A HUMAN LEUKOCYTE DIFFERENTIATION ANTIGEN FAMILY WITH DISTINCT α-SUBUNITS AND A COMMON β-SUBUNIT:

The Lymphocyte Function–Associated Antigen (LFA-1), the C3bi Complement Receptor (OKM1/Mac-1), and the p150,95 Molecule*

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Two human molecules with surprisingly similar molecular structures, LFA-1 and Mac-1/OKM1, have recently been found to be important in cytolytic T lymphocyte–mediated killing, and in complement receptor function, respectively. Human LFA-1 contains two subunits of M_r 177,000 and 95,000 (1), and OKM1/Mac-1 (2) has two polypeptides of strikingly similar size. Monoclonal antibodies (MAb) to human LFA-1 block antigen-specific CTL-mediated killing and T helper cell responses, as well as natural killing (1, 3). LFA-1 participates in the Mg^{+2}-dependent adhesion step of CTL-mediated killing (4). Human LFA-1 is a widely expressed leukocyte antigen present in lymphocytes, thymocytes, monocytes, granulocytes, and 37% of bone marrow cells (3). The participation of LFA-1 in natural as well as antigen-dependent killing, and its distribution on myeloid (3) as well as lymphoid cells, suggest that LFA-1 is not an antigen receptor but has a different and perhaps more general function in cell adhesion reactions.

MAb to human Mac-1(5) block adhesion by myeloid cells, mediated by the complement receptor type three (CR_3),[1] to C3bi-coated particles (6). Mac-1 may thus be identical to the CR_3. Human Mac-1 (5) and its homologue murine Mac-1 (7–9) appear identical to the human OKM1 and Mo1 antigens (2, 10–12) in cell distribution and structural characteristics. Mac-1, OKM1, and Mo1 are expressed on monocytes, granulocytes, and natural killer cells, and in contrast to LFA-1, are absent from lymphocytes.

A structural relationship between molecules implies similarities in the molecular mechanisms underlying their functions. Here, relationships between LFA-1, OKM1 (Mac-1), and further leukocyte cell surface antigens have been examined. The results show that human LFA-1, OKM1/Mo1, and a third novel

[1] Abbreviations used in this paper: CR_3, complement receptor type three; CTL, cytolytic T lymphocyte(s); D, dimensional; E, erythrocytes; MAb, monoclonal antibody(ies); PAGE, polyacrylamide gel electrophoresis; PBL, peripheral blood lymphocytes; pI, isoelectric point; PMA, 4β-phorbol 12β-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

molecule termed p150,95 each contain α- and β-subunits that are noncovalently associated in an $\alpha_1\beta_1$-structure. The α-subunits of M_r 177,000 to 150,000 of these three molecules differ, while the β-subunits of $M_r = 95,000$ are identical. The molecules have been characterized biochemically, immunochemically, and functionally.

Materials and Methods

Monoclonal Antibodies. Anti-LFA-1 MAb produced by the TS1 and TS2 subcloned hybridomas have been previously described (1). OKM1 MAb was purchased from Ortho Pharmaceutical (Raritan, NJ) or was the kind gift of Dr. G. Goldstein, as were OKM9 and OKM10 MAb. The 187.1 anti-mouse kappa chain hybridoma (13) was the kind gift of Dr. M. Scharff (Albert Einstein Medical School, NY).

Cells. Peripheral blood lymphocytes were isolated from normal adults by Ficoll-Hypaque density centrifugation (14) and removal of monocytes. Monocytes were purified by adherence to plastic tissue-culture dishes with subsequent release with xylocaine (15). Granulocytes were isolated from citrated blood by dextran sedimentation of erythrocytes followed by a Ficoll-Hypaque gradient (14). Remaining erythrocytes were lysed with hypotonic saline. Thymocytes were obtained from patients undergoing corrective cardiac surgery. CTL lines were as previously described (1). Cell lines JY, a B lymphoblastoid line, and U937, a monoblast-like line, were grown in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco), 2 mM glutamine, 50 μM 2-mercaptoethanol, and 50 μg/ml gentamycin. For induction, PMA dissolved in dimethylsulfoxide (14 μg/ml) was added to a final concentration of 2 ng/ml to cultures of U937 cells. 2/3 of the medium was replaced with fresh medium containing PMA every 2 d. For [^{35}S]methionine labeling, 10 ml of U937 cells at 4 $\times$ 10^5/ml were cultured in 60-mm tissue culture dishes to which they became adherent. For ^{125}I or fluorescent labeling, 5 $\times$ 10^5 cells/ml were cultured in Teflon beakers (Nalgene 1500), to which they did not adhere. For rosetting assays, 10^4 U937 cells/well were induced for 4 d in 96-well microtiter plates (Costar, Data Packaging, Cambridge, MA).

Labeling, Immunoprecipitation, and Electrophoresis of Cell Surface Proteins. Cells were iodinated using chloroglycoluril (IODO-GEN, Pierce Chemical Co., Rockford, IL) (16), except for granulocytes, which were enzymatically iodinated by the glucose oxidase/lactoperoxidase method (17). For biosynthesis studies, U937 cell induced with PMA for 3 d were washed with methionine-free RPMI 1640 + PMA + 10% dialyzed FCS, incubated with 1.2 ml of the same medium containing 140 μCi [^{35}S]methionine for 15 or 30 min, and then chased by rinsing and incubation in 5 ml of complete medium at 37°C for the indicated times. Triton X-100 lysates were prepared as described (16) (in 10 mM Tris pH 8.0, 0.14 M NaCl, 1% Triton X-100, 1% hemoglobin, 1.5 mM PMSF), except that [^{35}S]methionine-labeled Triton X-100 lysates were subjected to centrifugation at 11,000 g for 20 min in a microfuge, followed by addition of sodium deoxycholate to 1% and a second centrifugation at 100,000 g for 1 h. For immunoprecipitation, lysates were mixed with 75 μl of MAb-containing culture supernatants, 30 μl of OKM1 MAb at 100 μg/ml, or 20 μl of 1:100 OKM10 ascites. To isolate immune complexes, 100 μl (7.4 μg) of the 187.1 anti-mouse kappa chain MAb together with 50 μl of a 10% suspension of *Staphylococcus aureus* were used per MAb. In some experiments, antigens were isolated with monoclonal antibodies coupled to Sepharose CL-4B at 1 mg/ml or with purified MAb or ascites in conjunction with 187.1 MAb coupled to Sepharose (2.5 mg/ml). Immunoprecipitates were processed as previously described (18) and samples were subjected to SDS-PAGE and autoradiography with enhancing screens (19).

Dissociation of α- and β-Subunits by High pH Treatment. Cell lysates were mixed with a 6–10-fold excess (vol/vol) of 50 mM triethylamine pH 11.5, 0.5 M NaCl, and 0.1% Triton X-100 for 30 min at 0°C. Samples were neutralized by addition of 1/10 of final volume of 1 M Tris-HCl pH 6.8.

Cross-linking. Triton X-100 lysates were cross-linked with 0.1 mg/ml dithiobis-(succinimidyl propionate) (Pierce Chemical Co.) as previously described (20).

