A Novel Integrin Involved in Thymocyte–Thymic Epithelial Cell Interactions

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Summary

Thymocytes differentiate in the thymic microenvironment into immunocompetent T cell through the interaction with a variety of accessory cells, including thymic epithelial cells (TEC). TEC play an important role in the selection process presenting self antigens in association with major histocompatibility complex (MHC) molecules to the maturing T cells. The T cell receptor recognizes the self antigen–MHC complex, but other surface molecules help stabilize this interaction. Thus, the CD2/LFA-3 and LFA-1/intercellular adhesion molecule 1 pairs have been shown to participate in the binding between lymphoid cells and TEC. Here we describe an integrin of the very late activation antigen subfamily composed by the known β_1 chain and by a novel α chain. This adhesion molecule is expressed on the surface of medullary TEC and is involved in the adhesion between TEC and thymocytes, but not peripheral blood T lymphocytes.

The thymus provides the microenvironment for the differentiation of immature lymphoid cells into immunocompetent T cells. This maturation process requires the direct interaction between thymocytes and the nonlymphoid compartment of the thymus, which is comprised of a variety of accessory cells, including thymic epithelial cells (TEC),[1] macrophages, and interdigitating/dendritic cells (1–4). These cells may present antigens and secrete soluble factors (IL-1, granulocyte/macrophage [GM]-CSF, IL-3-like activity, and IL-6) effective on the maturation of T cells (5–8). The accessory cells are also thought to play an important role in the selection process through which only those thymocytes unable to recognize self antigens and hence directed against foreign determinants are allowed to survive, proliferate, and leave the thymus as mature T lymphocytes. Although the precise role of the different accessory cells in this process is still unclear, it is generally agreed that TEC are involved in the positive selection of thymocytes capable of recognizing self MHC molecules (9–11). It has also been proposed that TEC presenting self antigens in association with their MHC molecules participate in the removal of self-reactive cells from the pool of immature thymocytes (12, 13). Such crucial mechanisms require an intimate and extensive contact to ensure a precise selection.

Although the TCR/antigen-MHC interaction is the one that decides the fate of the cell, its instability, even with the contribution provided by CD4 or CD8 molecules (14–19), makes it unlikely that it alone may be able to sustain a strong interaction that requires an extensive cell-to-cell contact. In this connection, it has been shown that mature T lymphocytes and thymocytes bind to cultured TEC via their surface CD2 and LFA-1 molecules and that their ligands are LFA-3 and intercellular adhesion molecule 1 (ICAM-1), respectively (20–22). However, rosette formation between thymocytes and cultured TEC is not completely inhibited by mAbs to CD2 and to LFA-1. This observation suggests that other molecular interactions may participate in the binding event (21).

In this study, we describe a novel molecule of the integrin family, composed by the known β_1 chain and by an α chain different from those described so far. This molecule is expressed on the surface of epithelial cells of the thymic medulla, and participates in the binding of thymocytes, but not of mature T lymphocytes, to cultured TEC.

Materials and Methods

Cells. Thymic tissue was obtained from children (aged 6 mo to 3 yr) undergoing cardiac surgery. Thymic cell cultures were initiated by an explant technique, propagated at 37°C in 5% CO_2 in enriched medium and subcultured repeatedly for several months onto irradiated mouse 3T3 fibroblast feeder layers as described (23). The enriched epithelial culture medium consisted of a 2:1 (vol/vol) mixture of DMEM and Ham's F12 nutrient (Flow Laboratories,

[1] *Abbreviations used in this paper:* APAAP, alkaline phosphatase-antialkaline phosphatase; EGF, epidermal growth factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; ICAM-1, intercellular adhesion molecule 1; PAP, peroxidase-antiperoxidase; TEC, thymic epithelial cell; VLA, very late antigen.

Irvine, Scotland), supplemented with 10% FCS (PAA-Technogenetics, Milano, Italy), 0.4 μg/ml hydrocortisone (Sigma Chemical Co., St. Louis, MO), 10 ng/ml recombinant human epidermal growth factor (EGF; a gift from Dr. M. De Luca), 10^{-10} M cholera enterotoxin (Sigma Chemical Co.), 5 μg/ml insulin (Sigma Chemical Co.), 1.8×10^{-4} M adenine (Sigma Chemical Co.), 100 U/ml penicillin (Flow Laboratories), 100 μg/ml streptomycin (Flow Laboratories), 5 μg/ml human transferrin (Sigma Chemical Co.), 2×10^{-9} M 3,3',5 triiodo-L-thyronine sodium salt (Sigma Chemical Co.); 4 mM L-glutamine (Flow Laboratories). TEC were harvested on the second to third passage in vitro.

Thymocytes were obtained from thymus tissue by teasing and purified by centrifugation through Ficoll-Hypaque. When appropriate, thymocytes were activated with PHA-M (Gibco Laboratories, Grand Island, NY) at 10 μg/ml or with PMA (Sigma Chemical Co.) at 0.5 ng/ml in the presence of 0.5 μg/ml of antibodies to CD3 or to CD28. Cells were maintained in culture by adding 50 U/ml rIL-2 (Cetus Corp., Emeryville, CA).

Purified E-rosetting cells were isolated from peripheral blood according to methods described previously (24), and they are referred to as T-PBL. Long-term PHA-stimulated cell lines were initiated by adding PHA (Gibco Laboratories) at 10 μg/ml to PBMC, as described (25). These cells were maintained in culture by adding 50 U/ml rIL-2 (Cetus Corp.) twice weekly. Expression of 10.1.2 was tested weekly on these cells, up to 5 wk.

Purified B cells were obtained from human tonsils (26). Platelets were isolated from heparinized human blood after centrifugation through Ficoll-Hypaque and were identified by light scatter profile using a FacStar® cytofluorograph (Becton Dickinson & Co., Mountain View, CA). Primary cultures of human skin keratinocytes were kindly provided by Dr. M. De Luca. Umbilical cord endothelial cells at the second passage in vitro were obtained from Dr. E. Dejana.

mAb Production. The techniques for mAb production have been described previously (27). Briefly, BALB/c mice were immunized intraperitoneally at 7-d intervals with 5×10^6 TEC harvested after the second passage in culture and kept frozen until use. A mouse was killed 4 d after the third injection, and spleen cells were fused with P3X63Ag8U1 myeloma cells (P3U1) according to the method of Gefter et al. (28). 12 d after fusion, the supernatant of each culture well was assayed by ELISA with alkaline phosphatase-rabbit anti-mouse IgG and IgM antibodies (Zymed Laboratories, South San Francisco, CA), using 4×10^4 cells per well. Positive cultures were cloned in soft agar as described by Coffino et al. (29). The Ig fraction was purified from ascites by precipitation with 40% ammonium sulphate followed by gel filtration through an Ultrogel AcA34 column.

