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STUDIES ON ANTIGENS ASSOCIATED WITH THE ACTIVATION OF MURINE MONONUCLEAR PHAGOCYTES: KINETICS OF AND REQUIREMENTS FOR INDUCTION OF LYMPHOCYTE FUNCTION-ASSOCIATED (LFA)-1 ANTIGEN IN VITRO[1]

GIDEON STRASSMANN,[2]* TIMOTHY A. SPRINGER† AND DOLPH O. ADAMS*

*From the *Department of Pathology, Duke University Medical Center, Durham, NC 27710, and the †Laboratory of Membrane Immunochemistry, Dana Farber Cancer Institute, Harvard Medical School, Boston, MA 02115*

Macrophages activated and primed in vivo, although not resident or responsive macrophages, express the lymphocyte function associated (LFA)-1 antigen. By contrast, the biochemically related Mac-1 antigen is expressed on all populations of macrophages. In the present paper, we studied regulation of the LFA-1 antigen in vitro. LFA-1 could be induced in vitro on thioglycollate (TG)-elicited but not on proteose peptone (PP)-elicited or resident macrophages. Specifically, macrophage-activating factor (MAF), interferon-γ (IFN-γ), or picogram amounts of endotoxin (LPS) induced LFA-1 on TG-elicited macrophages following overnight incubation. Interferon, -α or -β, fucoidin, and colony-stimulating factor were not effective. While some levels of LFA-1 could be detected as soon as 10 hr, peak expression was observed after 16 to 32 hr of incubation. The induction could be completely abrogated by cycloheximide, suggesting that protein synthesis was required. These results indicate that the induction of LFA-1 on mononuclear phagocytes is closely regulated and that the requirements for such induction are distinct from but share certain similarities with induction of cytotoxic functions and expression of Ia antigen.

The functional development of murine mononuclear phagocytes can be achieved in vivo and in vitro (1). Exposure of macrophages to macrophage-activating factor (MAF)[3] in vitro results in an extensive remodeling of plasma membrane proteins, which gives rise to the acquisition of various functions and capacities (for review, see Reference 1). Among these, for example, are induction of selective tumor cell binding and competence for tumor cell lysis (1, 2), expression of class II major histocompatibility antigens (Ia antigen) (3), decreased expression of the F4/80 antigen (4), and reduction of the transferrin receptor (5). The inductive signals regulating each of these alterations are precise, so that activation is stringently regulated (1).

The lymphocyte-function associated (LFA)-1 antigen, which is involved in the cation-dependent adhesion between cytotoxic T lymphocytes (CTL) and targets, is present on B cells, myeloid cells, and T cells (6). Monoclonal antibodies (MAb) to this antigen precipitate a unique α-chain of 180,000 M_r and a β-chain of 95,000 M_r which is identical to the β-chain of Mac-1 (7, 8). The latter antigen is associated with function of the C3bi receptor (9). Deficiencies in the Mac-1, LFA-1 glycoprotein family have profound effects on host defense (10).

We have recently demonstrated that LFA-1 is also present on macrophages and, specifically, is expressed on activated macrophages (macrophages activated in vivo by bacillus Calmette-Guérin (BCG) and, to a lesser extent, on macrophages primed by pyran copolymer in vivo) (10a). Resident macrophages or responsive macrophages elicited by thioglycollate (TG) broth or proteose peptone (PP) did not express the antigen (10a). To analyze in detail the conditions which regulate LFA-1 expression on macrophages, we studied its expression in response to several potent activating signals. We here report that MAF, IFN-γ, or bacterial endotoxin (LPS) but not fucoidin or colony-stimulating factor (CSF) can induce expression of LFA-1. We further demonstrate the action of these inductive signals is dependent upon the precise population of macrophages tested, since TG-elicited but not PP-elicited or resident macrophages can be induced to express LFA-1.

MATERIALS AND METHODS

Mice. Inbred female, specific pathogen-free C57Bl/6J C3H/HeN and C3H/HeJ mice were obtained from the Trudeau Institute, Inc. (Saranac Lake, NY). Mice of 8 to 16 wk of age were used. The animals were kept in the Animal and Laboratory Isolation Facility at Duke University.

