MAPPING OF ANTIGENIC AND FUNCTIONAL EPITOPES ON THE α- AND β-SUBUNITS OF TWO RELATED MOUSE GLYCOPROTEINS INVOLVED IN CELL INTERACTIONS, LFA-1 AND MAC-1*

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Despite the importance of adhesive cell interactions in development and differentiation, progress in elucidating the underlying molecular mechanisms has been slow. The immune system has been extensively studied as a model, both because its cells circulate and can be readily obtained in suspension, and because adhesive interactions between T helper cells and macrophages (1, 2) and between T cytolytic cells and target cells (3, 4) are extremely important in the development, regulation, and expression of specific immunity. The interaction between T cytolytic cells (cytolytic T lymphocytes or CTL)[1] and target cells has proved particularly amenable to study. CTL are readily elicited to virus-infected cells or to histoincompatible foreign cells. CTL adhere to and lyse target cells bearing specific antigen. Adhesion to the target cells requires Mg^{+2}, while delivery of the lethal hit to the target requires Ca^{+2}.

Information on the molecular basis of the CTL-target interaction has been obtained with monoclonal antibodies (MAb). MAb to two molecules, Lyt-2,3 and LFA-1, block killing by inhibiting formation of the adhesion between the CTL and target cell (5–8). MAb to a large number of other CTL surface molecules, including some present in higher density, have no effect on killing (9, 10). It appears that LFA-1 and Lyt-2,3 are distinct from the antigen receptor, and together with it contribute to the avidity of the CTL for the target cell (6, 11–13). LFA-1 is present on B lymphocytes and myeloid cells as well as T lymphocytes (10), suggesting that it plays a more general role in adhesion than do antigen receptors, and might be a useful model of other adhesive interactions in cell biology.

Indeed, a second type of molecule involved in adhesive interactions, Mac-1, has been found to be structurally related to LFA-1 (14, 15). Mac-1 was originally defined as a differentiation antigen present on monocyte/macrophage, granu-

[1] *Abbreviations used in this paper:* CR₁, complement receptor type one; CR₃, complement receptor type three; CTL, cytolytic T lymphocyte(s); HoS, horse serum; MAb, monoclonal antibody(ies); NK, natural killer cells; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PEC, peritoneal exudate cell macrophages; PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecyl sulfate; SRBC, sheep erythrocytes.

locyte, and natural killer (NK) lineages in mice and humans, and absent from B and T lymphocytes (16, 17). Recently, it was discovered that MAb to Mac-1 selectively block the complement receptor type three (CR₃) on mouse and human macrophages and granulocytes (18). Anti-Mac-1 has no effect on macrophage Fc receptors or the complement receptor type one (CR₁), suggesting that Mac-1 is identical to or functionally associated with the adhesiveness of the CR₃. In an interesting resemblance to CTL adhesion, adhesion through the CR₃ but not the CR₁ or Fc receptor is Mg^{+2}-dependent (19, 20). The CR₃ is a physiologically important receptor on myeloid cells that is specific for the complement component C3bi or possibly its further degradation product C3d,g (α2D) (21). It mediates adherence to cells or particles opsonized through either the classical or alternative complement pathways with C3bi or C3d,g (22, 23). On activated macrophages, the CR₃ mediates phagocytosis (24, 25).

LFA-1 and Mac-1 appear very similar in overall structure. They contain α-subunits of M_r 180,000 or 170,000, respectively, and β-subunits of M_r 95,000. Both subunits are glycosylated and have surface exposure (15). In both antigens, the subunits are noncovalently associated in $\alpha_1\beta_1$-complexes (14, 15). Tyrosyl tryptic peptide mapping of purified antigens has shown that the β-subunits are very similar or identical, but the α subunits differ considerably (14). An antigen probably identical to LFA-1 was also shown to be related to Mac-1 by peptide mapping and MAb cross-reactivity (26). However, the peptide-mapping results should be interpreted with caution. Only the small proportion of peptides that contain tyrosine has thus far been mapped, and mapping is sensitive to a single amino acid change per peptide. Therefore, important homologies between the α-subunits as well as differences between the β-subunits might have been missed.

The MAb to LFA-1 that were selected for blockade of CTL-mediated killing (9, 27), and the MAb to Mac-1 that block CR₃ function (16, 18), do not cross-react (6, 14). LFA-1 and Mac-1 also bear shared antigenic epitopes, as shown both with cross-reactive MAb selected by immunoprecipitation (6, 26), and with conventional antisera (14). Whether the distinct and common epitopes are present on the α- or β-subunits has not been known.

Here, comparative studies have been carried out on these cell interaction molecules and their active sites. The degree of immunological cross-reactivity between the subunits of the LFA-1 and Mac-1 molecules is an important measure of their structural relationship. Therefore, conventional antisera have been raised to the purified Mac-1 and LFA-1 antigens and to the LFA-1 α-subunit, which recognize a broad range of antigenic epitopes, and have been used to test subunit crossreactivity. A panel of noncross-reactive MAb, as well as a newly obtained crossreactive MAb, have also been used to localize the distinct and shared epitopes to specific subunits. Both types of antibodies show that the α-subunits bear the noncross-reactive or "distinct" epitopes, whereas the β-subunits bear the common epitopes and are immunologically identical. Further, the specificity of MAb for particular epitopes on the α- and β-subunits has been correlated with their effect on T lymphocyte-mediated killing and macrophage complement receptor activity. Functional sites have been localized to the vicinity of epitopes on the α-subunits.

Materials and Methods

Antibodies. Monoclonal antibodies (MAb) recognizing unique determinants on the LFA-1 molecule were produced by the hybridomas M7/14 (9), M17/4, M17/5, and M17/7 (27); those specific for Mac-1 were obtained from the hybridomas M1/70 (16), M19/23 and M19/24. The latter M19 hybridomas were obtained by immunization of rats with *Corynebacterium parvum* macrophages and selection of hybrids for macrophage specificity using standard procedures (9, 28). The anti-β-chain M18/2 MAb (IgG2aK) was obtained by immunization of a rat with mouse CTL glycoproteins purified on lentil-lectin columns (29), followed by fusion of spleen cells with the mouse myeloma P3 × 63Ag8.6.5.3 cell line (28). The M18/2 hybrid was selected by immunoprecipitation of LFA-1. Hybridoma lines were stabilized by subcloning. Lines grown in RPMI 1640 medium + 10% FCS secreted 50–200 μg/ml of MAb and culture supernatants were used either directly or after purification of MAb Ig as described (17).

Polyclonal rat antisera to homogenous preparations of purified LFA-1 and Mac-1 antigens (15) were raised by intramuscular injection of 1 ml per rat of an emulsion in complete Freund's adjuvant containing 10 μg of purified antigen. The rats were boosted subcutaneously after 2 wk and 1 mo with 10 μg of antigen in incomplete Freund's adjuvant. An antiserum to the α-subunit of LFA-1, separated by preparative SDS-PAGE (30), was obtained by three different injections of ground and emulsified slices of gel containing the equivalent of 5 μg of LFA-1 α-subunit per boost.

