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Mycoplasma Membrane Lipoproteins Induce Proinflammatory Cytokines by a Mechanism Distinct from That of Lipopolysaccharide

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To gain a clear understanding of the mechanisms by which mycoplasmas induce the expression of proinflammatory cytokines in monocytic cells, we have studied the induction of interleukin-1β (IL-1β), tumor necrosis factor alpha, and IL-6 by mycoplasmas in three distinct human myelomonocytic cell lines in comparison with induction by lipopolysaccharide (LPS). HL-60 cell line did not release cytokines when induced with either LPS or mycoplasmas. In contrast to LPS, mycoplasmas failed to increase the weak levels of tumor necrosis factor alpha secreted by phorbol myristate acetate-differentiated U937 cells. In addition, Northern (RNA) blot analysis of cytokine expression in these cells showed that the induction of IL-1β by mycoplasmas involves, unlike that by LPS, posttranscriptional events. Interestingly, in THP-1 cells, cytokine induction pathways triggered by mycoplasmas remained operational under conditions where LPS pathways were abolished, suggesting functional independence. The study of cytokine-inducing activity displayed by distinct fractions derived from a series of different mycoplasma species demonstrated that lipid membrane constituents were largely responsible for these effects. Finally, we have demonstrated that tyrosine phosphorylation is a crucial event in the mycoplasma-mediated induction of proinflammatory cytokines in either THP-1 cells or human monocytes.

Mycoplasmas, the smallest self-replicating microorganisms, are pathogens capable of causing a wide variety of diseases, including acute respiratory illness, genitourinary tract or joint infections, and autoimmune disorders (19). A role for these organisms in the pathogenesis of AIDS has also been suggested recently (1). In addition, mycoplasmas have been demonstrated to have diverse and multiple effects on the cells of the immune system, including polyclonal activation of B and T cells, induction of expression of major histocompatibility complex class I and class II molecules, stimulation of macrophages, and cytotoxic activity of T cells and natural killer cells. Mycoplasmas also induce the production of cytokines such as interleukin-1 (IL-1), IL-2, IL-4, IL-6, interferons, tumor necrosis factor alpha (TNF-α), and granulocyte-macrophage colony-stimulating factor (21, 24).

One well-documented effect of mycoplasmas on immunocompetent cells is the in vitro production of proinflammatory cytokines (IL-1β, TNF-α, and IL-6) by monocytes and resident macrophages (2, 10, 12, 14, 15, 17, 22). These effects have been reported with distinct mycoplasma strains isolated from humans or animals by use of heterogeneous types of mycoplasma-derived materials (e.g., inactivated mycoplasmas, membrane fractions, or purified lipoproteins or lipid constituents) and different cell types (e.g., peripheral blood mononuclear cells, macrophages, monocytic cell lines, or astrocytes). However, the study of the specific mechanisms by which mycoplasmas may induce the production of proinflammatory cytokines has not been addressed extensively.

Because of the heterogeneity of any organismal monocyte/macrophage population, we decided to investigate the mechanisms by which mycoplasmas induce cytokine production in well characterized monocytic cell lines. For this purpose, we (i) studied the mycoplasma-derived fractions responsible for the cytokine induction from a series of distinct mycoplasma species, (ii) investigated the ability of mycoplasmas to induce mRNA expression of cytokines in the monocytic cell lines, (iii) compared the cytokine induction displayed by mycoplasma with respect to lipopolysaccharide (LPS) and phorbol 12-myristate 13-acetate (PMA) by desensitization assay, and (iv) analyzed the role of tyrosine phosphorylation in these events. Our results suggest that mycoplasmas can induce cytokine production by specific pathways which may overlap with, yet remain distinct from, those triggered by LPS.

MATERIALS AND METHODS

Reagents. PMA, LPS from *Escherichia coli* O55:B5 and polymyxin B were purchased from Sigma (France). Genistein and herbimycin A were from Gibco BRL (Cergy Pantoise, France). PMA was dissolved in dimethyl sulfoxide at 1 mg/ml, and LPS was dissolved in culture media at 10 mg/ml. Herbimycin A and genistein were dissolved in dimethyl sulfoxide at 1 and 20 mg/ml, respectively. Triton X-114 (TX-114) was obtained from Merck (Nogent sur Marne, France).

Mycoplasma culture and inactivation. *Mycoplasma fermentans* PG18, *Mycoplasma pneumoniae* FH-LIV, *Mycoplasma penetrans* GTU-54-6A1, and *Mycoplasma arginini* G230 were cultivated in medium containing 20% horse serum (Gibco BRL), 10% yeast extract (Gibco BRL), 1% glucose, and penicillin G at 1,000 U/ml. Glucose was substituted for arginine in the case of *M. arginini*. Mycoplasma quantification was performed as described by Rodwell and Whitcomb (20) and expressed as CFU per milliliter. Mycoplasmas were isolated by centrifugation, washed and resuspended in Hayflick medium, and inactivated either by heating at 60°C for 30 min or by X-ray exposure at 6,000 rads for 20 min. The efficiency of inactivation procedures was verified by negative results obtained following inoculation of inactivated mycoplasmas into culture media. Heat-inactivated mycoplasmas and X-ray-inactivated mycoplasma were stored at −70°C until used. The protein content of inactivated mycoplasma samples was determined by the micro-bicinchoninic acid assay (Pierce, Ansières, France). These preparations were tested by the *Limulus* assay (Haemachem, St. Louis, Mo.) for endotoxin contamination and found to contain ≤10 pg/ml.

