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Purification and Structural Characterization of LFA-1, a Lymphocyte Function-associated Antigen, and Mac-1, a Related Macrophage Differentiation Antigen Associated with the Type Three Complement Receptor*

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LFA-1, an antigen associated with antigen-specific T lymphocyte-mediated killing, and Mac-1, a macrophage differentiation antigen associated with type three complement receptor function, contain α chains of M_r = 180,000 and 170,000, respectively, and β chains of M_r = 95,000. The monoclonal antibodies defining these antigens do not cross-react. The LFA-1 and Mac-1 β chains are highly homologous or identical, whereas the α chains are highly different by tyrosyl tryptic peptide mapping (Kürzinger, K., Ho, M. K., and Springer, T. A. (1982) *Nature (Lond.)* 296, 668-670). T lymphoma cell lines express LFA-1 but not Mac-1 as shown by immunofluorescence and immunoprecipitation. Conversely, some macrophage-like lines express Mac-1 but not LFA-1. Other macrophage-like lines co-express Mac-1 and small amounts of LFA-1. Mac-1 and LFA-1 are present as separate molecules in these cells. [^{35}S]Methionine and [^{3}H]glucosamine are incorporated into both α and β chains of Mac-1 and LFA-1, showing both chains are endogenously synthesized and are glycoproteins. Cross-linking and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis experiments show that in both Mac-1 and LFA-1 the α and β chains are noncovalently associated in $\alpha_1\beta_1$ quaternary structures. By quantitative immunofluorescence flow cytometry, the EL-4 T lymphoma and P388D$_1$ macrophage-like lines were estimated to express 10^5 LFA-1 and 1.6×10^5 Mac-1 molecules/cell, respectively. From these sources the antigens have been purified to homogeneity in 200-400-μg quantities by monoclonal antibody affinity chromatography. The purified antigens contain only the α and β subunits.

In recent experiments designed to screen for surface molecules participating in T cell-mediated immunity, we described a novel murine cell surface antigen, LFA-1[1] (2, 3). MAb to LFA-1 block, in the absence of complement, a number of antigen-specific T cell responses (reviewed in Ref. 4). T lymphocyte-mediated killing is blocked by MAb bound to the effector cell, preventing adhesion-antigen recognition of the target cell. T helper cell proliferative responses are blocked at an early stage by MAb bound to the responding T cell. The anti-LFA-1 MAb is not toxic and inhibition is presumably mediated by steric hindrance or sequestration of the LFA-1 molecule. Blockade is specific, because antibodies bound to other surface components present in a higher number of sites/cell have little or no effect on function (5). This suggests that LFA-1 plays an important role in cell-mediated killing, although it probably is not an antigen receptor (4, 5). Similar conclusions have been reached with another MAb defining apparently the same antigen (6).

LFA-1 is expressed on B and T lymphocytes but not on thioglycolate-elicited peritoneal macrophages (5). This cell distribution contrasts to that of another antigen, Mac-1, which is found on murine macrophages, monocytes, granulocytes, and natural killer cells, but not on lymphocytes (7-10). The M1/70 anti-Mac-1 MAb also cross-reacts with an antigen with the same distribution on human cells (9).

Recently, anti-Mac-1 MAb was found to inhibit the murine and human macrophage/granulocyte type three complement receptors, which are specific for inactivated C3b or its degradation product C3d,g (Ref. 40 and reviewed in Ref. 4). Inhibition is specific, since it is not mediated by MAb binding to other macrophage antigens or by anti-LFA-1 MAb, and since the Fc receptor and the type one complement receptor specific for C3b are unaffected by anti-Mac-1 MAb.

Despite this distinctness in cell distribution and function, Mac-1 and LFA-1 resemble each other a great deal on the molecular level. Both antigens are composed of two chains, α and β. The α chains differ only slightly in molecular weight (180,000 *versus* 170,000) and the β subunits are of identical molecular weight, 95,000. The MAb defining Mac-1 and LFA-1 do not cross-react. However, conventional antisera to partially purified Mac-1 cross-react with LFA-1 (1), and a cross-reactive MAb has also been obtained (11). Furthermore, tryptic peptide mapping has shown that the M_r = 95,000 β chains are highly homologous or identical. The α chains, in contrast, are quite distinct (1, 11).

These studies showed that Mac-1 and LFA-1 are structurally related antigens. The selective expression of Mac-1 and LFA-1 in the monocytic and lymphoid lineages, respectively, of leukocyte differentiation is a particularly interesting feature. For a better understanding of the relationship of these antigens, characterization of their chemical and subunit structures is essential. Structural characterization of LFA-1 would also help to elucidate the molecular basis of T lymphocyte-mediated immunity. Purified molecules are required for these studies.

Here we report on the large-scale purification of both Mac-

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[1] The abbreviations used are: LFA-1, lymphocyte function-associated antigen one; MAb, monoclonal antibody(ies); PAGE, polyacrylamide gel electrophoresis; PEC, peritoneal exudate cells; SDS, sodium dodecyl sulfate; Mac-1, macrophage differentiation antigen associated with type three complement receptor.

1 and LFA-1. Employing MAb affinity columns, purifications have been accomplished in single-step procedures. To make purification practicable, rich sources for both antigens were found. Another purpose of this study was to compare Mac-1 and LFA-1 polypeptide chains as to site of synthesis, glycosylation, and quaternary structure. We also examined a panel of tumor cells for co-expression of Mac-1 and LFA-1 determinants and its underlying molecular basis.