Antibodies. The following reagents were used: W6/32 (anti-MHC class I) was from Dakopatts (Glostrup, Denmark); CK248 (anti-CD28) (30) was the gift of Dr. A. Poggi (Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy); MAR206 (anti-T_{11}-1) (31) was the gift of Dr. A. Moretta (Istituto di Anatomia Umana Normale, University of Genova, Italy); mAbs anti-CD1, anti-CD3, anti-CD4, and anti-CD8 were obtained from the American Type Culture Collection (ATCC; Rockville, MD); PTF-22.1 (anti-MHC class I), BT-2.9 (anti-MHC class II), and the mAb anti-CD31 were produced in our laboratory; AE1-AE3 (anti-human epithelial pankeratin) was from Menarini Diagnostici (Firenze, Italy); VIM 13.2 (anti-human vimentin) was from Biomedicals Ltd. (Liverpool, England); anti-β_1-peptide rabbit antiserum (anti-β_1 integrin chain) and anti-α_3-peptide rabbit antiserum (anti-α_3 integrin chain) were kindly provided by Dr. G. Tarone (Dipartimento di Genetica, Biologia e Chimica Medica, University of Torino). The polyclonal antisera to the β_1 and α_3 integrin subunits were prepared by immunizing rabbits against synthetic peptides reproducing amino acid sequences from the cytoplasmic domain of each subunit. The following peptides, obtained from Multiple Peptide System (San Diego, CA, USA), were used: β_1, CTTVVNPKYEGK; α_3, CRIQPSETERLTDDY. Peptides were coupled to hemocyanin with glutaraldehyde (approximate peptide/carrier molar ratio of 50:1), and rabbits were injected with 500 μg of the conjugate in CFA. Antibodies reacted specifically with the peptide sequence used for immunization, as determined by ELISA on peptide-BSA conjugates. The antibodies were specific for the appropriate subunit and did not show crossreaction with other integrin subunits as demonstrated by immunoprecipitation assays on different cell lines (G. Tarone, personal communication). TS2/7 (anti-α_1 integrin chain) (32) and B5G10 (anti-α_4 integrin chain) (33) were kindly provided by Dr. M. E. Hemler (Dana-Farber Cancer Center, Boston, MA); 10G11 (anti-α_2 integrin chain) (34) and GoH3 (anti-α_6 integrin chain) (35) were kindly provided by Dr. A. Sonnenberg (Central Laboratory of The Netherlands Red Cross Blood, Transfusion Service, Amsterdam, The Netherlands); anti-α_5-peptide rabbit antiserum (anti-α_5 integrin chain) (36) was kindly provided by Dr. E. Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA); NKI-M7 (anti-α_v integrin chain) (37) was kindly provided by Dr. C. G. Figdor (The Netherlands Cancer Institute, Division of Immunology, Amsterdam, the Netherlands).

Staining Procedures. Indirect immunofluorescence was carried out using cell suspensions. Briefly, cells were suspended in 100 μl of DMEM + 10% FCS and incubated with the appropriate dilution of each antibody for 30 min at 4°C in the presence of 100 μg of purified human Ig. After rinsing twice, cells were incubated for 30 min at 4°C in 70 μl of the same buffer containing 2 μg of affinity purified FITC-labeled goat anti-mouse IgG heavy and light chains (Cappel Laboratories, Turnhout, Belgium) or affinity purified FITC-labeled goat anti-rabbit IgG heavy and light chains (ICN ImmunoBiologicals, Costa Mesa, CA). After two washings, cells were analyzed with a FacStar® cytofluorograph (Becton Dickinson & Co.) and a Dialux fluorescence microscope (Leitz).

The peroxidase-antiperoxidase (PAP) and alkaline phosphatase-antialkaline phosphatase (APAAP) techniques (38, 39) were used for the immunocytochemical localization of the 10.1.2 antigen and of keratins in TEC culture monolayers and human tissues. Cryostat sections were put on gelatin-coated slides, air-dried, and incubated with the 10.1.2 mAb (undiluted culture supernatant) for 1 h at room temperature. For the APAAP staining, cells and tissues were subsequently incubated with rabbit anti-mouse Ig antibodies followed by AP-labeled mouse anti-rabbit Ig. AP activity was subsequently revealed by incubation with Naphthol AS-MX Phosphate (Sigma Chemical Co.). Peroxidase activity was revealed using diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co.) as substrate. All of the antibodies were purchased from Dakopatts.

TEC-Thymocyte Rosette Assay. Binding of TEC to thymocytes or to T-PBL was determined as described (20). Briefly, 2×10^6 thymocytes or T-PBL and 2.5×10^5 TEC were admixed in plastic tubes in 200 μl of RPMI 1640 (Flow Laboratories) supplemented with 5% FCS at 4°C, centrifuged for 5 min at 250 *g* at 4°C, and incubated for 20 min at 4°C. After gentle resuspension, samples were analyzed by microscopy, and TEC binding three or more thymocytes or T-PBL were scored as positive. In each assay, 200 TEC were enumerated and each determination was performed in duplicate. In experiments done using mAbs to inhibit TEC-thymocyte binding, the antibodies were added to each tube in a volume of 10 μl before addition of TEC at the concentrations indicated in Fig. 5.

Immunoprecipitation. Surface-iodinated TEC (6×10^6 cells)

were lysed with PBS, 0.5% NP-40 containing 1 μg/ml aprotinin, 1 μg/ml leupeptin, 100 μg/ml PMSF, 0.02 μg/ml soybean trypsin inhibitor (Sigma Chemical Co.), and centrifuged to remove nuclei and cell debris. The lysate was rotated overnight at 4°C with the antibodies indicated in Results. After incubation with the primary antibodies, immune complexes were recovered with Protein A–Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). To avoid nonspecific adsorption, incubation with primary antibodies was preceded by incubation of cell lysates with Protein A–Sepharose. After washing, antigens were eluted by boiling with an equal volume of 4% $NaDodSO_4$.

Biochemical Analyses. TEC were surface labeled with ^{125}I using lactoperoxidase as described (40). SDS-PAGE was carried out in slab gels in a discontinuous Tris buffer system (41). Proteins were recovered as described and iodinated with the chloramine T method according to Greenwood et al. (42). Peptic peptide maps were obtained as described (43). Briefly, the eluted chains were digested with pepsin (1:50 enzyme-to-protein weight ratio, 18 h at 37°C in 100 μl formic acid/acetic, acid/water buffer [1:4:45, vol/vol/vol]). Two-dimensional peptide maps were obtained by a technique described previously (43).

Results

Isolation of Human TEC. Long-term cultures of human TEC were derived by the method described in Materials and Methods. TEC were harvested after the second passage, and their epithelial nature was assessed by staining with a panel of antibodies against markers of human cells of different lineage. As shown in Table 1, cultured TEC were negative for vimentin and other markers of thymic lymphoid cells, fibroblasts, and macrophages, but were positive for keratins, proteins produced exclusively by epithelial cells.