Lipoprotein fraction preparation. Mycoplasma membrane lipoproteins were prepared as described previously (28). Briefly, 500 ml of mycoplasma culture was pelleted by centrifugation and resuspended in 5 ml of Tris-EDTA-buffered solution (50 mM Tris [pH 8], 0.15 M NaCl, 1 mM EDTA). TX-114 was added to

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a final concentration of 2% and mixed well, and the mixture was incubated at 4°C for 60 min. The TX-114 lysate was then incubated at 37°C for 10 min for phase separation and then centrifuged at 10,000 × *g* for 20 min. The upper aqueous phase was transferred to a second tube and replaced with an equal volume of Tris-EDTA-buffered solution. This phase separation was repeated twice. The final TX-114 phase was adjusted to the original volume by adding Tris-EDTA-buffered solution. Two and one-half volumes of ethanol was then added to precipitate membrane lipoproteins overnight at −20°C. Similarly, proteins from the aqueous phase were ethanol precipitated overnight at −20°C. Precipitated materials (i.e., lipoproteins from the TX-114 fraction and proteins from the aqueous phase) were recovered by centrifugation and resuspended in phosphate-buffered saline (PBS) by brief sonication. Protein content was determined by the micro-bicinchoninic acid assay. These preparations were preincubated for 2 h with polymyxin B at 1,000 U/ml prior to use in cell stimulation. The *Limulus* assay shows that both lipoprotein and protein fractions contain ≤10 pg of endotoxin per ml.

Cell culture and stimulation. The THP-1, HL-60, and U937 cell lines were obtained from the European Animal and Cell Culture Collection. Cells were cultured (37°C, 5% CO_2) in RPMI 1640 culture medium (Gibco BRL) containing 10% fetal calf serum (Gibco BRL), 2 mM L-glutamine, and antibiotics. Cells were tested every 2 weeks by a PCR-based detection assay for mycoplasma contamination (18). U937 cells were grown to a density of 0.6×10^6 to 0.7×10^6 cells per ml, while THP-1 and HL-60 cells were grown to a density of 1×10^6 to 1.5×10^6 cells per ml before stimulation. Cells were washed and resuspended at a density of 1.0×10^6 cells per ml and stimulated with either LPS, PMA, or mycoplasma preparations. Twenty-four-well plates (Costar, Brumath, France) were used for cytokine immunoassay determinations, while 75-cm^2 tissue culture flasks (Costar) were used for RNA isolations. THP-1 and HL-60 cells were stimulated with either LPS (5 μg/ml), PMA (10 ng/ml), or mycoplasma preparations. U937 cells were costimulated with either PMA (10 ng/ml) plus LPS (20 μg/ml) or PMA (10 ng/ml) plus mycoplasma preparations.

For THP-1 cell desensitization assays, cells were first preincubated with desensitizing stimuli (LPS, PMA, or heat-inactivated mycoplasmas) for 5 h. After two washes with medium, the cells were exposed to either LPS, PMA, or heat-inactivated mycoplasmas for 24 h. Controls consisted of cells which were treated only with desensitizing agent. Cytokine production was immunoassayed after 24 h for both control and challenged cells.

Human monocyte culture and stimulation. Monocytes were prepared from peripheral blood mononuclear cells by Ficoll-Hypaque density gradient centrifugation (Pharmacia LKB, Uppsala, Sweden). Peripheral blood mononuclear cells (5×10^7) were allowed to adhere to six-well tissue culture plates (Costar) for 1 h at 37°C, under 5% CO_2, in RPMI 1640 medium containing 1% human serum (CNTS, Paris, France), 2 mM L-glutamine, and antibiotics. To remove nonadherent cells, the wells were washed twice with prewarmed culture medium. Adherent cells (approximately 5×10^6 cells) were cultured (37°C, 5% CO_2) in 5 ml of culture medium with or without a stimulus. Human monocytes were stimulated with either LPS (10 ng/ml), PMA (10 ng/ml), or mycoplasma preparations. Cytokine production was monitored after 24 h of culture.

Assay for cytokines. After 24 h of stimulation, cells were lysed by two consecutive cycles of freezing and thawing. Thus, the samples represented the total amount of cytokines produced (both intracellular and those that have been released into the supernatant). The cytokine concentration of these samples was measured with IL-1β, IL-6, and TNF-α enzyme-linked immunoadsorbent assay (ELISA) kits from Immunotech (Marseille, France), R&D (Abingdon, United Kingdom), and Genzyme (Cambridge, Mass.), respectively. The assays were performed as described in the manufacturer's instructions.