Reagents. Protease peptone (PP) broth and Brewer's thioglycollate broth (TG), prepared according to manufacturer's instructions and endotoxin (Cat. No. 3121), were obtained from DIFCO Manufacturing Co. (Detroit, MI). Fetal bovine serum (FBS) was obtained from Sterile Systems, Inc. (Logan, UT, endotoxin 0.019 ng/ml). All reagents contained less than 0.25 ng/ml of endotoxin as quantified by the Limulus Amoebocyte Lysate assay (Cape Cod Associates, Woods Hole, MA). Fucoidin was purchased from Pfaltz and Bauer, Inc. (Stamford, CT). Macrophage-activating factor (MAF) was a generous gift from Dr. Robert Schreiber (Scripps Clinic and Research Foundation). This material was produced by a T cell hybridoma (Clone 24/G1) as described previously (11) and has recently been extensively purified and shown to be physically and functionally similar to murine IFN-γ (12). IFN-γ was produced at Genentech, Inc. (So. San Francisco, CA) from monkey COS-7 cells transfected with a plasmid containing the murine IFN-γ gene expressed under control

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[2] Correspondence address: Dr. G. Strassmann, Department of Pathology, Box 3712, Duke University Medical Center, Durham, NC 27710.
[3] Abbreviations used in this paper: MAF, macrophage activating factor; LFA, lymphocyte function-associated antigen; PEC, peritoneal exudate cells; MAb, Monoclonal antibodies; IFNγ, gamma interferon; LPS, bacterial endotoxin; TG, thioglycollate; PP, protease peptone, CSF, colony stimulating factor; CTL, cytotoxic T lymphocytes.

of the SV40 promoter (13). The interferon activity was estimated to be 15,000 units/ml in a cytopathic effect inhibition assay with encephalomyocarditis virus on murine L929 cells. Cycloheximide was purchased from Sigma (St. Louis, MO). Murine IFN-α and IFN-β were purchased from Lee-Biomolecular Research Inc. (San Diego, CA). All other reagents were of the highest grade commercially obtainable.

Macrophages. Inflammatory macrophages were obtained 3 days after i.p. injection of 1 ml of sterile TG or PP. Resident macrophages were obtained by peritoneal lavage of untreated animals. The peritoneal exudate cells (PEC) were obtained by lavage with 10 ml of cold HBSS supplemented with 10 units heparin/ml. The PEC were centrifuged at 250 × G for 5 min, resuspended in Eagle's minimal essential medium (EMEM) + 10% FBS (fetal bovine serum, Sterile Systems, Inc., Salt Lake City, UT). Aliquots of 2×10^5 macrophages in 100 μl of medium were plated in flat-bottom microtiter plates (Limbro 7600605). The plates were incubated at 37°C in 5% CO_2 for 2 hr and the monolayers were washed three times to remove nonadherent cells. We routinely found the cell monolayers to contain >95% macrophages as determined by morphology by Wright's stain or a histochemical assay for nonspecific esterase.

Treatment of macrophages in vitro. Macrophage monolayers were incubated at 37°C with 0.15 ml of EMEM + 10% FBS containing various agents for 18 hr to 3 days. Following this exposure period, macrophage monolayers were subjected to radioimmunoassay.

Monoclonal antibodies. The Mab used in this study were previously described (7, 8, 14). The M17/4 (α-LFA-1, IgG_{2a}) Mab was partially purified from culture supernatants by ammonium sulfate (2.2 M) precipitation and dialyzed extensively against 0.1 M Tris HCl buffer pH 7.8. The M1/70 (α-Mac-1, IgG_{2b}) and M5/114 (α-I-A^b, IgG_{2b}) and M3/38 (α-Mac-2, IgG_{2a}) were used as culture supernatants.