Purification of Mac-1 and LFA-1. Mac-1 and LFA-1 were purified from P388D$_1$ or EL-4 lysates on MAb-Sepharose-CL 4B columns (15) with the following modifications. The final elutions for the anti-LFA-1 M17/4 or M17/7 MAb columns were with 20 mM triethylamine, 0.1% TX-100, 1 mM PMSF, pH 11.0, and the M17/7-Sepharose column was additionally eluted with 50 mM triethylamine, 0.5 M NaCl, 0.1% TX-100, 1 mM PMSF pH 11.5. Aliquots of fractions were analyzed by SDS-PAGE and Coomassie Blue staining as described (15).

Iodination, Immunoprecipitation, and Electrophoresis of Cell Membrane Proteins. Hypaque-Ficoll purified C57BL/6J anti-P815 CTL (2 × 10^7 cells, viability >90%) and 4-d thioglycollate-induced peritoneal exudate cell macrophages (PEC) (2 × 10^7 cells, viability >95%) were iodinated using chloroglycoluril (IODO-GEN, Pierce Chemical Co., Rockford, IL) (31).

Purified LFA-1 and Mac-1 antigens, and LFA-1 α- and β-subunits were iodinated in solution using chloroglycoluril (31). Immunoprecipitation was performed in two steps. The MAb and polyclonal antisera were mixed with labeled cell lysates or purified antigens for 2 h at 4°C, followed by the addition of 40 μl of purified RG7/7.6 mouse anti-rat kappa chain coupled to Sepharose (2 mg/ml) (32). After shaking for 1½ h at 4°C, samples were washed and subjected to SDS-PAGE (16) and autoradiography with enhancing screens (33).

Immunoblotting. Unlabeled thioglycollate-elicited peritoneal exudate cell or P388D$_1$ lysates (3.3 × 10^7 cells/ml) or EL-4 lysates (5 × 10^7 cells/ml) were immunoprecipitated with ¼–⅙ volume of M1/70-Sepharose (2–3 mg/ml), M17/4-Sepharose (4 mg/ml), or M7/14-Sepharose (2 mg/ml). After shaking for 1½ h at 4°C, the washed immunoprecipitates were subjected to SDS 8% PAGE. Immunoprecipitates containing labeled ^{125}I-Mac-1 or ^{125}I-LFA-1 were included as positive controls for α- and β-chain transfer and to confirm positions of the subunits in immunoblots. The proteins were transferred at room temperature to nitrocellulose membranes (Millipore/Continental Water Systems, Bedford, MA) in an Electroblot apparatus (Bio-Rad Laboratories, Richmonds, CA) at 200 mA for 15 h with water circulation cooling (34). The nitrocellulose blots were then saturated with 3% BSA, 1% HoS (horse serum) in phosphate buffer (PBS) plus 0.02% (wt/vol) sodium azide at either room temperature overnight or 37°C for 1 h. Blots were then cut into strips and incubated with 5 ml of 1:10 diluted MAb or 1:1,000 dilutions of polyclonal antisera in plastic bags. Blots were incubated at 4°C or room temperature for 2–5 h with gentle shaking, then washed for 30 min with six changes of PBS, 1% BSA, 0.3% HoS. Strips were then incubated at room temperature for 2 h with ^{125}I MAb anti-rat kappa chain RG7/7.6 (32) (0.8–1 × 10^5 cpm/ml) in 100 ml of 3% BSA in PBS, and

washed for 30 min with four changes of PBS, 1% BSA, 0.3% HoS, and two changes of PBS. Dried blots were autoradiographed with intensifying screens (33).

Dissociation of Mac-1 α- and β-Subunits by High pH Treatment. ^{125}I-labeled Mac-1 (50,000 cpm/μl) was mixed with 10- to 50-fold (vol/vol) excess of the following solutions: 10 mM Tris-HCl, 140 mM NaCl, pH 7.8; 20 mM glycine-NaOH, pH 9.0; 20 mM glycine-NaOH, pH 10.0; 20 mM triethylamine, pH 11.0; or 50 mM triethylamine, pH 11.5. The mixtures were incubated for 30 min at specified temperatures and then neutralized by addition of 1/10 volume of 1 M Tris-HCl, pH 6.8. Samples were then assayed by immunoprecipitation with the different MAb and antisera. In other experiments, high pH treatment was carried out at a series of higher temperatures.

Absorption of Anti-Mac-1 Serum with Cell Lysates. P388D₁ macrophage and EL-4 T cell lines were lysed with Tris-HCl pH 7.8, 0.14 M NaCl, 1 mM PMSF, 1% TX-100 (lysis buffer) at cell concentrations of 5×10^7 cells/ml. Varying volumes of cell lysates were mixed with 10 μl of 1:200 diluted anti-Mac-1 serum, and samples were adjusted to 100 μl with lysis buffer and incubated overnight at 4°C. After centrifugation at 2,000 rpm × 20 min, the absorbed supernatants were tested for immunoprecipitation of ^{125}I-labeled Mac-1 antigen which had been previously dissociated by treatment at pH 11.5 at 30°C for 30 min.

CTL Generation and Assay. Generation of effector cells and inhibition of mouse CTL-mediated cytolysis using C57BL/6J anti-P815 (allogeneic) and C57BL/6J anti-rat BNLφ (xenogeneic) effector cells were performed and percentages of specific ^{51}Cr release and inhibition of killing activity were calculated as described (5, 27).

CR₃ Rosette Analysis. The rosetting assays were performed essentially as described by Beller et al. (18) but adapted to 50 μl final volume in 96-well plates (Costar, Data Packaging, Cambridge, MA). Briefly, 25×10^3 4-d thioglycollate-induced peritoneal exudate cells from C57BL/6J mice were plated in 40 μl of Hank's balanced salt solution, 10 mM HEPES per well. MAb were added in 5 μl to triplicate wells either undiluted or diluted with 10% BSA in Hank's balanced salt solution/HEPES. After 10 min at room temperature, 5 μl of an opsonized sheep erythrocyte (SRBC) suspension was added. The cultures were incubated for 30 min and then washed gently 4–5 times to remove nonadhered SRBC. Monolayers were fixed in 1% glutaraldehyde and the percentage of macrophages rosetting 5 or more SRBC was determined on an inverted microscope.

Opsonization of SRBC was carried out as previously described (18), except that the anti-Forssman antigen M1/87.27 IgM MAb (35) was used at the maximal nonagglutinating concentration of 1:32 to sensitize a 2% suspension of washed SRBC. C5-deficient A/J mouse serum was used as the complement source.