RNA isolation and analysis. At the times indicated, total RNA was extracted from 10^7 cells with the total RNA isolation kit from Bioprobe (Montrevil, France) as described in the manufacturer's instructions. Five to 10 μg of RNA was fractionated on 1.2% agarose–formaldehyde gels, transferred to a Hybond-N$^+$ filters (Amersham, Buckinghamshire, United Kingdom), and UV cross-linked. Complete human IL-1β and TNF-α cDNAs amplified by PCR were used as probes. The amount of RNA in each filter lane was normalized by probing for glyceraldehyde phosphate dehydrogenase (Clonetech, Palo Alto, Calif.). All probes were labeled by a multiprime DNA labeling system with [α-^{32}P]dCTP (Amersham). Filters were prehybridized in a mixture of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% sodium dodecyl sulfate (SDS), 5× Denhardt's solution, and 100 μg of denatured salmon sperm DNA per ml for 2 h at 64°C. An overnight hybridization was performed at 64°C in the same buffer containing the probe at 2.5×10^5 cpm/ml. The membrane was then washed twice for 10 min in 2× SSC–0.05% SDS at room temperature and once in 1× SSC–0.1% SDS at 64°C. Filters were exposed to Amersham Hyperfilm at −80°C for 2 to 72 h.

Detection of tyrosine phosphorylation by immunoblotting. For antiphosphotyrosine monoclonal antibody immunoblotting, 2×10^6 cells were incubated with LPS or mycoplasma fractions for the indicated times. After stimulation, the cells were washed with cold PBS and pellets were suspended in ice-cold lysis buffer (20 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1 mM Na_3VO_4, 1 mM *N*-ethylmaleimide, 1 μg of aprotinin per ml, 2.5 μg of leupeptin per ml, 10 μM pepstatin, 10 μg of trypsin inhibitor per ml). Total proteins from 4×10^5 cells were electrophoresed on an SDS–12% polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Molshein, France). The immunoblot was incubated with 1/1,000 antiphosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc., Lake Placid, N.Y.) for 1 h and then with 1/10,000 peroxidase-conjugated rabbit anti-mouse immunoglobulin G2b (Southern Biotechnology Associates, Inc.) for 45 min and washed and developed with an enhanced chemiluminescence kit (Amersham) as described in the manufacturer's instructions.

RESULTS

Mycoplasmas induce the production of IL-1β, TNF-α, and IL-6 by human monocytes and myelomonocytic cell lines. It has been demonstrated previously that some mycoplasma species and mycoplasma-derived fractions trigger the production of IL-1β, TNF-α, and IL-6 in human monocytes. We have studied the production of these cytokines in human monocytes derived from five healthy donors and stimulated by LPS or heat-inactivated *M. fermentans* (HIMf). HIMf (10^6 CFU/ml) was able to trigger the secretion of cytokines at levels comparable to those obtained with LPS. The responses of the different donors with regard to cytokine production were observed to be quite varied (data not shown). One of the five donors showed no TNF-α and a weak IL-β production in response to HIMf, while the IL-6 level remained comparable to that of other donors.

Given the variability in the responses observed with human monocytes, three different human myelomonocytic cell lines (HL-60, U937, and THP-1) were used to investigate the production of cytokines in response to mycoplasmas. Each of these three cell lines represents a unique stage of differentiation in that differing stimuli are necessary to induce the secretion of cytokines (3, 9, 26). The promyelomonocytic cell line HL-60 produces IL-1β and TNF-α in response to PMA, but it is insensitive to LPS (3). In contrast, the monocytic cell line THP-1 produces IL-1β, TNF-α, and IL-6 in response to either PMA or LPS (26). Finally, U937 cells must first be induced to differentiate with PMA before they are able to produce IL-1β, TNF-α, and IL-6 in response to LPS stimulation (9).

HIMf (10^6 CFU/ml) was used either alone or in combination with LPS or PMA to evaluate the production of cytokines by these cell lines. In HL-60 cells, HIMf was not able to induce cytokine production (Fig. 1A). In contrast, HIMf induced the synthesis of IL-1β, IL-6, and TNF-α in THP-1 cells at levels comparable to those obtained with LPS or PMA (Fig. 1B). Upon stimulation with HIMf alone, U937 cells did not synthesize cytokines. Costimulation of U937 cells with PMA and HIMf induced the secretion of IL-1β and IL-6 at levels comparable to those obtained with PMA-LPS. However, there was no increase in TNF-α on U937 cells costimulated with PMA plus HIMf relative to that of PMA alone (Fig. 1C). It has been shown that IL-1β protein is not processed efficiently by U937 cells stimulated with PMA-LPS (13). Accordingly, in both PMA-LPS- and PMA-HIMf-stimulated cells, the majority of this cytokine was found to be intracellular (data not shown).

When *M. fermentans* was inactivated by X-ray irradiation, a procedure which leaves the cell structure intact, no changes were observed in its ability to induce cytokines. The levels of IL-1β production by THP-1 and PMA-differentiated U937 cells stimulated with X-ray-inactivated *M. fermentans* in comparison with those stimulated with HIMf are shown in Fig. 1D. Similar levels of cytokine production were detected when live *M. fermentans* was used as the stimulus (data not shown).