Two-dimensional (2-D) Electrophoresis. For nonreducing-reducing 2D PAGE, immuno-precipitates were solubilized in Laemmli sample buffer containing 50 mM iodoacetamide for 5 min at 100°C and loaded on 0.3 cm × 11.5 cm 5% polyacrylamide tube gels (first dimension, nonreducing) (21). A stacking gel was included. After completion of electro-phoresis the gels were extruded from the tubes and equilibrated in 0.0625 M Tris pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, and 100 mM dithiothreitol at 21°C for 30 min and placed on 1.6-mm thick 7% polyacrylamide Laemmli slab gels (second dimension reducing). The 1% agarose SDS sample buffer solution used to seal the tube gels to the stacking gel contained 100 mM dithiothreitol.

2-D electrophoresis, with isoelectric focusing in the first dimension and SDS-PAGE in the second dimension, was carried out using the procedure of O'Farrell as described by Jones (22), except that Triton X-100 was used in place of NP-40. Ampholytes (LKB) were a 4:1 mixture of pH 5–7 and pH 3.5–10. pH gradients were measured by cutting parallel blank gels into 0.5-cm sections. Gel slices were soaked overnight at 21°C in 3 ml of distilled, deionized water in tightly capped tubes before metering the pH. pI marker proteins (Isogel pI markers, FMC Corporation) were also used.

Flow Cytometry. 10^6 cells were labeled with 50 μl TS1/12 or TS1/18 MAb culture supernatant containing $\sim$100 μg antibody/ml or with purified OKM1 MAb at 20 μg/ml, followed by washing and labeling with FITC-conjugated affinity-purified goat anti-mouse IgG, 70 μg/ml (Zymed Laboratories, South San Francisco, CA) as previously described (16). Antibody titrations confirmed saturation labeling.

CR_3 Rosetting Assay. Rosetting to U937 cells induced with PMA for 4 d was assayed in 10 mM HEPES-buffered Hank's balanced salt solution. Sheep erythrocytes (E) were sensitized with an IgM anti-Forssman MAb and C5-deficient A/J mouse serum to generate E-IgM-C. U937 cells were incubated with MAb for ½ h at 20°C before addition of E-IgM-C. Cells rosetting $\geq$5 E-IgM-C were scored as positive. Procedures were as previously described (23), except for washing. After addition of 200 μl of HEPES-Hank's, wells were sealed with tape (Dynatech Laboratories, Inc. Dynatech Corp., Alexandria, VA), inverted, and the trapped air bubble was gently gyrated. After 5 min the tape was removed and residual medium drained onto absorbent paper towels. Wells were washed thrice.

Results

Localization of Antigen Epitopes of the α- and β-Subunits of Human LFA-1. During studies on a panel of MAb to human LFA-1, one MAb was noted to immunopre-cipitate an extra band from ^{125}I-labeled CTL. While six of seven independent anti-LFA-1 MAb precipitated only the LFA-1 α (αL)- and β-subunits of 177,000 and 95,000 M_r, respectively (Fig. 1A, lanes 2–3 and 5–8), the TS1/18 anti-LFA-1 MAb additionally precipitated a chain of 150,000 M_r denoted αX (Fig. 1A, lane 4). The αX-chain was seen in some but not all preparations of human cytolytic T lymphocyte lines (cf. Fig. 2 of reference 1), perhaps due to variations in the presence of feeder cells.

The α- and β-subunits of human LFA-1 are not disulfide-linked, but are associated noncovalently in an $\alpha_1\beta_1$-complex (1). Previous work with mouse LFA-1 has shown the subunits can be dissociated with high pH (23). To determine whether the anti-human LFA-1 MAb bound to epitopes on the α- or β-subunits, the α- and β-subunits were separated by brief exposure to pH 11.5, followed by neutralization and immunoprecipitation. Five of the MAb precipitated the LFA-1 α-subunit of 177,000 M_r with no associated β-subunit (Fig. 1B, lanes 2, 3, 5, 7, and 8). The determinant recognized by the TS2/4 MAb (Fig. 1B, lane 6) was lost after the high pH treatment. The TS1/18 MAb (Fig. 1B, lane 4) precipitated the β-subunit with αX but no αL associated. The precipitation of αX in addition to β by TS1/18 appears due to the greater stability of the αXβ-complex than the
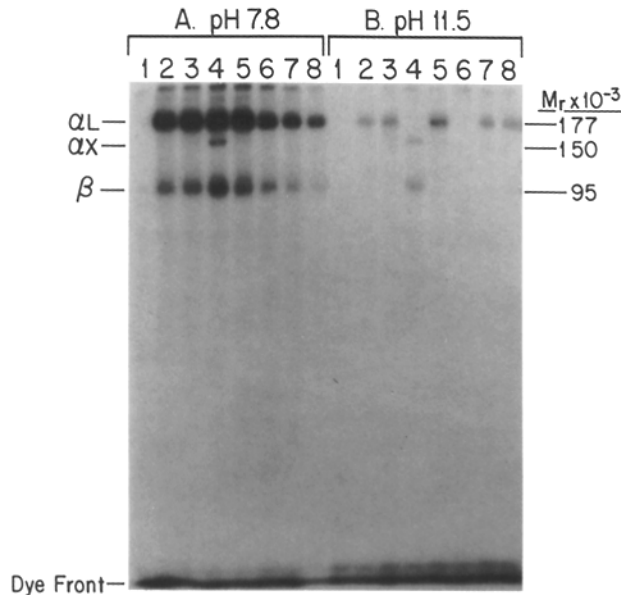
FIGURE 1.   Precipitation of native and pH-dissociated antigen from CTL by anti-LFA-1 MAb. ^{125}I-labeled CTL lysates were immunoprecipitated after treatment with pH 7.8 buffer as control (A) or at pH 11.5 to dissociate subunits (B) as described in Materials and Methods. Immunopreciptation was with the following MAb: X63 myeloma as control, lane 1; TS1/11, lane 2; TS1/12, lane 3; TS1/18, lane 4; TS1/22, lane 5; TS2/4, lane 6; TS2/6, lane 7, TS2/14, lane 8. Samples were subjected to SDS 10% PAGE and autoradiography. M_r was calibrated with previously described standards (16).

$\alpha L\beta$-complex to dissociation. The β-subunit clearly bears the TS1/18 determinant, since β is precipitated in the absence of αX from thymocytes and uninduced U937 cells (see Fig. 4 below). Thus, out of seven different anti-LFA-1 MAb, five recognize epitopes on the α-subunit and one recognizes an epitope located on the β-subunit.

The anti-β MAb precipitated three α-chains of M_r 177,000, 165,000, and 150,000 in association with the β-subunit from granulocytes (Fig. 2A, lane 3). The 177,000 M_r α-subunit co-migrated identically with αL precipitated by an anti-LFA-1 α MAb (Fig. 2A, lane 4). The α-subunit of 150,000 M_r appeared identical to αX described above. Interestingly, the α-subunit of 165,000 M_r (Fig. 2A, lane 3) co-migrated identically with the α-subunit precipitated by anti-OKM1 MAb (Fig. 2 A, lane 2). The anti-OKM1, anti-β, and anti-LFA-α MAb all precipitated β-subunits of identical M_r 95,000 (Fig. 2A, lanes 2–4).

