1987

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Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 3802–3806, June 1987
Immunology

Induction of murine interleukin 1: Stimuli and responsive primary cells

(dendritic cells/macrophages/lymphocyte mitogenesis/lipopolysaccharide)

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Communicated by Zanvil A. Cohn, January 14, 1987

ABSTRACT **An interleukin 1_α (IL-1_α) cDNA probe and an IL-1 responsive T-cell clone (D10.G4; half-maximal stimulation, 0.1–1 pM) have been used to study the production of IL-1 by primary murine cell populations, particularly macrophages and dendritic cells. Spleen and peritoneal macrophages produced IL-1 mRNA and released biologically active IL-1 when challenged with lipopolysaccharide (LPS). Induction of IL-1 was evident over a dose range of 0.01–10 μg of LPS per ml, and maximal mRNA levels were maintained from 4 to 20 hr. Several other stimuli did not induce IL-1 in cultured macrophages, including phorbol 12-myristate 13-acetate, γ-interferon, Con A, macrophage colony-stimulating factor, IL-3, cachectin, and activated T cells. Activated T cells could markedly reduce the response of peritoneal macrophages to LPS. When other cell types were compared with macrophages, keratinocytes had high levels of IL-1 mRNA, apparently in response to endogenous LPS. However B and T lymphocytes did not yield detectable IL-1 during proliferative responses to LPS and Con A, respectively, while dendritic cells produced little or no IL-1 when challenged with a battery of stimuli. Therefore, IL-1 may not be required for the potent accessory function of dendritic cells in lymphocyte mitogenesis. The results indicate that macrophages and dendritic cells have different secretory capacities. The macrophage is the principal leukocyte that synthesizes IL-1, and select stimuli increase and decrease the levels of macrophage IL-1 mRNA.**

Interleukin 1 (IL-1) is a 16-kDa polypeptide that mediates several aspects of acute and chronic inflammation (1). The events that are influenced by IL-1 include synthesis of acute phase proteins by hepatocytes, release of degradative enzymes from connective tissue cells, and proliferation of lymphocytes (1). Among the leukocytes, macrophages, lymphocytes, and dendritic cells have all been implicated in production of IL-1 (2–8).

RNA blotting and a sensitive IL-1 bioassay have recently become available to study the production of IL-1. We have used these assays on primary cell populations with an emphasis on a comparison of macrophages and dendritic cells. These leukocytes have different roles in cell-mediated immunity. The macrophage functions as an effector cell in phagocytosis and anti-microbial resistance, while the nonphagocytic dendritic cell acts as a potent, if not essential, accessory cell for induction of antigen-specific sensitized T lymphocytes (9–11). The capacity of these two cell types to synthesize cytokines such as IL-1 has not been compared in detail. We report that macrophages, but not dendritic cells, make IL-1, and we define some of the stimuli that increase and decrease the levels of macrophage IL-1 mRNA.

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MATERIALS AND METHODS

Mice. BALB/c × DBA/2 (C × D2)F_1, B6.H-2k, ICR, C3H/He (The Trudeau Institute, Saranac Lake, NY), and BALB/cAnN (The Rockefeller University) mice of both sexes were used with similar results.

Medium. RPMI 1640 medium (GIBCO) was supplemented with 10% heat-inactivated fetal calf serum (Sterile Systems, Logan, UT)/1 mM glutamine/streptomycin (10 μg/ml)/penicillin (100 units/ml)/gentamycin (20 μg/ml)/50 μM 2-mercaptoethanol.

Cytokines. Murine recombinant IL-1_α (rIL-1_α) [specific activity, 6×10^6 units/mg in the standard thymocyte mitogenesis assay (12)] was provided by P. Lomedico (Hoffmann–La Roche). Murine recombinant γ-interferon (rIFN-γ) (6×10^6 units/mg) was provided by Genentech (South San Francisco, CA), and human recombinant cachectin/tumor necrosis factor (10^8 units/mg) was from B. Beutler and A. Cerami (The Rockefeller University). Conditioned media from the WEHI-3 cell line and L cells were used as sources of IL-3 and macrophage colony-stimulating factor, respectively.