MATERIALS AND METHODS

Cells—Four-day thioglycollate-induced PEC macrophages and unstimulated or concanavalin A-stimulated spleen cells were obtained from C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) as described (5, 7). The following tumor cell lines were from the Salk Cell Distribution Center (now available from American Type Culture Collection, Kensington, MD) unless otherwise indicated: the lymphoma lines EL-4 (12), YAC (13), RDM-4 (14), TIMI.4G.1.3 (15), and RIE/TL8×.1 (16); the myeloid leukemia line C1498.3 (17); the myeloblast line M1 (18) (gift of Dr. P. Ralph, Sloan-Kettering, Rye, NY); the monocyte/macrophage-like lines (reviewed in Ref. 18) $P388D_1$ and its variant clones P388D1-E2 and $P388D_1$-E8; WEHI-3, J774 and its 5′ nucleotidase-positive and negative clones $J774N^+$ and $J774N^-$ (gifts of Dr. P. Edelson, Children's Hospital, Boston, MA); RAW-264.7 and 426C (gifts of Dr. P. Ralph), and WEHI-3A; and the mastocytoma line P815 (19). Tumor cells were grown in culture in Dulbecco's modified Eagle's medium supplemented with either 10% horse serum or 5–10% fetal calf serum (Gibco). For the purpose of antigen purification, $P388D_1$ cells were grown in spinner flasks in medium containing 10% horse serum. EL-4 cells were grown as ascites: 2×10^7 cells were injected intraperitoneally in C57BL/6J mice and allowed to grow for 7 days to a final density of $3–4 \times 10^8$ cells/mouse.

Antibodies—MAb recognizing LFA-1 were produced by the hybridomas M7/14 (2) or M17/4 (41); those specific for Mac-1 were obtained from the hybridoma M1/70 (7). A polyclonal rat antiserum to partially purified Mac-1 which cross-reacts with LFA-1 was obtained as described elsewhere (1).

Immunofluorescence—Labeling of cells, immunofluorescence flow cytometry, and calculation of data were as in Ref. 5. Briefly, cells were labeled with saturating amounts of monoclonal antibodies, then with fluorescein isothiocyanate-labeled, affinity-purified, rabbit $F(ab')_2$ anti-rat IgG, absorbed with mouse IgG. Cells were analyzed on a Becton-Dickinson FACS II. Mean fluorescence intensity was determined by integration and expressed relative to the intensity of glutaraldehyde-fixed sheep red blood cell standards which were analyzed at a series of gain settings.

Radioactive Labeling and Immunoprecipitation—Cells were surface-iodinated using chloroglycoluril (IODO-GEN, Pierce) as described (5). For biosynthetic labeling of adherent cells (PEC and macrophage-like tumor cell lines), 10^7 cells were plated on 25-cm^2 tissue culture flasks and incubated overnight at 37 °C. Nonadherent cells were washed off and adherent cells labeled with 200 μCi of L-[^{35}S]methionine (New England Nuclear), in 1 ml of methionine-free Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal calf serum (Gibco) for 6 h. The adherent cell layer was washed 3 times in 0.14 M NaCl, 0.01 M $NaPO_4$, pH 7.3, and detergent-solubilized as described (5). Cells growing in suspension (concanavalin A-stimulated spleen cells and T lymphoma tumor cell lines) were washed and suspended to a density of 10^7 cells/ml in the above labeling medium. Final volumes of 1 ml were incubated in 22-mm wells of 12-well culture plates (Costar, Cambridge, MA) for 6 h. Then the cells were washed 3 times in 0.14 M NaCl, 0.01 M $NaPO_4$, pH 7.3 and detergent-solubilized. After centrifugation at $100,000 \times g$ for 1 h, the supernatant was recovered. For carbohydrate labeling, a modification of the above medium was used containing the regular concentration of methionine but only 0.9 mM glucose instead of 11 mM, and 150 μCi/ml of D-[^{3}H]glucosamine hydrochloride (New England Nuclear). For immunoprecipitation, 10–20 μl of MAb coupled to Sepharose CL-4B (1–2 mg of protein/ml of wet gel) were incubated with cell lysate in a final volume of approximately 200 μl. After shaking for 2 h, the beads were washed 3 times with 0.01 M Tris-HCl, pH 8, 0.1% hemoglobulin, 0.1% Triton X100, 0.14 M NaCl, once with 0.01 M Tris-HCl, pH 8, 0.14 M NaCl, and once with 0.05 M Tris-HCl, pH 6.8. After addition of sample buffer and heating in a boiling water bath for 5 min, the samples were subjected to SDS-PAGE as described (7). Iodinated molecules were visualized by autoradiography with enhancing screens (20), and ^{3}H- and ^{35}S-labeled molecules by fluorography after impregnation with either 2,5-diphenyloxazole (21) or EN^{3}HANCE (New England Nuclear).

Cross-linking—Surface-iodinated PEC and EL-4 cells were lysed with Triton X100 as previously described (5) in the absence of carrier protein. Lysates were dialyzed *versus* 0.1 M NaCl, 0.05 M sodium borate, pH 9, and incubated with 0.1 mg/ml of dithiobis(succinimidyl propionate) (Pierce) (22) at 21 °C for 1 h. After dialysis *versus* 0.14 M NaCl, 0.01 M Tris-HCl, pH 7.5, aliquots were immunoprecipitated and analyzed by two-dimensional SDS-PAGE.

Two-dimensional SDS-PAGE—Washed immunoprecipitates were solubilized in 5 μl of 6 mM Tris, 5 mM glycine, 0.5% SDS, 5 mM iodoacetamide, 25% (w/v) glycerol, and loaded on 1.7 × 125-mm tube gels (first dimension, nonreducing) containing 5% acrylamide, 0.13% methylenebisacrylamide, 0.12 M Tris, 0.1 M glycine, 0.1% SDS (no stacking gel). The electrophoresis buffer was 0.01 M Tris, 0.33 M glycine, 0.1% SDS. After completion of electrophoresis, the gels were extruded from the tubes, equilibrated in 0.38 M Tris-HCl, pH 8.8, 0.1% SDS, 100 mM dithiothreitol at 21 °C for 10 min and placed directly on 1.6-mm slab gels (second dimension, reducing conditions) prepared according to Ref. 23. No agarose layer or stacking gel was used.