Expression of IL-1β and TNF-α mRNAs in stimulated THP-1 and U937 cells. To investigate the kinetics of cytokine induction by mycoplasmas in monocytic cell lines, we studied the expression of IL-1β and TNF-α mRNA in THP-1 and U937 cell lines. As shown in Fig. 2, THP-1 cells showed similar kinetics for IL-1β and TNF-α mRNA induction after stimula-

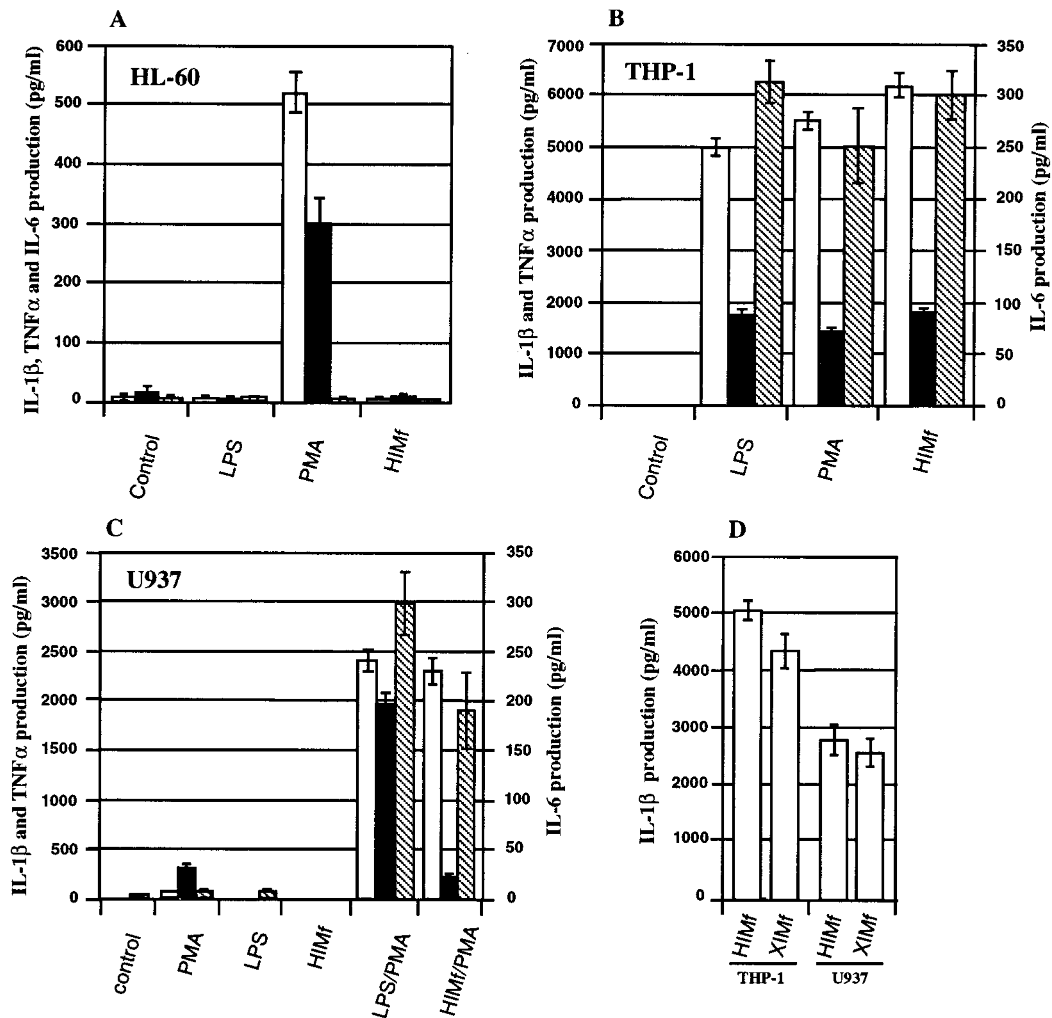

FIG. 1. Proinflammatory cytokine production by monocytic cell lines. (A) HL-60 cells (10^6/ml) were stimulated with either PMA (10 ng/ml), LPS (20 μg/ml), or HIMf (10^6 CFU/ml). (B) THP-1 cells (10^6/ml) were stimulated with either PMA (10 ng/ml), LPS (5 μg/ml), or HIMf (10^6 CFU/ml). (C) U937 cells (2×10^6/ml) were stimulated with either PMA (10 ng/ml), LPS (20 μg/ml), HIMf (10^6 CFU/ml), PMA (10 ng/ml)–LPS (20 μg/ml), or PMA (10 ng/ml)–HIMf (10^6 CFU/ml). (D) Comparison of IL-1β induction in THP-1 and PMA-differentiated U937 cells stimulated by HIMf and X-ray-inactivated *M. fermentans* (XIMf). Cytokine production was measured by ELISA 24 h after stimulation. Data are means ± standard deviations for triplicate cultures, which were assayed in duplicate. Symbols: □, IL-1β; ■, TNF-α; ▧, IL-6.