To determine which subunits bore the antigen epitopes, the ^{125}I-granulocyte lysates were subjected to high pH to dissociate subunits, followed by neutralization and immunoprecipitation. The anti-OKM1 MAb precipitated the OKM1 α (αM)-chain with no β-chain (Fig. 2B, lane 2), showing that the OKM1 epitope is on the αM-chain. The OKM1 β-chain is therefore precipitated from native antigen preparations (Fig. 2A, lane 2), due to noncovalent association with the αM-chain. The TS1/18 anti-β MAb precipitated the β-subunit only (Fig. 2B, lane 3) and the TS1/22 anti-LFA-1 α MAb precipitated the αL-subunit only (Fig. B, lane 4), confirming results obtained with the dissociated subunits from CTL.
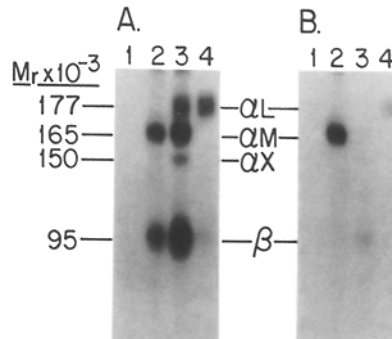
FIGURE 2. Precipitation of native and pH-dissociated antigen from granulocytes. ^{125}I-labeled granulocyte lysates were treated at neutral pH to preserve native subunit structure (A) or at pH 11.5 to dissociate subunits (B) as described in Materials and Methods. Lysates were then immunoprecipitated with X63 as control, lane 1; anti-OKM1 MAb, lane 2; TS1/18 anti-β MAb, lane 3; or TS1/22 anti-LFA-1 α MAb, lane 4. Immunoprecipitates were subjected to SDS 7% PAGE and autoradiography. Only the relevant portion of the gel is shown.

Identical results have also been obtained with high pH-dissociated monocyte lysates (results not shown).

The above experiments suggested that the anti-β MAb precipitated the same LFA-1 and OKM1 antigens as precipitated by anti-αL and anti-αM MAb, respectively. This hypothesis was tested with preclearing experiments. Granulocyte lysates were precipitated (precleared) with one MAb, then the precleared supernatants were subjected to immunoprecipitation with a second MAb. The amount of OKM1 antigen precipitated by the anti-OKM1 MAb after control preclearing with X63 (Fig. 3, lane 1) was greatly reduced by preclearing with the anti-β MAb (Fig. 3, lane 4). Both the α- and β-subunits were precleared. Conversely, the amount of αM precipitated by the anti-β MAb (Fig. 3, lane 2) was greatly reduced after preclearing with anti-OKM1 (Fig. 3, lane 5). The amount of β-subunit precipitation by anti-TS1/18 was also decreased. This showed that a substantial amount of the total β present in granulocytes is associated with the OKM1 α-subunit, as would be expected from the relative amount of the three α-subunits (Fig. 3, lane 2). The amount of αL and αX precipitated by the anti-β TS1/18 MAb was unaffected by preclearing with the OKM1 MAb (Fig. 3, lane 5 compared with lane 2). Similar results were obtained with LFA-1. The TS1/18 anti-β MAb precleared precipitation of LFA-1 by the TS1/22 anti-LFA-1 α MAb (Fig. 3, lane 9 compared with lane 7). The TS1/22 anti-α MAb precleared αL-chain precipitation by the TS1/18 MAb, while having no effect on αM or αX precipitation (Fig. 3, lane 10 compared to lane 2). These results prove that the αM- and αL-subunits recognized by the OKM1 and TS1/22 MAb, respectively, are associated with β-subunits bearing a common epitope recognized by the TS1/18 MAb.

The TS1/18 anti-β MAb was the only MAb studied here that was cross-reactive. The six antibodies that precipitated LFA-1 but not the αX-subunit (Fig. 1A) also did not precipitate OKM1 (not shown). The recently described OKM9 and OKM10 MAb have the same specificity as OKM1 MAb for monocytes and granulocytes but not lymphocytes, and define topographically distinct epitopes
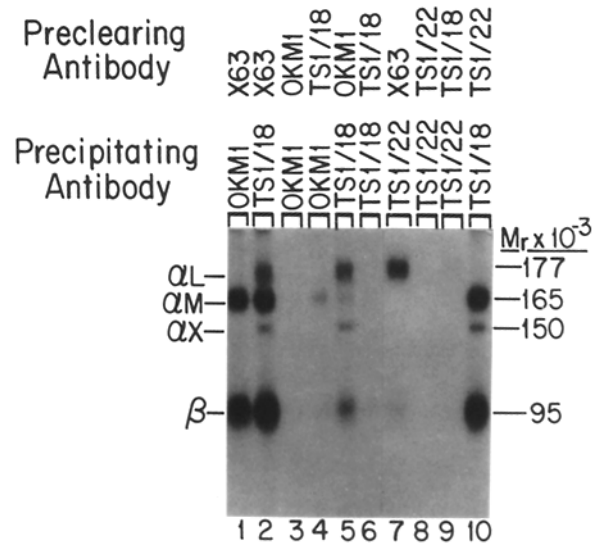
FIGURE 3. The same OKM1 or LFA-1 molecules are precipitated by anti-β or the relevant anti-α MAb. ^{125}I-granulocyte lysates were first immunoprecipitated with the indicated "preclearing antibody." X63 myeloma antibody was used as a control. The resulting precleared supernatants were then immunoprecipitated with the indicated "precipitating antibody." The latter immunoprecipitates were subjected to SDS 7% PAGE and autoradiography. Only the upper portion of the gel is shown.

on the OKM1 molecule (24). OKM9 (not shown) and OKM10 MAb (Fig. 8 below) precipitated OKM1 but not LFA-1 or αX.

$\alpha_1\beta_1$-Subunit Structures. To examine the subunit structure of these antigens, cross-linking experiments were carried out. Monocytes which bear OKM1, LFA-1, and in particular abundance, αX (Fig. 6A below), were used in these studies. Detergent-solubilized lysates were subjected to cross-linking with the cleavable reagent dithiobis(succinimidyl proprionate) and immunoprecipitated with either an anti-αM MAb, an anti-αL MAb, or the anti-β MAb. The cross-linked, immunoprecipitated material was analyzed by 2-D nonreduced-reduced, SDS-PAGE in which the cross-linker was cleaved with 0.1 M dithiothreitol before electrophoresis in the second dimension, thereby dissociating the cross-linked products into their constituent polypeptide chains (Fig. 4, a, c, and e). In the first dimension cross-linked products of M_r 250,000–270,000 were found for OKM1 (Fig. 4a), LFA-1 (Fig. 4c), and the three antigens precipitated by the anti-β MAb (Fig. 4e). Upon reduction in the second dimension the cross-linked OKM1 and LFA-1 products were cleaved into the OKM1 α- and β-chains of M_r 165,000 and 95,000 (Fig. 4a) and the LFA-1 α- and β-chains of M_r 177,000 and 95,000 (Fig. 4c), respectively. The fact that the α- and β-bands are vertically aligned with each other in the second dimension, below the diagonal of uncross-linked proteins, indicates that both subunits are present in the cross-linked products. The molecular weights of cross-linked products in the first dimension of M_r 260,000 for OKM1 and M_r 270,000 for LFA-1 show that they are of the composition $\alpha_1\beta_1$.