Lipopolysaccharide (LPS). Boivin preparations of *Escherichia coli* 05:B55 and *Salmonella typhosa* LPS were from Difco. Purified LPS from *E. coli* K-12, rough mutant 94 mm, was from List Biological Labs (Campbell, CA).

IL-1 Bioassay. The D10.G4.1 T cell clone was passaged as described (13). A standard curve was constructed relating D10 growth to rIL-1 dose (units determined in the thymocyte assay). This curve was related to the dose–response curve of various samples. Half-maximal stimulation of D10 cell growth was observed at 0.01–0.1 unit of rIL-1_α per ml (0.1–1 pM) or 0.3% (vol/vol) LPS-stimulated macrophage culture supernatant.

Phenol/Chloroform Extraction (14) of Cytoplasmic RNA. Cells in suspension were washed in Hanks' balanced salt solution without Ca^{2+} or Mg^{2+}. Adherent cells were rinsed in RPMI 1640 medium and dislodged with a cell scraper (Costar, Cambridge, MA) in Hanks' balanced salt solution. After centrifugation, cells were resuspended in 1 ml of TSM buffer (0.14 M NaCl/10 mM Tris·HCl, pH 8.6/1.5 mM $MgCl_2$) plus 5% vanadyl ribosyl complex (VRC, New England Biolabs) and centrifuged for 3 min at 1500 × *g*. The pellet was resuspended in 3–4 vol of lysis buffer (TSM/0.5% Nonidet P-40/10% VRC), mixed in a Vortex, set on ice for 3 min, and nuclei were removed by centrifugation of 2000 × *g* for 4 min. The cytoplasmic supernatant was added to an equal vol of proteinase K (200 μg/ml) (Merck) in 0.2 M Tris·HCl, pH 7.5/25 mM EDTA/0.3 M NaCl, and incubated at 37°C for 30 min. RNA was extracted twice with an equal vol of

Abbreviations: LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; IFN, interferon; rIFn, recombinant IFN; IL-1 and -3, interleukins 1 and 3; EA, erythrocytes coated with antibody.

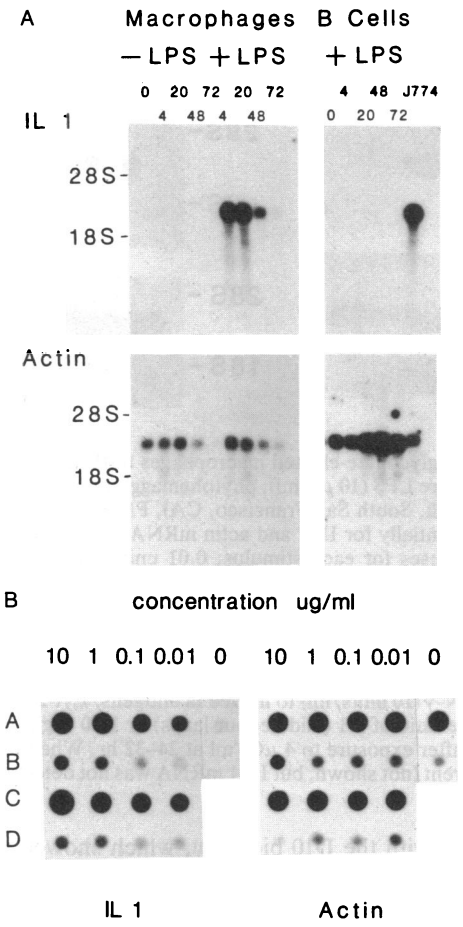


FIG. 1. LPS induction of IL-1 mRNA in peritoneal macrophages. (*A*) Kinetics of the response in thioglycollate macrophages and spleen B cells. Cells were cultured with or without LPS (10 μg/ml) for 0, 4, 20, 48, and 72 hr. The B-cell proliferative response was 130,000 cpm per 3×10^5 cells after an 8-hr pulse of [^{3}H]thymidine (4 μCi/ml; 1 Ci = 37 GBq) on day 3. RNA (5 μg) (yield per 10^7 cells) was electrophoresed and hybridized with p1301 IL-1$_\alpha$ cDNA. The blots were exposed for 2 days. For the lane with IL-1 mRNA on the far right, we applied 0.25 μg of RNA from the J774 mouse macrophage cell line stimulated with LPS for 6 hr. (*B*) Dose response of two LPS preparations. Dot blots were prepared using 5 μg (rows A and C) and 0.5 μg (rows B and D) samples of RNA from thioglycollate-elicited macrophages that had been cultured for 20 hr with graded doses of LPS from *S. typhosa* (rows A and B) and *E. coli* K-12 (rows C and D).