Purification of MAb and Coupling to Sepharose—M1/70 and M7/14 IgG were purified as described previously (3, 9). The same applies to M17/4 IgG, except that the Sephadex G-200 step was omitted which resulted in a preparation of 65% pure IgG. Pure and semi-pure MAb and normal rat IgG (Miles Laboratories) were coupled to CNBr-activated Sepharose CL-4B (Pharmacia) at 4–5 mg of protein/ml of wet gel according to the method of Cuatrecasas (24).

Purification of Mac-1—$P388D_1$ cells were grown to 8 liters in spinner culture and yielded 7.7×10^9 cells. All subsequent steps were at 4 °C or on ice. Cells were harvested by centrifugation at $350 \times g$ for 20 min, washed three times with 300 ml of 0.14 M NaCl, 0.01 M $NaPO_4$, pH 7.3, and lysed at a density of 5×10^7 cells/ml in 0.5% Triton X100, 20 mM Tris-HCl, pH 8.0, at 4 °C, 1 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetamide, 0.2 units/ml of Aprotinin (Sigma), 0.025% NaN_3 (lysis buffer). After 10 min, nuclei were pelleted at $4,300 \times g$ for 15 min and the supernatant was then cleared of detergent-insoluble material by centrifugation at $100,000 \times g$ for 45 min (25, 26).

The supernatant ($100,000 \times g$) was passed in series through columns of 1 ml each of CNBr-activated, glycine-quenched Sepharose CL-4B, normal rat IgG-Sepharose CL-4B, and M1/70 IgG-Sepharose CL-4B. The columns were loaded, washed, and eluted at flow rates of 10–15 ml/h. After loading, the M1/70 column was washed with 30 ml of lysis buffer and then with 20 ml each of buffers of pH (at 4 °C) 9.0 (20 mM glycine/NaOH), pH 10.0 (20 mM glycine/NaOH), and pH 11.0 (20 mM triethylamine/HCl), containing 0.5% Triton X100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, and 0.025% NaN_3. Eluates of pH 9 and higher were immediately neutralized by collection into tubes containing 1 M Tris-HCl, pH 6.7. Fractions of about 2 ml were collected.

For SDS-PAGE, aliquots of individual or pooled fractions were precipitated with 5 volumes of acetone. After 14 h at −20 °C, the protein was pelleted by centrifugation at $500 \times g$ for 15 min, redissolved in reducing sample buffer, and analyzed by SDS-PAGE and Coomassie blue staining (27). SDS-PAGE showed that eluates of pH 9 and 10 did not contain any Mac-1, but several unrelated bands. The pH 11 eluate contained Mac-1 slightly contaminated by substances of M_r = ~200,000 and 35,000. These fractions were dialyzed, reapplied to the M1/70 column (50–60% of the material rebound), and eluted by buffer of pH 11. After this second cycle, Mac-1 was free of apparent contaminants.

Purification of LFA-1—1.3×10^{10} EL-4 cells, grown as ascites, were washed three times in 0.14 M NaCl, 0.01 M $NaPO_4$, pH 7.3, lysed, and processed as above with the following alteration: sodium deoxycholate (Sigma) of 0.5% final concentration was added between the low and high speed centrifugation (28). An M17/4 Sepharose CL-4B column was substituted for the M1/70 column. Columns were first washed with lysis buffer containing both Triton X100 and sodium deoxycholate, followed by pH 9 buffer with Triton X100 only. In this step only very little material was eluted and this was unrelated to LFA-1. The pH 10 step was omitted. Pure LFA-1 was eluted with the pH 11 buffer as described for Mac-1, except also containing 0.5 M NaCl.

Protein Determination—For protein determination by the method of Lowry *et al.* (29), acetone precipitates were solubilized in 0.3% SDS in a boiling water bath. Bovine serum albumin, subjected to acetone precipitation in parallel, was used as a standard.

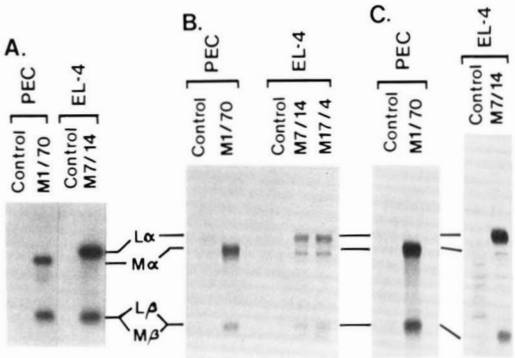

FIG. 1. **Surface and biosynthetic labeling of Mac-1 and LFA-1.** 4-day thioglycollate-elicited PEC, 3-day concanavalin A-stimulated spleen cells, P388D$_1$ or EL-4 tumor cells were either *A*, ^{125}I surface-labeled or, *B*, internally labeled with [^{3}H]glucosamine or, *C*, [^{35}S] methionine as described under "Materials and Methods." Cell lysates were immunoprecipitated as indicated with Sepharose-coupled anti-Mac-1 MAb M1/70, the anti-LFA-1 MAb M7/14 or M17/4, or with normal rat IgG-Sepharose as control. The immunoprecipitates were analyzed on SDS gels of 10 (*A*), 8 (*B* and *C* (PEC)), and 7% (*C*, (EL-4)) polyacrylamide, respectively. *Lα*, LFA-1α; *Lβ*, LFA-1β, *Mα*, Mac-1α, *Mβ*, Mac-1β. Molecular weights mentioned in the text were determined with standards as previously described (5).

