FUNCTIONAL CHARACTERISTICS OF PEYER'S PATCH LYMPHOID CELLS*

II. Lipopolysaccharide is Thymus Dependent

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In the preceding paper we showed that Peyer's patch cell suspensions from normal mice are deficient in an accessory adherent cell type required for the induction of immune responses (1). Humoral and cell-mediated immune responses can be induced in these cultures after the addition of adherent peritoneal exudate cells (APEC)[1] or 2-mercaptoethanol (ME). Peyer's patches from C57BL/6 mice contain antigen-sensitive B and T cells, while Peyer's patches from congenitally athymic (nude) mice contain a highly purified B-cell population. Peyer's patch lymphoid cells are a well-defined source of antigen-sensitive cells which can be used to examine the requirements for induction in vitro of immune responses to specific antigens.

It has been reported that bacterial lipopolysaccharide (LPS) is a thymus-independent antigen (2–4) capable of stimulating DNA synthesis in B cells (5–8), as well as stimulating immune responses to various antigens (9–12) and monovalent haptens (13) in T-cell depleted spleen cultures. In this paper we report experiments which demonstrate that LPS does not stimulate DNA synthesis, or affect the induction of primary humoral immune responses to heterologous erythrocytes in Peyer's patch cultures, unless T cells are present in addition to B cells and adherent cells or ME. The observed thymus dependence of LPS is interpreted in terms of a model in which two signals are required for B-cell induction (14).

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[1] *Abbreviations used in this paper*: APEC, adherent peritoneal exudate cells; Con A, concanavalin A; FBS, fetal bovine serum; FudR, fluorodeoxyuridine; HRBC, horse red blood cells; LPS, lipopolysaccharide; ME, 2-mercaptoethanol; MEM, Eagle's minimum essential medium; PFC, plaque-forming cells; SRBC, sheep red blood cells; TdR, thymidine.

Materials and Methods

Mice.—Congenitally athymic (nude) mice (nu/nu) and heterozygous nude mice (nu/+) were bred and used exactly as described in the preceding paper (1). C57BL/6 mice were purchased from Jackson Laboratories, Bar Harbor, Maine. All mice were used at 7–9 wk of age.

Peyer's Patch Cultures.—In vitro immune responses were studied in Peyer's patch cell suspensions from unimmunized C57BL/6 or nude mice prepared exactly as described previously (1). Peyer's patch cultures contained 1×10^7 Peyer's patch cells in 1 ml of Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS) and antigen as specified in the text. Only batches of FBS designated as normal (15) were used (batch J76207 from Reheis Chemical Corp., Chicago, Ill.).

Peritoneal Adherent Cells.—APEC were prepared as described previously (1). 5×10^5 seeded peritoneal exudate cells were used per culture. APEC obtained from unimmunized C57BL/6 or nude (nu/nu) mice were added to cultures of C57BL/6 or nude Peyer's patches, respectively, as indicated in the text.

2-Mercaptoethanol.—ME was obtained from Matheson, Coleman and Bell, Norwood, Ohio and used in culture at a concentration of 10^{-4} M.

Mitogens.—Salmonella typhosa (W0901) LPS was obtained from Difco Laboratories, 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. LPS was identical to that previously used by Dr. J. Watson, Salk Institute, San Diego, Calif. (12, 13). Maximal stimulation of DNA synthesis by LPS was found to occur between 5 μg and 10 μg per culture. Concanavalin A (Con A) obtained from Calbiochem, Los Angeles, Calif. was dissolved at a concentration of 1 mg/ml in phosphate-buffered saline (pH 7.2) and stored frozen.

Irradiation.—Peyer's patch cell suspensions from unimmunized C57BL/6 or heterozygous nude mice were irradiated with 2,000 R as described previously (1). These cells were used as the source of C57BL/6 or nude T cells (16, 17).