preequilibrated phenol/chloroform (1:1) and once with chloroform/isoamyl alcohol (24:1). RNA was precipitated with 2.5 vol of 100% ethanol overnight at −20°C, pelleted by centrifuging at 12,000 × *g* for 10 min, resuspended in water, and stored at −20°C. RNA yields were 0.3–2.0 μg per 10^6 cells as determined by absorbance at 260 nm. RNA quality was assessed by A_{260}/A_{280} ratios and by electrophoresis in ethidium bromide/agarose gels to compare 28S and 18S ribosomal RNA bands.

RNA Blots. Samples were quantitated by A_{280} and RNA blot analyses were performed as described (15) using GeneScreen filters (New England Nuclear). For dot blots, samples were adjusted to 3× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate)/7.4% formaldehyde, heated to 60°C for 5 min, applied to filters with a dotting manifold (Bio-Rad), and processed as described above. p1301 IL-1$_\alpha$ cDNA plasmid (12) (provided by P. Lomedico) and a chicken actin probe (16) were labeled with [^{32}P]dCTP (New England Nuclear) using a nick-translation reagent kit (Bethesda Research Laboratories). Specific activities were $2–4 \times 10^7$ cpm per μg of DNA. Blots were hybridized overnight with 10^6 cpm/ml and washed as described (15). Filters were exposed 2–5 days to Fuji film with intensifying screens. To visualize actin mRNA on the IL-1 blots, we stripped the latter with 5 mM Tris·HCl, pH 8.0/0.002% polyvinylpyrrolidone (40 kDa)/0.002% bovine serum albumin/0.002% Ficoll (400 kDa) for 2 hr at 70°C, and then hybridized with the actin probe.

Table 1. Kinetics of IL-1 release from LPS-induced macrophages and lymphocytes

Time in culture, hr	IL-1 activity, units/ml			
	Macrophages − LPS	Macrophages + LPS	T cells + LPS	B cells + LPS
4	0.35	0.95	0.001*	<0.001*
20	0.66	44.8	0.001*	<0.001*
48	0.47	38.2	0.002*	0.001*
72	0.44	32.4	0.004*	0.001*

Thioglycollate-elicited peritoneal macrophages, T cells, or B lymphocytes (10^6) were cultured in 1 ml of medium in 16-mm diameter wells with or without LPS (10 μg/ml). Graded doses of culture supernatant were assayed for activity in the D10 bioassay (see Fig. 4 for standard curves and assay conditions).
*Sample in which the IL-1 level was less than half-maximal (≈0.01 unit of rIL-1 per ml) at 10% (vol/vol).

Cells. Low-density spleen adherent populations were separated into dendritic cells (Fc receptor negative) and macrophage (Fc receptor positive)-enriched populations using erythrocytes coated with antibody (EA; see ref. 17). The EA$^-$ cells were >95% dendritic cells and <3% B cells and macrophages, while EA$^+$ cells had equal percentages of macrophages and B lymphocytes and <10% dendritic cells. Erythrocytes were lysed with 0.83% NH_4Cl. Peritoneal macrophages were obtained by adhering 3×10^7 resident or 10^7 elicited (1 ml of Brewer's thioglycollate, Difco, 5 days earlier) peritoneal cells on 100-mm plastic culture dishes. At 2 hr, nonadherent cells were removed by washing. B cells were Sephadex G-10 nonadherent spleen lymphocytes that were treated with anti-thy-1, lyt-1, lyt-2 monoclonal antibody and complement (clones HO 13.4, C3PO, and HO 2.2 from the American Type Culture Collection). B cells were cultured at 1 and 3×10^6 cells per ml to test for IL-1 release and mRNA, respectively. Keratinocyte monolayers were obtained from mouse epidermal suspensions (18). The trace epidermal Langerhans cell component was nonadherent (18). T lymphocytes were nylon wool nonadherent spleen and lymph node suspensions treated with anti-Ia (B21-2, American Type Culture Collection, no. TIB 229) and rabbit complement (Pel-Freez).