Results

Distinct and Common Epitopes on the LFA-1 and Mac-1 Molecules. A panel of 9 MAb and 3 conventional antisera were tested for cross-reactivity by immunoprecipitation of LFA-1 and Mac-1 from lysates of ^{125}I-labeled cytolytic T lymphocytes and macrophages, respectively (Fig. 1). Four independent MAb to LFA-1 obtained by selection for blockade of CTL-mediated killing precipitated LFA-1 from CTL (Fig. 1A, lanes 2–5), but nothing from macrophages (Fig. 1B, lanes 2–5). Four independent MAb to Mac-1 selected for macrophage-specificity precipitated Mac-1 from macrophages (Fig. 1B, lanes 7–10), but nothing from CTL (Fig. 1A, lanes 7–10). These two groups of MAb thus recognize noncrossreactive or "distinct" epitopes on the LFA-1 and Mac-1 molecules. In contrast, the M18/2 MAb precipitated LFA-1 and Mac-1 equally well (Fig. 1A and B, lane 6). This MAb thus defines a crossreactive or "common" epitope. Conventional antisera, raised to Mac-1 or LFA-1 purified by MAb affinity chromatography, were also cross-reactive (Fig. 1A and B, lanes 12 and 13).

Interestingly, the antibodies which recognize common epitopes precipitated
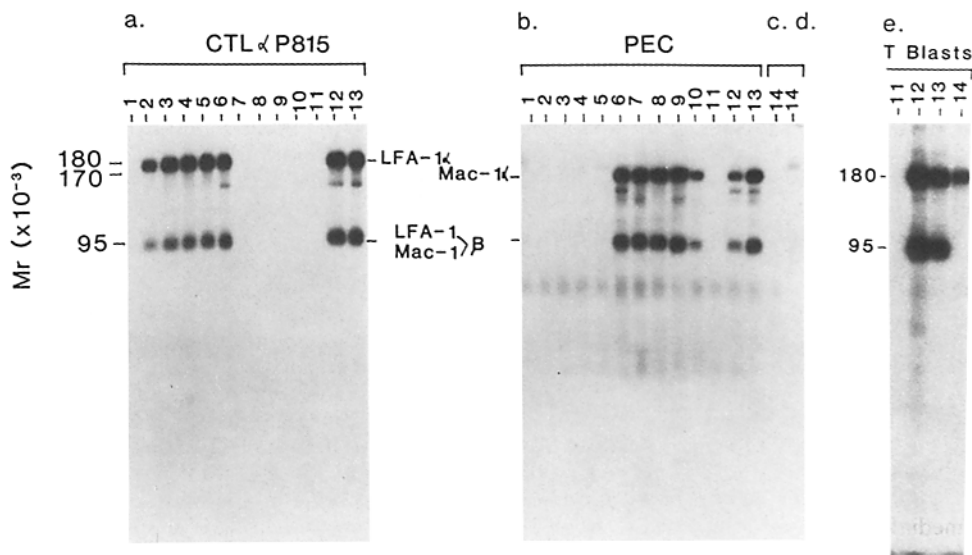
FIGURE 1. LFA-1 and Mac-1 bear distinct and common antigenic determinants. Spleen cells from B6 mice primed with P815 and restimulated *in vitro* (CTL anti-P815) (*A* and *D*), 4-day thioglycollate-elicited peritoneal exudate cells (PEC) (*B* and *C*), and 4-day Con A-stimulated spleen cells (T cell blasts) (*E*) were labeled with ^{125}I. Cell lysates were immunoprecipitated with 100 μl of supernatants of NSI plus 50 μg/ml normal rat IgG as control (lane *1*); the anti-LFA-1 M7/14, M17/4, M17/5 and M17/7 MAb (lanes *2–5*, respectively); the M18/2 MAb (lane *6*); the anti-Mac-1 M1/70, M19/23, M19/24, and M19/1 MAb (lanes *7–10*, respectively); or with 1 μl normal rat serum (lane *11*); or antisera to purified Mac-1 (lane *12*), LFA-1 (lane *13*), or LFA-1 α-chain (lane *14*). Immune complexes were precipitaed with anti-rat kappa chain MAb coupled to Sepharose. Reduced samples were subjected to SDS 10% PAGE and autoradiography.

an additional α-like band of 155,000 M_r from CTL and macrophage preparations (Fig. 1*A* and *B*, lanes *6, 12, 13*). This band is the same M_r in both cell preparations, while the M_r's of the LFA-1 and Mac-1 α-chains differ. Since it is precipitated by crossreactive but not by noncross-reactive MAb (Fig. 1), the 155,000 M_r band does not appear to be a degradation product. Trypsin and papain degradation products of Mac-1 are precipitated equally efficiently by crossreactive and non-crossreactive MAb (data not shown). Therefore, this band may represent a third member of the LFA-1 and Mac-1 family.

Another conventional antiserum was raised to LFA-1 α-chain which had been separated from LFA-1 β-chain by preparative SDS-PAGE. Anti-LFA-1 α chain serum precipitated LFA-1 α-chain, with little or no associated β-chain from CTL, and no Mac-1 from macrophages (Fig. 1*C* and *D*, lane *14*). The amount of LFA-1 α-chain precipitated varied from high in some preparations (Fig. 1*E*, lane *14*), to none in others (not shown), probably due to differences in the amount of α-chain which has become dissociated from the β-subunit. The anti-LFA-1 α-chain serum appears predominantly specific for free LFA-1 α-chain as opposed to the native LFA-1 $α_1β_1$-complex.

Localization of Epitopes with Immunoblotting.   To localize epitopes to the α- or β-subunits, unlabeled subunits were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with the rat antibodies to LFA-1 or Mac-1,

then with a second ^{125}I-labeled anti-rat kappa chain MAb (32). The β-subunits of both Mac-1 and LFA-1 reacted with anti-Mac-1 serum (Fig. 2A, lanes 2 and 5), with anti-LFA-1 serum (Fig. 2A, lanes 3 and 6), and with the cross-reactive M18/2 MAb (Fig. 2B, lanes 2 and 3). This shows that common epitopes recognized by conventional antibodies and by a monoclonal antibody are present on the β-subunits. Reaction of antibodies with the β-subunits was found after SDS-PAGE under nonreducing conditions (Fig. 2A-C), but not under reducing conditions (Fig. 2D). This showed that the β-subunits contain disulfide bonds, and that they are important for proper renaturation.

None of 8 MAb that defined specific epitopes reacted in immunoblotting, despite use of nonreducing conditions (data not shown). The anti-Mac-1 serum reacted with the Mac-1 α-chain (Fig. 2A, lane 2; Fig. 2C, lanes 1 and 3; Fig. 2D, lanes 5 and 7), but not with the LFA-1 α chain (Fig. 2A, lane 5; Fig. 2C, lane 2; Fig. 2D, lane 6). Reactivity of anti-Mac-1 serum with the Mac-1 α-chain was retained even after reduction (compare Fig. 2C and Fig. 2D).