Radioimmunoassay. Macrophage monolayers were incubated for 30 min at 4°C with 100 μl/well of 2% normal mouse serum, 8% normal horse serum, and 0.02% sodium azide in PBS. The fluid was aspirated and 25 μl of Mab diluted in PBS containing 8% horse serum and 0.02% sodium azide (referred to as "diluent") were added for 2 hr in the cold. After three washes with 150 μl of the diluent, 30 μl (1×10^5 cpm) of ^{125}I-labeled F(ab')$_2$ of goat anti-rat IgG_{HL} (Cappel Labs, Malvern, PA) were added per well. ^{125}I (Amersham) labeling of antibodies was performed by the chloramine T technique. Specific activity of several batches prepared was 2.8 to 4.2 μCi/μg. After 2 hr in the cold, the plates were washed three times with diluent (150 μl) and the monolayers were dissolved by the addition of 100 μl of 0.25% SDS per well. The content of each well was transferred into a tube and counted in a Packard gamma scintillation spectrometer. Triplicate wells were used per each point tested. Standard error did not exceed 7%. The addition of either 1×10^5 or 2×10^5 cpm of ^{125}I-F(ab')$_2$ of goat anti-rat IgG to Mac-1-coated TG-induced macrophages did not result in significant change of cpm bound to cells (data not shown). These results indicate that saturation of antibody-coated macrophages by the radioactive reagent could be achieved with the lower amount of radioactivity, e.g., 1×10^5 cpm/well. As a negative control, macrophage monolayers received 25 μl of 2% normal rat serum. Cells thus treated bind between 100 and 400 cpm. In previous experiments, (10a) the results obtained in this assay agreed closely with those obtained by flow cytometry.

RESULTS

Induction of LFA-1 by MAF and IFN-γ but not with IFN-α and IFN-β. Lymphokines are known regulators of various macrophage functions and capacities (1). Since macrophages activated or primed in vivo express LFA-1 (10a), it was initially of interest to determine whether this antigen can be modulated in vitro on various LFA-1$^-$ macrophage populations in response to various activating signals. TG-elicited macrophages incubated at 37°C for 18 hr with T cell hybridoma-derived MAF can be induced to express LFA-1 antigen (Figure 1*A*). MAF induced the expression of LFA-1 in a dose-dependent manner, and the most effective dose was a 1:100 dilution. MAF at a dilution of 1:1562, which is equivalent to approximately 0.5 U/ml of IFN-γ (15), still induced significant elevation of LFA-1 (Figure 1*A*). Fucoidin (100 mg/ml), which was previously shown to serve as a second signal for macrophage cytotoxicity (16), could not induce LFA-1 expression (Figure 1*A*). Recombinant IFN-γ induced LFA-1 to a similar although lower extent, in a dose dependent way; optimum induction was observed when recombinant IFN-γ was used at 20 to 100 U/ml (Figure 1*B*). Lower doses of IFN-γ, such as 4 or 0.8 units/ml, were either less effective or not effective, respectively. The inductive capacity of IFN-γ appears to be relatively specific, since either 100 or 1000 U/ml of either IFN-α or IFN-β could not induce LFA-1 expression (Figure 2).

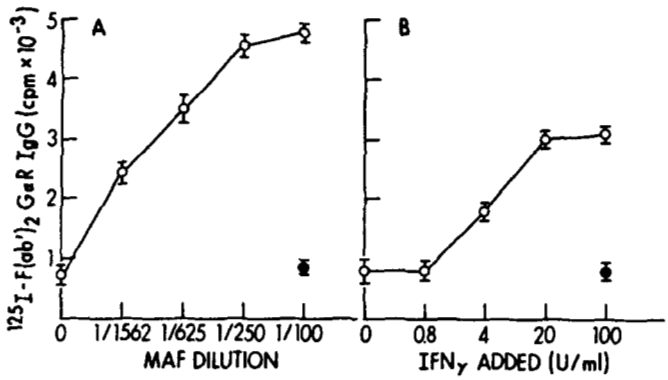

Figure 1. Induction of LFA-1 by MAF and IFN-γ. Two × 10^5 TG-elicited macrophages were incubated for 18 hr with various doses of (*A*) MAF and (*B*) IFN-γ. Macrophages were then subjected to radioimmunoassay with various dilutions of α-LFA-1 MAb (see *Materials and Methods*). Optimal amount of radioactivity bound to macrophages at 1:500 dilution of LFA-1 MAb is shown. Closed circles indicate the expression of LFA-1 following treatment with fucoidin (100 μg/ml). Similar results were obtained in eight experiments.