RESULTS

IL-1 Production by LPS-Stimulated Macrophages. Peritoneal macrophages could transiently (<16 hr) express some IL-1$_\alpha$ mRNA upon isolation, so that cells were cultured overnight before use in most experiments. The 24-hr cultures expressed little or no IL-1 mRNA but were induced with LPS (Fig. 1*A Left*). The mRNA was a single 2.6-kilobase (22S) message that comigrated with the IL-1$_\alpha$ mRNA from the LPS-induced macrophage cell line J774. mRNA was detectable at 1 hr (not shown), maintained plateau levels for 4–20 hr, and decreased markedly by 48 hr (Fig. 1*A*). LPS from *S. typhosa* and purified LPS from an *E. coli* strain K-12 rough mutant had comparable IL-1 inducing activity with plateau levels of mRNA at 10 ng/ml (Fig. 1*B*). Polymyxin B, a cyclic peptide that binds and neutralizes LPS, reduced the IL-1-inducing activity of LPS ≈100-fold but did not alter macrophage viability, total RNA yield, or actin mRNA levels (not shown).

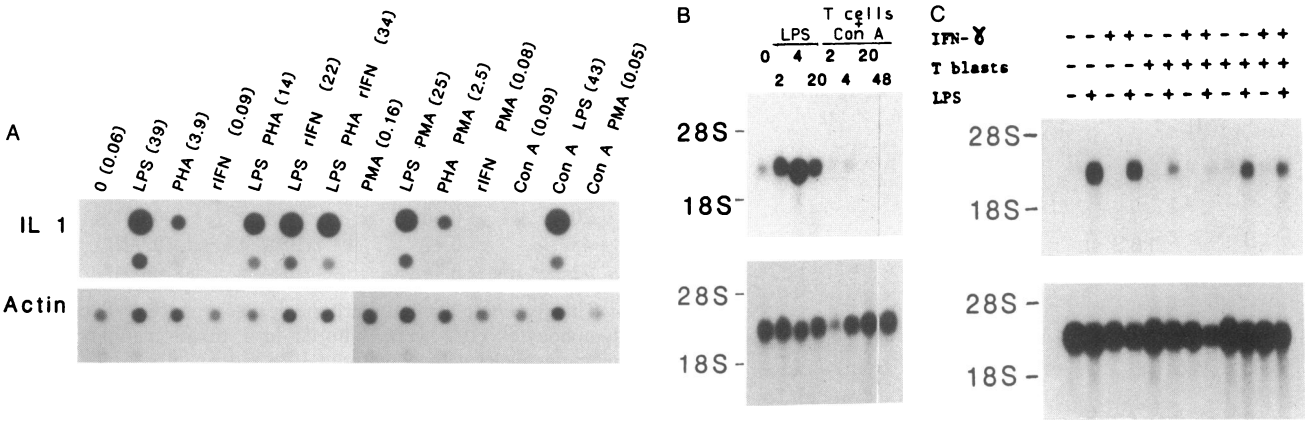
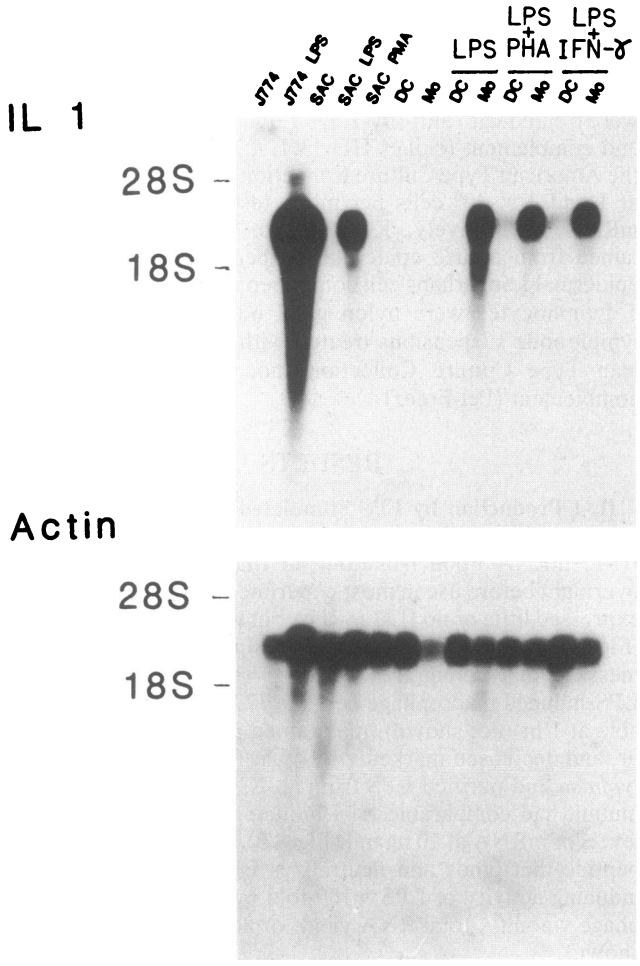