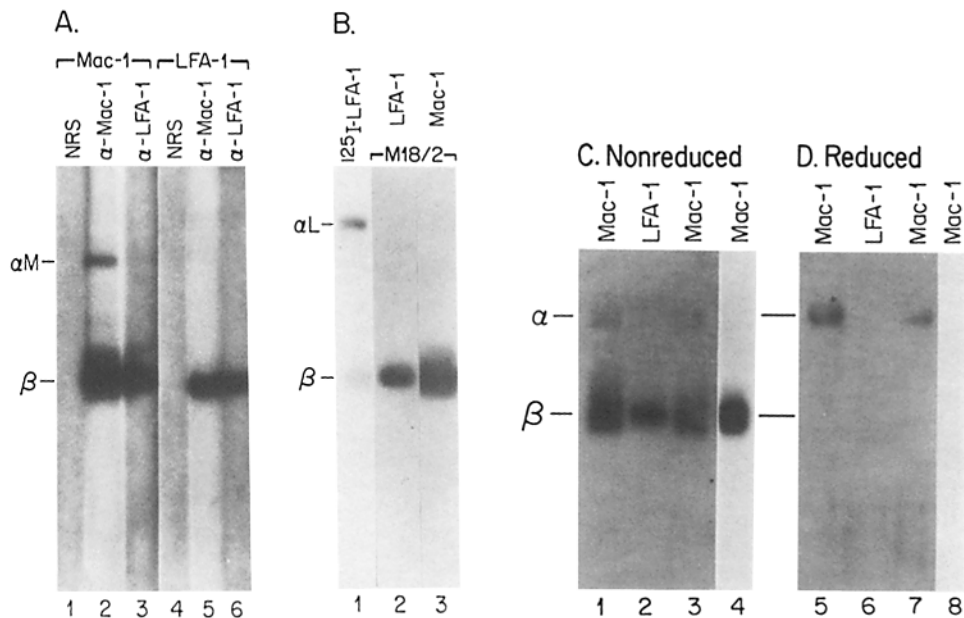*Localization of Specific LFA-1 Epitopes to the α-Subunit.* The subunit reactivity

FIGURE 2. Localization of cross-reactive determinants on the β-subunits by immunoblotting. Unlabeled Mac-1 and LFA-1 antigens were purified from thioglycollate-induced Mφ and EL-4 lysates, respectively, using the anti-Mac-1 M1/70 MAb and the anti-LFA-1 M17/4 MAb coupled to Sepharose. The immunoprecipitates were subjected to SDS-8% PAGE under nonreducing (A–C) or reducing (D) conditions and transferred to nitrocellulose blots. In B, lane 1, ^{125}I-LFA-1 was run side by side with unlabeled antigens and transferred to nitrocellulose as a positive control for α- and β-transfer and as a M_r marker. Strips of the nitrocellulose blots containing LFA-1 or Mac-1 antigen as indicated in the figure were incubated with 1/1,000 diluted normal rat serum (A, lanes 1 and 4), anti-Mac-1 serum (A, lanes 2 and 5; C, lanes 1–3; D, lanes 5–7), anti-LFA-1 serum (A, lanes 3 and 6), or 1/10 diluted M18/2 MAb (B, lanes 2 and 3; C, lane 4; D, lane 4). In A and B, antigen from 42 μl of lysate was used per strip. In C and D, the LFA-1 antigen was from 750 μl lysate/lane, while the Mac-1 antigen was from a lysate volume of 250 μl (lane 1), 120 μl (lane 3), 370 μl (lane 5), or 190 μl (lane 7). ^{125}I anti-rat kappa chain MAb was used as second antibody. Blots were autoradiographed.

of MAb which bind to noncross-reactive epitopes on LFA-1 was studied with two different anti-LFA-1 MAb coupled to Sepharose. During large scale purification, LFA-1 from EL-4 cells was absorbed to M17/4 or M17/7 MAb-Sepharose affinity columns. Upon elution of the M17/4 column with pH 11 buffer, small amounts of (α chain and large amounts of β appeared in early fractions (Fig. 3A, lanes $1-2$), while α was greatly enriched in later fractions (Fig. 3A, lanes $4-6$). Similarly, when the M17/7 column was used, pH 11 buffer eluted largely β with only small amounts of α (Fig. 3B, lanes $1-8$), while pH 11.5 eluted α and β (Fig. 3B, lane 11), followed in later fractions by very high ratios of α to β or α only (Fig. 3B, lanes $12-14$). Thus the α-chain can remain bound to the M17/4 or M17/7 MAb after β is dissociated from α, showing that α bears the LFA-1-specific antigenic determinants.

It appears that this antigenic epitope is lost after α elutes from the MAb-Sepharose column, probably due to a conformational change when the subunits become dissociated. This was shown using fractions eluted from the affinity columns and labeled with ^{125}I, and which were enriched for LFA-1 α-chains (Fig. 4A, lane 2) or β-chains (Fig. 4A, lane 3). The ^{125}I-labeled LFA-1 α could not be rebound by the same LFA-1 MAb with which it was purified (Fig. 4B, lane 9), but was strongly bound by anti-LFA-1 α-serum (Fig. 4B, lane 7). A positive control showed the anti-LFA-1 MAb precipitated the $\alpha_1\beta_1$-complex from the preparation enriched for β-chains (Fig. 4C, lane 9). In contrast, the M18/2 anti-β MAb precipitated large amounts of free β chain in addition to the $\alpha_1\beta_1$-complex (Fig. 4C, lane 10).

Epitopes on the Dissociated Mac-1 α- and β-Subunits. The Mac-1 α- and β-subunits remain associated after elution from anti-Mac-1 MAb-Sepharose at pH 11. To determine conditions for dissociation, purified ^{125}I-labeled Mac-1 was treated at various pH's for 30 min. at 0°C, neutralized, and samples were tested by
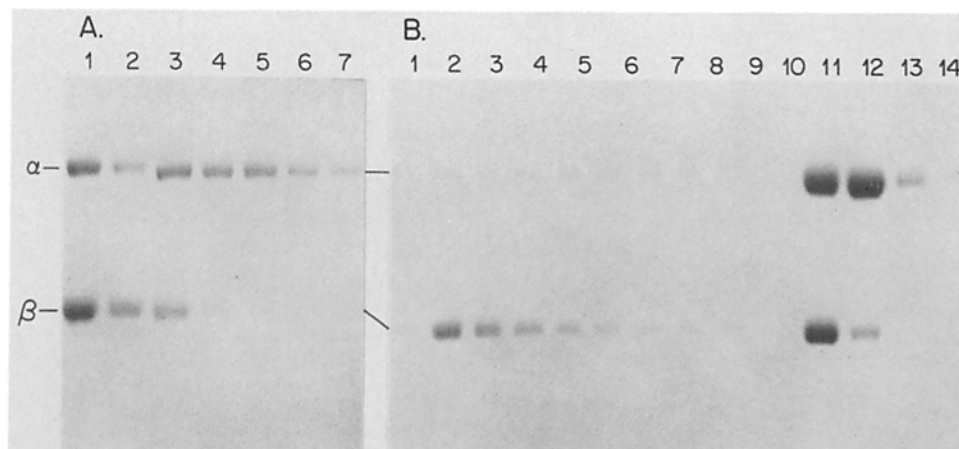