Production of mAbs to Surface Antigens of Cultured TEC. The spleen of a mouse immunized with cultured TEC was fused with P3U1 myeloma cells. Hybridoma supernatants were tested by an ELISA standard method for the presence of antibodies to surface determinants of cultured TEC. Positive supernatants were then tested by the same method on cultured foreskin fibroblasts and 3T3 cells, and by indirect immunofluorescence on thymocytes and PBL. Hybridoma supernatants that reacted only with TEC were cloned in soft agar and injected intraperitoneally in mice for production of mAbs. Among the antibodies obtained, 10.1.2 (IgG1 isotype) stained strongly all cultured TEC, while being consistently negative on freshly isolated thymocytes, PBL (both resting and after short-term culture with PHA), platelets, and B cells (Fig. 1). This mAb was next tested on a panel of cell lines and found positive on cells of epithelial origin (HeLa cells and primary cultures of human skin keratinocytes) and on an endothelial cell line.

Fig. 2 shows an immunofluorescence analysis of 10.1.2 molecule expression on cultured T cell lines. After initial stimulation with PHA and culture with rIL-2, the levels of 10.1.2 expression on PBL slowly increased with time. The mean fluorescence intensity increased minimally after 2 wk of activation. However, after 3–4 wk of culture, 10.1.2 was expressed by 73% of the cultured cells, albeit with a low fluorescence intensity. In contrast, the 10.1.2 molecule was not detected on thymocytes even after 3–4 wk of culture.

Table 1. *Phenotypic characterization of Cultured Human TEC*

Antibody	Specificity	Percent positive cells
AE1-AE3	Human epithelial pan-keratin	95–100
PTF-22.1	MHC class I	95–100
BT-2.9	MHC class II	10
mAb anti-CD31	CD31	0
VIM 13.2	Human vimentin	1–2
MAR206	CD2	0
mAb anti-CD1	CD1	0
mAb anti-CD3	CD3	0
mAb anti-CD4	CD4	0
mAb anti-CD8	CD8	0

Viable cell suspensions were stained in a surface immunofluorescence assay followed by FACS® analysis. Antikeratin and antivimentin reagents were used to stain fixed cytocentrifuge cell preparations.

Tissue Distribution of the Antigen Recognized by the 10.1.2 mAb. The reactivity of 10.1.2 with TEC used for immunization was tested also by APAAP staining of TEC culture monolayers. Virtually all of the TEC were stained by both the immunofluorescent and the APAAP technique (Fig. 3 *a*), whereas granulocytes, monocytes, and bone marrow cells failed to react with the 10.1.2 mAb. Thymocytes from both prenatal and postnatal thymuses were also negative.

Six thymuses from subjects of different age (between the 22nd week of gestation and 6 mo after birth) were tested for their reactivity with the 10.1.2 mAb using cryostat sections and the APAAP and PAP techniques. Only medullary epithelial cells and epithelial cells in the outer layers of the thymic corpuscles were positive (Fig. 3 *b*.) TEC in the cortex were consistently negative, as well as thymocytes, macrophages, and other hemic cells. Interlobular blood vessels and arterioles and venules at the cortico-medullary junction, as well as blood vessels in other tissues, were stained at the endothelial level; the endothelial localization of the 10.1.2 mAb was prominent in the lymph node high endothelial venules (Fig. 3 *c*).

We subsequently tested a variety of human tissues (skin, thyroid gland, lung, salivary glands, kidney, liver, bone marrow, spleen, and lymph nodes) for their reactivity with the 10.1.2 mAb. Epithelial cells of the lung, thyroid, and kidney were positive. Of note was the reactivity of the superficial and lateral aspects of the cells in the basal layer of the epidermis, whereas the deep surface of the basal cells facing the basement membrane was unstained (Fig. 3 *d*). These data indicate that the 10.1.2 mAb reacts with a surface molecule expressed on medullary TEC, certain epithelial cells, and the endothelia.

The 10.1.2 mAb Inhibits the Binding of Thymocytes to Cultured TEC. The involvement of the molecule recognized by the 10.1.2 mAb in the thymocyte-TEC interaction was

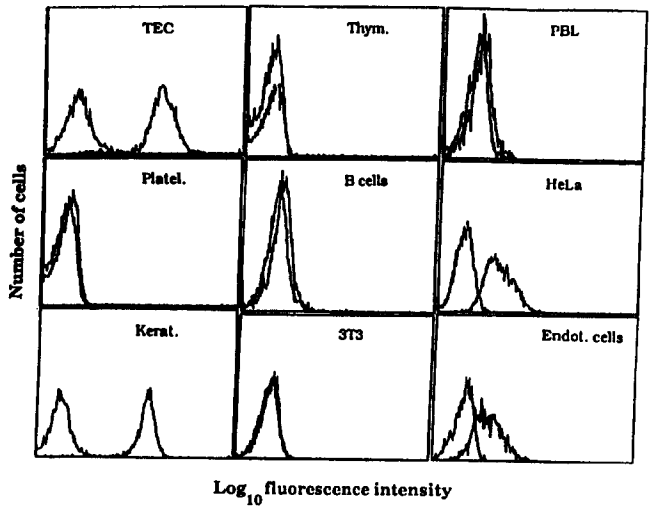

Figure 1. Expression of 10.1.2 on different cell types. Cells were stained with the 10.1.2 mAb followed by FITC-labeled anti-mouse Ig antiserum. TEC, keratinocytes, HeLa, and endothelial cells express the 10.1.2 antigen.

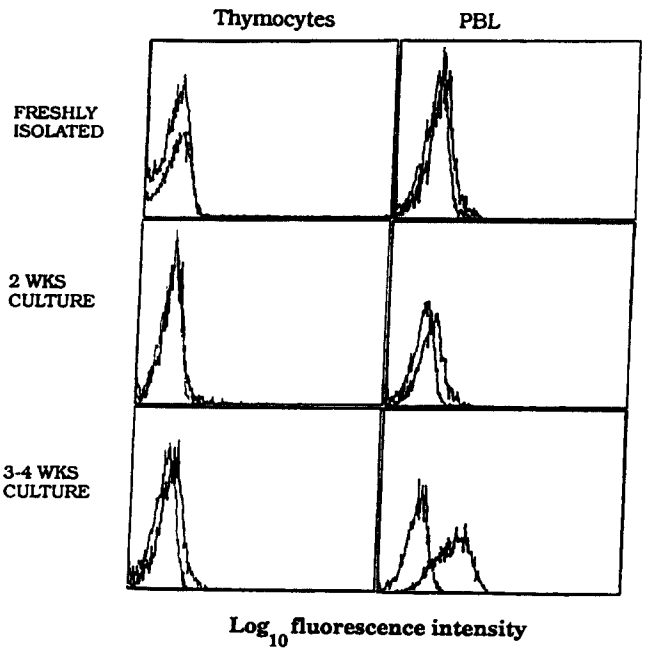

Figure 2. Expression of the 10.1.2 molecule on cells in long-term culture. Thymocytes or PBL were maintained in culture with rIL-2 after initial stimulation with PHA. Cells were stained with the 10.1.2 mAb followed by FITC-labeled goat anti-mouse Ig antiserum as described in Materials and Methods. PBL, but not thymocytes, express the 10.1.2 determinant after long-term culture.

