enhancing effect of LPS on the antigen-dependent re-
sponse was as much as 30-fold and was not correlated with the height of the
response in any one culture to SRBC alone (cf. exps. 3–5).

Enhancement of Secondary In Vitro Antibody Responses to Particulate SRBC

TABLE II

Enhancement of Primary In Vitro IgM And IgG Anti-SRBC Antibody Responses by LPS*

Exp.	Strain	In vitro antigen	Anti-SRBC Antibody Response			
			IgM PFC		IgG PFC	
			No LPS	LPS	No LPS	LPS
				200 μg/ml		*200 μg/ml*
1	BALB/c	None	150	1,600	20	1,450
		SRBC	3,600	11,320	3,420	12,120
2	DBA/2	None	93	1,180	53	320
		SRBC	2,040	2,760	1,110	1,770
3	C57BL/6	None	140	1,700	160	450
		SRBC	1,050	30,000	1,880	16,050
4	CAF₁	None	160	1,676	53	718
		SRBC	720	7,920	220	5,920
5	BALB/c	None	0	732	0	585
		SRBC	216	10,017	216	6,075

* Unprimed spleen cells (3.33×10^6 cells/ml) from various mouse strains were cultured
for 4 days in the absence or presence of SRBC (1%), without or with LPS (200 μg/ml). The
figures presented are PFC to SRBC per 10^7 cultured cells.

by LPS.—The contrasting effects of LPS on primary in vitro antibody responses to DNP-KLH (Table I) and SRBC (Table II), respectively, might be related to differences in presentation of antigenic determinants on these two antigens inasmuch as one is predominantly particulate while the other is in soluble form. This possibility is testable by comparing the effects of LPS on responses to soluble and particulate SRBC antigens, respectively.

In this experiment, spleen cells from SRBC-primed BALB/c mice were cultured in the presence of 3 log increment doses of particulate (intact) or soluble SRBC either without LPS or with LPS added in one of three different concentrations. The resulting IgM (left panel) and IgG (right panel) secondary anti-SRBC responses are illustrated in Fig. 6. Several points are noteworthy about the data: the magnitudes of the secondary responses in both antibody classes obtained in the absence of LPS varied with the dose of antigen employed, and the highest IgG response elicited by soluble SRBC was comparable with that obtained with particulate SRBC. The effect of adding LPS to these cultures, however, was quite clearly related to the form of in vitro SRBC antigen employed. Thus, in the cultures containing soluble SRBC, the addition of LPS
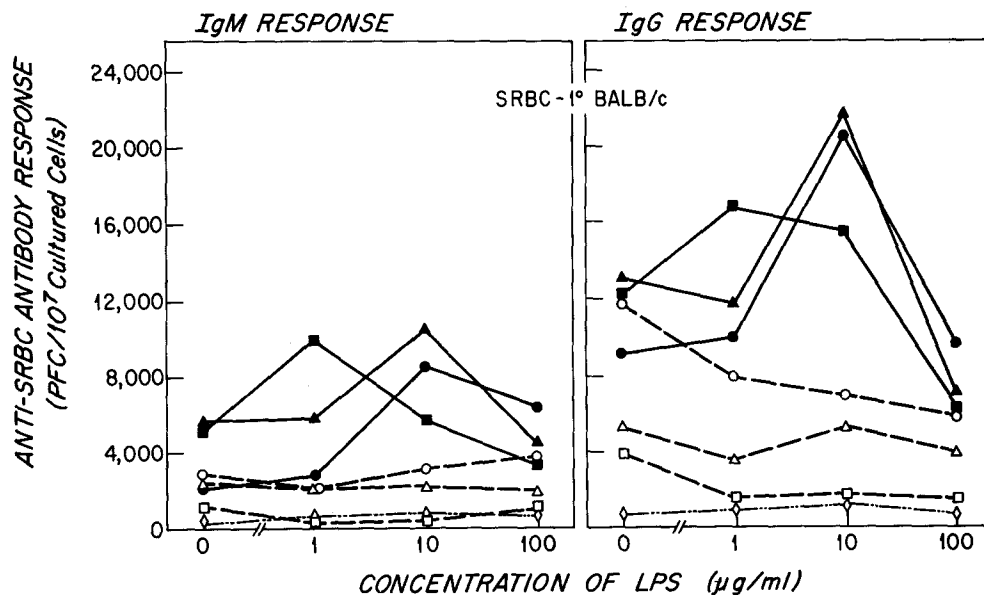