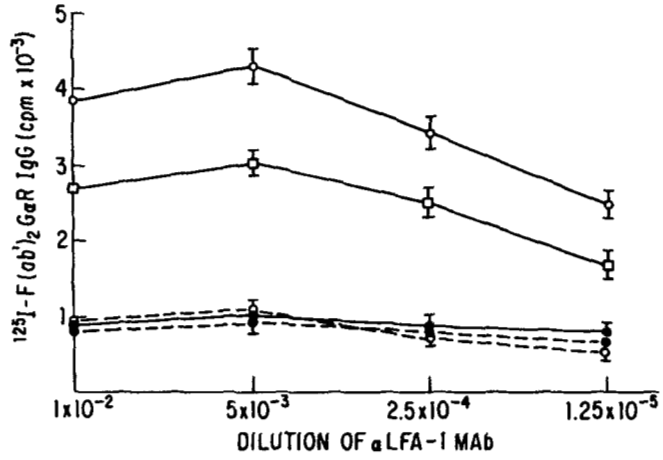

Figure 2. Differential capacity of interferons to induce LFA-1. Two × 10^5 TG-elicited macrophages were treated with medium (●—●), 1000 or 100 U/ml IFN-β (●— —●), 1000 or 100 U/ml IFN-α (○— —○), MAF 1:100 (○——○), or 20 U/ml IFN-γ (□——□) for 18 hr and analyzed for LFA-1 expression. Similar results obtained in two experiments.

LFA-1 could be detected as early as 6 to 10 hr of incubation with either MAF or IFN-γ (Figure 3). Maximum expression of the antigen was observed between 16 and 32 hr. Again, IFN-γ was somewhat less effective than MAF, because macrophages incubated with MAF reach a higher and faster plateau than with IFN-γ (1.2×10^{10} vs 7.8×10^9 molecules of secondary ^{125}I-reagent bound per macrophage). This result suggests the possibility that IFN-γ contains inhibitory activity for induction of LFA-1. Mixing of MAF with increasing amounts of IFN-γ, however, did not produce either suppression or augmentation of LFA-1 induction (data not shown).

To determine whether expression of LFA-1 requires protein synthesis, MAF-treated macrophages, with or without 1 μM cycloheximide, were incubated for 16 hr.

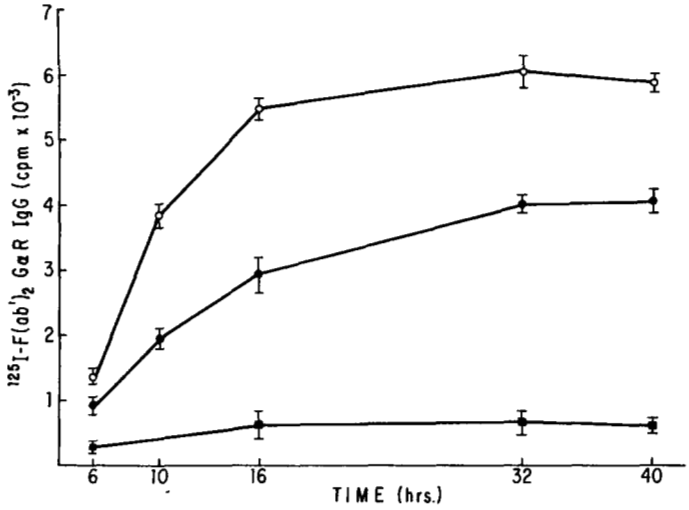

Figure 3. Time requirements for LFA-1 induction. Two × 10^5 TG-elicited macrophages were incubated with 1:100 dilution of MAF (○—○), 20 U/ml IFN-γ (●—●), or medium (■—■) for various periods of time as indicated and analyzed in radioimmunoassay with 1:500 dilution of α-LFA-1 MAb. Similar results obtained in two experiments.