tested using an in vitro rosette inhibition assay. When incubated with cultured TEC at 4°C, allogeneic thymocytes and T-PBL spontaneously bind to TEC (reference 44, and our own data). Under these conditions, up to 40% of the TEC bind several lymphoid cells, forming rosettes that can be easily enumerated under the microscope. In our experiments, TEC were admixed with freshly isolated thymocytes or T-PBL at a 1:10 ratio in the presence of different concentrations of 10.1.2, W6/32 (anti-MHC class I), and MAR206 (anti-CD2) mAbs. The latter represented a positive control, as it has been shown that anti-CD2 antibodies inhibit this type of rosette by interfering with the CD2/LFA-3 interaction (20). In Fig. 4, we show that rosette formation between thymocytes and TEC is inhibited more effectively by the 10.1.2 mAb than by anti-CD2. However, this higher rate of inhibition is not necessarily significant, because the extent of inhibition by anti-CD2 antibodies depends on the type of mAb used, probably reflecting differences in affinity and/or epitope specificity (20). The same results in the rosette inhibition assay were obtained when resting thymocytes were substituted for by thymocytes activated by incubation with anti-CD3 or anti-CD28 antibodies that displayed a mature phenotype ($CD3^+$, $CD4^+CD8^-$, or $CD4^-CD8^+$). By contrast, the interaction between T-PBL and TEC was not affected by the 10.1.2 mAb under experimental conditions in which the anti-CD2 mAb yielded 40% inhibition of rosette formation.

Biochemical Characterization of the Molecule Recognized by the 10.1.2 mAb. Culture TEC were surface labeled with ^{125}I, lysed in PBS 0.5% NP-40, and the lysate was immunoprecipitated with 10.1.2. Fig. 5 shows the electrophoretic pattern obtained when the immunoprecipitate was analyzed in SDS-PAGE. Under nonreducing conditions, two bands were present with an apparent molecular mass of 150–160 and 110–120 kD. Under reducing conditions, a single band with an apparent molecular mass of 130 kD was visible. Experiments in which the two bands were eluted from a nonreducing gel, and run again in SDS-PAGE after reduction with mercaptoethanol, showed that the single band observed under reducing conditions was indeed composed by the 150–160- and 110–120-kD bands, which, upon reduction, comigrate with a nearly identical molecular mass (data not shown).

The 10.1.2 Molecule Is a Member of the Integrin Family. The involvement of the molecule recognized by the 10.1.2 mAb in the thymocyte-TEC interaction suggests that it may function as an adhesion molecule. Among the several groups of adhesion molecules so far characterized, only a subset of the integrin family, namely the very late antigens (VLAs), have a subunit composition similar to that of the 10.1.2 molecule. This set consists of several related molecules, many of them sharing the same 110-kD (β_1) chain, noncovalently linked to a variety of α chains ranging in molecular mass from 140 to 200 kD (45–49). The β_1 chain of the VLAs was considered a good candidate because in SDS-PAGE it behaves exactly as the 110–120-kD band of the 10.1.2 molecule and migrates to a position corresponding to 130 kD upon reduction (Fig. 5). Sequential immunoprecipitation was then carried out from a lysate of TEC surface labeled with ^{125}I. The lysate was precleared three times with an anti-β_1 antiserum and subsequently immunoprecipitated with 10.1.2. Fig. 6 shows a SDS-PAGE analysis of the material immunoprecipitated by 10.1.2 before and after preclearing with the anti-β_1 antiserum or with an unrelated antiserum (anti-κ Ig chain).