RESULTS

Biosynthetic Labeling of Mac-1 and LFA-1—Previous experiments with ^{125}I-labeled material showed that both anti-Mac-1 and anti-LFA-1 MAb precipitate two polypeptide chains (5, 7). Since it was possible that one or both of the chains could be exogenously acquired as, for example, by a receptor-ligand interaction, it was important to test whether both chains could be biosynthetically labeled. Mouse PEC or EL-4 T lymphoma cells were internally labeled with [^{35}S] methionine, lysed, immunoprecipitated, and analyzed by SDS-PAGE (Fig. 1*C*). The M1/70 anti-Mac-1 MAb precipitated two polypeptide chains from PEC, an α chain of M_r = 170,000 and a β chain of M_r = 95,000; the M7/14 anti-LFA-1 MAb also precipitated two polypeptide chains from EL-4, an α chain of M_r = 180,000 and a β chain of M_r = 95,000 (Fig. 1*C*). These are the same structures as obtained after surface iodination (Fig. 1*A*), showing that both chains are biosynthesized.

To determine whether the Mac-1 and LFA-1 polypeptide chains are glycosylated, cells were biosynthetically labeled with D-[^{3}H]glucosamine. Both α and β chains of Mac-1 were labeled with [^{3}H]glucosamine (M1/70 precipitate from PEC, Fig. 1*B*). Two different MAb, M7/14 and M17/4, were used to isolate LFA-1 from glucosamine-labeled EL-4 cells. Strongly labeled α and β polypeptides were seen with mobilities lower than the Mac-1 α chain and identical with the Mac-1 β chain, respectively (Fig. 1*B*). These polypeptides had molecular weights identical with that seen after ^{125}I-labeling of LFA-1 (Fig. 1*A*). In addition, weaker bands of M_r = 10,000 lower than the α and β bands were seen. These correspond to α and β chain precursors which have been detected after [^{35}S]methionine pulse-chase labeling of LFA-1.[2] Tunicamycin, which blocks *N*-glycosylation, inhibited the appearance of immunoprecipitable [^{35}S]methionine-labeled Mac-1 and LFA-1 (data not shown).

Both Antigens Exist as $\alpha_1\beta_1$ Complexes—The presence of α and β polypeptides in Mac-1 and LFA-1 was confirmed by all labeling techniques tested. Precipitation by a monoclonal antibody of two different polypeptide chains could be because 1) the polypeptide chains are noncovalently associated, or 2) the chains bear identical or cross-reactive antigenic determinants. To test whether the α and β subunits were noncovalently associated, detergent lysates of ^{125}I surface-labeled cells were treated with the cleavable cross-linking reagent dithiobis(succinimidyl propionate) (22) and immunoprecipitated. Cross-linked material was subjected to two-dimensional, nonreduced-reduced SDS-PAGE (Fig. 2). In the first dimension, cross-linked Mac-1 migrated as a fuzzy band of M_r = 240,000 to 290,000, centering at M_r = 260,000 (Fig. 2*A*). Upon reduction in the second dimension, the cross-linked product was cleaved into the Mac-1 α and β chains of M_r = 170,000 and 95,000. Similar results were obtained with LFA-1, except that the estimated molecular weight of the cross-linked product was somewhat lower, M_r = 245,000 (Fig. 2*B*). Since the α and β bands are vertically aligned with each other in the second dimension, both are present in the cross-linked product. The cross-linked Mac-1 product has an M_r = 260,000, in good agreement with the predicted molecular weight of an $\alpha_1\beta_1$ structure of 265,000. The molecular weight of the LFA-1

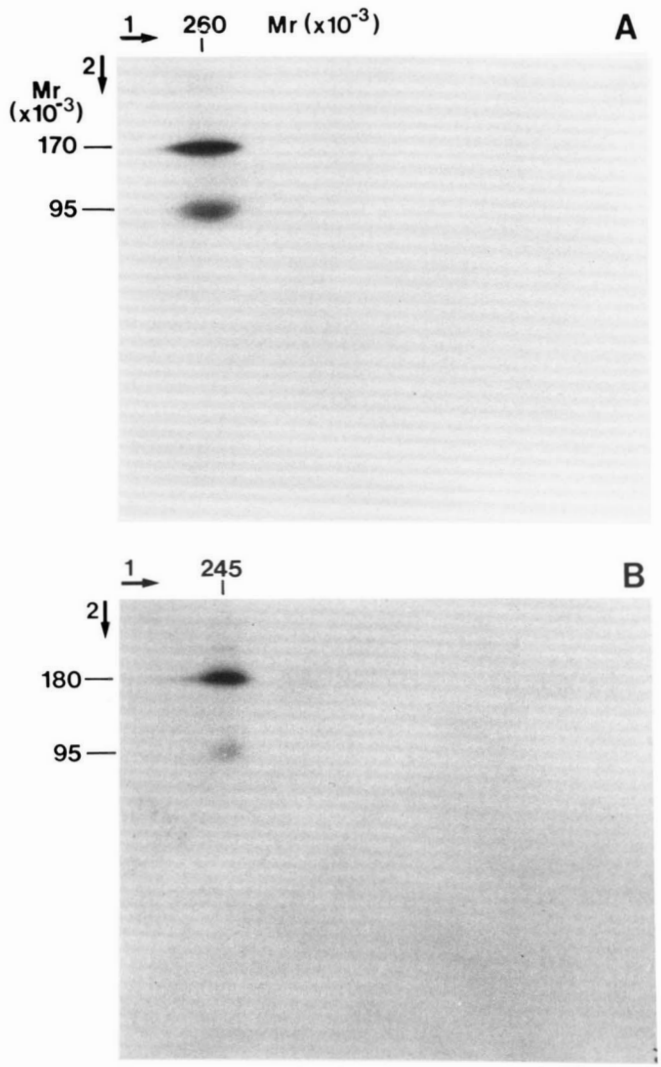

FIG. 2. **Two-dimensional electrophoresis in SDS gels of cross-linked Mac-1 (*A*) and LFA-1 (*B*).** Samples were cross-linked as described under "Materials and Methods," immunoprecipitated, and electrophoresed in 5% first dimension tube gels (nonreducing) and 8% second dimension slab gels (reducing). The molecular weights of the cross-linked species were determined in a parallel experiment by electrophoresis in one-dimensional slab gels with molecular weight markers. Molecular weights above 200,000 were determined by extrapolation. *1*, direction of first dimension; *2*, direction of second dimension.