FIG. 2. LPS is the principal inducer of IL-1 in macrophages. (*A*) Response of thioglycollate-elicited macrophages to the indicated stimuli. Macrophages (10^7) were cultured for 20 hr with single or multiple stimuli. The doses were LPS (10 μg/ml), phytohemagglutinin (PHA) (2 μg/ml) (Burroughs Wellcome, Research Triangle Park, NC), IFN-γ (100 units/ml) (Genentech, South San Francisco, CA), PMA (10 ng/ml) (Sigma), Con A (3 μg/ml) (Pharmacia). Top row, 5; bottom row, 0.5 μg of RNA blotted sequentially for IL-1 and actin mRNAs. The amounts of IL-1 activity in the culture medium (units/ml in the D10 bioassay) are listed in parentheses for each stimulus; 0.01 unit of rIL-1_α per ml is a half-maximal stimulus for the D10 bioassay (see Fig. 4*A*). (*B*) Lack of IL-1 in macrophage–T-cell cocultures. Peritoneal adherent cells (3×10^6) were cultured for the indicated times with LPS (10 μg/ml) (left) or with 3×10^7 purified T cells plus Con A (3 μg/ml) (right). For each lane, RNA was separately extracted from the macrophage-rich adherent fraction (shown) and nonadherent cells (T cells plus some macrophages; only the 48-hr time point is shown on the far right). [^{3}H]Thymidine uptake of 300,000 T cells plus 30,000 macrophages was 43,000 cpm (exposure, 4 μCi/ml at 48–60 hr). (*C*) Lack of IL-1 in macrophages cultured with IFN-γ or with alloreactive T lymphoblasts. Thioglycollate-elicited macrophages (1.2×10^7) from B6.H-2k mice were cultured 3 days with or without IFN-γ (10 units/ml) to induce Ia antigens. Lyt-2$^-$ alloreactive T blasts (C × D2 anti-B6.H-2k; ref. 10) were added for 18 hr at a T-cell/macrophage ratio of 1:1 (middle four lanes) or 1:10 (right four lanes). [^{3}H]Thymidine uptake of 30,000 T blasts plus 10,000 macrophages was 98,000 cpm after exposure to 4 μCi/ml at 24–32 hr. When present, the LPS dose was 10 μg/ml. RNA was extracted from the adherent (shown) and nonadherent (not shown, but IL-1 mRNA was not detectable) layers and 5 μg of RNA was loaded per lane.

LPS stimulated B cells to proliferate actively but did not induce IL-1_α mRNA (Fig. 1*A Right*). The finding was confirmed with the D10 bioassay, which showed that macrophages released high levels of IL-1, while B and T cell populations were essentially inactive (Table 1). Whereas IL-1_α mRNA reached plateau levels 4 hr after addition of LPS to macrophages (Fig. 1*A*), the cytokine was just detectable in the medium at that time (Table 1).