Figure 3. Differential elution of LFA-1 α- and β-subunits from MAb immunoadsorbents. Lysates were prepared from EL-4 cells and applied to Sepharose columns coupled with the anti-LFA-1 M17/4 or M17/7 MAb. The columns were washed and eluted with high pH buffers. Analyses of aliquots of fractions eluted with pH 11.0 buffer from the M17/4 (A, lanes $1-7$) and M17/7 (B, lanes $1-10$) columns and subsequent elution of the M17/7 column with pH 11.5 buffer (B, lanes $11-14$) were performed by SDS-PAGE and Coomasie Blue staining.
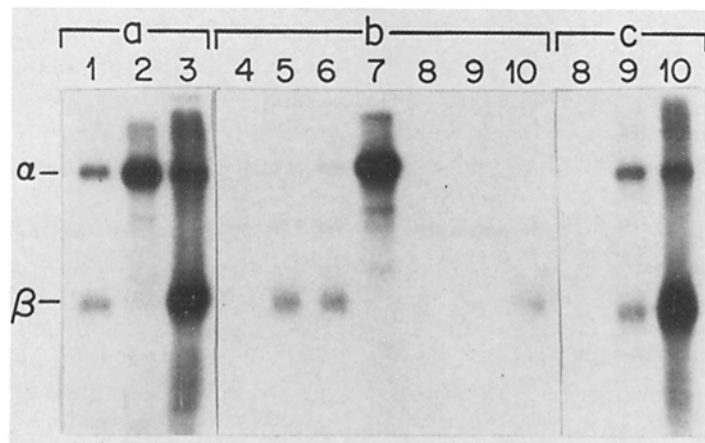
FIGURE 4. Immunoprecipitation of ^{125}I-labeled LFA-1 preparations enriched in the α- or β-subunit. Preparations of LFA-1 containing similar amounts of α and β, enriched in α, or enriched in β (corresponding to fractions analyzed in Fig. 3A, lanes 1 and 6; and Fig. 3B, lane 2, respectively) were labeled with ^{125}I and analyzed by SDS-10% PAGE and autoradiography either directly (A, lanes 1, 2, and 3, respectively) or after immunoprecipitation. Preparations enriched in α (B) or β (C) were mixed with nonimmune rat serum as control, lane 4; anti-Mac-1 serum, lane 5; anti-LFA-1 serum, lane 6; anti-LFA-1 α serum, lane 7; and the following MAb: NSI culture supernatant as control, lane 8; anti-LFA-1 M17/7 MAb, lane 9; and anti-LFA-1 β-chain M18/2 MAb, lane 10. Anti-rat kappa chain MAb coupled to Sepharose was used as second antibody.
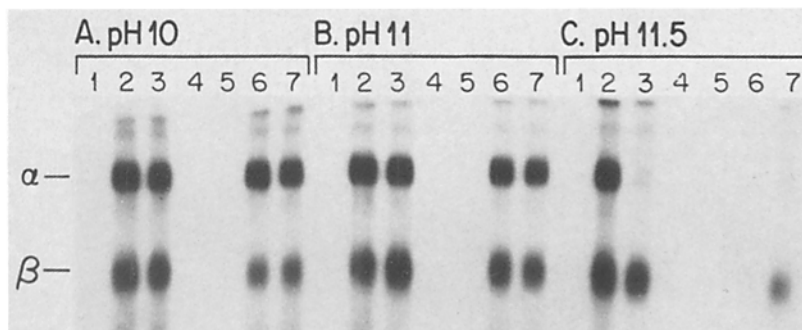

FIGURE 5. pH-Dependent dissociation of the Mac-1 α- and β-subunits. ^{125}I-labeled, purified Mac-1 (1 μl) was diluted with 50 μl of solutions of pH 10.0 (A), pH 11.0 (B), and pH 11.5 (C) for 30 min. at 0°C. The samples were neutralized and tested by immunoprecipitation with nonimmune rat serum as control, lane 1; anti-Mac-1 serum, lane 2; anti-LFA-1 serum, lane 3; anti-LFA-1 α serum, lane 4; and the following MAb: NSI culture supernatant as control, lane 5; anti-Mac-1 M19/23 MAb, lane 6; anti-β chain M18/2 MAb, lane 7. Immune complexes were isolated with anti-rat kappa chain MAb-Sepharose and subjected to SDS 10%-PAGE and autoradiography.

immunoprecipitation with the antisera and MAb to Mac-1. The results after treatment at pH 7.8 and 9 (not shown), pH 10 (Fig. 5A), and pH 11 (Fig. 5B) were identical: the Mac-1 α- and β-subunits remained associated as shown by precipitation of both subunits by the M18/2 anti-β MAb (Fig. 5A, B, lane 7) and the M19/23 Mac-1-specific MAb (Fig. 5A and B, lane 6). Treatment at pH 11.5 caused dissociation of the α- and β-subunits, as shown by precipitation of the β-

subunit but no α-subunit by anti-LFA-1 serum and the M18/2 anti-β MAb (Fig. 5C, lanes 3 and 7). Concomitantly with dissociation, the determinant defined by the Mac-1-specific M19/23 MAb was lost (Fig. 5C, lane 6). The Mac-1 antiserum precipitated both α and β, showing that it contained two populations of antibodies, one reactive with the α-subunit and the other reactive with the β-subunit (Fig. 5C, lane 2).

Non-cross-reactivity of Mac-1 and LFA-1 α-Chains. The conventional antisera to Mac-1 and to LFA-1 α-chain were used to test for the presence of any shared epitopes on the Mac-1 and LFA-1 α-chains. ^{125}I-Mac-1 antigen that had been completely dissociated by treatment with pH 11.5 at 25°C as verified with the anti-β MAb (Fig. 6E, lane 9) and α-chain enriched, dissociated ^{125}I-LFA-1 preparations were used. Serial dilutions of antisera showed the LFA-1 α-chain was precipitated by as little as 1/25 μl of anti-LFA-1 α-serum (Fig. 6D, lane 4), while up to 4 μl gave no precipitation of the Mac-1 α-chain (Fig. 6C, lane 1). This showed specificity for the LFA-1 α-chain was >100-fold. Conversely, 4 μl of anti-Mac-1 serum did not precipitate LFA-1 α (Fig. 6B, lane 1), but 1/125 μl precipitated the Mac-1 α-chain (Fig. 6A, lane 5), showing specificity for Mac-1 α was >500-fold. In contrast, β-chain specific antibodies in anti-Mac-1 serum precipitated Mac-1 β-chain and small amounts of β-chain present in the enriched LFA-1 α-chain preparation equivalently at a concentration range of 4 μl to 1/ 125 μl (Fig. 6A and B, lanes 1–5). The anti-LFA-1 α-serum did not precipitate any LFA-1 or Mac-1 β-chain, confirming it was completely specific for LFA-1 α.