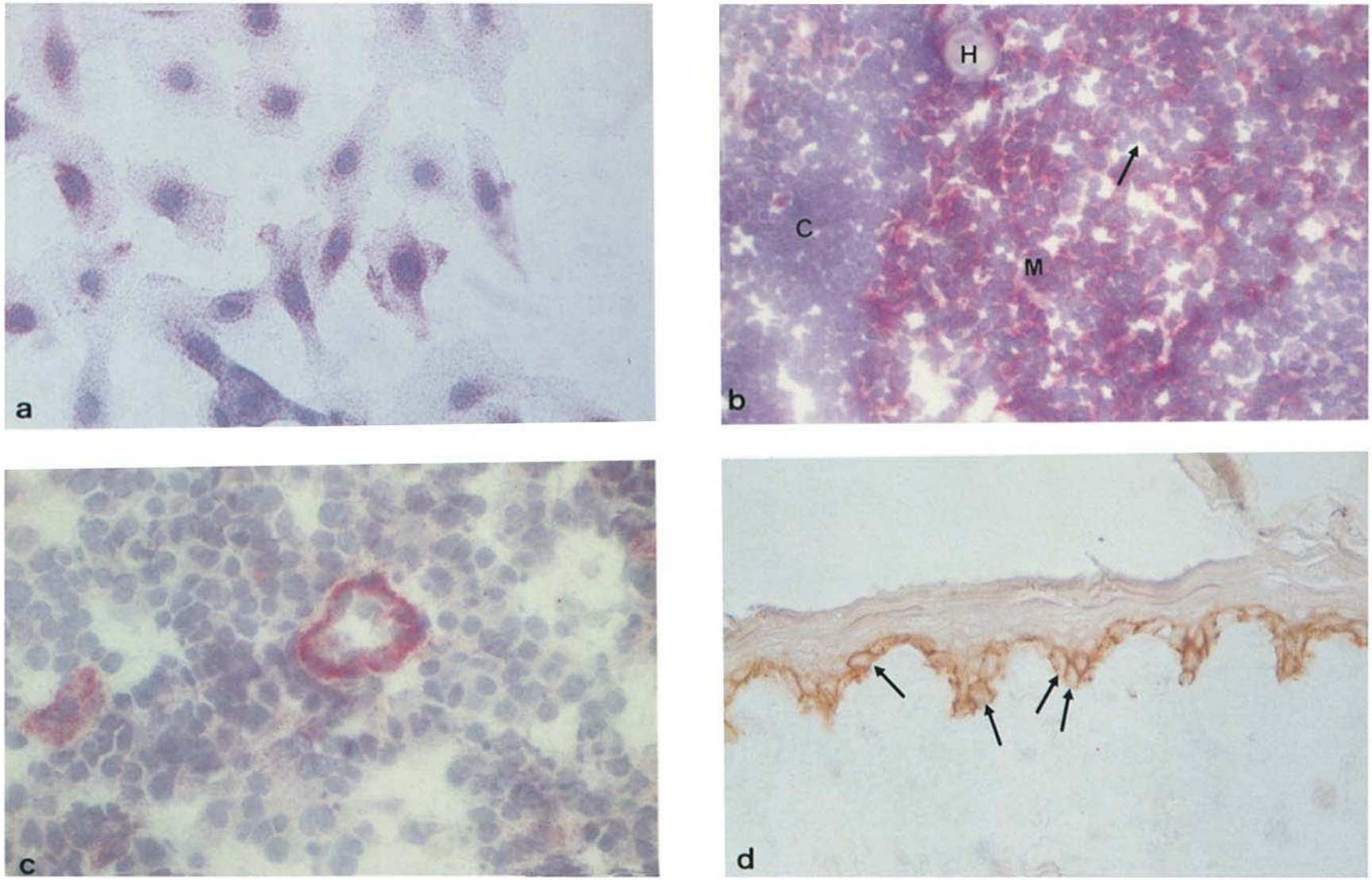

Figure 3. Tissue distribution of 10.1.2 antigen as shown by immunocytochemical methods. (*a*) Cultured TEC display a microgranular pattern of staining indicating the surface localization of the antigen. (*b*) Section of human thymus showing the localization of the antigen in the medullary region (*M*) and lack of staining in the cortex (*C*) and in Hassall's corpuscles (*H*). Arrow points to negative medullary thymocytes. (*c*) Human lymph node section. The endothelium of a high endothelial venule is labeled by the 10.1.2 antibody. (*d*) The 10.1.2 antibody stains selectively the basal layer of the human epidermis. Of note is the lack of staining at the deep aspect of the basal cells. Tissues in *a*, *b* and *c* were stained with the APAAP method; *d* is a PAP preparation.

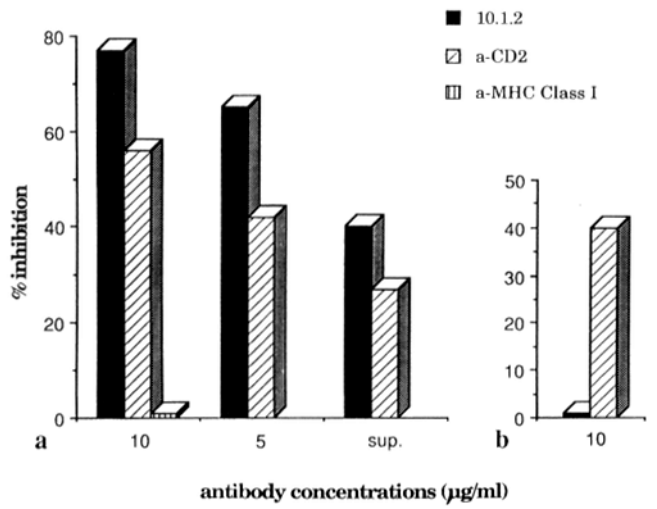

Figure 4. Incubation with the 10.1.2 mAb inhibits rosette formation between TEC and thymocytes (*a*) but not between TEC and T-PBL (*b*). Two additional mAb were used as positive and negative controls: MAR206 (anti-CD2) and W6/32 (anti-MHC class I). Each mAb was present in each sample during the assay at the concentrations indicated (sup. is the 10.1.2 hybridoma cells supernatant). The results are the mean of three experiments using resting thymocytes.

Only a very faint band is visible after preclearing with the anti-β_1 antiserum, suggesting that the molecule recognized by the 10.1.2 mAb contains the β_1 chain. In Fig. 6, a third band with an apparent molecular mass of 83 kD is visible in the profile obtained with both the 10.1.2 antibody and the anti-β_1 antiserum. This band, which is sometimes prominent in SDS-PAGE patterns of integrin molecules (see Figs. 9 and 10 *b*), has been previously identified as a fragment of α subunits (33, 50). To confirm that β_1 and the 110-kD band present in the molecule recognized by the 10.1.2 mAb

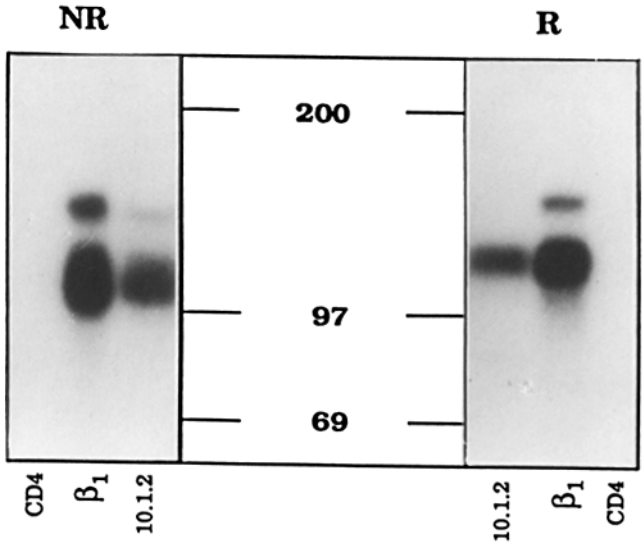

Figure 5. SDS-PAGE analysis of the protein precipitated from ^{125}I-labeled cultured TEC by the 10.1.2 mAb and by an anti-β_1 antiserum. After immunoprecipitation, samples were run in a 6% polyacrylamide gel under reducing (*right*) and nonreducing (*left*) conditions.

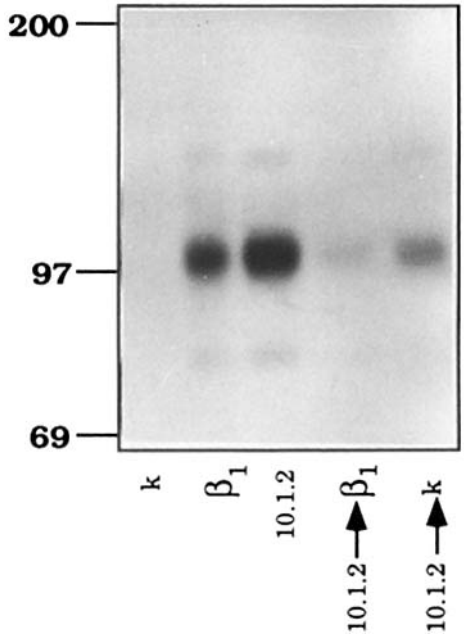

Figure 6. A comparison of the 10.1.2 protein and the β_1 VLA-subunit under nonreducing conditions in a 6% polyacrylamide gel. The lysate of ^{125}I surface-labeled TEC was precleared three times with an anti-β_1 antiserum or with a control antiserum (anti-κ Ig chain), and immunoprecipitated with the 10.1.2 mAb. From *left* to *right*: anti-human Ig κ chain, anti-β_1, 10.1.2, 10.1.2 after preclearing with anti-β_1, 10.1.2 after preclearing with anti-human Ig κ chain.