[2] M. K. Ho and T. A. Springer, unpublished data.

product was 245,000, within 11% of the molecular weight of an $\alpha_1\beta_1$ structure of 275,000. These results show that for both Mac-1 and LFA-1, the α and β polypeptides are subunits which are noncovalently associated into $\alpha_1\beta_1$ structures.

Expression of Mac-1 and LFA-1 on Tumor Cell Lines—To find the best source for purification of Mac-1 and LFA-1 antigens, and to test whether some tumor cells might express both Mac-1 and LFA-1, a panel of cell lines was examined by immunoprecipitation and immunofluorescent flow cytometry. Tumor cell lines were labeled with [^{35}S]methionine, and lysates containing equal quantities of incorporated radioactivity were subjected to immunoprecipitation and SDS-PAGE. The EL-4 (Fig. 3, *lane 1*) and YAC (not shown) T lymphoma lines were found to biosynthesize LFA-1 but not Mac-1. The P815 mastocytoma line synthesized lower quantities of LFA-1 than the T lymphomas, and no Mac-1 (Fig. 3, *lane 5*). Four macrophage-like lines showed the opposite pattern. RAW-264.7 and WEHI-3A (Fig. 3, *lanes 7* and *8*) and WEHI-3 and 426C (not shown) expressed Mac-1 but not LFA-1. The macrophage-like cell lines P388D$_1$, its subclones E$_2$ and E8, and J774 expressed large quantities of Mac-1, and additionally, trace quantities of LFA-1 (Fig. 3, *lanes 2–4* and *6*). The LFA-1 α chain precipitated from the macrophage-like lines had a mobility identical with that isolated from EL-4 and P815 (Fig. 3, *lanes 1b–6b*). As shown by the higher molecular weight of the LFA-1 than the Mac-1 α chain, in P388D$_1$, its subclones, and J774, M1/70 and M7/14 determinants are expressed on separate molecules. A myeloid leukemia line, C1498.3 (Fig. 3, *lane 10*) and an immature myeloid progenitor line, M1 (Fig. 3, *lane 9*) synthesized neither Mac-1 nor LFA-1.

To further quantitate antigen expression, cell lines were labeled with saturating amounts of MAb and mouse IgG-absorbed, affinity-purified, fluoresceinisothiocyanate-rabbit F(ab')$_2$ anti-rat IgG, and subjected to flow cytometry (Table I). Labeling was performed under saturating conditions, so that fluorescence intensity was proportional to the number of antigen sites/cell. Using published values for H-2 antigen site numbers, and correcting for the amount of fluoresceinisothiocyanate-anti-rat IgG bound to different rat IgG subclasses, the number of molecules/cell has been estimated (Table I). All lines examined expressed LFA-1. The EL-4 and YAC T lymphomas expressed the highest amount. The quantity of LFA-1 expression was less than that of H-2, except for R1E/TL8×.1 which is an H-2$^-$, TL$^-$ mutant (30). None of the T lymphomas or the mastocytoma line expressed significant quantities of Mac-1, in agreement with the immunoprecipitation data. In contrast, the P388D$_1$ macrophage-like line bore about 160,000 Mac-1 molecules/cell. This was in good agreement with previous findings that thioglycollate-elicited macrophages express 1.6×10^5 Mac-1 sites/cell (8) and that they express the same quantity of Mac-1 as P388D$_1$ (7).

Purification of Mac-1 and LFA-1 by means of Affinity Chromatography—The fact that the M1/70 and M7/14 MAb coupled to Sepharose CL-4B could be used to immunoprecipitate Mac-1 and LFA-1, respectively, suggested they might be effective affinity reagents for large scale isolation of these antigens. The tumor cell line EL-4 was chosen for isolation of LFA-1. It expressed about 10^5 molecules/cell (Table I), 1.3-fold and 6-fold more LFA-1 than concanavalin A-stimulated spleen cells or normal spleen cells, respectively (5). Three to four-fold more EL-4 than spleen cells/mouse can be obtained by growth as an ascites tumor. EL-4 can alternatively be

