Immunology 1992 77 477–482

Polyclonal activation of B lymphocytes by lipopolysaccharide requires macrophage-derived interleukin-1

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Accepted for publication 21 July 1992

SUMMARY

Lipopolysaccharide (LPS) is a potent murine polyclonal B-cell activator which induces cellular proliferation and IgM secretion. The precise role of activated macrophages in the induction of LPS-dependent, B-cell responses has been unclear. Although early reports concluded that the LPS effect occurs independently of other cell types, other studies have suggested that adherent macrophages exert either potentiating or inhibitory effects. In the present study, B-cell mitogenesis and IgM production were measured in primary spleen cell cultures after removing adherent cells by a variety of experimental procedures. B-cell activation by LPS was found to be strictly dependent on the presence of adherent macrophages. Antibody neutralization and cytokine reconstitution studies demonstrated that macrophage-derived interleukin- (IL-1) is a necessary co-factor for LPS-induced polyclonal activation.

INTRODUCTION

When incubated with lipopolysaccharide (LPS), murine B lymphocytes undergo blast transformation, proliferation and differentiation into IgM-secreting cells.[1-3] Lipopolysaccharide stimulates a large number of cells (> 30%) in an antigen non-specific manner, and results in the synthesis and secretion of polyclonal antibodies with diverse specificities.[2,3] The precise cellular events which underlie the activation of B cells by LPS are only partially understood. Early studies indicated that the LPS effect occurs independently of T cells and macrophages,[4,5] but subsequent investigations have suggested inhibitory,[6] potentiating,[7] or obligatory roles for accessor cells.[8,9] Lipopolysaccharide can synergize with antigen or T cells to promote B-cell response in a variety of experimental systems.[10-13]

In the present study, the role of adherent macrophages in LPS-induced B-cell activation was re-examined in the context of the following considerations. First, the concentration of LPS required to induce polyclonal activation exceeds by more than two orders of magnitude the amount required to induce maximally the production of macrophage-derived cytokines.[2,14] Second, because of the extreme biological potency of macrophage products such as tumour necrosis factor (TNF) and interleukin-1 (IL-1), it is likely that in B-cell cultures that are incompletely depleted of macrophages, LPS activation occurs in a milieu of macrophage-derived cytokines. The present study has confirmed that polyclonal B-cell activation requires activated macrophages[8,9] and determined that IL-1 is a necessary signal for both LPS-induced mitogenesis and immunoglobulin production.

Abbreviations: EU, endotoxin units; IFN-γ, interferon-γ; IL-1α, interleukin-1α; IL-6, interleukin-6; LPS, lipopolysaccharide; PEC, peritoneal exudate cells; TNF-α, tumour necrosis factor.

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MATERIALS AND METHODS

Preparation of cells

Female BALB/c mice aged 8–16 weeks were used in these studies (Charles River, Wilmington, MA). Spleen cell suspensions were prepared according to a standard protocol and contaminating erythrocytes were removed by NH_4Cl lysis.[15] Spleen cells were cultured at a density of $1{\cdot}5 \times 10^6$/ml in RPMI-1640 supplemented with 10% foetal calf serum (FCS) (heat-inactivated and endotoxin-free), 2 mM L-glutamine, and 50 μg/ml gentamicin. Adherent cells were removed by a variety of procedures including adherence on plastic tissue culture flasks,[15] passage over Sephadex G-10,[16] complement lysis[15,17] and by a combination of procedures. For adherence, washed spleen cell suspensions were cultured overnight in T-100 flasks at a density of $1{\cdot}5 \times 10^6$ cells/ml. Non-adherent cells were collected by gentle pipetting. For purification over Sephadex G-10, crude cell suspensions ($1{\cdot}5 \times 10^8$ cells/ml) were loaded onto sterile columns of resin (with a constant bed volume of 15 ml of resin/ml of cell suspension) which were pre-equilibrated with phosphate-buffered saline (PBS)/10% FCS. Non-adherent cells were eluted with 1 bed volume of RPMI/10% FCS. In experiments which combined both treatments, adherence to plastic followed column chromatography. Complement lysis was performed as described previously.[17] Briefly, spleen cells (3×10^7/ml) were incubated with 30 μg/ml of rat monoclonal antibodies to pan-Mac and Mac-1 antigens (Bioproducts, Indianapolis, IN) for 30