The above experiments showed that the dissociated α-subunits were not cross-reactive, while the dissociated β-subunits were cross-reactive. It was next tested
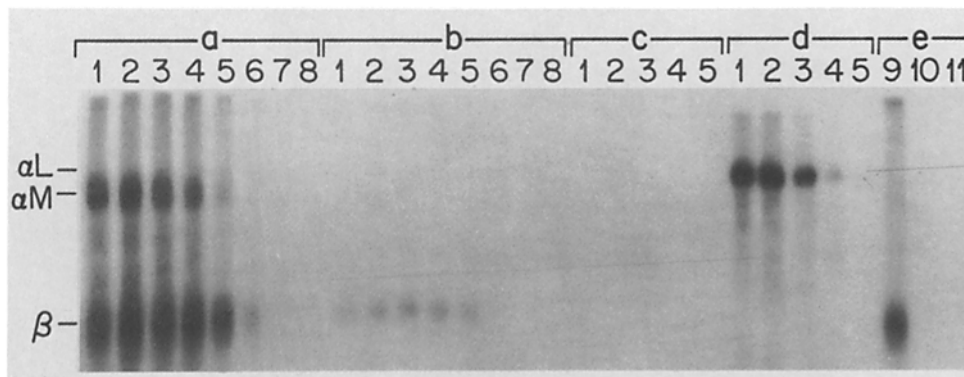

FIGURE 6. Lack of cross-reaction between the α-subunits with conventional sera to Mac-1 and to LFA-1 α-chain. Serial dilutions of anti-Mac-1 serum (A and B) and anti-LFA-1 α chain serum (C and D) were tested for immunoprecipitation of ^{125}I-labeled purified Mac-1 α- and β-subunits that had been dissociated at pH 11.5 at 25°C for 30 min (A and C) or of ^{125}I-labeled, pH-dissociated LFA-1 preparation that was enriched in α chains (B and D). The amounts of sera were 4 μl (lane 1), 1 μl (lane 2), 1/5 μl (lane 3), 1/25 μl (lane 4), 1/125 μl (lane 5), 1/ 625 μl (lane 6), 1/3,125 μl (lane 7), or 1/15,625 μl (lane 8) diluted in a final volume of 20 μl. Immunoprecipitation of dissociated ^{125}I-Mac-1 (E) with the M18/2 anti-β-chain MAb (lane 9) and the M19/23 anti-Mac-1 MAb (lane 10) showed Mac-1 dissociation was complete. Nonimmune rat serum (4 μl) was used as a control for specific immunoprecipitation (lane 11). Immune complexes were isolated with anti-rat kappa chain MAb-Sepharose and subjected to SDS 8%-PAGE and autoradiography.

whether this also held for the α- and β-subunits in the native $\alpha_1\beta_1$-complex. Anti-Mac-1 serum was used, which had been raised to the $\alpha_1\beta_1$-complex and reacted with the native complex as well as the dissociated α- and β-subunits. Anti-Mac-1 serum was absorbed with cells of the Mac-1$^+$ P388D$_1$ macrophagelike line or the LFA-1$^+$ EL-4 T lymphoma line (15), which had been solubilized with Triton X-100 to expose intracellular as well as extracellular portions of the Mac-1 and LFA-1 molecules. The absorbed serum was then tested for immunoprecipitation of the dissociated Mac-1 α- and β-subunits that were quantitated by scanning of autoradiograms. The anti-β activity in the serum was removed to the same extent by P388D$_1$ or EL-4 lysates (Fig. 7B), suggesting that all β-determinants recognized by the anti-Mac-1 serum are common to Mac-1 and LFA-1. Equivalent absorption by P388D$_1$ and EL-4 was in agreement with previous findings that they have similar densities of Mac-1 and LFA-1, respectively (15). P388D$_1$ lysates but not EL-4 lysates absorbed the reactivity of the anti-Mac-1 serum with the Mac-1 α-subunit (Fig. 7). Mac-1 and LFA-1 remain in the native $\alpha_1\beta_1$-complex after solubilization with Triton X-100 (15). No dissociation of the Mac-1 subunits in Triton X-100 was seen at neutral pH (cf. Fig. 5). Thus, it appears that the anti-Mac-1 antibodies that immunoprecipitate the dissociated α- and β-subunits were absorbed by the native $\alpha_1\beta_1$-complex. These results confirm the absence of common determinants on the α-chains of the native Mac-1 and LFA-1 molecules, and show the β-subunits are immunologically identical.

Differential Functional Effects of MAb to α- and β-Epitopes. The above studies showed that antibodies specific for Mac-1 or LFA-1 recognized epitopes on the α-subunits while cross-reactive antibodies recognized β-subunit epitopes. It was of great interest to explore whether antibodies binding to epitopes on the α- and β-subunits differed in their functional effects. Preliminary experiments utilizing
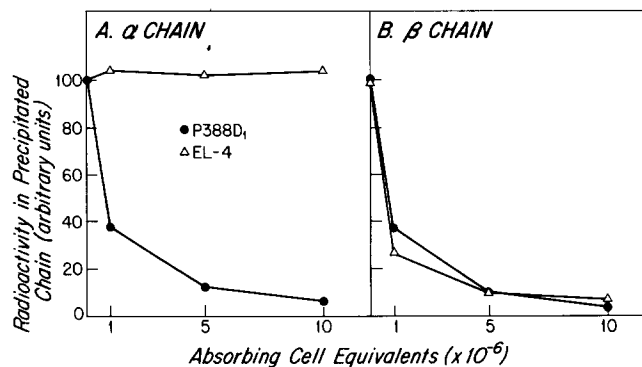

FIGURE 7. Absorption of anti- α- and anti- β-antibodies in anti-Mac-1 serum by P388D$_1$ (Mac-1$^+$) or EL-4 (LFA-1$^+$) cell lines. Anti-Mac-1 serum (10 μl of a 1/200 dilution) was adsorbed with 100 μl of lysis buffer (no absorption control) or with the indicated number of P388D$_1$ (●) or EL-4 (△) cells in 100 μl of lysis buffer as described in materials and methods. The absorbed antisera were used to immunoprecipitate ^{125}I-purified Mac-1 α- and β-subunits which had been dissociated by treatment with a 50-fold volume excess of pH 11.5 triethylamine for 30 min at 30°C and neutralized. Immunoprecipitates were subjected to SDS-10% PAGE and autoradiography with hypersensitized film and enhancing screens (33). The amount of precipitated Mac-1 α-chain (A) or β chain (B) was quantitated by scanning the autoradiograms. Lack of precipitation by the M19/23 MAb and precipitation of the β-subunit only by the M18/2 MAb confirmed that chain dissociation was complete (not shown).