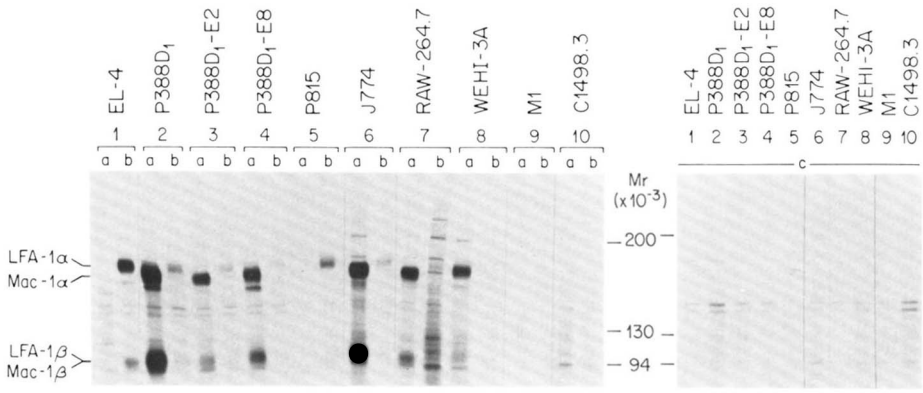

FIG. 3. **Screening by immunoprecipitation for expression of Mac-1 and/or LFA-1 by tumor cell lines.** Tumor cells grown as ascites (EL-4) or in culture (other cell lines) were biosynthetically labeled with [^{35}S]methionine for 6 h. Lysates were immunoprecipitated with Sepharose-coupled MAb M1/70 (*a*), M7/14 (*b*) or with normal rat IgG-Sepharose (*c*) as control. Immunoprecipitates were analyzed by SDS-8% PAGE and fluorography. Specific immunoprecipitates and controls were run on separate gels.

TABLE I

Mac-1, LFA-1, and H-2 sites on tumor cells

Antibodies used for labeling were M1/70, IgG2b, anti-Mac-1; M7/14, IgG2a, anti-LFA-1; M1/42 (26), IgG2a, anti-H-2. Fluoresceinisothiocyanate-anti-rat IgG was used as the second step reagent.

Cells	Fluorescence intensity[a]				Molecules/cell[b] × 10^{-3}		
	Mac-1	LFA-1	H-2	Background[c]	Mac-1	LFA 1	H-2
Spleen	0.1	4.7	29	(0.8)	<0.6	16	(100)
EL-4 T lymphoma	0.1	30	101	(1.2)	<0.6	100	350
YAC T lymphoma	0.0	31	92	(1.3)	0	110	320
RDM-4 T lymphoma	0.2	5.3	284	(0.8)	<1.3	18	980
R1E/TL8×.1 T lymphoma	0.0	17	0.0	(1.2)	0	59	0
P815 mastocytoma	0.5	12.6	100	(1.4)	<3.2	43	340
P388D$_1$ macrophage-like line	25.5	18.6	77.5	(7.6)	160	64	270

[a] Fluorescence intensity is expressed relative to that of glutaraldehyde-fixed sheep red blood cells. Background fluorescence has been subtracted.

[b] Molecules/cell were calculated using an estimate of 10^5 H-2 molecules/spleen cell (5). The ratio of fluoresceinisothiocyanate-anti-rat IgG bound to IgG2b has been found to be 0.54 of that bound to IgG2a, as determined with MAb binding to the same site on the same cell surface molecule (39).[5] Values for Mac-1 were adjusted accordingly.

[c] Background was determined using the nonbinding M1/69.16.11HK MAb in the first step.

grown to large quantities in suspension cultures. P388D$_1$ was the strongest producer of Mac-1 as judged by immunoprecipitation and immunofluorescence. P388D$_1$ could not be grown in good yield as an ascites tumor. Although this line is adherent in stationary cultures, it grew in suspension in spinner cultures, reaching a density of 10^9 cells/liter. Thus, P388D$_1$ was selected for large scale production of Mac-1 antigen.

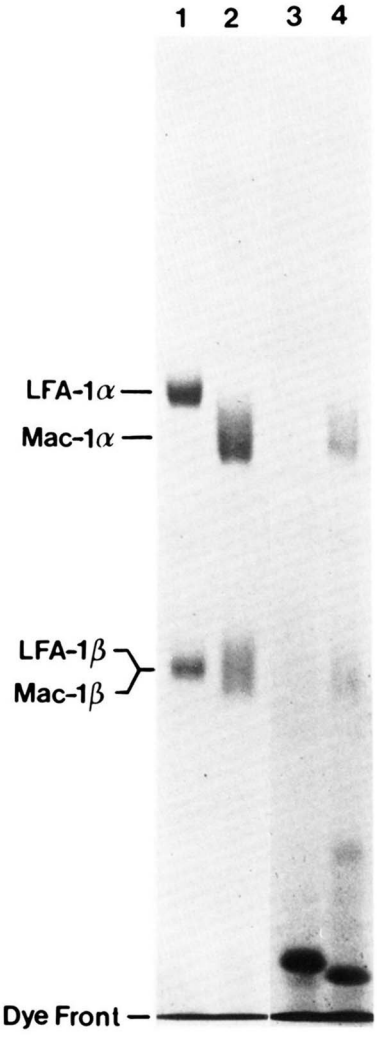

FIG. 4. **Analysis of Purified LFA-1 and Mac-1 by SDS-PAGE.** LFA-1 and Mac-1 were purified by MAb affinity chromatography as described in the text. Aliquots corresponding to 2.5 μg of purified LFA-1 (1, 3) or 3.5 μg of purified Mac-1 (2, 4) were either acetone-precipitated (1, 2) or incubated with Sepharose beads conjugated to the relevant MAb as described under "Materials and Methods." The acetone precipitates or the washed Sepharose-MAb immune complexes were dissolved in SDS sample buffer, subjected to SDS-6% PAGE, and subsequently stained with Coomassie blue. The *bands* at the *bottom* of *lanes 3* and *4* represent the heavy chains of M17/4 and M1/70 IgG.