min at 37°. This was followed by washing and incubation with 3 ml of rabbit complement (Pel-Freeze, Rogers, AR) for 45 min at 37°. Adherence to plastic removed 24±8% of input cells, Sephadex G-10 chromatography removed 49±11% of input cells, and the combination of both procedures removed 65±9% of input cells ($n=4$). Complement lysis removed 31±5% of input cells ($n=3$). The presence of macrophage-like cells was determined morphologically by specific staining with α-naphthyl esterase and counterstaining with haematoxylin.[18] The percentage of esterase-positive cells was determined after enumeration of discrete numbers of cells collected by cytospin. Cell viability was measured by trypan blue exclusion and exceeded 95%.

Preparation of conditioned media

Peritoneal exudate cells (PEC) were isolated from BALB/c mice after elicitation with thioglycollate.[19] For these studies, BALB/c mice were injected intraperitoneally with 1·5 ml of thioglycolate 3 days prior to peritoneal lavage with 8 ml of sterile, endotoxin-free saline. Collected cells were washed twice and then cultured in RPMI/10% FCS at a density of 2×10^6 cells/ml. Cells were allowed to adhere overnight on standard tissue culture plates. Cultures were washed five times with Hanks' balanced salt solution prior to the addition of 10 ml of either fresh medium or medium containing LPS. The monocytic cell line RAW 264.7 (RAW) was cultured to confluence in RPMI/10% FCS.[20] PEC and RAW cells were activated by adding LPS at a concentration of 1 μg/ml for 18 hr.[14] At the end of this time, cell-free supernatants were prepared by removing the media, centrifuging and filtering the supernatant through a 0·22 μm syringe filter unit (Millipore, Inc., Harrow, U.K.). Control conditioned media (without LPS addition) was tested for the presence of endotoxin by the Limulus Amebocyte Lysate test (Whittaker Bioproducts, Walkersville, MD).[21] Cultures were eliminated from further study when positive for >0·1 EU/ml of medium.

Assay of mitogenesis and immunoglobulin production

Spleen cell suspensions were activated with LPS by adding LPS (*Escherichia coli* serotype 026:B6; Difco Laboratories, Detroit, MI) at a final concentration of 50 μg/ml. This concentration was observed to induce maximal cell proliferation under the culture conditions utilized for this study. For the assay of mitogenesis, triplicate 0·2 ml spleen cell cultures ($1·5\times10^6$ cells/ml) were established in 96-well flat-bottom microtitre plates. At 24-hr time intervals, 1 μCi of [^{3}H]methyl-thymidine (2 Ci/mmol; New England Nuclear, Wilmington, DE) was added in a 20 μl aliquot for the final 24 hr of culture. Cells were lysed by freeze thawing and the tritium incorporation into DNA was measured by liquid scintillation spectrometry after collecting and washing the cell lysates on filter discs (Harvard Apparatus, Watertown, MA). Experimental data are expressed as per cent [^{3}H]thymidine incorporation relative to control LPS-induced spleen cell incubations which were performed concurrently. At 72 hr of culture (the time of peak [^{3}H]thymidine incorporation), total incorporation in LPS-stimulated cultures ranged from 1·5 to $2·5\times10^4$ c.p.m. Background (uninduced) incorporation was $<5\times10^2$ c.p.m.