were indeed identical, the two bands were eluted from the gel, iodinated in SDS with Chloramine T (see Materials and Methods), digested with pepsin in the presence of equal amounts of a carrier protein (BSA) to ensure an identical enzyme to protein ratio (1:50), and their peptic peptide patterns were compared by two-dimensional microfingerprinting on silica gel plates (see Materials and Methods). Fig. 7 shows that the two patterns are nearly identical with most of the peptides superimposable. Minor differences observed are likely related to small amounts of contaminant proteins or to the inherent power of the technique that separates peptides identical but in slightly different states of oxidation, thus enhancing differences (51). Taken together, these results indicate that the β_1 chain and the 110–120-kD chain present in the molecule recognized by the 10.1.2 mAb are the product of the same gene, even though the possibility that the two polypeptide chains are the result of an alternative splicing, which seems to occur in this family of proteins, cannot be ruled out on the basis of the available data.

The 150–160-kD Chain of the 10.1.2 Molecule Is Not Identifiable as any of the Characterized Integrin α Chains. Seven different β_1 subunit–associated α chains have been characterized so far: α_1, α_2, α_3, α_4, α_5, α_6, and α_v (48, 49), some of which comigrate with β_1 upon reduction. It is clear from Fig. 5 that the anti-β_1 antiserum precipitates a mixture of integrins with several different α chains, some of which do not change behavior in SDS-PAGE under reducing conditions, whereas the 10.1.2 mAb reacts with a single molecule, as indicated

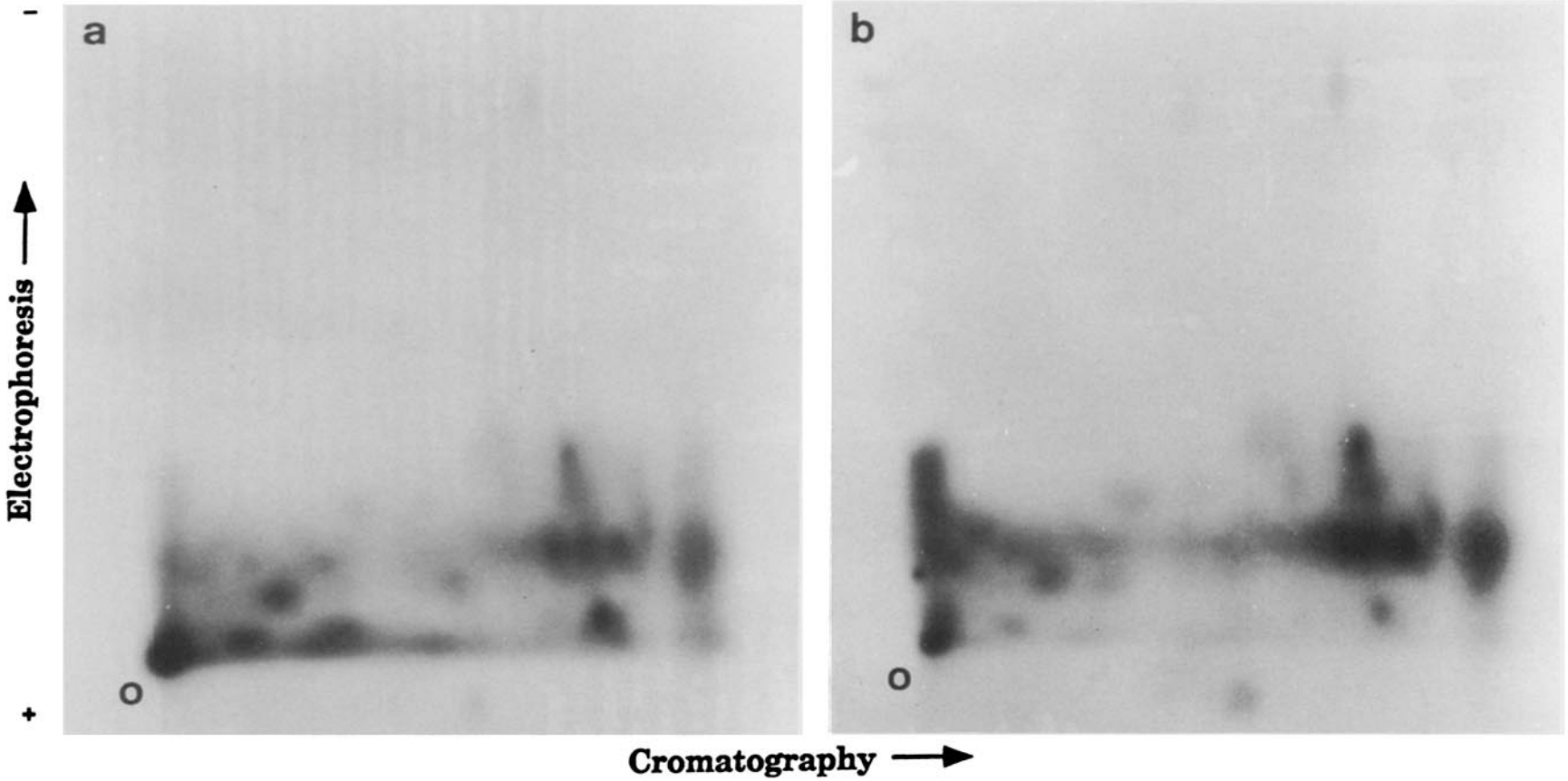

Figure 7. Autoradiographs of peptide maps of ^{125}I-labeled 10.1.2 β chain (*a*) and β_1 VLA subunit (*b*). Lysates obtained from ^{125}I-labeled TEC were immunoprecipitated with the mAb 10.1.2 or with the anti-β_1 antiserum as described in Materials and Methods. The immunoprecipitated proteins were run on SDS-PAGE under nonreducing conditions, and the bands of 110 kD were eluted and digested with pepsin. The resulting peptides were separated by two-dimensional microfingerprinting on silica gel plates. The origin of the electrophoresis is indicated (*o*).