FIG. 6. Enhancement of secondary in vitro IgM and IgG anti-SRBC antibody response by LPS. Spleen cells from BALB/c mice primed 2 mo previously with 10^8 SRBC intraperitoneally were cultured for 4 days in the presence of varying doses of particulate (0.001–0.1%) or soluble (1:1000–1:10) SRBC either with or without LPS (1, 10, or 100 μg/ml). Final cell concentration was 3.33 × 10^6 cells/ml. The IgM (left panel) and IgG (right panel) anti-SRBC-PFC responses are shown. ◇‑‑‑‑‑◇, No antigen. Particulate SRBC: ●—●, 0.1%; ▲—▲, 0.01%; and ■—■, 0.001%. Soluble SRBC: O‑‑‑O, 1:10; △‑‑‑△, 1:100; and □‑‑‑□, 1:1000.

affected the IgM response only appreciably at the lowest dose of antigen (1:10 dilution) and the highest dose of LPS (100 µg/ml) employed. The IgG antibody response to soluble SRBC tended to be inhibited by increasing concentrations of LPS. This was also true for both IgM and IgG responses to particulate SRBC at the dose of 100 µg/ml of LPS. However, lower doses of LPS (1 or 10 µg/ml) significantly augmented the responses in both antibody classes, but, especially in the IgG class, to particulate SRBC, the extent of which was related to the concentration of antigen used.

Failure of LPS to Completely Substitute for Helper Cell Function in Primary In Vitro Anti-SRBC Antibody Responses.—In the last experiment we examined the extent to which LPS might "substitute" for helper T cells in an in vitro primary response to particulate SRBC. The data from two representative experiments using unprimed spleen either untreated or depleted of T lymphocytes by treatment with anti-θ-serum plus complement are presented in Table III. Once again, note that the antigen-induced primary response is significantly enhanced by this optimal dose of LPS. Anti-θ-serum treatment markedly reduced not only the response to antigen alone, but the effect of LPS on the antigen-induced response as well. The fact that the anti-SRBC antibody response of anti-θ-treated cells to SRBC is nevertheless still enhanced by LPS as compared with the response of such cells to SRBC alone argues as much for an effect of the LPS on residual helper T-cell function as directly on B cells. Moreover, although not presented here, we were able to completely reconstitute

TABLE III

Failure of LPS to Completely Substitute for Helper Cell Function in Primary In Vitro Anti-SRBC Responses *

Exp.	Strain	Cells	In vitro antigen	Anti-SRBC Antibody Response			
				IgM PFC		IgG PFC	
				No LPS	LPS	No LPS	LPS
					200 µg/ml		*200 µg/ml*
1	BALB/c	Untreated	None	150	1,600	20	1,450
			SRBC	3,600	11,320	3,420	12,120
		Anti-θ-treated	None	30	350	0	50
			SRBC	500	1,820	500	1,240
2	DBA/2	Untreated	None	93	1,180	53	320
			SRBC	2,040	2,760	1,110	1,770
		Anti-θ-treated	None	80	519	40	253
			SRBC	240	800	40	460

* Unprimed normal or anti-θ-serum treated spleen cells (3.33 × 10^6 cells/ml) from BALB/c or DBA/2 mice were incubated with or without SRBC (1%), in the absence or presence of LPS (200 µg/ml) for 4 days. Figures presented are PFC per 10^7 cultured cells.

the antibody response to SRBC in exp. 2 with a factor(s) obtained from mixed lymphocyte cultures (manuscript in preparation).[1] Finally, other experiments on the secondary in vitro anti-DNP antibody response of anti-θ-treated spleen cells also indicate that LPS cannot substitute for helper cell function.