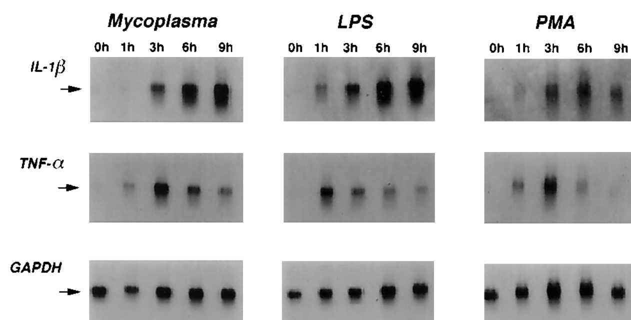

FIG. 2. Northern blot analysis of cytokine transcripts in THP-1 cells. THP-1 cells were stimulated with either LPS (5 μg/ml), HIMf (10^6 CFU/ml), or PMA (10 ng/ml). Total RNA was extracted from 10^7 cells at the indicated times and analyzed by Northern blot hybridization for expression of IL-1β and TNF-α. As a control for equivalent lane loading, constitutively expressed glyceraldehyde phosphate dehydrogenase (GAPDH) transcripts were detected.

tion with HIMf, LPS, or PMA. TNF-α mRNA levels reached a peak about 3 h after stimulation, while IL-1β mRNA increased continuously up to 9 h poststimulation.

As shown above, U937 cells need to be costimulated subsequently with PMA and either LPS or HIMf to produce cytokines efficiently. To study the time course of mRNA expression of cytokines in this cell line, cells were first incubated with PMA for 6 h and then washed and challenged with LPS or HIMf for the indicated time. Under these conditions, cytokine levels similar to those shown in Fig. 1C could be detected (data not shown). As shown in Fig. 3, in U937 cells treated with PMA, TNF-α mRNA levels peaked within 1 to 3 h, whereas IL-1β mRNA increased continuously over a 6-h period. Under these conditions, no IL-1β and little TNF-α were detected by ELISA (Fig. 1C). LPS induced a second increase in IL-1β mRNA when added to the PMA-differentiated U937 cells (Fig. 3). Stimulation of PMA-differentiated U937 cells with HIMf, however, did not show any effect on IL-1β mRNA expression (Fig. 3). Additionally, neither HIMf nor LPS was able to in-

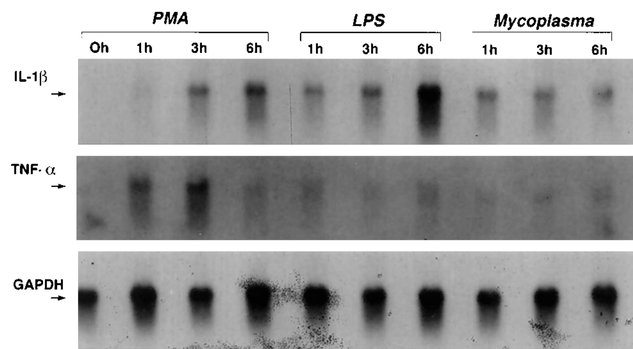

FIG. 3. Northern blot analysis of cytokine transcripts in U937 cells. U937 cells were induced with PMA (10 ng/ml) for 6 h and then, after washing, stimulated with either LPS (20 μg/ml) or HIMf (10^6 CFU/ml). Total RNA was extracted from 10^7 cells at the indicated times for each stimulus and analyzed by Northern blot hybridization for expression of IL-1β and TNF-α. As a control for equivalent lane loading, constitutively expressed glyceraldehyde phosphate dehydrogenase (GAPDH) transcripts were detected.

duce TNF-α mRNA expression in PMA-differentiated U937 cells.

Investigation of mycoplasma cytokine induction pathways by THP-1 desensitization assay. LPS-stimulated THP-1 cells have been shown to be refractory to further LPS stimulation for at least 7 h after the initial stimulation, demonstrating a phenomenon known as desensitization (4, 5). It follows that THP-1 cells stimulated with LPS should also be refractory to other inducers that trigger the same signal transduction pathways as those of LPS (6). We have studied the specificity of homologous desensitization of IL-1β and TNF-α production in THP-1 cells pretreated with LPS, PMA, or HIMf. Desensitization was monitored by observing the differences in cytokine production between the cells which were exposed to desensitizing agent only and the cells which were exposed to desensitizing agent and then challenged with the same or different inducers. The results are shown in Table 1. As expected, in THP-1 cells, LPS and PMA induced specific desensitization but failed to cross-desensitize. THP-1 cells treated with PMA or HIMf were responsive to additional stimulation by HIMf or PMA, respectively. Cells pretreated with HIMf for 5 h became refractory to LPS, and conversely, HIMf was able to stimulate TNF-α production in cells desensitized with LPS. The expression profiles of IL-1β were more complex. Although IL-1β production following HIMf secondary stimulation did not reach the levels obtained with PMA, it was significantly greater than that observed with LPS. These results suggest that (i) HIMf and PMA trigger distinct pathways to induce IL-1β and TNF-α in THP-1 cells and (ii) even if LPS and HIMf do have some common cytokine induction pathways, HIMf may bypass the LPS-desensitized pathways, allowing the induction of TNF-α and, to a lesser extent, IL-1β in THP-1 cells.