Assay.—On the 4th day after culturing, the number of direct hemolytic plaque-forming cells (PFC) in Peyer's patch cultures was determined using a microscope slide assay (18). The same sheep red blood cells (SRBC) as used in culture were used in the slide assay to determine PFC against SRBC. In each experiment, Peyer's patch cell suspensions immunized with SRBC or no antigen were examined for PFC directed against SRBC, and PFC directed against horse red blood cells (HRBC).

DNA Synthesis.—Peyer's patch cultures were prepared containing 5×10^6 cells in 1 ml of MEM containing 5% FBS. Duplicate cultures were incubated for 40 h after which 10^{-7} M fluorodeoxyuridine (FudR), 10^{-6} M thymidine (TdR), and 0.5 μCi of [^{3}H]TdR (52 Ci/mmol, New England Nuclear, Boston, Mass.) (15) were added and cultures were incubated for an additional 5 h. Cells were collected by centrifugation, the DNA was precipitated with 5% trichloroacetic acid, and the precipitate collected by membrane filtration. Radioactive measurements were made after the addition of a toluene-Liquifluor scintillation fluid to dried filters.

RESULTS

Effect of LPS on DNA Synthesis.—LPS stimulated DNA synthesis only when T cells were present in cultures also containing B cells, and APEC or ME. The data presented in Table I describe the effect of 5 μg/ml LPS on DNA synthesis in cultures of C57BL/6 and nude Peyer's patch cells. The following was consistently observed: (*a*) LPS did not stimulate DNA synthesis in nude Peyer's patch cultures previously shown to functionally contain only antigen-sensitive B cells (1). (*b*) LPS did not stimulate DNA synthesis in nude cultures to which APEC or ME were added. (*c*) LPS did not significantly stimulate

TABLE I

*Effect of LPS 5 μg/ml on DNA Synthesis in C57BL/6 or Nude (nu/nu) Peyer's Patch Cultures**

Strain	Cell types in culture‡			ME	CPM/culture	
	B	T§	APEC		LPS 5 μg/ml	No LPS
nu/nu	+	−	−	−	3	10
nu/nu	+	−	+	−	1	0
nu/nu	+	−	−	+	1	20
C57BL/6	+	+	−	−	120	43
nu/nu	+	+	−	−	4	2
nu/+‖	+	+	−	−	5	14
C57BL/6‖	+	+	−	−	8	11
C57BL/6	−	−	+	−	10	22
C57BL/6	+	+	+	−	610	55
nu/nu	+	+	+	−	240	18
C57BL/6	+	+	−	+	19,000	1,900
nu/nu	+	+	−	+	13,000	1,000

* No SRBC were added to culture.

‡ nu/nu Peyer's patches were used as a population of B cells while C57BL/6 Peyer's patches were used as a population of B and T cells.

§ As a source of T cells for nu/nu cultures, 2.5×10^6 irradiated heterozygous nude (nu/+) Peyer's patch cells were added to 2.5×10^6 nude (nu/nu) Peyer's patch cells.

‖ nu/+ or C57BL/6 Peyer's patches exposed in vitro to 2,000 R before culture were considered to contain T cells as discussed.

DNA synthesis in C57BL/6 cultures which were previously shown to contain both antigen-sensitive T and B cells (1) or in nude Peyer's patch cultures to which irradiated heterozygous nude Peyer's patch cells were added as a source of T cells. (*d*) LPS did not stimulate DNA synthesis in irradiated heterozygous nude Peyer's patch cells or in irradiated C57BL/6 Peyer's patch cells which had cooperating T-cell activity (1). (*e*) LPS did not stimulate DNA synthesis in cultures of APEC. (*f*) LPS produced a 7–12-fold stimulation of DNA synthesis in cultures containing T cells, B cells, and APEC or ME. In contrast to LPS, 2 μg/ml of Con A stimulated a 10-fold increase in DNA synthesis in C57BL/6 Peyer's patch cultures in the absence of APEC or ME.