In the cross-linked anti-β precipitate (Fig. 4e), the identity of cross-linked α-chains was confirmed by comparison of their mobility and intensity in side-by-
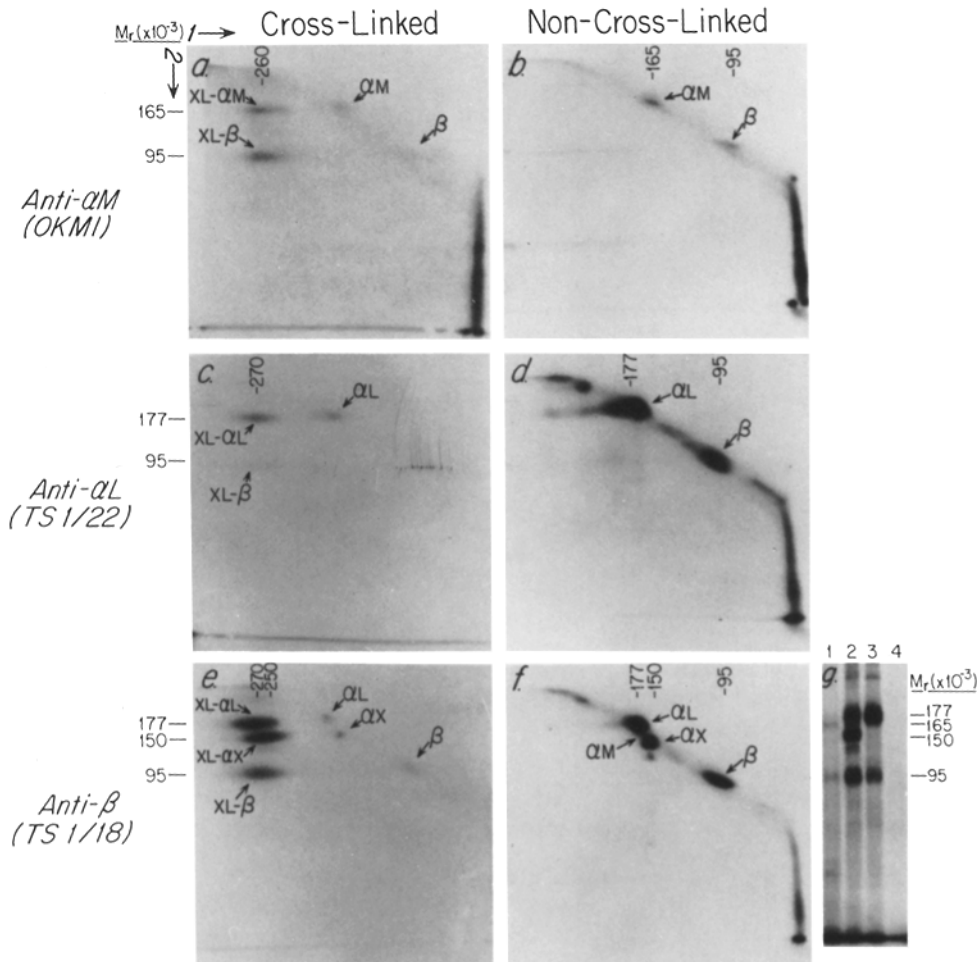
FIGURE 4. 2-D SDS-PAGE of cross-linked and noncross-linked OKM1, LFA-1 and p150,95. Cross-linked or uncrosslinked lysates (150 μl), as indicated in the figure, were immunoprecipitated with anti-OKM1 α MAb (using 100 μl of 0.1 mg/ml OKM1 and 100 μl of 187.1-Sepharose) (a and b), TS1/22 anti-LFA-1 α MAb (using 100 μl of TS1/22-Sepharose) (c and d), and TS1/18 anti-LFA-1 β MAb (using 100 μl of TS1/18-Sepharose (e and f). Immunoprecipitates were subjected to SDS-PAGE under nonreducing conditions in 5% polyacrylamide tube gels (first dimension) followed by electrophoresis under reducing conditions in 7% polyacrylamide slab gels (second dimension). Control immunoprecipitates with rat IgG Sepharose (1 mg/ml) run in parallel gels showed no specific spots (data not shown). The molecular weights of the cross-linked species were determined by SDS 5% PAGE in one dimensional slab gels under nonreducing conditions with molecular weight markers. Molecular weights in the reduced dimension were determined with markers loaded to the left of the focusing gel. The streaks visible in (b) and (f) are due to leakage of these markers. In (g), the mobilities and intensities of the different α chains precipitated by anti-β and anti-β MAb were compared using the same cross-liked lysate preparation in a reducing one dimensional slab system identical to that employed in the second dimension of a–f. Antigens were isolated with OKM1 and 187.1-Sepharose (lane 1), TS1/18-Sepharose (lane 2), TS 1/22-Sepharose (lane 3), and rat IgG-Sepharose as control (lane 4).
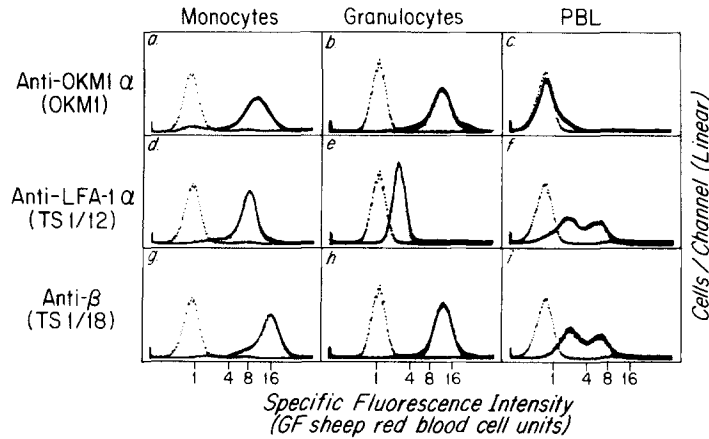
FIGURE 5. Immunofluorescence flow cytometry of peripheral blood monocytes, granulocytes, and lymphocytes. Cells were labeled with saturating concentrations of monoclonal antibodies OKM1, TS1/12, and TS1/18 (continuous curves) or X63 control antibody (dotted curves), followed by fluorescein isothiocyanate-anti-mouse IgG, and analyzed on a FACS II. Relative fluorescence intensity was calibrated with glutaraldehyde fixed sheep erythrocyte standards (16). Multiple peaks on PBL indicate subpopulations of cells with differing antigen densities.

side 1-D SDS-PAGE of the three different cross-linked immunoprecipitates under reducing conditions (Fig. 4g). αL and αX were much more prominent than αM in the anti-β precipitate (Fig. 4g, lane 2). αL (Fig. 4g, lane 3) migrated near to αM (Fig. 4g, lane 1) and obscured αM in the analysis of anti-β precipitates in 1-D (Fig. 4g, lane 2) and 2-D (Fig. 4e). In 2-D SDS-PAGE, cross-linked αX migrated at a slightly lower M_r than cross-linked αL in the first dimension (Fig. 4e), as expected from the lower M_r of an αXβ- than an αLβ-complex. Cross-linked αX was vertically aligned with cross-linked β. This, and the M_r 250,000 of the cross-linked product in the first dimension, show that αX is associated with β in an $αX_1β_1$-complex. Henceforward, this molecule will be designated according to the molecular weights of its polypeptides as p150,95.