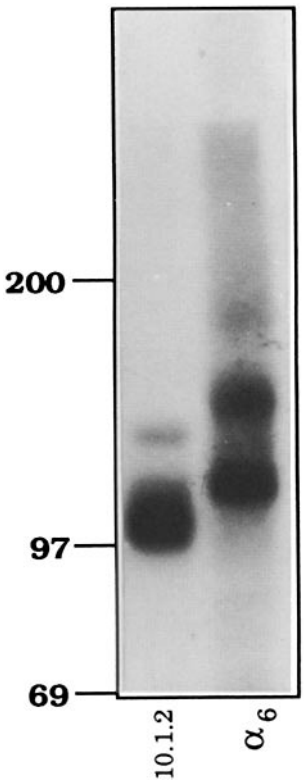

Figure 8. A comparison of 10.1.2 and α_6 proteins immunoprecipitated from ^{125}I-labeled TEC. TEC lysates were immunoprecipitated using the 10.1.2 mAb or the GoH3 mAb, specific for the VLA α_6 chain. Samples were analyzed in 6% SDS-PAGE under nonreducing conditions.

by the sharper and fainter α chain band that, upon reduction, comigrates with β_1. The 10.1.2 mAb must then be directed against the α subunit. The identity of the 150–160-kD chain precipitated by the 10.1.2 mAb with three of the known α chains could be ruled out on the basis of their migration in SDS-PAGE. Thus, α_1 migrates in SDS-PAGE as a band of 200 kD, which under reducing conditions appears as a protein band of 210 kD. Moreover, an anti-α_1 antibody (TS2/7) was unable to precipitate any band from surface-labeled TEC. The electrophoretic pattern obtained with the mAb GoH3, which is specific for α_6, shows several bands, none

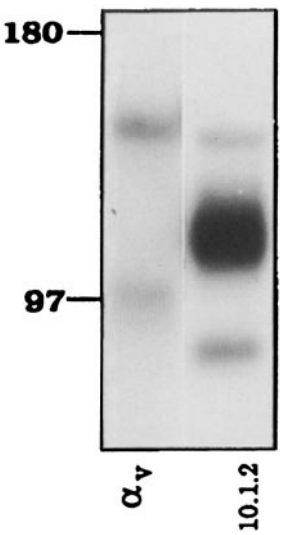

Figure 9. A comparison of 10.1.2 and α_v proteins immunoprecipitated from ^{125}I-labeled TEC. TEC lysates were immunoprecipitated using the 10.1.2 mAb or the NKI-M7 mAb, specific for the VLA α_v chain. Samples were analyzed in 6% SDS-PAGE under nonreducing conditions.

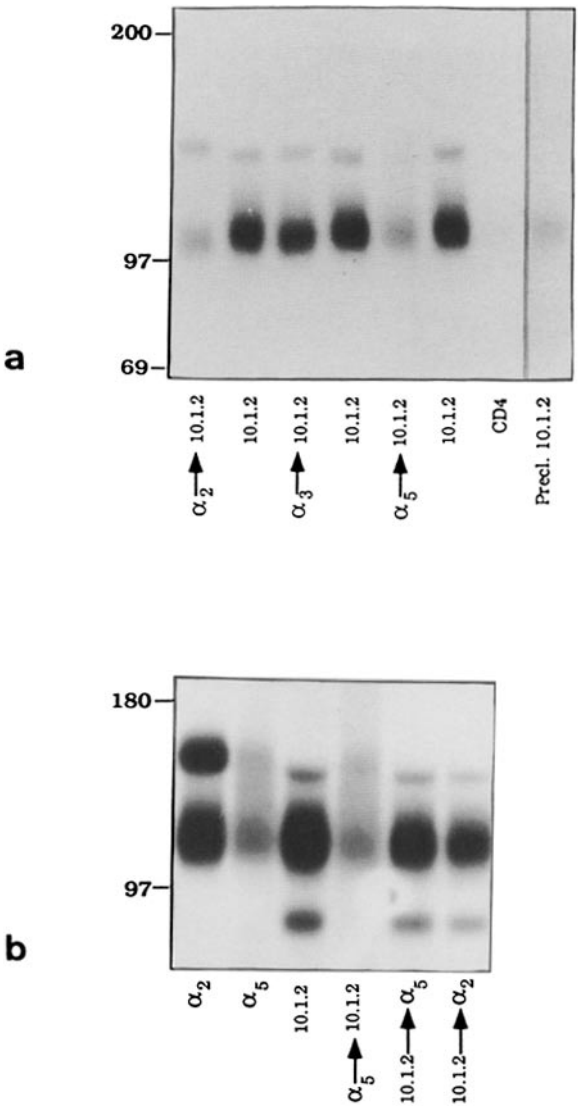

Figure 10. A comparison of 10.1.2 α subunit and the α_2, α_3, α_5 integrin chains. (*a*) A lysate of ^{125}I surface-labeled TEC was precleared repeatedly with the 10.1.2 mAb. The precleared lysate was divided into aliquots. Each aliquot was immunoprecipitated with one of the following antibodies: 10G11 mAb (anti-α_2 integrin chain), anti-α_3 peptide rabbit antiserum (anti-α_3 integrin chain), anti-α_5 peptide rabbit antiserum (anti-α_5 integrin chain). For a better comparison, an aliquot of 10.1.2 immunoprecipitated from the original lysate was loaded on the gel every two lanes. An anti-CD4 antibody was used as control. The material precipitated by the 10.1.2 mAb after the third preclearing was loaded in the last lane to the right. (*b*) From *left* to *right*: material immunoprecipitated from a lysate of ^{125}I surface labeled TEC with the anti-α_2, anti-α_5, and 10.1.2 antibodies without preclearing; material immunoprecipitated by an anti-α_5 antiserum after three rounds of preclearing with 10.1.2; material immunoprecipitated by 10.1.2 after three rounds of preclearing with anti-α_5 or anti-α_2. Samples were analyzed in 6% SDS-PAGE under nonreducing conditions.

of which corresponds to the two bands precipitated by the 10.1.2 mAb (Fig. 8). The α_v chain could also be ruled out since it is associated with a β chain of a molecular mass lower than that of the β_1 chain and compatible with that of β_2 or β_5 (Fig. 9). The α_4 chain was also ruled out because it is not expressed by TEC (data not shown). For the remaining