TABLE 1. Desensitization of cytokine induction pathways in THP-1 cells

Desensitizing agent	Cytokine measured	% Cytokine produced[a]			
		None[b]	LPS	PMA	HIMf
LPS	IL1-β	100	110 ± 6	173.8 ± 2.3	150 ± 3
	TNF-α	100	102 ± 1	218 ± 4	214 ± 5
PMA	IL1-β	100	128 ± 1	106 ± 1	141 ± 2.3
	TNF-α	100	171 ± 4	112 ± 1.2	192 ± 5.5
HIMf	IL1-β	100	115 ± 1.7	173 ± 1	114 ± 1.2
	TNF-α	100	101 ± 1.5	211 ± 5	101 ± 2

[a] The indicated percentages of cytokine production were normalized to control levels from cells treated only with the respective desensitizing agent. Values are means ± standard deviations for four separate experiments.
[b] Inducer.

TABLE 2. Cytokine production by THP-1 cells and PMA-differentiated U937 cells stimulated with TX-114 or aqueous fractions extracted from different mycoplasma species

Mycoplasma species-derived fraction[a]	Cytokine produced (pg/ml)[b]				
	IL-1β		IL-6		TNF-α by THP-1
	U937[c]	THP-1	U937[c]	THP-1	
M. fermentans					
TX-114	2,755 ± 115	5,500 ± 122	250 ± 65	166 ± 42	3,230 ± 143
Aqueous	0	0	0	0	0
M. arginini					
TX-114	6,100 ± 130	5,240 ± 210	260 ± 20	120 ± 15	2,280 ± 200
Aqueous	0	0	0	0	0
M. pneumoniae					
TX-114	0	0	0	0	320 ± 54
Aqueous	0	0	0	0	0
M. penetrans					
TX-114	3,220 ± 189	4,020 ± 155	300 ± 45	545 ± 65	2,890 ± 130
Aqueous	0	0	0	0	0

[a] TX114, membrane lipoprotein fraction; aqueous, protein fraction.
[b] Values are means ± standard deviations of three experiments.
[c] U937 cells were costimulated with PMA and mycoplasma-derived fractions.

Lipoprotein fractions derived from a series of mycoplasma species are responsible for the induction of cytokines in monocytic cell lines. To determine whether the component responsible for the cytokine induction by monocytic cell lines was hydrophobic or hydrophilic, four mycoplasma species were used for TX-114 phase fractionation. Three of the mycoplasma species used are human contaminants (*M. fermentans*, *M. penetrans*, and *M. pneumoniae*), and one is commonly found in cell cultures (*M. arginini*). These mycoplasma strains have been shown to induce cytokine production in human monocytes (11). The quantity of proteins fractionated into the aqueous and the TX-114 phases was measured by a colorimetric assay, and about 1 μg of protein was used to stimulate the cells. Samples were incubated with polymyxin B (1,000 U/ml) for 2 h before their addition to cell line cultures to eliminate the effect of potential LPS contamination. The results displayed in Table 2 show that only the TX-114 fractions retained the capacity to significantly induce the production of cytokines by PMA-differentiated U937 cells and by THP-1 cells. The pretreatment of the TX-114 fractions with proteinase K did not significantly change the ability of these preparations to induce cytokines (data not shown), thus indicating that the lipid fraction is largely responsible for the activity. It should be noted that TX-114 fractions derived from *M. pneumoniae* were unable to induce the production of either IL-1β or IL-6 by the two cell lines and were able to induce only small amounts of TNF-α in THP-1 cells.

Tyrosine phosphorylation is a key event in mycoplasma induction of cytokines. It is well documented that tyrosine phosphorylation plays a crucial role in mediating the extracellular stimulation that triggers cytokine production in immune cells. To analyze the importance of these events in cytokine induction by mycoplasmas, THP-1 cells and fresh human monocytes were pretreated with the specific tyrosine kinase inhibitor herbimycin A at a concentration that does not affect cell viability as judged by microscopic analysis (5 to 10 μM, 18 h) (27). Untreated or herbimycin A-treated cells were then stimulated