Some uncross-linked α- and β-chains were present on the diagonal in all cross-linked immunoprecipitates (Fig. 3, a, c, and e). Since uncross-linked α-chains were present in anti-β precipitates and vice versa, the uncross-linked material is due to the lack of 100% efficiency in cross-linking.

Parallel experiments, with the cross-linking omitted, were carried out to determine whether any normally occurring disulfide-linkages were present. For OKM1 (Fig. 4b), LFA-1 (Fig. 4d), and OKM1, LFA-1, and p150,95 together (Fig. 4f), the α- and β-subunits were seen along the diagonal, again confirming the noncovalent association of the α- and β-subunits in each of these three antigens.

Immunofluorescent Staining by Anti-α and Anti-β MAb. The expression of αL-, αM-, and β-subunits on peripheral blood lymphocytes, monocytes, and granulocytes, was quantitated with immunofluorescence flow cytometry. Monocytes and granulocytes (Fig. 5, a and b) expressed the OKM1 αM-subunit but only a small percentage of lymphocytes, probably corresponding to "null" cells (11), were

weakly positive for αM (Fig. 5c). Lymphocytes were strongly αL⁺ and demonstrated two subpopulations differing in quantitative expression (Fig. 5f). Monocytes also expressed the LFA-1 α-subunit (Fig. 5d), and granulocytes were weakly LFA-1 α positive (Fig. 5e). The ratio of OKM1 α to LFA-1 α expression was 1.5:1 on monocytes and 7:1 on granulocytes, respectively. On monocytes and granulocytes, the fluorescence intensity given by the anti-β MAb (Fig. 5, g and h) was greater than for either anti-αL or anti-αM alone. On lymphocytes, which express only LFA-1, the fluorescence intensity given by anti-αL and by anti-β MAb was identical (Fig. 5, f and i).

α- and β-Structures on Other Cell Types.   The expression of LFA-1, OKM1, and other members of this family sharing the same β-subunit was examined by immunoprecipitation in a number of other types of cells. Blood monocytes expressed both the OKM1 and LFA-1 antigens (Fig. 6A, lanes 2 and 4). The anti-β MAb precipitated from monocytes αL, αM, and in greater quantities than had been seen in other cell types, αX (Fig. 6A, lane 3). A weak band migrating below αX was also seen (Fig. 6A, lane 3). In the JY B lymphoblastoid cell line, LFA-1 was precipitated by the anti-LFA-1 α and anti-β MAb (Fig. 6B, lanes 4 and 3), but no OKM1 was seen (Fig. 6B, lane 2). A band possibly corresponding to αX was precipitated by the anti-β MAb (Fig. 6B, lane 3). In thymocytes, only LFA-1 was precipitated (Fig. 6C, lanes 3 and 4). In the U937 immature monoblast-like cell line, LFA-1 was precipitated by anti-α and anti-β MAb, but little or no OKM1 or p150,95 were present (Fig. 6D, lanes 3 and 4). Phorbol esters induce maturation of U937 cells (25). After 1 d of culture of U937 cells with phorbol ester, αX in addition to αL was detected with the anti-β MAb (Fig. 4E, lane 3). OKM1 gradually increased after 1 d of culture reaching maximum levels at day 5, while LFA-1 gradually decreased (data not shown and cf. Fig. 7).

αX did not appear to be a degradation product of αM or αL because of its differing expression during maturation of U937 and its lack of multiple epitopes present on αM and αL. Furthermore, the same amount of αX was obtained whether monocytes were lysed in the standard buffer containing phenylmethylsulfonyl fluoride (PMSF), or in buffer containing additionally aprotinin, iodoacetic acid, and N-ethyl maleimide; and whether the lysates were dialyzed overnight at 4°C, or held at −80°C. Moreover, αX was not generated when ¹²⁵I-
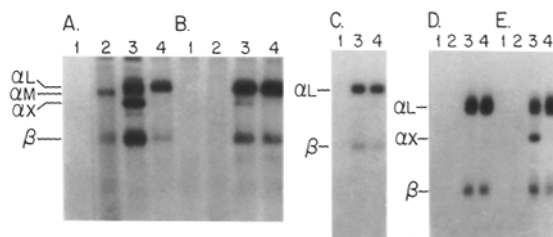

FIGURE 6.   Immunoprecipitation of OKM1, LFA-1, and related antigens from different cell sources. Lysates of ¹²⁵I-labeled monocytes (A), JY B lymphoblastoid cells (B), thymocytes (C), U937 myelomonocytic cells (D), or U937 myelomonocytic cells cultured 1 d with phorbol ester (E) were immunoprecipitated with X63 as control (lane 1), anti-OKM1 α MAb (lane 2), TS1/18 anti-LFA-1 β MAb (lane 3), or TS1/22 anti-LFA-1 α MAb (lane 4). Immunoprecipitates were subjected to SDS 10% (A–C, C is a separate gel) or 7% (D and E) PAGE and autoradiography. Only the upper portion of gels are shown.

labeled OKM1 isolated with OKM10 MAb and anti-kappa MAb Sepharose was mixed with a lysate of unlabeled monocytes prepared under standard conditions, and held overnight at 4°C (data not shown). Thus, it is extremely unlikely that αX is an artifact of proteolysis occurring during antigen isolation in vitro.

Biosynthesis Studies.   It was important to further test whether αX was an in vivo degradation product of αL or αM, or whether it represented a de novo biosynthetic product. It was also of interest to determine whether the α- and β-subunits of a given antigen were derived from separate precursors, or from a single precursor by proteolysis. For biosynthesis studies, U937 cells induced with phorbol ester for 3 d were pulse-labeled with [^{35}S]methionine for 0.25 or 0.5 h, chased for varying periods of time with "cold" methionine, and then lysed and subjected to immunoprecipitation. For comparison to mature polypeptide chains on the cell surface, cells surface-labeled with ^{125}I were also immunoprecipitated.