Comparison of LPS with Other Stimuli. A large number of agents have been considered to be active in inducing IL-1 (1). Many proved to be weak or inactive when tested on primary peritoneal (Fig. 2) and spleen (see below) macrophages. The stimuli included Con A, phorbol 12-myristate 13-acetate (PMA), and IFN-γ either alone or in combination (Fig. 2*A*). Phytohemagglutinin induced ≈1/10th the level of IL-1 as LPS, but we could not rule out contamination of the phytohemagglutinin with some LPS. None of the stimuli synergized with LPS. Resident and thioglycollate-elicited macrophages behaved similarly (not shown).

Several approaches were taken to test whether T cells could induce IL-1 from macrophages, but none were effective. These included T cells added to macrophages in the presence of Con A (Fig. 2*B*), the conditioned medium from Con A-stimulated rat spleen cells (data not shown) and rIFN-γ (Fig. 2*C*), and alloreactive T lymphoblasts (Fig. 2*C*). In the macrophage–T-cell coculture experiments (Fig. 2 *B* and *C*), we observed clustering of the two cell types and significant T-cell proliferation (Fig. 2, legend) but little or no IL-1 mRNA. Remarkably, the addition of alloreactive T blasts reduced the level of IL-1 mRNA in response to LPS but had no effect on cell viability or actin mRNA levels (Fig. 2*C*).

FIG. 3. Spleen macrophages but not dendritic cells, make IL-1 mRNA. Spleen low-density adherent cells (SAC) were cultured 20 hr with LPS (10 μg/ml), PMA (10 ng/ml), or LPS plus phytohemagglutinin or IFN-γ. RNA was extracted from unfractionated SAC, or from dendritic cell (DC)-enriched and macrophage–B-cell-enriched (Mo) components of SAC prepared by a one-step rosetting technique (17). RNA from the J774 cell line (1 μg) or RNA from the primary cells (3 μg) was loaded on the lanes. This experiment was performed three times with similar results.