immunofluorescence and binding of ^{125}I-labeled MAb to intact cells showed that both anti-α and anti-β MAb bind to cell surface epitopes, that the α- and β-chain epitopes are topographically distinct as shown by lack of competitive inhibition, and that concentrations of MAb used in functional experiments gave saturation of binding (data not shown). Preclearing experiments confirmed that anti-β and the appropriate anti-α MAb bound to the same population of Mac-1 or LFA-1 molecules (not shown). Inhibitory effects were tested in two different cytolytic T lymphocyte assays employing B6 mouse killer T lymphocytes specific for antigens on allogeneic P815 mouse target cells or xenogeneic BN lymphoma rat target cells, respectively. The anti-LFA-1 α-chain MAb gave >85% of inhibition of T cell-mediated cytolysis in both the allogeneic and the xenogeneic killing systems, but the anti-β-chain M18/2 MAb had no effect (Table I). MAb were similarly tested for inhibition of adherence of erythrocytes sensitized with IgM and complement to the complement receptor type 3 (CR$_3$) on macrophages. The M1/70 and M19/23 MAb specific for Mac-1 gave strong inhibition of the percentage of macrophages forming rosettes with C3bi-sensitized erythrocytes (Table I). The M1/70 and M19/23 MAb recognize the same or spatially related epitopes, since they mutually inhibit each other's binding (not shown). The M19/24 MAb recognizes a spatially distinct epitope on the α-chain, since it does not cross-compete with M1/70 or M19/23 (not shown). Interestingly, M19/24 did not inhibit the CR$_3$ (Table I). The anti-β-chain M18/2 MAb did not block CR$_3$ activity, but in contrast, enhanced the percentage of rosetting macrophages by

TABLE 1

Effect of MAb on CTL-Mediated Killing and Complement Receptor Activity

Specificity	MAb	A. Killing Activity				B. E-IgM-C Rosetting	
		B6 Anti-BNLØ		B6 anti-P815			
		Specific release	Inhibition	Specific release	Inhibition	Rosetting	Inhibition
		%	%	%	%	%	%
Control	M1/69HK	52.5	—	51.0	—	75	—
Anti-LFA-1 α	M17/4	0.6	99	3.3	94	71	5
Anti-LFA-1 α	M17/5	0.9	98	2.3	96	74	1
Anti-LFA-1 α	M17/7	0.4	99	8.1	85	71	5
Anti-β	M18/2	49.0	6	62.6	0	94	-25
Anti-Mac-1 α	M1/70	51.2	0	51.8	3	8	89
Anti-Mac-1 α	M19/23	55.0	0	61.8	0	3	96
Anti-Mac-1 α	M19/24	59.0	0	58.9	0	71	5

(A) Secondary (1-1) xenogeneic C57BL/6J (B6) mouse anti-rat BNLØ CTL (E:T=10:1) or primary (0-1) allogeneic anti-P815 CTL (E:T=2:1) were pretreated with an equal volume of MAb in hybridoma culture supernatants for 15 min at room temperature, then ^{51}Cr-labeled target cells were added and the assay was completed. The spontaneous release of ^{51}Cr in wells containing the appropriate culture supernatant but with effector cells omitted was 3% and 14%, respectively. Percent of inhibition of specific ^{51}Cr release is expressed relative to CTL treated with culture supernatant of the inactive M1/69 HK hybridoma which has lost its specific L chain.

(B) Macrophages adherent to microtiter wells were pretreated for 10 min. at room temperature with 10% hybridoma culture supernatant. Then sheep erythrocytes coated with IgM (E-IgM) or with IgM followed by C5-deficient complement (E-IgM-C) were added and the assay was completed. Wells with E-IgM had 0% rosettes. Percent of inhibition of rosetting is expressed relative to cultures with M1/69 HK culture supernatants.

25% (Table I). This enhancing effect has been repeatedly demonstrated and is considerably more dramatic (up to 100%) when erythrocytes are suboptimally sensitized with complement and control rosetting is <50%.[2] As expected, MAb specific for Mac-1 or LFA-1 had no effect on CTL-mediated killing or CR_3-mediated adherence, respectively (Table I).

Discussion

Cell-cell adhesions are of crucial importance in the development and differentiation of higher organisms. Here, two molecules involved in adhesive interactions have been compared and their functional sites have been localized. Previously, the structural basis of the common and distinct epitopes (14, 26) on the Mac-1 and LFA-1 molecules had been unknown. The present studies show that the immunological cross-reactivity between the Mac-1 and LFA-1 antigens is due to their β-subunits. To explore the widest possible range of antigen epitopes, conventional antisera were raised to the purified Mac-1 and LFA-1 antigens. Crossreactions occurred only with the β-subunit of the heterologous antigen, as shown by immunoblotting after SDS-PAGE and by immunoprecipitation after dissociation of subunits by high pH treatment. A cross-reactive MAb was also shown to bind to the LFA-1 and Mac-1 β-subunits. Moreover, conventional sera had equal titer for LFA-1 and Mac-1 β-subunits, and cell lysates containing LFA-1 were as effective as those containing Mac-1 in absorbing antibodies to the Mac-1 β-subunit present in anti-Mac-1 serum. These results show the LFA-1 and Mac-1 β-subunits are immunologically identical. Previous studies had shown the β-subunits have essentially identical tyrosyl tryptic peptide maps, but differences among nontyrosyl peptides, which are normally more numerous, could not be ruled out (14, 26). Taken together, these findings now show there is a high degree of identity between the Mac-1 and LFA-1 β-subunits, and that they could be encoded by a single gene.

The Mac-1 and LFA-1 α-subunits have been shown to bear the distinct epitopes. Despite the use of polyclonal antisera at concentrations 100- to 500-fold higher than required for reaction with the homologous α-subunits, no cross-reaction between the α-subunits was found. Anti-α-chain activity of anti-Mac-1 serum was absorbed by Mac-1^{+} macrophage lysates but not by LFA-1^{+} lymphocyte lysates. Furthermore, distinct determinants recognized by MAb to LFA-1 were located on the α-subunit as demonstrated by differential elution from MAb-Sepharose columns with dissociative high pH treatment. It can be deduced from the non-cross-reactivity of the α-subunits and the complete cross-reactivity of the β-subunits as shown by conventional antisera, that the MAb-defined, distinct epitopes on Mac-1 are also present on the α-subunit. The non-cross-reactivity shows there are extensive differences between the α-subunits, in agreement with tyrosyl tryptic map data (14, 26). It should be emphasized that many homologous proteins show no cross-reactivity or peptide map identities. A single amino acid substitution per peptide or determinant would generate differences. As a general rule for globular proteins, 60% sequence homology is required for cross-reaction to occur (36, 37). In analogy to other protein families with shared subunits, e.g.