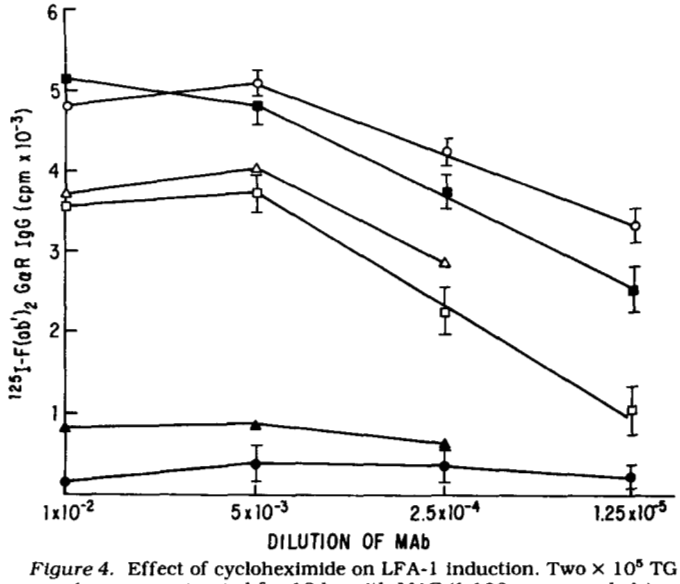

Figure 4. Effect of cycloheximide on LFA-1 induction. Two × 10^5 TG macrophages were treated for 16 hr with MAF (1:100, open symbols) or MAF plus 1 μM cycloheximide (closed symbols). The expression of several antigens was analyzed. Circles indicate LFA-1 (M17/4 MAb), squares indicate Mac-2 (M3/38 MAb), and triangles indicate I-A^b (M5/114 MAb). Similar results obtained in three experiments.

Induction by MAF of both LFA-1 and I-A^b was sensitive to the drug (Figure 4). On the other hand, levels of Mac-2 expression remained unchanged, suggesting that the inhibitory activity of cycloheximide is not due to nonspecific toxicity. The data suggest that protein synthesis is essential for the induction of the LFA-1 α-chain on macrophages.

Induction of LFA-1 by LPS. Macrophages primed in vivo, which are capable of binding but not lysing tumor cells (17), express less (~50%) LFA-1 than do BCG-activated macrophages (10a). Since bacterial endotoxin plays an important role in pushing primed macrophages to the activated state, the ability of LPS to induce LFA-1 synthesis on macrophages was also analyzed. LPS induces expression of LFA-1 on TG-elicited macrophages in a dose-dependent fashion (Figure 5). Maximum inductive capacity of LPS was obtained at 2 or 10 ng/ml, but

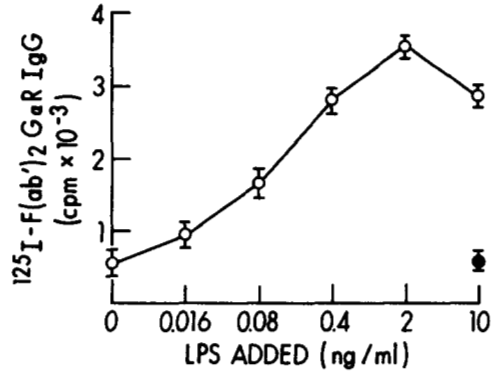

Figure 5. The capacity of LPS to induce LFA-1. Various doses of LPS were incubated with 2 × 10^5 TG macrophages. After 18 hr incubation, cells were analyzed in radioimmunoassay with 1:500 dilution of LFA-1 MAb. Closed circles indicate expression of LFA-1 on macrophages exposed to CSF. Similar results were obtained in four experiments.

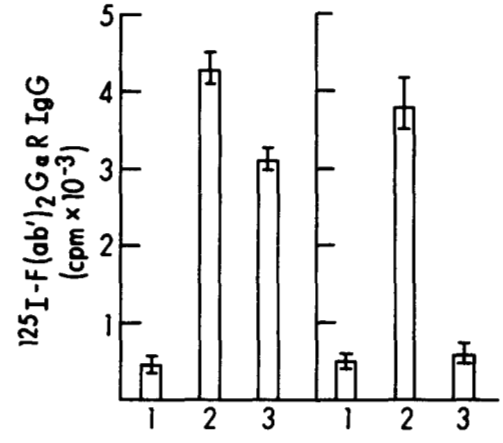

Figure 6. The inability of TG macrophages from C3H/HeJ mice to be induced by LPS. Cells from C3H/HeN (*left*) or C3H/HeJ (*right*) mice were incubated with medium (1), MAF 1:100 (2), and LPS 10 ng/ml (3) for 18 hr and were analyzed for LFA-1 expression with 1:500 dilution of the MAb. Similar results were obtained in two experiments.