The biosynthesis of LFA-1 was studied using an anti-αL MAb (Fig. 7C). The αL-chain was derived from a precursor of 170,000 M_r denoted α'L (Fig. 7C), lanes *18–20*). The mature αL-chain was first seen at 1 h of chase, in greater quantities at 2 h, and was the only product at 4 and 22 h of chase (Fig. 7C, lanes *20–23*). Mature β-chain was weakly seen at 4 and 22 h of chase (Fig. 7C, lanes
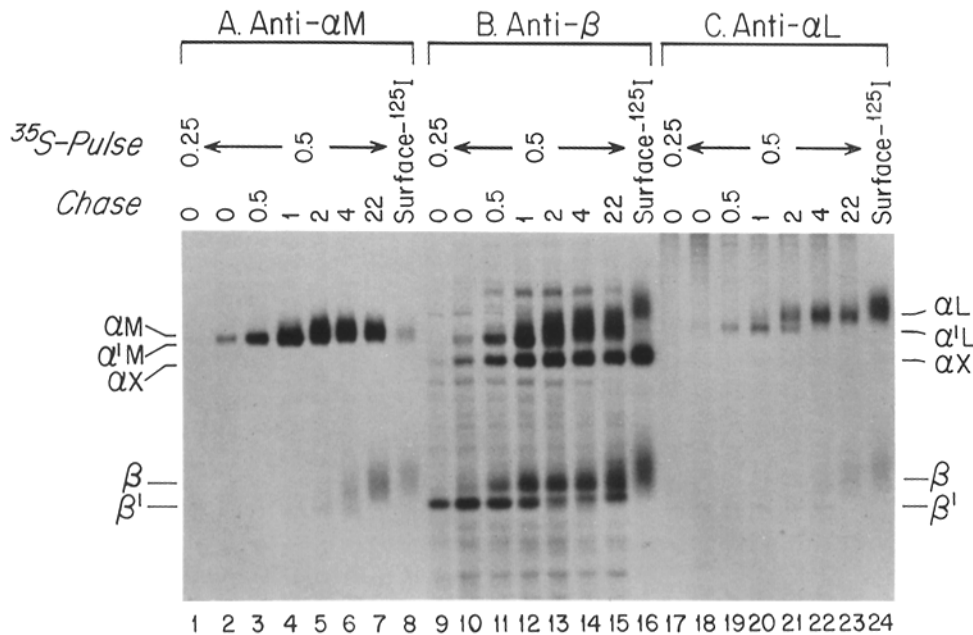

FIGURE 7.   Biosynthesis of LFA-1, OKM1, and p150,95. U937 cells cultured in PMA for 3 d in petri dishes were pulse-labeled with [^{35}S]methionine for 0.25 or 0.5 h, then washed and chased with "cold" methionine for the times indicated in the figure in hours. Alternatively, U937 cells were cultured in PMA for 3 d in Teflon beakers and surface-labeled with ^{125}I as indicated in the figure. Lysates were immunoprecipitated with the anti-αM OKM10 MAb (A), the anti-β TS1/18 MAb (B), or the anti-αL TS1/22 MAb (C), and subjected to SDS 7% PAGE and fluorography (39) with an enhancing screen. There was less ^{125}I-αM than ^{35}S-αM in anti-αM and in the anti-β precipitate relative to other antigens; these measurements of the amount of OKM1 accumulated on the surface and the rate of synthesis would be expected to differ because OKM1 is only starting to be induced at day 3.

22 and *23*).

The pattern of OKM1 biosynthesis appeared similar (Fig. 7*A*). An α'M-precursor of 160,000 M_r was seen after chase for 0–0.5 h (Fig. 6*A*, lanes *2* and *3*) and was partially (1–2 h of chase) and then fully converted (4 and 22 h of chase) to the mature αM-subunit (Fig. 6*A*, lanes *4–7*). The α'M- and αM-chains could be seen to be clearly resolved from one another in shorter autoradiogram exposures (not shown). A β-chain precursor (β') of M_r 90,000 was seen weakly at 1 h of chase and more strongly at 2 and 4 h of chase (Fig. 6*A*, lanes *4–6*). Conversion to the mature β-subunit of M_r 95,000 was seen at 4 h and was complete by 22 h (Fig. 6*A*, lanes *6* and *7*). These findings suggest that association of the β'-precursor with the α'M- or αM-subunit does not occur until 1 h, and precedes processing to the mature β-subunit. As expected, the [^{35}S]labeled mature αM- and β-subunits (Fig. 7*A*, lane *7*) were identical in molecular weight to the ^{125}I-labeled cell surface subunits (Fig. 7*A*, lane *8*).

The anti-β precipitates were more complex (Fig. 7*B*). Both α'M and α'L could be seen after chase for 0.5 or 1 h (Fig. 7*B*, lanes *11* and *12*), showing that association with β' or β can precede α-subunit processing. After 4–22 h of chase, α'M and α'L gave rise to higher molecular weight αM- and αL-subunits (Fig. 7*B*, lanes *14* and *15*) identical to those seen in anti-αM and anti-αL precipitates. The β'-precursor was readily seen in anti-β precipitates beginning at 0 h of chase, and was largely although not completely converted to the mature β-subunit between 1 and 22 h of chase.

Additionally, αX was present in the anti-β precipitates. The αX-precursor was seen at 0 h of chase, did not change in molecular weight during 22 h of chase (Fig. 7*B*, lanes 10–15), and was identical in size to ^{125}I surface-labeled αX (Fig. 7*B*, lane 16). The αX-precursor was clearly distinct in size from α'M and α'L and appeared with the same kinetics. Thus, it does not appear to be related by processing or degradation to α'L, α'M, αL, or αM.

Isoelectric Focusing of the LFA-1, OKM1, and p150,95 Molecules.   Differences and identities among the subunits of the LFA-1, OKM1, and p150,95 molecules isolated from monocytes were examined by 2-D isoelectric focusing–SDS-PAGE (Fig. 8). αL, αM, αX, and β each focused as discrete series of spots. Multiple closely spaced spots are typical of glycoproteins with sialic acid charge heterogeneity. The OKM1 α-subunit focused at pI 5.9–6.1, while the β-subunit focused at pI 5.2–5.5 (Fig. 8*A*). The LFA-1 α-subunit was more acidic than that of OKM1, focusing at pI 5.1–5.5 (Fig. 8*B*). The β-subunit of LFA-1 focused identically to that of OKM1.

The anti-β precipitate contained bands corresponding to αM, αL, αX, and the β-subunit (Fig. 8*c*). The positions of αM and αL isolated with the anti-β MAb (Fig. 8*c*) were the same as after isolation with anti-α MAb (Fig. 8,*a* and *b*, respectively). The αX-band focused between pI 5.8 and 6.1, closer to αM than to αL. Importantly, the pattern seen for the β-subunit of all three antigens together (Fig. 8*c*) was superimposable with that seen for the individual OKM1 and LFA-1 β-subunits in Fig. 8,*a* and *b*, respectively. This shows that the β-subunits of LFA-1, OKM1, and p150,95 exactly co-migrate in isoelectric focusing, and it therefore appears that they are identical.