For the assay of immunoglobulin production, duplicate 1 ml aliquots of cells ($1·5\times10^6$ cells/ml) were cultured in 24-well flat-bottom plates. At 24 hr time intervals, the culture supernatant was removed and cleared of cells by centrifugation (12,000 ***g*** for 30 seconds). Supernatants were analysed for the presence of IgM by antigen capture ELISA. Briefly, flat-bottom microtitre plates were coated with 0·1 ml of goat anti-murine IgM antibody (μ-chain specific; Cappel Laboratories, West Chester, PA) at a final concentration of 10 μg/ml in PBS for 2 hr at room temperature. After washing with PBS/0·05% Tween-20 and blocking with a solution of PBS/2% goat serum/1% bovine serum albumin (BSA) (1 hr at room temperature), 100 μl of diluted culture media (1/10–1/100) was added. The plates were incubated overnight at 4° and then developed by adding alkaline phosphatase-linked anti-murine IgM (μ-chain specific) at a 1/10,000 dilution for 2 hr at 37°. The plates were washed and then incubated with *p*-nitrophenyl phosphate (1 mg/ml in 0·1 M diethanolamine buffer, pH 9·8). Immunoglobulin M concentrations were calculated by comparison to a standard curve obtained by measuring in triplicate, for each assay set, seven standard concentrations of purified mouse IgM (range 1–200 ng/ml). Experimental data are expressed as per cent IgM production relative to LPS-induced control spleen cell incubation performed concurrently. Total IgM production in LPS-stimulated cultures ranged from 10 to 15 μg/ml. Background (uninduced) levels of IgM were <0·1 μg/ml.

Cytokine studies

Antibody neutralization studies were performed by adding to cultures, at the same time as LPS, aliquots of the following neutralizing, cytokine-specific antibodies: rabbit polyclonal anti-interleukin-1α antibody (the generous gift of Dr L. Moldawer, Cornell Medical College), goat polyclonal anti-interleukin-1β antibody (R & D Systems, Minneapolis, MN), rabbit polyclonal anti-tumour necrosis factor-α antibody (the generous gift of Dr B. Sherry, Picower Institute),[22] monoclonal anti-interferon-γ antibody, and monoclonal anti-interleukin-6 antibody (both from Genzyme, Cambridge, MA). Duplicate experiments compared fivefold differences in antibody concentrations and all assays were performed in triplicate. Final concentration of antibody was 8 μg/ml and 42 μg/ml for monoclonal antibodies and 18 μg/ml and 90 μg/ml for polyclonal antibody (calculated as the total IgG fraction). Recombinant murine interleukin-1α (8×10^6 U/mg) was obtained from Genzyme. As indicated, IL-1α was added to spleen cell cultures which had been depleted of adherent cells by complement lysis. IL-1α was measured in PEC- and RAW-conditioned media by radioimmunoassay using a commercially available kit (Cytokine Sciences, Boston, MA). Prostaglandin synthesis was inhibited by the addition of indomethacin (Sigma Chemical Co., St Louis, MO) to cell cultures at a final concentration of 1 μM.[23]

RESULTS

An LPS concentration of 50 μg/ml was found to stimulate maximally [^{3}H]thymidine incorporation and IgM synthesis under the cell culture conditions utilized in this study. To assess the adherent cell requirement for these responses, spleen cell suspensions were treated with a variety of procedures to remove macrophages prior to incubation with LPS. Adherent cells were removed by incubation on plastic, passage over Sephadex G-10, complement-mediated lysis and a combination of procedures. In three representative experiments, it was observed that adherence to flasks reduced the esterase positive population from 7·0 to ≈1·0%. Column chromatography reduced the

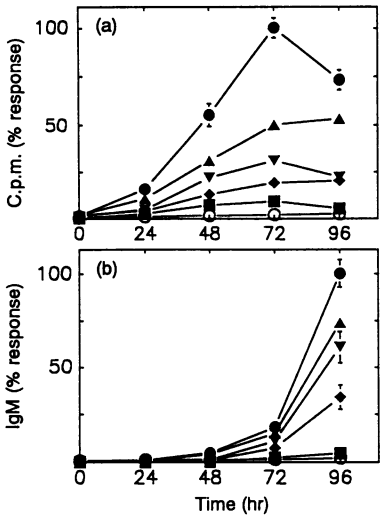

Figure 1. LPS-induced polyclonal activation studied as a function of time. (a) Spleen cell proliferation measured by [^{3}H]thymidine incorporation. (b) IgM synthesis measured by antigen capture ELISA. Control spleen cells (●). Spleen cells depleted of adherent cells by incubation on plastic (▲), passage over Sephadex G-10 (▼), by both procedures successively (◆), and by complement-mediated lysis (■). Control cells incubated without LPS (○). Results are expressed as per cent [^{3}H]thymidine incorporation and IgM production compared to the LPS-induced spleen cell control incubations. Maximal and background responses are as described in Materials and Methods. Maximal responses ranged from 1·5 to 2·5 × 10^4 c.p.m. for [^{3}H]thymidine incorporation (at 72 hr), and 10–15 μg/ml for IgM production (at 96 hr).