After conditions for binding and elution of Mac-1 had been found in small scale experiments, large scale purification was started from 7.7×10^9 P388D$_1$ suspension-cultured cells. Whole cells were solubilized in Triton X100 in the presence of protease inhibitors. Membrane preparation was omitted to increase antigen yield. The supernatant (100,000 × *g*) was passed through two precolumns to remove nonspecifically binding material, then a column of M1/70-Sepharose CL-4B. Bound material was eluted with buffers of stepwise increasing pH. Each elution of differing pH was collected as 10 fractions, which were analyzed by SDS-PAGE and Coomassie Blue staining. Mac-1, together with small amounts of impurities, eluted beginning at pH 10 and predominantly at pH 11. The Mac-1 M_r = 170,000 and 95,000 α and β polypeptides always coeluted (not shown). The eluates were neutralized, reapplied to the M1/70-Sepharose column, and eluted with pH 11 buffer. Purified Mac-1 was obtained which contained the α and β subunits free of contaminants as shown by SDS-PAGE (Fig. 4, *lane 2*). Immunoprecipitation of this material with M1/70-Sepharose showed that it was authentic Mac-1, and that about 40% of it had retained its antigenicity (Fig. 4, *lane 4*). The preparation contained 210 μg of protein, *i.e.* 36% of the calculated amount of Mac-1 protein in the starting material (Table II). Purification in relation to the detergent lysate was by a factor of 1430.

Purification of LFA-1 from EL-4 cells by the above procedure, using M17/4 Sepharose CL-4B, yielded the LFA-1 α and β chains, but contaminated with other proteins. Therefore, the procedure of Brown *et al.* (28) was adopted in which sodium deoxycholate was added to the Triton X100 lysate. Much less material was nonspecifically adsorbed to the columns. Buffer of pH 11 and 0.5 M NaCl coeluted the LFA-1 α and β subunits essentially free of contaminants (Fig. 4, *lane 1*). This preparation contained 420 μg of protein, *i.e.* 70% of the calculated amount of LFA-1 protein in the starting material (Table II). Purification in relation to the detergent lysate was 1250-fold. Less than 5% of the purified LFA-1 could be immunoprecipitated or be rebound to the M17/4 column (Fig. 4, *lane 3*). The LFA-1 antigenic determinant was more vulnerable to pH 11 elution than that of Mac-1. In other experiments, LFA-1 could be eluted at pH 10 with partial retention of antigenic activity.

DISCUSSION

Recently we described two novel murine cell surface antigens, Mac-1 and LFA-1. The Mac-1 antigen was identified with a monoclonal antibody which bound to macrophages but not lymphocytes (7). The LFA-1 antigen was identified by screening for MAb which would block antigen-specific, T lymphocyte-mediated killing (2). The MAb defining Mac-1 and LFA-1 do not cross-react, and the antigens have distinct cellular distributions. However, recent studies have shown that Mac-1 and LFA-1 are structurally related (1, 11). It thus was of interest to further compare Mac-1 and LFA-1 structurally, and to purify antigens in quantities sufficient for chemical characterization.

TABLE II
Purification of Mac-1 and LFA-1

Antigen	Source	Molecules/cell[a]	Theoretical yield	Actual yield[b]	Yield	Initial protein[c]	Purification
			mg		%	*mg*	
Mac-1	7.7×10^9 P388D$_1$ cells	1.6×10^5	0.54	0.21	39	837	1550
LFA-1	13×10^9 EL-4 cells	1.0×10^5	0.60	0.42	70	752	1250

[a] See Table I.
[b] Determined by Lowry assay.
[c] Detergent extract (100,000 × *g*).

LFA-1 appears crucially important for several lymphocyte functions which require cell-cell contact. Monoclonal antibodies to LFA-1 block cytotoxic T lymphocyte-mediated killing of target cells by binding to the effector cell and inhibiting adhesion to target cells (3). LFA-1 thus appears to participate in the Mg^{+2}-dependent, antigen recognition-adhesion step of CTL-mediated killing. LFA-1 also plays a crucial role in antigen-specific T helper cell responses (3). Only one other antigen, Lyt-2,3, has so far been described to play an important role in T lymphocyte-mediated killing (reviewed in Ref. 4). Further investigation of the biochemistry of LFA-1 is an important stepstone to an understanding of the molecular basis of T lymphocyte-mediated immunity. LFA-1 is the first T lymphocyte surface molecule important in cell-mediated immunity to be purified to homogeneity.

Subsequent to its identification as a differentiation antigen, Mac-1 has been found to be either identical with or closely associated with the function of the macrophage/granulocyte type three complement receptor (footnote 1, reviewed in Ref. 4).[2] Anti-Mac-1 MAb and its $F(ab')_2$ fragment specifically block this receptor but have no effect on the type one complement receptor or the Fc receptor. The type three complement receptor, which is specific for C3bi or C3d,g, was only recently distinguished from the type one and type two receptors specific for C3b and C3d, respectively (reviewed in Ref. 31). The type three complement receptor has not been previously isolated or characterized. Testing of purified Mac-1 for complement receptor activity should allow the nature of its relationship to the type three complement receptor to be definitively established.

So far, insight into the structure of Mac-1 and LFA-1 has been gained through surface iodination and immunoprecipitation. Immunoprecipitation of both antigens shows α chains of M_r = 170,000–180,000 and β chains of M_r = 95,000. With surface-labeled material it was possible that one or both polypeptides were externally acquired in a ligand-receptor interaction such as seen with transferrin and its receptor (32). Biosynthetic labeling dismissed this possibility. [^{35}S]Methionine was incorporated into both α and β chains of Mac-1 and LFA-1. Both α and β chains are also glycosylated in Mac-1 and LFA-1, as demonstrated by incorporation of [^{3}H]glucosamine. In some [^{35}S]methionine and [^{3}H]glucosamine labeling experiments, bands of about M_r = 10,000 lower than α and β were also noted. These are the only bands seen after 5-min pulse labeling, and thus represent the biosynthetic precursors of the α and β chains.[2] Mac-1 and LFA-1 were also found to have a two-chain structure after purification to homogeneity. Coomassie blue staining suggested the α and β chains are present in approximately equimolar quantities.