Effect of LPS on Induction of Immune Response to SRBC:—The data presented in Table II describe the effect of 5 μg/ml LPS on the induction of primary in vitro humoral immune responses to SRBC in C57BL/6 or nude Peyer's patch cultures. The following was noted: (*a*) No PFC were obtained when LPS was added to cultures containing nude Peyer's patch cells. (*b*) Nude Peyer's patch cells to which APEC and LPS were added had low levels of PFC, while nude Peyer's patch cells to which ME and LPS were added had no anti-SRBC PFC. (*c*) Neither C57BL/6 cultures nor nude cultures to which irradiated heterozygous nude Peyer's patch cells were added, as a source of T cells, could be induced to produce a primary in vitro immune response against SRBC by

TABLE II

*Effect of LPS 5 μg/ml on Immune Responses to SRBC in C57BL/6 or Nude (nu/nu) Peyer's Patch Cultures**

Strain	Cell types in culture‡			ME	Anti-SRBC PFC/culture‖
	B	T§	APEC		
nu/nu	+	−	−	−	0
nu/nu	+	−	+	−	25
nu/nu	+	−	−	+	0
nu/nu	+	+	−	−	14
C57BL/6	+	+	−	−	15
C57BL/6	+	+	−	+	3,282
nu/nu	+	+	−	+	5,280
nu/nu	+	+	+	−	1,463
C57BL/6	+	+	+	−	192
C57BL/6¶	+	+	+	−	1,235

* SRBC and LPS 5 μg/ml were added to each culture.

‡ nu/nu Peyer's patches were used as a population of B cells while C57BL/6 Peyer patches were used as a population of B and T cells.

§ As a source of T cells for nu/nu cultures, 5×10^6 irradiated heterozygous nude (nu/+) Peyer's patch cells were added to 5×10^6 nude (nu/nu) Peyer's patch cells.

‖ Anti-SRBC PFC responses in the absence of LPS are listed in Table I of the preceding paper.

¶ C57BL/6 Peyer's patch cells treated with antitheta serum before cuture.

the addition of LPS. (*d*) In vitro immune responses were induced only when LPS was added to cultures containing B cells, T cells, and ME or APEC. Cell viability was increased by LPS only in cultures containing B cells, T cells, and ME. (*e*) LPS consistently enhanced the immune response to SRBC in nude cultures restored with T cells, and APEC or ME (for comparisons see preceding paper). LPS usually suppressed the immune response to SRBC when added to cultures of C57BL/6 Peyer's patches containing APEC and consistently suppressed the immune response when added to C57BL/6 cultures containing ME (for comparisons see preceding paper). Reduction of the T-cell population of C57BL/6 Peyer's patches by antitheta treatment before the addition of LPS and APEC resulted in a significantly greater anti-SRBC response than observed in parallel cultures not treated with antitheta serum. When 5×10^4, 5×10^5, or 5×10^6 irradiated heterozygous nude Peyer's patches were added as a source of T cells to cultures of nude Peyer's patches containing APEC and LPS, the magnitude of the SRBC response correlated directly with the number of irradiated heterozygous Peyer's patch cells (i.e., T cells) added.

Background Responses:—DNA synthesis was stimulated in Peyer's patch cultures when ME was added to C57BL/6 or nude cultures containing T and B cells (Table I). Irradiation of heterozygous nude Peyer's patch cells before addition to nude cultures did not significantly affect the stimulation of DNA synthesis by ME. ME did not stimulate DNA synthesis in cultures in which both B and T cells were irradiated before culture (Table I).

Background PFC were detected in cultures containing T and B cells to which ME was added. In these cultures, background PFC directed against SRBC equaled 4% of the total anti-SRBC PFC observed when SRBC antigen was used in culture. LPS did not induce anti-SRBC PFC in the absence of SRBC and did not increase the background anti-SRBC PFC response obtained with ME. After induction with SRBC, PFC directed against HRBC comprised 0.1% of the anti-SRBC response. LPS did not significantly increase the number of PFC reactive with HRBC during induction to SRBC antigens.