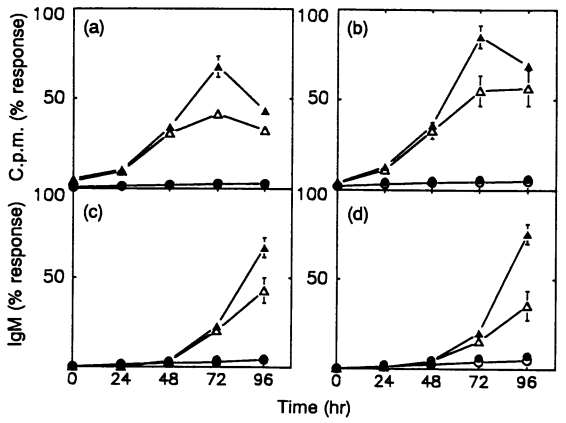

Figure 2. LPS-induced spleen cell proliferation and IgM synthesis after supplementation of adherent cell-depleted cultures with monocyte conditioned media (to a final concentration of 5% conditioned media). LPS-induced spleen cell proliferation (a) and IgM synthesis (c) after supplementation with LPS-stimulated (▲) and unstimulated (△) PEC-conditioned media. LPS-induced spleen cell proliferation (b) and IgM synthesis (d) after supplementation with LPS-stimulated (▲) and unstimulated (△) RAW-conditioned media. Control (no LPS) adherent cell-depleted spleen cell cultures supplemented with LPS-stimulated (●) and unstimulated (○) monocyte-conditioned media. Maximal responses are as described in Materials and Methods (1·5–2·5 × 10^4 c.p.m. for [^{3}H]thymidine incorporation at 72 hr, and 10–15 μg/ml for IgM production at 96 hr).