*Inhibition of the CR*₃.   MAb to OKM1 were tested for inhibition of the CR₃-
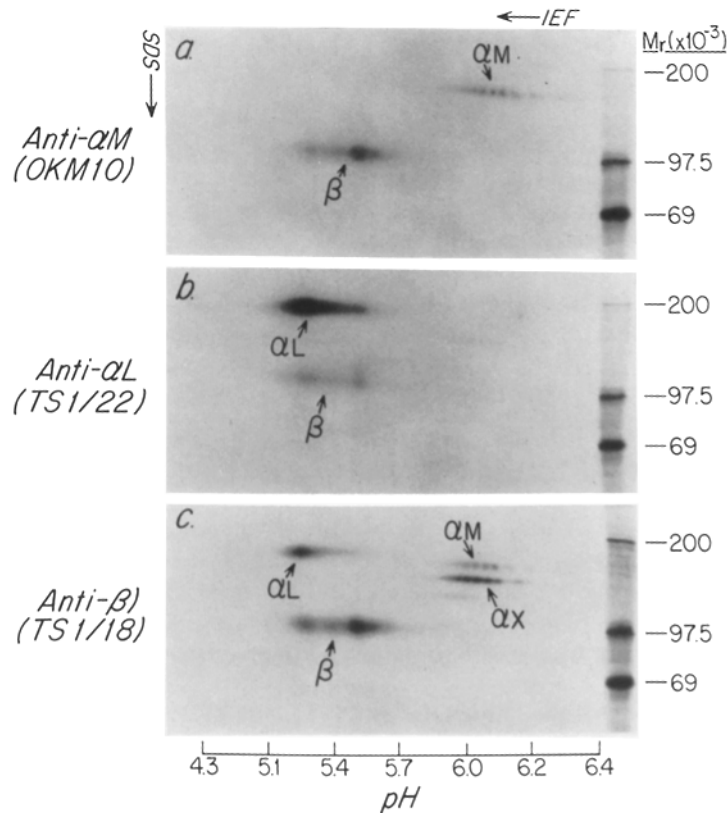
FIGURE 8.   Isoelectric Focusing SDS-PAGE 2-D Gel Electrophoresis. Lysates (75 μl) of ^{125}I-labeled monocytes were immunoprecipitated with (a) the anti-OKM1 OKM10 MAb (30 μl of 1:100 ascites) and 30 μl 187.1-Sepharose, (b) TS1/22-Sepharose (30 μl), and (c) TS1/18-Sepharose (30 μl). Immunoprecipitates were subjected to isoelectric focusing in the first dimension followed by SDS 7% PAGE under reducing conditions in the second dimension. Focusing was for 10,000 Volt-hours. Identical pI's were estimated for the α- and β-subunits in a parallel run after focusing for 5,000 Volt-hours, showing equilibrium was reached.

mediated rosetting of E-IgM-C by U937 cells induced for 4 d with PMA (Fig. 9). Antibody to the CR₁ had little or no effect on E-IgM-C rosetting, confirming that rosetting was due to the CR₃ as previously reported for these indicator cells (6, 26). The CR₃ was completely inhibited by the OKM1 and OKM10 anti-αM MAb and only slightly by the OKM9 putative anti-αM MAb and the TS1/18 anti-β MAb in a dose-dependent fashion. The TS1/22 anti-αL MAb, and the W6/32 anti-HLA-A,B MAb, which bind to induced U937 cells had no effect, demonstrating the specificity of this inhibition.

Discussion

This report describes the interrelations among a family of three human leukocyte differentiation antigens. The three antigens have very similar subunit structures. They contain an α-subunit of M_r 177,000 to 150,000, depending on the antigen, and a β-subunit of M_r 95,000. The LFA-1, OKM1, and p150,95 α-subunits are designated αL = 177,000 M_r, αM = 165,000 M_r, and αX = 150,000
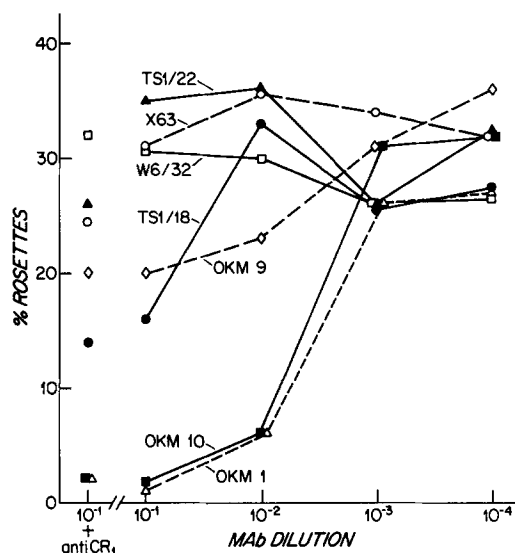
FIGURE 9. Inhibition of the complement receptor type three. Induced U937 cells were incubated for ½ h with MAb at the indicated dilutions, then challenged with E-IgM-C, and rosetting cells were enumerated microscopically as described in Methods. Stock antibodies from which dilutions were made were culture supernatants for W6/32, TS1/18, TS1/22, and X63, 200 μg/ml purified antibody for OKM1, 1:50 ascites for OKM9, and 1:100 ascites for OKM10. Anti-CR₁ was added at 12 μg/well. U937 cells challenged with E-IgM control cells gave 0% rosetting.

M_r, respectively. In each antigen the subunits are noncovalently associated in an $\alpha_1\beta_1$ quaternary structure. The OKM1 antigen had previously been reported to contain two polypeptide chains of 155,000 and 94,000 M_r (2), in reasonable agreement with the M_r reported here, but it was not previously known to which chain(s) the MAb bound or whether the chains were noncovalently associated. Subunit association is stable at neutral pH in the mild detergents Triton X-100 and deoxycholate, but is disrupted by pH 11.5 and by SDS. The subunits of p150,95 are more stable to high pH dissociation than those of LFA-1 and OKM1.

The ability of high pH to dissociate subunits with retention of most antigenic determinants played a key role in the assignment of distinct and cross-reactive antigen epitopes to particular subunits. The TS1/18 MAb precipitates the dissociated β-subunit and cross-reacts with the native LFA-1, OKM1, and p150,95 $\alpha\beta$-complexes, demonstrating that their β-subunits bear a common antigen epitope. Furthermore, the β-subunits of the three different antigens exactly co-migrate in 2-D isoelectric focusing–SDS-PAGE. Since isoelectric focusing is sensitive to a single charge difference, it appears that the β-subunits are identical.

The three α-subunits differ in immunochemical, physicochemical, and biosynthetic properties. The 5 MAb found here to recognize the LFA-1 α-subunit appear to define four distinct epitopes as shown by cross-inhibition studies (27). Since none of these anti-αL MAb cross-react with OKM1 or p150,95, the αL subunit bears multiple determinants not present on αM or αX. The αM-subunit bears the OKM1 epitope, which is not present on αL or αX. Furthermore, anti-

OKM9 and anti-OKM10 MAb, which recognize two other epitopes on the OKM1 molecule (24), do not cross-react with LFA-1 or p150,95. αX is distinct in its lack of four epitopes present on αL and three epitopes present on αM. Further characterization of p150,95 would be facilitated by MAb specific for the αX-subunit. Besides these multiple differences in antigen epitopes, the three α-subunits differ in physicochemical properties, as shown by 2-D isoelectric focusing-SDS-PAGE.

The biosynthetic precursors of αL, αM, αX, and β are distinct and appear with similar kinetics, suggesting that they are not interrelated by processing and are products of distinct genes. The α'L-, α'M-, and β'- but not α'X-precursors increase in size during maturation, perhaps due to carbohydrate processing. The precursors can assemble into the $\alpha_1\beta_1$-complexes before processing. The utilization of separate precursors for each subunit would require only a single β-gene for all three types of $\alpha\beta$-complexes. The same biosynthetic pathway has been previously demonstrated for the mouse Mac-1 and LFA-1 antigens (28).