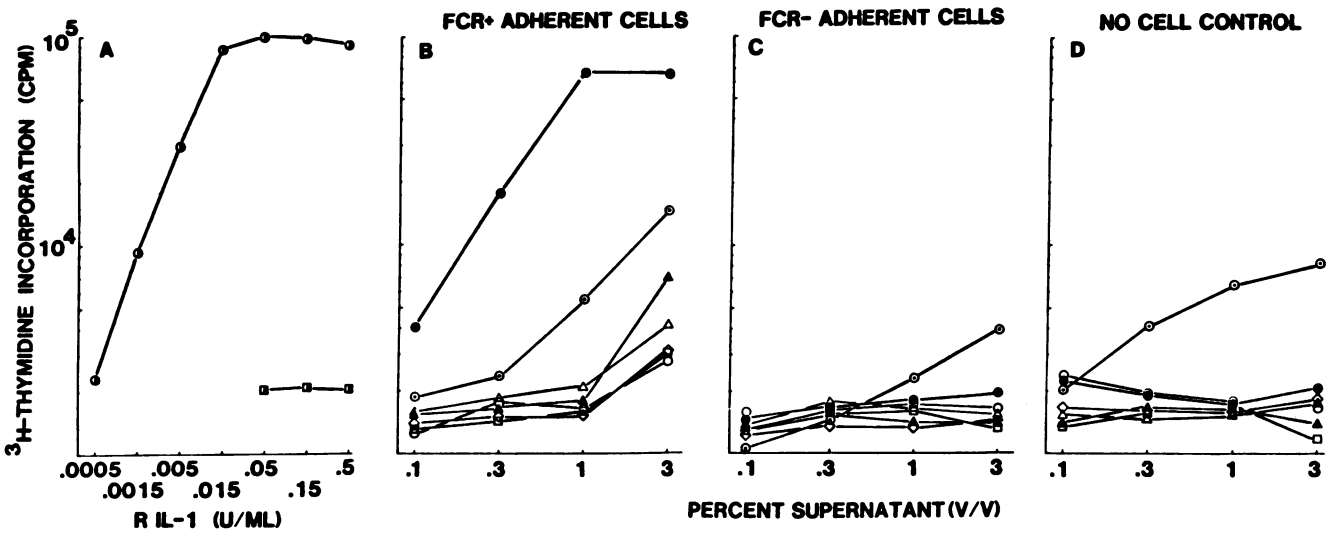


FIG. 4. Spleen macrophages but not dendritic cells release IL-1 biologic activity. Data are means of triplicate cultures of 2×10^4 D10 cells exposed to graded doses of conditioned medium and exposed to [^{3}H]thymidine at 48–62 hr (18). As in Fig. 3, Fc receptor positive and negative fractions were prepared by a one-step rosetting method. (*A*) Dose response of rIL-1 (◑) vs. *E. coli* control (◨) (no IL-1 DNA insert) protein in inducing the proliferation of D10 cells. (*B*) Release of IL-1 from Fc receptor positive spleen adherent cells following culture with a panel of stimuli: ●, 10 μg of LPS per ml; □, 100 units of IFN-γ per ml; △, 1 ng of cachectin/tumor necrosis factor per ml; ◇, 30% (vol/vol) WEHI-3 conditioned medium; ▲, 30% (vol/vol) L-cell conditioned medium; ⊙, 10 ng of PMA per ml; ○, no stimulus. The small effect seen with PMA is also evident in the medium control (see *D*). (*C*) Lack of IL-1 release from stimulated Fc receptor negative dendritic cells. (*D*) Medium control—i.e., wells cultured with the stimuli but in the absence of cells.

We did not assay the macrophage–T-cell medium for IL-1 biologic activity, since the D10 cell line would respond to the IL-2 that was released by the stimulated lymphocytes. We conclude that LPS is the principal agent that induces IL-1 in mouse peritoneal macrophages, while sensitized T cells can reduce the LPS effect.

Comparison of Spleen Macrophages and Spleen Dendritic Cells. Adherent spleen cells contain Fc receptor negative dendritic cells and Fc receptor positive macrophages and B lymphocytes. The former induce T-cell responses to several antigens *in vitro* and *in vivo* (9–11, 19). Spleen adherent cells, like peritoneal cells, could be induced to express IL-1 mRNA by LPS (Fig. 3). When the populations were separated into dendritic cells and macrophage–B-cell-enriched fractions after LPS stimulation, the latter synthesized IL-1 mRNA (Fig. 3). The trace amount of IL-1$_\alpha$ mRNA in the Fc receptor negative dendritic cell-enriched fraction could be attributed to residual macrophages (<3% of the total cells). Since spleen B cells did not produce IL-1 (Fig. 1, Table 1), the active Fc receptor positive cell was likely the macrophage.

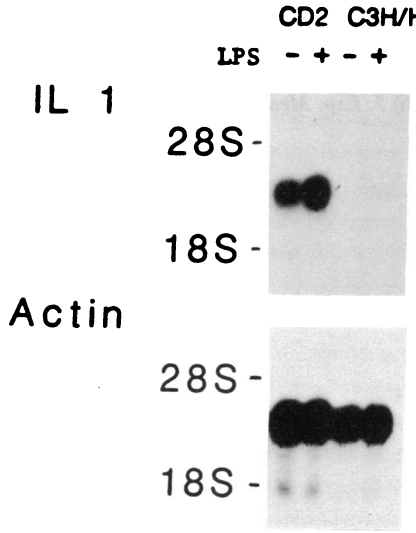


FIG. 5. Expression of IL-1 mRNA in keratinocytes. Keratinocyte monolayers were cultured for 48 hr with or without exogenous LPS (10 μg/ml). The cells were from LPS responsive (C × D2)F$_1$ and nonresponsive (C3H/HeJ) mice. RNA was extracted, and 5 μg was electrophoresed and probed for IL-1 and actin mRNAs.

The dendritic cell- and macrophage-enriched fractions of spleen-adherent cells were stimulated with a variety of individual cytokines, PMA, or LPS. When the conditioned medium was tested for IL-1 in the sensitive D10 bioassay, only macrophages released active IL-1 when exposed to LPS but not other stimuli (Fig. 4). Dendritic cells did not release IL-1 (Fig. 4), and frozen–thawed lysates of dendritic cells lacked activity (not shown). When unfractionated adherent cells were treated with monoclonal antibody 33D1 and complement to deplete dendritic cells (9), the production of IL-1 in response to LPS was not reduced (not shown). Thus, IL-1 primarily is a macrophage rather than a dendritic cell product.