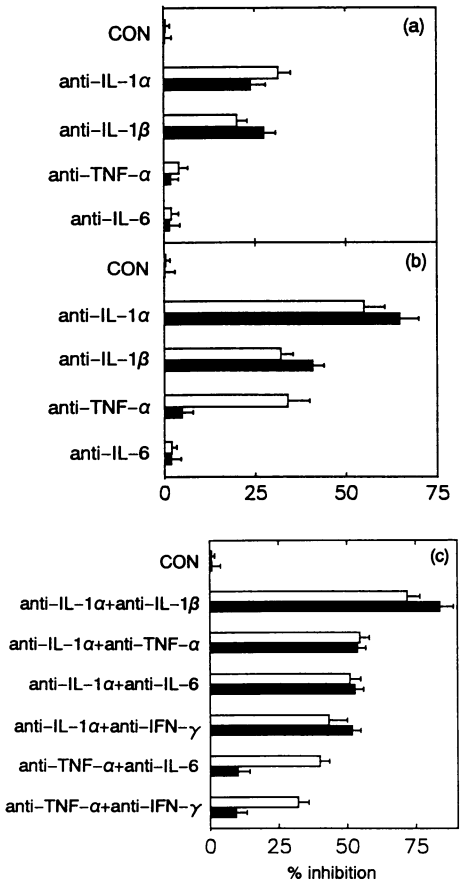

Figure 3. (a, b) The effect of anti-cytokine neutralizing antibodies on LPS-induced spleen cell proliferation and IgM synthesis. [^{3}H]thymidine incorporation (□) (measured at 72 hr) and IgM production (■) (measured at 96 hr). (a) Con: (no antibody) and the following anti-cytokine antibodies: anti-IL-1α, anti-IL-1β, anti-TNF-α and anti-IL-6. (b) Antibodies added at a fivefold greater amount. Con: (no antibody), anti-IL-1α, anti-IL-1β, anti-TNF-α, and anti-IL-6. Antibody amounts are described in Materials and Methods. Data shown are representative of two experiments. Control incubations ranged from 1·5 to 2·5 × 10^4 c.p.m. for [^{3}H]thymidine incorporation, and 10–15 μg/ml for IgM production. (c) The effect of pairwise addition of anti-cytokine antibodies on LPS-induced polyclonal activation. Thymidine incorporation (□) (measured at 72 hr) and IgM production (■) (measured at 96 hr). The addition of anti-IFN-γ mAb alone was not observed to effect either [^{3}H]thymidine incorporation or IgM production (not shown). The amount of antibodies added are described in Materials and Methods and are equivalent to the amounts used in (b). Control incubations ranged from 1·5 to 2·5 × 10^4 c.p.m. for [^{3}H]thymidine incorporation, and 10–15 μg/ml for IgM production.

esterase positive population to ≈0·35%. The combination of both adherence procedures or complement lysis reduced the macrophage/monocyte population to <0·1%. As shown in Fig. 1, the removal of adherent macrophages from spleen cell suspensions resulted in the progressive diminution of the LPS-induced mitogenesis and IgM secretion responses. In the case of physical adherence, a ≈2 log depletion of the adherent cell population diminished the mitogenic response by 83% and the total IgM produced by 67%. It is apparent that the LPS response is extremely sensitive to the presence of small numbers of adherent cells or adherent cell factor(s). Complement-mediated cell lysis with specific anti-macrophage antibodies was

Table 1. Immunoreactive IL-1α in macrophage-conditioned media. Conditioned media was prepared and 0·1 ml aliquots subjected to competitive radioimmunoassay as described in Materials and Methods. Assay sensitivity was 300 pg/ml (defined as 95% B/B_0). Duplicate aliquots were assayed

	Interleukin-1α (ng/ml)	
	−LPS	+LPS
PEC	<0·3	2·2±0·9
RAW 264·7	1·2±0·2	5·1±2·8

Values shown are the mean±SEM of triplicate determinations.

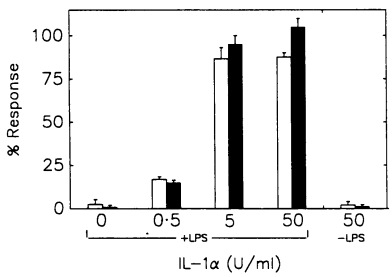

Figure 4. Restoration of [^{3}H]thymidine incorporation (□) and IgM synthesis (■) in LPS-stimulated (50 μg/ml) adherent cell-depleted spleen cell cultures by recombinant murine IL-1α. [^{3}H]Thymidine incorporation and IgM synthesis were measured at 72 and 96 hr respectively. Maximal responses ranged from 1·5 to 2·5 × 10^4 c.p.m. for [^{3}H]thymidine incorporation and 10–15 μg/ml for IgM production.

observed to be the most effective procedure for removing the stimulatory activity of adherent cells and diminished the proliferative and IgM synthesis responses by >95%.

It was reasoned that the adherent cell requirement for LPS-induced polyclonal activation might be replaced by media conditioned with LPS-stimulated macrophages, and such reconstitution was observed in a previous study.[9] Conditioned media were prepared from thioglycollate-elicited murine PEC and the cell line RAW 264·7, two monocytic cell types that produce inflammatory mediators in response to LPS stimulation.[14,24] Elicited macrophage and monocyte cell cultures were incubated with LPS under conditions sufficient to induce maximally cytokine release (1 μg/ml, 18 hr).[14] The addition of media conditioned by LPS-stimulated PEC and RAW cell cultures together with LPS (50 μg/ml) was observed to reconstitute the mitogenesis and IgM synthesis responses of adherent cell-depleted spleen cultures (Fig. 2). Unstimulated conditioned media augmented the spleen cell response to LPS to a lesser degree than conditioned media obtained from LPS-stimulated monocytes. The effect observed with unstimulated media may have resulted from small amounts of macrophage-derived products which are present constitutively in conditioned media.