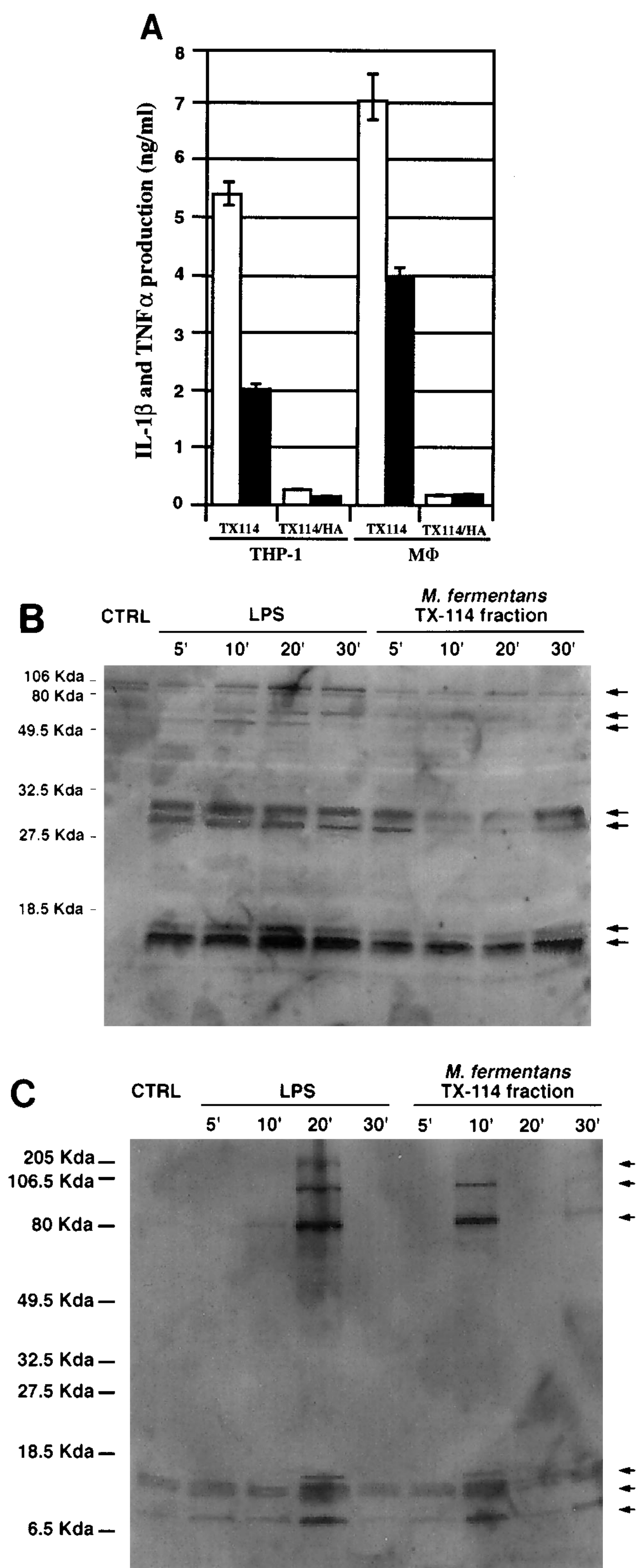

FIG. 4. Stimulation of tyrosine phosphorylation in THP-1 cells and human monocytes (MΦ) treated by *M. fermentans*-derived TX-114 fraction. (A) Cells were pretreated for 2 h with 5 μM herbimycin A (HA) and then stimulated for 24 h with either *M. fermentans*-derived TX-114 fraction or LPS. IL-1β and TNF-α production by THP-1 cells and human monocytes was monitored in cell supernatant by an ELISA as described in Materials and Methods. Symbols: □, IL-1β; ■, TNF-α. (B and C) THP-1 cells (B) or human monocytes (C) were treated for 5, 10, 20, and 30 min with either *M. fermentans* TX-114-derived fraction or LPS. Cell lysates were resolved by SDS–14.5% polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and probed with antiphosphotyrosine monoclonal antibody 4G10 as described in Materials and Methods. CTRL, tyrosine phosphorylation pattern of untreated cells. Molecular mass markers (in kilodaltons) are shown on the left.

with the *M. fermentans*-derived TX-114 fraction or with LPS. As shown in Fig. 4A, herbimycin A pretreatment completely inhibited IL-1β and TNF-α production. Similarly, herbimycin A pretreatment blocked IL-6 production (data not shown). This observation was supported by identical experiments with genistein, another tyrosine kinase inhibitor (data not shown). Moreover, herbimycin A and genistein were shown to inhibit cytokine production in U937 cells when costimulated with PMA and mycoplasmas (HIMf or TX-114 fractions [data not shown]).

To further investigate the role of tyrosine phosphorylation in mycoplasma stimulation, THP-1 cells and fresh human monocytes were treated with the *M. fermentans*-derived TX-114 fraction. Cell lysates were analyzed for tyrosine phosphorylation by Western blotting (immunoblotting) as described in Materials and Methods. In addition, the tyrosine phosphorylation patterns for LPS stimulation were analyzed and compared with those of mycoplasma-treated cells. As shown in Fig. 4B and C, the major phosphorylated proteins in the two cell types were essentially the same for both LPS and *M. fermentans*-derived TX-114 fraction stimulation; however, some high-molecular-weight proteins were slightly phosphorylated only with LPS. These results as well as the desensitization experiments suggest that mycoplasma stimulation uses some, but not all, of the pathways used by LPS.