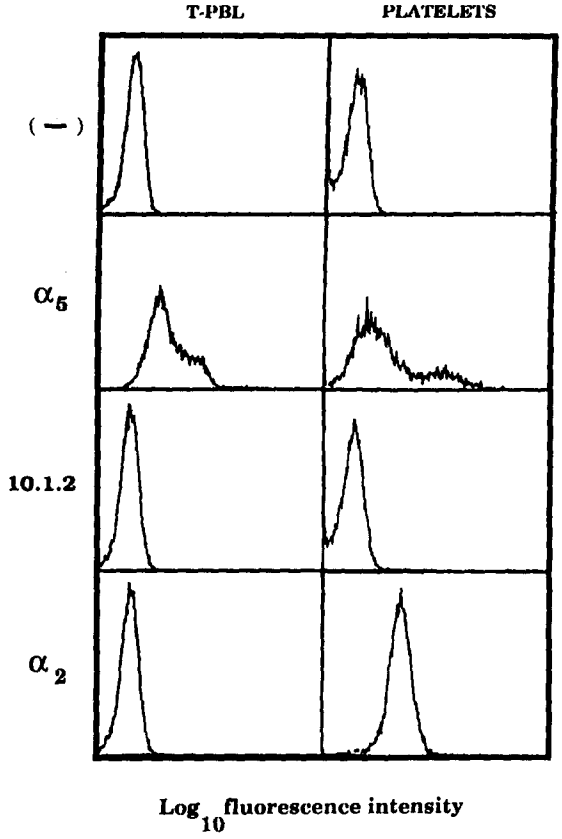

Figure 11. Pattern of expression of VLA-5, 10.1.2, and VLA-2 on T-PBL and platelets. Cells were stained with an anti-α_5-peptide rabbit antiserum, the 10.1.2 mAb, and the 10G11 (anti-α_2 integrin chain) antibody followed by FITC-labeled goat anti-mouse or anti-rabbit Ig antiserum as described in Materials and Methods.

α chains, sequential immunoprecipitation was carried out from surface-labeled TEC. Cell lysates were precleared three times with 10.1.2 and then immunoprecipitated with antibodies directed against the various α chains. As shown in Fig. 10 *a*, the anti-α_2, anti-α_3, and anti-α_5 antibodies were capable of precipitating bands corresponding to the molecule against which they are directed, even after extensive preclearing with 10.1.2. However, the level of radiolabeled bands obtained with anti-α_5 and anti-α_2 antibodies was rather low. Therefore, another lysate was immunoprecipitated with anti-α_2, anti-α_5, and 10.1.2 to assess the amount of each individual integrin before preclearing. As shown in Fig. 10 *b*, the amount of radioactivity precipitated by the anti-α_5 antiserum is indeed very low even before preclearing, and is not much reduced after three rounds of preclearing with the 10.1.2 antibody. When the preclearing was performed with anti-α_2 or anti-α_5, the intensity of the bands immunoprecipitated by the 10.1.2 antibody was again only slightly reduced. Moreover, the molecular mass of the α_2 subunit is higher than that of the 10.1.2 α subunit (see Figs. 10, *a* and *b*). The α_5 band is heavier than the 10.1.2 α subunit and has a smeared appearance, suggesting heterogeneity in its carbohydrate moiety.

As shown in Fig. 11, the pattern of expression of the three integrins VLA-2, VLA-5, and 10.1.2 on T-PBL and platelets is also different. Thus, 10.1.2 is absent from both T-PBL and platelets, VLA-2 is present on platelets only, and VLA-5 is present on both T-PBL and platelets. Similar findings have already been reported for the distribution of VLA-2 and VLA-5, although the profile obtained with a mAb on platelets indicated a more homogeneous distribution of VLA-5 (49) than that revealed by the polyclonal antiserum used in our study.

Therefore, we conclude that the 10.1.2 mAb is directed against an integrin α chain that does not correspond to any of the integrin α chains characterized so far.

Discussion

The determinant recognized by the mAb 10.1.2 belongs to a surface molecule expressed by medullary TEC, which is involved in the adhesion between thymocytes and TEC. Other TEC receptors participating in this interaction have already been described, and their ligands on thymocytes identified. Thus, the CD2/LFA-3 ligand pair has been shown to participate in the interaction between TEC and thymocytes, either resting or activated (20, 21), whereas the LFA-1/ICAM-1 system appears to be involved in the binding of activated thymocytes only (22). The 10.1.2 molecule is then part of an adhesion system of increasing complexity that stabilizes the interaction between these two types of cells in the thymus. It is of note that the binding between the two ligands of each pair (CD2/LFA-3, LFA-1/ICAM-1, 10.1.2/?) is not strong enough to keep the two cells together, as indicated by the finding that rosette formation can be blocked using antibodies that interfere with one pair of ligands only (reference 20, and our own data). CD2 and LFA-1, along with other adhesion molecules, are present also on the surface of other cell types, for instance, mature T lymphocytes, where they help stabilize a variety of cell-cell interactions (20, 52–57). It is therefore likely that different combinations of several complementary ligands, none of which can mediate a binding strong enough to support a functional interaction between two cells, are used to promote specific contacts between different types of cells. In this respect, it is of note that the ligand of 10.1.2 is present on both resting and activated thymocytes, but is absent on T-PBL. Thus, the 10.1.2 mAb, unlike anti-CD2 antibodies, is unable to inhibit rosette formation between T-PBL and TEC. The absence of the 10.1.2 molecule from cortical TEC also suggests the possibility of differential binding mechanisms of thymocyte subpopulations to cortical and medullary TEC. On the other hand, this molecule is also expressed by other epithelial and endothelial cells, where it mediates presumably different types of binding, perhaps in combination with other adhesion molecules.

As the molecule recognized by the 10.1.2 mAb appears to be a cell-cell adhesion protein, the finding that it is a member of the integrin receptor family is not surprising. Integrins are a growing family of surface molecules consisting of two chains, α and β, involved in cell-cell and cell–extracellular matrix interactions (45). At least 11 different α chains and six different β chains, encoded by two families of related genes,

have been described so far, and they associate in various combinations forming several subsets of heterodimeric receptors with different specificity (49, 58). The 10.1.2 molecule is composed by the β_1 chain typical of the VLA subset, originally described on activated T lymphocytes after several weeks in culture (59). The 10.1.2 molecule is indeed expressed by T cells after long-term culture. However, the α chain does not correspond to any of those already described. Although this latter conclusion relies mainly on depletion experiments, other evidences point to the same direction: α_2 migrates in SDS-PAGE as a band of 160 kD under nonreducing conditions and as a band of 165 kD (and not 130 kD) under reducing conditions, and is expressed by platelets (the 10.1.2 mAb does not stain platelets), where it represents one of the collagen receptor subunits; α_5 is the fibronectin receptor on platelets and is expressed by resting $CD4^+$ T cells (which do not bear the 10.1.2 molecule); α_6 not only shows different bands in SDS-PAGE but is also part of the laminin receptor of the platelets; α_v has an electrophoretic behavior different from that of 10.1.2 and is expressed on fibroblasts that are negative for 10.1.2.

Preliminary experiments in which the 10.1.2 mAb was used to inhibit the adhesion of TEC to the culture dish and to purified laminin failed to show any effect of the antibody, even at high concentration (100 μg/ml), suggesting that this integrin is primarily involved in cell-to-cell contact. This contention is reinforced by the finding that the 10.1.2 molecule is absent in the deep aspect of the basal epidermal cells, which faces the basement membrane. Furthermore the finding that the molecule is not expressed by thymocytes, either resting or activated, indicates that this receptor is not homotypic but binds to an as yet unidentified ligand.

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