Lipopolysaccharide induces the production of a variety of macrophage-derived products including prostaglandins, IL-1, TNF, IL-6, colony-stimulating factors (CSFs), and macrophage inflammatory proteins (MIP).[14,24,25] The addition of indomethacin (1 μM) to LPS-stimulated cultures did not significantly inhibit either cellular proliferation or IgM production, arguing against a role for cyclooxygenase products as positive mediators of polyclonal activation (data not shown).

To identify the particular macrophage-derived cytokines which might promote LPS responses, neutralizing anti-cytokine antibodies were added to spleen cell cultures (Fig. 3). Anti-interleukin-1α antibody showed the greatest inhibitory activity, blocking both mitogenesis and antibody production in a dose-dependent manner. Despite the use of large amounts of available antibody, greater than 60–70% inhibition of either mitogenesis or antibody secretion was not observed. This may have been due to antibody non-reactivity with IL-1β, which binds to the same receptor as IL-1α,[26] or the presence of additional stimulatory cytokines. Anti-IL-1β-specific antibody inhibited mitogenesis and IgM production, but to a lesser degree than the anti-IL-1α antibody that was used.

To address the possible role of other macrophage-derived cytokines in the induction of LPS responses, the effect of a variety of additional anti-cytokine antibodies was studied. TNF is a major product of LPS-activated macrophages,[27] readily augments the production of other cytokines,[24] and induces IL-1 secretion by an autocrine pathway.[28] A high concentration of neutralizing anti-TNF-α antibody was found to inhibit partially spleen cell proliferation without significantly affecting IgM production (Fig. 3a, b). Although TNF-α augments IL-1 production,[28] it also may be acting as a mitogen (or co-mitogen) for a non-IgM secreting subpopulation present in the spleen cell cultures. The combination of anti-TNF-α and anti-IL-1α antibodies was no more effective than anti-IL-1α antibody alone in inhibiting spleen cell mitogenesis and IgM secretion (Fig. 3c). IL-6 plays an important role in the differentiation of B cells into plasma cells,[29] but does not appear to play a role in LPS-inducible responses since neutralizing monoclonal antibody did not effect either thymidine incorporation or IgM production. The pairwise combination of anti-IL-6 antibody with either anti-IL-1α or anti-TNF antibodies also did not inhibit B-cell responses to LPS to any greater degree than either anti-IL-1α or anti-TNF antibody alone (Fig. 3c).

It is conceivable that in the present experimental system, macrophage-derived cytokines first augment T-cell responses, which in turn may promote B-cell activation.[30–32] The role of one T-cell-derived cytokine, IFN-γ,[33] was examined. Neutralizing monoclonal anti-IFN-γ antibody resulted in no significant inhibition of the polyclonal activation response, even when added in combination with other anti-cytokine antibodies (Fig. 3c).

To delineate further the role of IL-1 in LPS-induced polyclonal activation, the macrophage-conditioned media used to reconstitute adherent cell-depleted cultures (Fig. 2) was assayed for the presence of IL-1α by radioimmunoassay. As shown in Table 1, the concentration of immunoreactive IL-1α increased markedly in both RAW and PEC cell cultures after stimulation with LPS.

The role of macrophage-derived IL-1 in LPS-induced polyclonal activation was confirmed by adding recombinant murine IL-1α to spleen cell cultures which had been depleted of macrophages by complement lysis. As shown in Fig. 4, IL-1α restored both the mitogenesis and antibody production response of LPS in a dose-dependent fashion. An almost complete restoration of the LPS response was found with 5 U/ml. Given a specific activity of recombinant IL-1α of 8 U/ng, this would

correspond to approximately 0·6 ng/ml of IL-1α present in spleen cell culture. This value agrees well with the amount of immunoreactive IL-1α found to be present in the conditioned media used in the reconstitution experiment (0·1–0·25 ng/ml, after calculating for media dilution).