Although they can be readily distinguished by their antigenic and physicochemical properties, the three α-subunits share certain characteristics. They possess a region which is complementary to the β-subunit, conferring the specificity for the α-β interaction, and their molecular weights differ by <20%. In analogy to other protein families that share a common subunit, such as the class I histocompatibility antigens, hemoglobins, and immunoglobulins, it is predicted that the αL-, αM-, and αX-subunits are homologous in amino acid sequence and arose by gene duplication.

The expression of the αL-, αM-, and αX-subunits is selectively regulated in leukocyte differentiation. Lymphoid cells, i.e. thymocytes, peripheral blood lymphocytes, T lymphoma cells (unpublished observations), B lymphoblastoid lines, and CTL, are LFA-1$^+$, OKM1$^-$, and low or negative in p150,95. Granulocytes and monocytes express all three antigens, but granulocytes express OKM1 > LFA-1 > p150,95, while blood monocytes express similar quantities of all three molecules. In the mouse, as in the human, lymphoid cells are LFA-1$^+$ Mac-1$^-$ (29) and monocytes are LFA-1$^+$ Mac-1$^+$ (Springer unpublished). More differentiated thioglycollate-elicited macrophages are LFA-1$^-$ Mac-1$^+$ (29). An interesting subject for further research is the mechanism that coordinates the reciprocal relationship between αM and αL expression during monocyte maturation, and the selective expression of the OKM1, LFA-1, and p150,95 α-subunits in leukocyte differentiation.

The previously described murine LFA-1 and Mac-1 molecules (4) are identical in cell distribution, function, and structure to human LFA-1 and OKM1/Mac-1. Furthermore, the murine LFA-1 and Mac-1 α-subunits bear distinct, noncrossreactive epitopes and the β-subunits bear common, completely cross-reactive epitopes as shown with both monoclonal and conventional antibodies (23). Peptide mapping has also shown the Mac-1 and LFA-1 α subunits differ while the β-subunits are identical (29, 30). The finding that human LFA-1 and OKM1 also have shared β-subunits confirms the homology between the mouse and human families. The conservation of this family, its functions, and its subunit organization in evolution reinforces its importance. Although an αX equivalent has not been definitively characterized in the mouse, a third type of α-subunit of

155,000 M_r has been found to be precipitated by an anti-β MAb (23).

It is interesting to compare the functional activities of human LFA-1 and OKM1/Mac-1. The function of the novel p150,95 molecule is currently unknown. The human LFA-1 antigen appears to participate in several types of cell-cell interactions. MAb to it block both antigen-specific T lymphocyte–mediated killing and natural killing. Blockade is due to binding of anti-LFA-1 MAb to the CTL effector rather than to the target cell (3). An independently obtained MAb to human LFA-1 has also been reported to block antigen-specific and natural killing (31). Anti-LFA-1 MAb also inhibit T cell proliferative responses to antigen and to mitogen (3, 31). In the mouse system, anti-LFA-1 MAb has been shown to block the adhesion of CTL to target cells (4).

Human Mac-1, OKM1, and Mol, which appear to be equivalent, were originally defined as differentiation antigens present on monocytes, granulocytes, and "null" cells with NK and ADCC activity, but absent from lymphocytes. OKM1 and Mol were defined with mouse anti-human MAb (10–12); human Mac-1 was defined with a cross-reactive rat anti-mouse Mac-1 MAb (5). The anti-Mac-1 MAb has been shown to block the mouse and human receptor for C3bi, termed the complement receptor type three (CR_3) (6). This suggests Mac-1 and the CR_3 may be identical. It was found here that the OKM1 and OKM10 MAb to the αM-subunit also inhibit the CR_3. The OKM9 MAb, which binds to a different epitope (24) presumably on the αM-subunit, and the TS1/18 MAb, which binds to the β-subunit, were much less inhibitory. These differences suggest that the C3bi ligand-binding site may be on the α-subunit near the OKM1 and OKM10 epitopes. Anti-Mol (32) and anti-OKM1 and OKM10 MAb, but not anti-OKM9 MAb (33), have independently been found to block the CR_3.

Despite the differences in function between Mac-1/OKM1/Mol and LFA-1, there are also common features. Both molecules act to strengthen adhesions between effector cells and target cells. In both cases, it appears that the participation of additional cell surface molecules is required for optimal functional activity. LFA-1 acts together with the antigen receptor in CTL-mediated killing (1, 4). Similarly, the CR_3 acts synergistically with the Fc receptor to stimulate phagocytosis by macrophages and granulocytes (34) and killing by antibody-dependent cytotoxic effectors (35). Another interesting parallel is that both CTL-target cell adherence (36) and adherence through the CR_3 (37, 38) are dependent on divalent cations. In contrast, CR_1 and FcR adherence are not divalent cation-dependent (38).

The findings on the relationship between the human LFA-1 and OKM1/Mac-1 molecules allow some predictions about structure-fucntion relationships. The identities between the β-subunits and differences between the α-subunits suggest that the α-subunits govern the specificity of cell adhesion reactions, i.e. bear ligand-binding sites. The identical β-subunit is predicted to mediate a common function. It might be involved in the divalent cation-dependence and might function in signal transduction or in regulation.

Summary

The human lymphocyte function-associated antigen-1 (LFA-1), the complement receptor-associated OKM1 molecule, and a previously undescribed mole-

cule termed p150,95, have been found to be structurally and antigenically related. Each antigen contains an α- and β-subunit noncovalently associated in an $\alpha_1\beta_1$-structure as shown by cross-linking experiments. LFA-1, OKM1, and p150,95 α-subunit designations and their molecular weights are αL = 177,000 M_r, αM = 165,000 M_r, and αX = 150,000 M_r, respectively. The β-subunits are all = 95,000 M_r. Some MAb precipitated only LFA-1, others only OKM1, and another precipitates all three antigens. The specificity of these MAb for particular subunits was examined after subunit dissociation by high pH. MAb specific for LFA-1 or OKM1 bind to the αL- or αM-subunits, respectively, while the cross-reactive MAb binds to the β-subunits. Coprecipitation experiments with intact $\alpha_1\beta_1$-complexes showed anti-α and anti-β MAb can precipitate the same molecules. In two-dimensional (2D) isoelectric focusing-SDS-PAGE, the α subunits of the three antigens are distinct, while the β-subunits are identical. Biosynthesis experiments showed αL, αM, and αX are synthesized from distinct precursors, as is β. The three antigens differ in expression on lymphocytes, granulocytes, and monocytes. During maturation of the monoblast-like U937 line, αM and αX are upregulated and αL is downregulated. Some MAb to the α subunit of OKM1 inhibited the complement receptor type three. LFA-1, OKM1, and p150,95 constitute a novel family of functionally important human leukocyte antigens that share a common β-subunit.

Note added in proof: The LFA-1, Mac-1/OKM1/Mol, p150,95 family appears clinically important. Several patients with recurrent bacterial infections have defective granulocyte adherence functions and lack specific high molecular weight proteins (40–43). Studies on one of these patients show his granulocytes lack the cell surface proteins normally precipitated by anti-αM and anti-β MAb (44).

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