significant LFA-1 levels could still be observed in macrophages treated with as little as 80 pg/ml of LPS when compared to macrophages cultured in medium alone or in 10% CSF. The inductive ability of LPS was found to be cycloheximide sensitive as was shown for MAF (data not shown). Finally, incubation for 18 hr with either LPS or MAF can induce a further increase in the level of LFA-1 on pyran-primed macrophages (data not shown).

In order to confirm the observation that LPS can induce synthesis of LFA-1, we analyzed induction of the antigen on macrophages derived from LPS-nonresponder C3H/HeJ mice or their normal counterparts, C3H/HeN mice. Macrophages from C3H/HeN mice could be induced to express LFA-1 by LPS (10 ng/ml) or MAF (1:100), while macrophages from C3H/HeJ mice could be induced by MAF but not with endotoxin (Figure 6).

Inability to induce LFA-1 in PP-elicited or resident macrophages. Activation for cytolysis can be induced in a variety of elicited macrophages (including those elicited by PP or TG) but not in resident peritoneal macrophages (1). To assess the macrophage requirement for induction of LFA-1, we exposed resident macrophages and another type of responsive macrophage to MAF and to IFN-γ. LFA-1 could not be induced by optimal doses of MAF (1:100) or IFN-γ (20 U/ml) on PP-elicited or resident macrophages

(Figure 7). LPS also could not induce LFA-1 expression on these macrophages (data not shown). As a control for possible cell dissociation of cells from the monolayer during culture, expression of Mac-1 was checked and found to be similar on both PP-elicited and resident macrophages (Figure 7). Furthermore, the same preparation of MAF readily increased LFA-1 on TG macrophages in these experiments (data not shown). The inability of PP or resident macrophage populations to be induced by either MAF or interferon could not be altered by prolonged incubation, because LFA-1 could not be induced by MAF on macrophages grown for 3 days in culture (data not shown).

DISCUSSION

We have recently found that LFA-1 is selectively expressed on macrophages primed and activated in vivo but not on resident or responsive macrophages (10a). In this study, we analyzed the specific signals regulating the expression of LFA-1 on macrophages and present evidence that the α-chain of LFA-1 can be induced in vitro by MAF, IFN-γ, or LPS (Figures 1, 3, 5, and 6). Recently, IFN-γ was shown to share functional and immunochemical properties with MAF (12). In our hands, the preparation of MAF appeared somewhat more potent than did IFN-γ for induction of LFA-1 (Figures 1 and 3), although the basis of this difference was not explained. LFA-1 could be detected as soon as 6 hr after exposure to inductive signals, but peak expression was observed between 16 and 32 hours on TG-elicited macrophages treated with lymphokine (Figure 3). Furthermore, induction of LFA-1 by MAF or LPS could be abrogated by cycloheximide (Figure 4), which indicates that protein synthesis is necessary for expression of the LFA-1 α-chain.

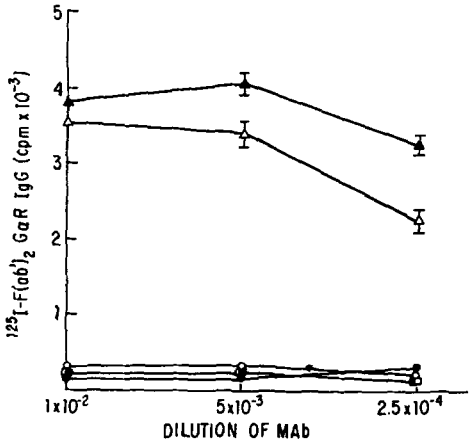

Figure 7. PP and resident macrophages cannot express LFA-1 after MAF or IFN-γ treatment. Resident macrophages were treated with IFN-γ 20 U/ml (□——□) or MAF 1:100 (○——○) and analyzed for LFA-1 expression or Mac-1 expression (△——△). Closed figures indicate the same treatment and analysis for PP-induced macrophages. Similar results were obtained in three experiments.