DISCUSSION

The findings in the present study confirm those reports which have proposed an obligatory role for adherent macrophages in the murine polyclonal B-cell response to LPS.[8,9] Depletion procedures based on successive rounds of physical adherence were found to be only partially effective in abrogating the LPS response; a 100-fold depletion of adherent cells (by histochemical criteria) diminished LPS activation by only 70%. This finding points to the extreme sensitivity of LPS activation to the presence of adherent macrophages, and agrees with a limiting dilution analysis that suggested that 30–1000 bone marrow-derived macrophages are necessary for a B-cell response to LPS.[9] It is likely that in studies which demonstrated adherent-cell independence, small numbers of residual adherent cells were sufficient to reconstitute LPS responsiveness.

The adherent cell requirement could be replaced by conditioned media from LPS-activated macrophages, suggesting that soluble macrophage-derived factors were sufficient to provide the co-stimulatory activity for polyclonal activation. Antibody neutralization studies implicated IL-1 as a co-factor in LPS-induced polyclonal activation, and exogenously added IL-1α reconstituted the LPS response in adherent cell-depleted spleen cell cultures. The sensitivity of the B-cell response to macrophage-derived factors most likely accounts for the high antibody concentrations required to inhibit the LPS response in the present study.

A role for additional macrophage-derived cytokines in LPS-induced polyclonal activation was not demonstrated in the present study. Although TNF-α is a major product of LPS-activated macrophages,[27] and augments the production of other cytokines via both autocrinic and paracrine pathways,[24,28] the addition of anti-TNF-α antibody was found to inhibit LPS-induced spleen cell proliferation without affecting total antibody production. Anti-TNF-α antibody also did not potentiate the inhibition of anti-IL-1α antibody. Because IgM production was unaffected by anti-TNF-α antibody, it seems unlikely that the anti-TNF effect was due simply to the inhibition of an autocrine pathway. One possibility is that TNF-α is acting as a co-mitogen for a subpopulation of spleen cells. Kehril *et al.*[34] have reported that TNF-α augments the proliferation of human tonsilar B cells when co-cultured with *Staphylococcus aureus* as a mitogen. Immunoglobulin production was not affected in this system, unless cultures were additionally supplemented with IL-2. Although IL-1 may act directly on B cells, it also may activate T cells to provide additional B-cell signals, via T-cell-derived cytokines or cell surface molecules. Interleukin-1 augments T-cell-dependent responses in a number of experimental systems, and may promote polyclonal B-cell activation through the release of B-cell-activating factors such as IL-4, IL-5, and IL-6.[31,32,35,36] Alternatively, macrophages or IL-1 may provide co-stimulation by suppressing inhibitory T-cell or natural killer (NK) cell effects. Splenic T cells were not excluded in the present experimental system and delineation of the proximate target of macrophage-derived IL-1 will require additional cell fractionation and reconstitution studies.

These experiments complement previous studies which have shown that IL-1 plays an important role in B-cell activation. Interleukin-1 promotes pre-B-cell maturation,[29,37] synergizes with IL-2 (along with IFN-γ) in the induction of sheep red blood cell (RBC) responses[38] and potentiates the proliferative effects of IL-4.[35] Interleukin-1 can replace the macrophage in the B-cell response to thymus-independent antigens[39] and in the human system, in which an accessory cell requirement has long been recognized,[40] monocyte-derived IL-1 plays an essential role in the activation responses of peripheral blood monocytes to pokeweed mitogen.[41]

Polyclonal activation may represent an untoward sequelae of chronic infection and tissue invasion, and result in a variety of immunopathological and autoimmune phenomena.[42] A large body of experimental data has established that many of the profound inflammatory and immunostimulatory effects of microbial products rely on the induction of cytokines mediators.[24–26] Investigation of the cytokine networks involved in these responses should lead to improved strategies in combating the adverse effects of chronic immune stimulation.

ACKNOWLEDGMENTS

I am grateful to Dr A. Cerami for helpful discussions and to L. Chui and S. Robinson for technical assistance. This work was supported by the Arthritis Foundation.

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