DISCUSSION

In these studies we have analyzed in detail the effects of LPS on cells involved in antibody production in vitro. It has been previously reported by others that addition of LPS to cultures of unprimed mouse spleen cells induces spontaneous (e.g. antigen-independent) antibody production to a variety of haptenic and erythrocyte antigens (3, 5, 16, 17). We have likewise demonstrated spontaneous synthesis of anti-DNP (Table I) and anti-SRBC (Table II) antibodies following exposure of unprimed spleen cells to an appropriate dose of LPS. This antigen-independent induction of antibody synthesis by LPS most likely reflects, as suggested by others (3, 5, 16, 17), a direct effect of this substance on B lymphocytes.

However, this antigen-independent effect of LPS on B cells does not explain the biological adjuvanticity of this molecule on antigen-induced immune responses. Thus, the evidence presented here demonstrates that LPS exerts a considerable effect on T-cell-mediated helper function. This conclusion derives from the following series of observations: (a) Spleen cells from mice primed to DNP-KLH develop considerably enhanced secondary in vitro anti-DNP antibody responses when exposed to LPS in the presence of antigen, provided the antigen employed is the DNP derivative of the original immunizing carrier, i.e. KLH (Figs. 1–3). Incorporation of a heterologous carrier conjugate, such as DNP-OVA or DNP-ASC completely fails to induce antibody responses irrespective of whether or not optimal doses of LPS are also present. (b) Similarly, whereas unprimed spleen cells fail to develop primary anti-DNP responses to DNP-proteins in the presence of LPS (Table I), spleen cells from mice that were previously primed to carrier protein develop augmented primary responses to the DNP derivative of the specific immunizing carrier and these responses are significantly enhanced further by the addition of LPS (Fig. 4). Again, further enhancement by LPS is dependent upon the use of the DNP conjugate of the original immunizing carrier; heterologous carrier conjugates fail to induce responses. (c) Finally, cooperative anti-DNP secondary responses to DNP-FγG obtained with admixtures of DNP-KLH-primed B cells and FγG-primed helper T cells were enhanced by LPS to varying degrees depending on the frequency of FγG-primed T cells incorporated into the culture (Fig. 5). Thus, increasing the concentration of FγG helper T cells resulted in development of anti-DNP responses of progressively higher magnitude in the absence of LPS which were clearly related in an increasing linear fashion to the concentration of FγG cells added. The addition of LPS to such cultures further enhanced the

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[1] D. Armerding and D. H. Katz. 1973. Manuscript in preparation.

magnitude of responses observed but maintained the linear increase related to the increasing frequency of FγG-specific cells.

Taken collectively these observations are interpreted by us to indicate that the biological action of LPS in vitro may be predominantly manifested on the *function* of B cells or T cells or both depending on whether or not: (*a*) antigen is present; (*b*) the antigen is particulate or soluble; and (*c*) the cells are primed. In the absence of antigen, we contend that LPS acts primarily on B lymphocytes. In the presence of antigen, however, the data show that LPS influences helper T cell function in a highly significant manner and we believe that it is this influence that is predominantly responsible for the adjuvant effect of LPS on antigen-specific antibody responses. This conclusion derives in large part from the demonstrated effects, discussed above, of LPS on in vitro responses requiring the presence of carrier-primed helper T cells.