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[2] P. Simon, F. Sanchez-Madrid, and T. Springer, manuscript in preparation.

the hemoglobins, immunoglobulins, and class I histocompatibility antigens, the α-chains are predicted to be homologous at the level of amino acid sequence.

MAb-defined epitopes on the LFA-1 and Mac-1 α-subunits were lost after dissociation from β with high pH. Conversely, the antisera prepared to SDS-denatured, purified LFA-1 α reacted with the free LFA-1 α-chain but not with the $\alpha_1\beta_1$-complex. A similar dependence of HLA α-chain conformation on association with the β-2m subunit has been reported (38). The most likely interpretation is that conformational changes occur in α after dissociation from β, but it is also possible that all these antibodies recognize epitopes in the region of αβ-contact. A proportion of the antibodies to the Mac-1 $\alpha_1\beta_1$-complex were reactive with the free Mac-1 α-chain. These may be high affinity antibodies which can "capture" the dissociated α-chain in a conformation resembling the native one. Intact disulfide bonds were required for proper renaturation of β-chain epitopes after SDS-PAGE, but no dependence of β-epitope conformation on association with the α-chain was detected.

The finding that epitopes could be localized to the α- and β-chains raised the question of whether MAb binding to different epitopes would differ in their effects on function. Two anti-Mac-1 α MAb (M1/70 and M19/23) which compete for the same topographic determinant on the α subunit strongly inhibited CR_3 activity. The M19/24 MAb which recognizes a second, topographically distinct epitope on the Mac-1 α-chain, did not show any inhibitory effect. Furthermore, binding of the M18/2 MAb to Mac-1 β-subunit did not inhibit complement receptor activity. A functionally important site has thus been localized to the vicinity of the M1/70-M19/23 epitope on the α-subunit. This may be a receptor site for binding of the C3bi ligand. In a similar fashion, three anti-LFA-1 MAb that recognize identical or closely related epitopes (27) on the α-subunit gave strong inhibition of CTL-mediated killing, while the anti-β MAb gave no inhibition.

Functional sites on the LFA-1 molecules and Mac-1 thus both map to the vicinity of specific epitopes on the α-subunits. The findings emphasize the importance of these sites, but do not rule out the possibility that additional functional sites might be found with appropriate MAb. The simplest interpretation is that the α-subunit sites defined here function in adhesion, and that α-chain divergence reflects specialization for binding of distinct ligands. Mac-1 may bind C3bi, while the ligand for LFA-1 remains unidentified. A ligand for LFA-1 might normally be present on target cells, or deposited on them by CTL.

It is possible that further members of the LFA-1 and Mac-1 family exist which are specialized for binding of other ligands. The α-like chain of 155,000 M_r precipitated by anti-β-antibodies might represent a distinct member of this family. It is of interest that a 150,000 M_r glycoprotein has been shown by MAb blocking studies and by its deficiency in Bernard Soulier syndrome to be involved in platelet adhesion to the subendothelial surface (39).

The Mac-1 and LFA-1 antigens are selectively expressed on some leukocytes, such as thioglycollate-elicited macrophages and lymphocytes, respectively, and are co-expressed on others, such as granulocytes and NK cells. The MAb used to define and to distinguish between these antigens have now been shown to recognize epitopes on the α-subunit, which can be thought of as "differentiation"

epitopes. The LFA-1 and Mac-1 gene family appears ideally suited for studies on the regulation of gene expression during leukocyte differentiation. There are at least two α-chain genes and one β-chain gene. Biosynthesis studies have shown the mature antigens are derived from the assembly of the separate α- and β-precursors, implying the α- and β-genes are separate (40). Cloning of the genes would allow studies on how α-genes are selectively expressed and how α- and β-expression is coordinated. A particularly interesting system is the M1 myelomonocytic cell line, which is Mac-1$^-$ but becomes strongly Mac-1$^+$ after maturation to a macrophage phenotype is induced with corticosteroids or lymphokines (41). Cloning should be facilitated by the antisera described here that react with the separated α- and β-subunits. They would be expected to bind to the nascent chains on polyribosomes, allowing isolation of specific mRNA's (42), and to allow immunoprecipitation of in vitro translation products.

The human homologues of LFA-1 and Mac-1 have been found. Human LFA-1 is important in anti-HLA-DR and anti-HLA-A,B CTL-mediated killing and appears identical to murine LFA-1 in subunit composition and cell distribution (43). The rat anti-mouse Mac-1 M1/70 MAb cross-reacts with human Mac-1 and locks the human CR$_3$ (18). The OKM1 MAb also blocks the CR$_3$,[3] suggesting human Mac-1 and OKM1 are identical as was expected from their cell distributions (17, 44). Recently, a MAb has been found that cross-reacts with human LFA-1 and Mac-1 (OKM1) antigens, and the epitope it recognizes has been localized on the β-subunit of both antigens.[3] The functional significance of this protein family and its subunit organization is emphasized by conservation in evolution.

Summary

Mouse Mac-1, a complement receptor-associated surface structure on macrophages, and LFA-1, a function-associated structure on lymphocytes, comprise a novel family of leukocyte differentiation antigens participating in adhesive cell interactions. Mac-1 and LFA-1 contain α-subunits of 170,000 and 180,000 M_r, respectively, and β-subunits of 95,000 M_r noncovalently associated in $\alpha_1\beta_1$ complexes. The structural relation between the α- and between the β-subunits, and the location of functionally important sites on the molecules, have been probed with antibodies. Both non-cross-reactive and cross-reactive monoclonal antibodies (MAb) and antisera prepared to the purified molecules or the LFA-1 α-subunits were used. Reactivity with individual subunits was studied by immunoprecipitation after dissociation induced by high pH treatment, or by immunoblotting after SDS-PAGE. Cross-reactive epitopes on Mac-1 and LFA-1 were found to be present on the β-subunits, which were immunologically identical. Non-cross-reactive epitopes that are distinctive for Mac-1 or LFA-1 were localized to the α-subunits. MAb to LFA-1 α-subunit epitopes inhibited CTL-mediated killing. Two MAb to Mac-1 α-subunit epitopes but not a third MAb to a spatially distinct α-epitope inhibited complement receptor function. Neither function was inhibited by a MAb binding to a common β-subunit epitope. Therefore, sites of Mac-1 and LFA-1 involved in their respective adhesion-related functions, as well as

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[3] F. Sanchez-Madrid, J. Nagy, E. Robbins, P. Simon, and T. Springer, manuscript submitted.

distinctive structural features, have been localized to the α-subunits.

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