Definitive evidence on the relationship between the α and β chains was provided by cross-linking experiments. For both LFA-1 and Mac-1, a cross-linked complex was obtained, which was demonstrated to contain 1 α subunit and 1 β subunit by nonreduced-reduced two-dimensional SDS-PAGE. It is highly likely that this reflects the native state of the antigens since mild detergents such as Triton X100 have been shown to preserve the subunit association of other integral membrane proteins (33). Cross-linking was complete, *i.e.* no uncross-linked α or β bands were left. This implies that there are no free α or β chains on the cell surface and the two subunits must be synthesized and expressed on the surface in a highly coordinate fashion.

Screening of tumor cell lines showed that T lymphomas expressed LFA-1 but not Mac-1, as do normal T lymphocytes. Conversely, some macrophage-like lines expressed Mac-1 but not LFA-1, resembling thioglycollate-elicited peritoneal exudate macrophages. Of particular interest were some other macrophage-like lines which coexpressed large amounts of Mac-1 and small amounts of LFA-1. Some normal cells also coexpress Mac-1 and LFA-1, since bone marrow cells are 50% Mac-1$^+$ (7), 75% LFA-1$^+$ (5). Double labeling experiments have shown the Mac-1$^+$ subpopulation in bone marrow is completely included within the LFA-1$^+$ subpopulation.[3] Immunoprecipitation experiments with the tumor cells coexpressing Mac-1 and LFA-1 showed that the Mac-1 and LFA-1 determinants are present on separate molecules. The absence of molecules with both determinants suggests that determinants are not on carbohydrate moieties attached at multiple sites on the polypeptide chains. It also suggests that Mac-1 and LFA-1 do not form mixed dimers containing both types of α subunits, which is consistent with the lack of homodimers in the cross-linking experiments.

Another purpose of screening tumor lines was to identify the best sources for antigen isolation. The EL-4 and P388D$_1$ cell lines were found to express about 10^5 and 1.6×10^5 molecules/cell of LFA-1 and Mac-1, respectively. Compared to H-2 histocompatibility antigens, which are a major surface component, LFA-1 and Mac-1 are present on EL-4 and P388D$_1$ in lower quantities on a molar basis, but in higher quantities on a weight basis. The antigens were purified by a rapid MAb-Sepharose affinity chromatography procedure. As described recently for several other antigens (25, 28, 34, 35), it was unnecessary to first isolate membranes. This simplified the method and added to the speed of the procedure. The supernatant (100,000 × *g*) of Triton X100 extracts from up to 1.5×10^{10} cells could be passed through two precolumns to remove material binding nonspecifically and through the MAb column without difficulties. Purified antigen could be obtained in 2 days with column loading being the rate-limiting step (up to 24 h). Extensive washes at pH 8 did not get rid of material nonspecifically bound to the affinity column. Differential elution with stepwise increasing pH, however, could desorb unrelated molecules and finally elute the specifically bound antigen in almost pure form at pH 11. Complete purification could be obtained for Mac-1 by a second cycle of affinity chromatography. Addition to the Triton X100 extract of 0.5% sodium deoxycholate (28) greatly reduced nonspecific binding to the column and made binding to and elution from the column such an effective purification step that a second column passage became superfluous. Thus, 400 μg of LFA-1 could be isolated with high apparent purity in a single column step. The high yield of 70% was accompanied by a purification factor of 1250 *versus* the detergent extract.

As seen in the SDS-PAGE analysis, purified Mac-1 and LFA-1 contain α and β subunits. At no step during purification did the subunits elute separately, a further indication of the tight association of α and β polypeptides. Both Mac-1 subunits give rise to broader bands in SDS-PAGE than those of LFA-1. This could be due to microheterogeneities in the carbohydrate content of individual molecules. About 40% of the purified Mac-1 antigen retained its antigenicity whereas less than 5% of LFA-1 did so. The reason for the lack of antigenicity of purified LFA-1 lies in the elution conditions used, since more antigenicity was retained in other experiments with pH 10 elution. LFA-1 antigenicity was also partially destroyed by derivatization with cross-linking reagents, whereas Mac-1 was unaffected.[4] LFA-1 appears to have a less stable antigenic determinant than Mac-1.

Tryptic peptide mapping has shown that the LFA-1 and Mac-1 β chains are highly homologous or identical, whereas the α chains are highly different (1, 11). Together with the

[3] T. A. Springer, unpublished data.

[4] T. A. Springer and K. Kürzinger, unpublished data.

studies reported here, this establishes that Mac-1 and LFA-1 comprise a novel family of antigens. The antigens contain distinct, glycosylated α subunits noncovalently associated with homologous or identical glycosylated β subunits in $\alpha_1\beta_1$ structures. The α subunits are distinct in molecular weight and bear distinct antigenic determinants whether isolated from different cells or the same cells.

A major outstanding question is whether the α chains are homologous in amino acid sequence, but at a level not detectable by peptide mapping. Homology of the α chains would be predicted by analogy to other protein families which have shared homologous or identical subunits, such as the hemoglobins, immunoglobulins, and histocompatibility antigens. The availability of purified Mac-1 and LFA-1 will allow this question to be answered by amino acid sequencing studies. Since both Mac-1 and LFA-1 are function-associated, detailed chemical characterization can also yield important information about their structure-function relationships.

The membrane organization of Mac-1 and LFA-1 can now also be investigated with purified material. The organization of LFA-1 is of particular interest with respect to its participation in cell-mediated immunity. With total monomeric M_r = 265,000–275,000, Mac-1 and LFA-1 are among the most complex leukocyte surface antigens yet described. It is quite possible that the membrane organization of these antigens will differ from that of previously described smaller leukocyte surface antigens, such as HLA (36), Thy-1 (37), and immunoglobulin (38).

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