The conclusions drawn from our studies appear to be at variance with many of the recently reported observations on the effects of LPS on lymphocytes. There are, however, plausible explanations for certain of the seemingly contradictory results. Firstly, it is evident that the biological activity of LPS on cells of the immune system is multifaceted and varies with the conditions under which it is employed. The highly preferential mitogenic effect of LPS on B lymphocytes under normal conditions in vitro has been amply documented (3–8). Moreover, the stimulation by LPS of antibody production in relatively pure B lymphocyte populations in vitro (5, 8 15–17) and in relatively thymus-deprived mice in vivo (10, 11, 13) has been reported. These observations have created the impression that, insofar as antibody production is concerned, LPS acts on B cells in a biologically analogous manner to the obligate second signal normally provided by specific T lymphocytes. On the other hand, certain studies on adjuvant properties of LPS in vivo have demonstrated that the presence of T cells is required for such effects to be observed. This was shown by Allison and Davies (9) in antibody responses of thymus-deprived mice to bovine serum albumin, and by Hamaoka and Katz (14) in adoptive transfer secondary anti-DNP responses to DNP-carrier conjugates. The studies reported herein provide the first documentation of the effect of LPS on T-cell helper function in an in vitro antibody response system.

How, then, can these discrepancies be reconciled? One immediately apparent point is that the effects of LPS on lymphocytes in the absence of exogenous antigen are qualitatively and quantitatively distinct from its effects in the presence of exogenous antigen. This reasoning follows from several observations made by others and ourselves: (*a*) Stimulation of antibody production by LPS in the absence of antigen is a phenomenon easily demonstrable in vitro with either unprimed or primed spleen cells, but does not occur to any appreciable extent in the intact animal (14); (*b*) The in vitro antigen-independent phenomenon follows a kinetic pattern that is distinctly different from the kinetics of antigen-dependent antibody responses, and the latter follow identical kinetics

both in the presence or absence of LPS (Fig. 3); (c) The antigen-independent LPS effect is clearly multispecific, since antibodies of varied determinant specificities are induced (16), whereas the antigen-dependent LPS influence is highly specific for the antigen employed as shown in the present report. In the latter regard, it should be reiterated that, with the notable exception of particulate erythrocyte antigens, the addition of antigen to unprimed cells diminishes the LPS-induced spontaneous antibody production in a highly specific fashion (reference 16, and data herein); and (d) Finally, and most importantly, as shown in the studies presented here, the effect of LPS on antigen-induced in vitro responses is generally dependent upon and clearly magnified by the presence and frequency of carrier-specific helper T cells.

In the context of what has been reported previously by others, it is important to place our results of the effects of LPS on anti-θ-treated spleen cells in proper perspective (Table III). Firstly, the primary in vitro response of untreated spleen cells to SRBC was markedly enhanced by LPS (in our hands). This was a consistent finding in many experiments and was not dependent upon unusually low responses to SRBC in the absence of LPS in order to be manifested as has been reported by Sjöberg et al. (15).[2] Secondly, relative depletion of T cells from such unprimed spleens by anti-θ-serum treatment not only diminished the response to SRBC alone, but also resulted in a significant depression in the absolute magnitude of the LPS-enhanced response to SRBC. What deserves emphasis in this regard is that while the level of response of the T-cell-depleted population to SRBC plus LPS may be comparable with the normal primary response of untreated cells to SRBC alone (cf exp. 1, Table III), it is, nevertheless, still markedly lower (by almost 10-fold) than the response of untreated cells to SRBC plus LPS. If, in fact, the predominant action of LPS was on B cells, one would expect to see as good or better response with anti-θ-treated cells in which a relative enrichment (as compared with untreated spleen) of B cells exists. In published reports of others (8) this difference between responses of untreated vs. T-cell-depleted spleen to SRBC plus LPS is often not presented for comparison and can result in erroneous interpretations of the data.

Clearly, the data presented here indicate that LPS does, indeed, influence helper T cell function. It should be stressed that we have deliberately chosen to refer to the biological action of LPS on helper T cell *function* rather than on T cells themselves. This is necessitated by the fact that there is no evidence defining which cell is directly and predominantly affected by LPS under these circumstances. What our studies presented here have precisely shown is that the potentiating action of LPS on antigen-induced antibody responses in vitro is helper T cell-dependent under these conditions. These results could reflect one or more possible effects of LPS on T cells, B cells and/or macrophages as follows.

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[2] Moreover, we have obtained identical results in cultures established in classical Petri dishes thus ruling out possible differences on the basis of the microculture system.