DISCUSSION AND SUMMARY

This study shows that LPS is not mitogenic in cultures containing B cells, or B cells and accessory adherent cells or ME, unless T cells are present. This observation rules out models of induction of antibody synthesis in which it is assumed that the delivery of a mitogenic signal by the interaction of LPS with the membrane of the B cell is in itself sufficient for B-cell induction (19). Further, it makes unlikely the proposed extrapolation of such a model to other so-called thymus-independent antigens, e.g., PVP, levan, dextran, and SIII (19).

The mitogenic action of LPS appears to be due to its ability to complete an inductive stimulus to B cells (13). We interpret the observed thymus dependence of the B-cell response to LPS in light of a model in which two signals are obligatory for B-cell induction (14). The first signal in the inductive pathway is delivered to the antigen-sensitive cell via a conformational change in the receptor upon interaction with antigen. The second signal is delivered via the thymus-derived cooperating system. Since LPS can induce immune responses to both immunogenic and nonimmunogenic ligands (9–13) we envision that one signal is delivered to the B cell via specific binding of the ligand to the B-cell antigen receptor, while a second signal is delivered as a result of T-cell cooperation via membrane-bound LPS. This has been termed abnormal induction (20). In this example LPS is the foreign membrane-bound determinant in question although histocompatibility antigens (21, 22), viral determinants, or surface bound lectins could act similarly.

In light of the above model, one observation should be pointed out. LPS inhibits the induction of a SRBC response in normal Peyer's patch cells to which adherent cells or ME is added. This inhibition appears to be a T-cell-mediated effect because it is abolished by partial depletion of the T-cell population by antitheta treatment. Since the induction of IgM producing PFC is being measured, the T-cell-dependent LPS inhibition could act either (a) by induction of T-cell "suppression" (23, 24) of the normal cooperating system required for a SRBC response, or (b) by the induction of such high levels of cooperating function (13) as to be inhibitory to a SRBC IgM response.

Our observations contrast sharply with prior reports which describe LPS as a thymus-independent antigen (2–4) and a B-cell mitogen (5–8) capable of stimulating immune responses in the absence of T-cell cooperation (2–12).

This demonstration of the thymus dependence of LPS stimulation has been possible because Peyer's patches from congenitally athymic (nude) mice are functionally a highly purified B-cell population devoid of T cells and accessory adherent cells. In this respect, earlier studies relied on nude spleen cultures and spleen cultures from thymectomized, lethally irradiated, and bone marrow-reconstituted mice (3, 4, 6–13). These spleen cultures which contain B cells and accessory adherent cells are recognized to be deficient but not devoid of the thymus-derived contribution to the inductive stimulus (12, 13). It could be argued that the presence of T cells and adherent cells is in fact required for the antigen-specific effect and not for the LPS effect. However, this is unlikely since our experiments show that LPS is not directly mitogenic for B cells and does not stimulate background anti-SRBC PFC. It seems unlikely that Peyer's patch antigen-sensitive cells differ from antigen-sensitive cells in the spleen in their mechanism of induction. We have shown that Peyer's patch B cells can be specifically induced by antigen, and Peyer's patch T cells mediate cooperating and killer functions. Alternately, the possibility that Peyer's patch B cells were not stimulated by LPS as a result of prior cryptic exposure to LPS (13) in the intestinal tract was excluded since cultures containing B cells, T cells, and adherent cells or ME were stimulated to DNA synthesis by LPS.

The reason that certain antigens appear to be thymus independent may be that their repeating polymeric nature permits inductive interactions at very low levels of thymus-derived cooperation (see reference 20 for quantitative considerations). It has been stated that the inductive properties of all thymus-independent antigens are directly related to their ability to act as B-cell mitogens (19). The observation that LPS is thymus dependent for its B-cell mitogenic activity makes us question the thymus independence of any antigen.

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