IL-1 mRNA Expression in Keratinocytes. Epidermal cells release a cytokine that is similar to IL-1 (20). Keratinocyte monolayers that were depleted of Ia positive dendritic cells were prepared (18) and analyzed for IL-1$_\alpha$ mRNA. Keratinocytes from (C × D2)F$_1$ mice had significant levels of IL-1 mRNA. The amounts increased slightly upon addition of LPS. Since exposure to LPS (from the Gram-negative bacteria in mouse skin) during the preparation of epidermal cell suspensions was likely, we asked if cells from LPS unresponsive C3H/HeJ mice also had IL-1. None was found, and no response to LPS was observed (Fig. 5). Thus, the expression of IL-1 mRNA in primary mouse keratinocytes is likely a response to LPS.

DISCUSSION

Prior work on the functional properties of dendritic cells has revealed a lack of phagocytic function but an important accessory role in initiating cell-mediated immunity (reviewed in ref. 19). Little is known about dendritic cell secretory function, particularly with respect to cytokines, the polypeptides that mediate several components of inflammation and

immunity. The recent isolation of IL-1 cDNA clones and the IL-1 responsive D10.G4 cell line allows one to follow the production of IL-1 with a specificity and sensitivity that was not previously possible. Using RNA blotting and the D10 bioassay on primary cells, we detect little or no IL-1 production by dendritic cells (Figs. 3 and 4). Since IL-1 is not produced by neutrophils (21) or proliferating B and T lymphocytes (Figs. 1 and 2; Table 1), it seems that the macrophage is the principal leukocyte that makes IL-1.

The stimuli for macrophage IL-1 gene expression seem quite restricted, however. LPS is such a potent inducer (Fig. 1*B*) that the presence of small numbers of macrophages or small amounts of LPS severely complicates the analysis of IL-1 inducing stimuli and responsive primary cells. Con A, PMA, macrophage colony-stimulating factor, IL-3, IFN-γ, cachectin, and T cells do not trigger spleen and peritoneal macrophages (Figs. 2 and 4). Since we have only studied cultured tissue macrophages, some of the inactive agents may stimulate other populations of mononuclear phagocytes such as the blood monocyte. We also note little or no IL-1 mRNA (experiments not shown) in peritoneal macrophages elicited by periodate, thioglycollate, or bacillus Calmette–Guérin mycobacteria *in situ* (at day 5, the height of the inflammatory response).

The effect of T cells on the induction of IL-1 is under further study. A subpopulation of T cells, or a particular developmental stage of the macrophage such as the blood monocyte, may be required to observe IL-1 induction. This possibility is being pursued with human blood monocytes and a rabbit anti-IL-1 antibody to identify individual monocytes with IL-1 (22). The mixing of sensitized T cells with macrophages can clearly reduce IL-1_α mRNA (Fig. 2*C*). It remains to be determined whether this inhibition is due to a soluble T-cell factor.

Our results question the need for IL-1 in lymphocyte activation. B cells proliferating in response to LPS, and T lymphocytes proliferating in association with macrophages, do not necessarily produce the cytokine (Figs. 1*A* and 2 *B* and *C*). Dendritic cells, which are active accessory cells for a variety of T-dependent immune responses (9–11), also do not appear to make IL-1 (Figs. 3 and 4). Exogenous rIL-1 has little direct effect on T cells even in the presence of lectin or antigen (13). Therefore, IL-1 may not be essential for lymphocyte mitogenesis. Recent experiments indicate that IL-1 amplifies the capacity of dendritic cells to stimulate T cells (13). This amplification may account for some of the previous observations on the lymphocyte-activating properties of this cytokine.

We thank Drs. P. Lomedico and C. A. Janeway for providing the IL-1_α cDNA probe and the D10 cell line, and Dr. A. Granelli-Piperno for help with the RNA blotting. This work was supported by Grant AI 13013 from the National Institutes of Health.

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