T Cells.—The functional mechanism of LPS potentiation of specific helper T-cell function may be considered in at least two ways: (*a*) LPS-induced increase in the rate of proliferation of T cells; and/or (*b*) increased efficiency of individual helper T cells in their exertion of regulatory influences on B lymphocytes. Available data indicate that LPS alone does not induce a proliferative response in T cells (3–8). However, to our knowledge, the possibility of whether or not LPS can appreciably affect antigen-induced T cell proliferation has not been heretofore explored. This issue, currently under investigation in our laboratory, remains open until definitive data are obtained. This possibility must be taken into account in considering interpretations of data obtained in cultures of spleen cells from congenitally athymic or nude mice. Hence, it is conceivable that although such spleen cells may appear to be devoid of T lymphocytes by usual criteria of mitogenic stimulation by Concanavalin A or other T cell mitogens, there may exist immature T cells capable of induction under appropriate conditions such as the simultaneous presence of LPS and antigen. Indeed, quite recently it has been established that LPS is one of several agents (including thymus extracts and polyadenylic:polyuridylic acid) capable of inducing generation of T-cell surface antigen markers on immunoglobulin-negative lymphocytes from spleens of nude mice.[3] We ourselves have recently demonstrated that thymus extracts exert qualitatively identical functional effects as LPS on helper T cells in our culture system.[1] Failure to observe a comparable functional effect with Concanavalin A plus antigen (8) may reflect qualitative differences in the capabilities of T cells in varying stages of maturation to respond to such influences. Such failures do not, therefore, rule out the existence and participation of T cells in these effects. Moreover, the recently published observations of Watson et al. (26) that nude spleen cells respond to trinitrophenyl (TNP) conjugates of glycyglycine in the presence of LPS are not definitive since it is possible that such reagents may stick to LPS forming a T-cell-independent antigen, a point demonstrated directly by others (27).

B Cells.—LPS could act by perhaps lowering their threshold for activation which could result in a lower requirement for necessary T-cell help (thus making a limiting number of specific T lymphocytes sufficient in this regard). Alternatively, LPS could increase the proliferative rate of a few B cells which have been successfully triggered by antigen in the presence of limited T cell participation. Nonetheless, the participation of specific T cells is not circumvented in the process of B cell triggering by either possibility.

Macrophages.—The interrelationship of the effects of LPS on macrophage function and the ultimate expression of B or T cell activity must be borne in mind as a point of great importance. A possibility exists that macrophages release a factor in culture that is either necessary for or contributes significantly to any regulatory influence that LPS might have on T cells (28).

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[3] M. P. Scheid, M. K. Hoffman, K. Komuro, U. Hammerling, J. Abbott, E. A. Boyse, G. H. Cohen, J. A. Hooper, R. S. Schulof and A. L. Goldstein. 1973. Differentiation of T cells induced by preparations from thymus and by nonthymic agents. *J. Exp. Med.* **138**:1027.

We conclude, therefore, that the biological action of LPS in influencing antibody responses should not be construed simply as reflecting an exclusive B cell effect. The adjuvant properties of LPS on antigen-induced immune responses are quite clearly dependent upon the participation of T-cell helper function. Whether or not the ultimate biological effects observed are determined by direct or indirect action(s) on the functions of one or more cells of the immune system remains to be elucidated.

SUMMARY

The present studies were undertaken to analyze the nature of the effect of bacterial lipopolysaccharide (LPS) on antibody production in vitro. We have done this by making comparative studies of the effects of LPS on in vitro primary and secondary antibody responses to soluble hapten-protein conjugates and to particulate and soluble sheep erythrocyte antigens. The results obtained demonstrate that the biological action of LPS in vitro may be predominantly manifested on the function of B lymphocytes or T lymphocytes depending on the conditions employed. In the absence of antigen, LPS appears to act primarily on B lymphocytes. In the presence of antigen, however, the data presented here show that LPS significantly influences specific helper T-cell function and it is this latter influence that is predominantly responsible for the adjuvant effects of LPS on antigen-specific antibody responses.

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