The induction of LFA-1 has selectivity in two regards. First, other activating compounds such as IFN-α, IFN-β, CSF, or fucoidin could not induce LFA-1 (Figures 1, 2, and 5). Second, not all LFA-1$^-$ populations of macrophages (i.e., PP-elicited and resident macrophages) could be induced to synthesize LFA-1 (Figure 7). This differential responsiveness could not be altered by prolonged incubation, because the cells remained unresponsive to lymphokine even after 3 days in culture.

Of note, LPS could also induce expression of LFA-1. LPS stimulates macrophages to secrete a variety of molecules, including products of the cyclooxygenase pathway (18) and lysosomal enzymes (19), and to suppress humoral and cellular immune responses (20, 21). Here, we show that LPS in picogram amounts induces expression of the LFA-1 antigen (Figure 5). This result was further supported by use of LPS-nonresponder C3H/HeJ mice; TG-elicited macrophages from this mouse strain were able to respond to MAF but not to LPS (Figure 6).

Expression of two other activation-associated functions by macrophages is regulated by some of the same signals. Expression of Ia molecules, previously shown to be necessary for macrophage-T cell interreactions, is induced by lymphokines (22, 23); one lymphokine which regulates Ia expression has been further identified as IFN-γ (24, 25). Activation of macrophages for cytolytic function is induced by sequential application of IFN-γ and then LPS (1). Thus, activation of macrophages in three distinct ways is regulated by IFN-γ. Regulation of lytic function, expression of LFA-1, and expression of Ia are, however, quite distinct (Table I). First, in contrast to Ia molecules which cannot be induced by LPS, LFA-1 synthesis could be induced by endotoxin (Figure 5). In fact, LPS inhibits induction and maintenance of Ia expression by IFN-γ in vitro, possibly by stimulation of prostaglandin production by the macrophages (26). The induction of lytic competence requires IFN-γ plus LPS (1, 27). Second, Ia antigen can be induced on PP-elicited and resident macrophages (3). Lytic function can be induced in multiple types of elicited macrophages (27), while only macrophage elicited by TG could be induced to express LFA-1 (Figure 7).

The LFA-1 antigen, which comprises a unique α-chain (180,000 M_r) and a β-chain identical to Mac-1 β-chain (95,000 M_r) (7), plays an important role in the Mg^{++}-dependent, adhesion step between CTL and targets (6). LFA-1 has been hypothesized to contribute to the overall avidity between CTL and targets (for review, see Reference 6). The presence of LFA-1 on B cells, T cells, and myeloid cells (28) suggests that this molecule may play a more general role in adhesion reactions (6). Indeed, LFA-1 has also been found to function in natural killing (29,

TABLE I

Induction of cytolytic function, Ia expression, and LFA-1 expression in vitro

	Cytolytic Funcion	Ia	LFA-1
Macrophages that can be induced	Responsive macrophages, elicited by many stimuli	Resident and responsive macrophages	Selected populations of responsive macrophage (i.e., TG-elicited macrophages)
Effect of IFN-γ	Prepares macrophages for LPS	Induces Ia	Induces LFA-1
Effect of LPS	Drives IFN-γ-primed macrophages to lytic function	Down-regulates Ia	Induces LFA-1

30) and antibody-dependent cellular cytotoxicity (30). Recently, patients with a severe defect in several adhesion-dependent leukocyte functions were described (10, 30, 31); LFA-1 and Mac-1/OKM-1 were undetectable on the polymorphonuclear cells and monocytes of these patients. The role of LFA-1, which is present on activated macrophages, remains to be established fully. The existence of deficiencies in these molecules emphasizes further the importance of understanding the factors governing their expression.

In sum, the present data indicate that LFA-1, member of a related family of important recognition molecules, is strictly and closely regulated. Although LFA-1 is expressed on macrophages activated in vivo for the expression of Ia molecules and for cytolytic function, closer analysis indicates that each of these three recognition functions or molecules is distinctly controlled (Table I). These data provide further support for the contention that complex and fine regulation of macrophage surface proteins (for review, see Reference 32) is essential to the control of their multiple recognition functions.

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