DISCUSSION

It is well-known that mycoplasmas or substances isolated from these organisms have the potential to activate murine and human immune cells. While one of the best-described activities consists of the induction in cytokine production by monocytic cells, the mechanisms by which mycoplasmas exert this effect remain poorly understood. To further study these mechanisms, we have first compared the cytokine-inducing activities displayed by different mycoplasma preparations and LPS on three different human myelomonocytic cell lines. The cytokine production by both mycoplasmas and LPS correlated with the stage of cell line differentiation, but interestingly, mycoplasma preparations, unlike LPS, were unable to induce the secretion of TNF-α by PMA-differentiated U937 cells. The study by Northern (RNA) blotting of the induction of cytokine messenger induced by either LPS or mycoplasmas also showed a marked difference between these two stimuli in U937 cells. Whereas LPS was able to induce a second wave of IL-1β messenger in PMA-differentiated U937 cells via a second pathway, no increase of messenger could be demonstrated with mycoplasmas. Curiously, the levels of IL-1β synthesis in these two situations were similar, thus suggesting that in these cells, mycoplasmas regulate the production of IL-1β at a posttranscriptional level. These results indicate that mechanisms by which mycoplasmas stimulate cytokine expression are distinct from those triggered by LPS.

THP-1 desensitization assays further support the hypothesis that mycoplasmas trigger IL-1β and TNF-α production by pathways distinct from those of LPS or PMA. Mycoplasmas and PMA did not induce reciprocal desensitization, suggesting that the cytokine induction by these two agents is regulated by

distinct signals. LPS could not generate desensitization to mycoplasmas, whereas mycoplasmas abolished LPS stimulation. This strongly supports the hypothesis that cytokine induction by mycoplasmas involves biochemical pathways which allow them to bypass the LPS desensitization. The desensitization assay constitutes a versatile system that has been used in the study of very different biological responses. Although the exact nature of this type of system remains unclear, the activation of specific receptors has been implicated in some desensitization assays (23). It could be interesting to investigate the existence of specific cellular targets mediating the mycoplasma activity. A recent article (12) reported that *M. fermentans* lysate material does not stimulate monocytes through CD14 to induce the production of TNF-α. In addition, it has been demonstrated that in contrast to LPS, mycoplasmas can induce the production of cytokines by human monocytes in the absence of serum (10).

From our results obtained with either membrane lipoprotein fractions or total soluble proteins derived from four distinct mycoplasma species, some conclusions can be deduced. First, the capacity of mycoplasmas to induce proinflammatory cytokine seems to be a general phenomenon shared by very distant species. Second, this activity is apparently restricted to membrane components. Third, lipid constituents appear to be largely responsible for the induction of these cytokines. An *M. fermentans*-derived high-molecular-weight material (MDHM), recently characterized as a membrane-associated lipid-like component, has been shown to induce cytokine expression and production in macrophages (14, 15, 17). MDHM was able to induce only weak mRNA cytokine expression in THP-1 cells while having no detectable effect on cytokine secretion (17). In our hands, HIMf as well as membrane lipids or lipoproteins triggered cytokine production at high levels in this same cell line. These results suggest that MDHM alone does not suffice to trigger cytokine release from THP-1 cells and that additional lipid membrane components could be required. Interestingly, an *M. fermentans*-derived 48-kDa lipoprotein has been shown recently to induce TNF-α release by human monocytes (12). Although *M. pneumoniae* has been shown to induce proinflammatory cytokines in human monocytes (11, 16), a TX-114-derived fraction from this mycoplasma was inactive when added to PMA-differentiated U937 cells and poorly active on THP-1 cells. These results may be explained by the expression of a specific receptor(s) on human monocytes that is absent or poorly expressed in myelomonocytic cell lines.

Few investigators have approached the study of the signal transduction pathways involved in cytokine induction by mycoplasmas. In THP-1, mycoplasmas have been demonstrated to induce TNF-α by a Ca^{2+}-dependent, but not protein kinase C-dependent, biochemical pathway (25). Results presented in this report with two protein tyrosine kinase inhibitors demonstrate that tyrosine phosphorylation is a crucial event in the mycoplasma-mediated triggering of proinflammatory cytokines in either THP-1 or human monocytes (Fig. 4). In the case of peripheral blood mononuclear cells, protein tyrosine kinase has also been shown to be involved in the LPS cytokine induction pathway (7, 8). The investigation by immunoblotting of the tyrosine phosphorylation induced in THP-1 and monocytes stimulated by either LPS or the membrane lipoprotein TX-114-derived fraction from *M. fermentans* allowed the detection of very similar patterns for the two stimuli, although unlike LPS, mycoplasmas were unable to induce the phosphorylation of some high-molecular-weight proteins. These results together with those obtained in the desensitization assay suggest that mycoplasmas and LPS share some similar transduction pathways triggered through different monocytic cell surface molecules to induce proinflammatory cytokines. The characterization of the molecules involved in the interaction between mycoplasma and eukaryotic cells should help to better understand the immunomodulatory activity of these organisms and should help to elucidate the mechanism of pathogenicity of some species or facilitate the application of mycoplasma preparations as clinical immunomodulators.

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