

A Novel $\beta 4, \alpha 6$ Integrin-associated Epithelial Cell Antigen Involved in Natural Killer Cell and Antigen-specific Cytotoxic T Lymphocyte Cytotoxicity

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Summary

Efficient immune responses require interactions between cell adhesion molecules on lymphocytes and counter-receptors on antigen presenting cells or target cells. While target-specific receptors or ligands have not been identified for natural killer (NK) cells, cell adhesion molecules have been implicated in the interaction between NK cell effectors and tumor cell targets. Herein, we describe monoclonal antibodies (mAbs) against a carcinoma cell line that efficiently block the cytolytic activity of interleukin 2-activated NK cell lines and clones. L280 mAb reacts with secretory epithelial cells in normal human tissues, but does not react with hematopoietic cells or other tissue types. Biochemical analysis revealed that L280 mAb immunoprecipitates the $\beta 4, \alpha 6$ integrin, as well as a novel 98-kD glycoprotein, and probably reacts with a carbohydrate epitope on these molecules. Involvement of the L280 antigen in cellular immunity is not restricted to NK cell-mediated cytotoxicity. L280 mAb also efficiently inhibits alloantigen-specific cytotoxicity against Colo-205 cells mediated by human histocompatibility leukocyte antigen (HLA)-A2 alloantigen specific $\alpha\beta$ -TCR⁺ and $\gamma\delta$ -TCR⁺ cytotoxic T lymphocyte (CTL) clones. Additionally, we demonstrate that L280 mAb blocks cytotoxicity mediated by influenza peptide-specific HLA-restricted CTL clones. These data indicate that the antigen recognized by L280 mAb is important in both NK and CTL function, and that an as yet unidentified receptor for this epithelial antigen is present on both NK and T lymphocytes. The restricted expression of L280 antigen indicates that this molecule may be important in immune reactions in epithelial tissues.

T lymphocytes recognize antigen via the CD3/T cell antigen receptor complex (1). In addition to the antigen-specific receptor, other T cell surface molecules are essential for interaction of helper T cells with APC or CTL with target cells (2–4). These have been referred to as “accessory” or “cell adhesion” molecules. Cell adhesion molecules present on the effector T cell can interact with cellular ligands or “counter-receptors” on the membranes of APC or target cells. Recent studies have indicated that a vast array of membrane structures are involved in inter-cellular binding and signaling. Interaction of these accessory molecules can be critical to the generation of an immune response. For example, alloantigen specific helper T cells fail to produce cytokines in response to HLA-DR7 antigen expressed in murine L cells. However, cotransfection of HLA-DR7 and CD54 (ICAM-1), a cellular ligand for CD11/18 (LFA-1), restores T cell responsiveness (5). Thus, antigen-specific recognition alone may be insufficient to trigger effector function, since additional accessory molecules are required for an efficient response.

NK cells are a population of lymphocytes identified by the

phenotype CD3^ε⁻, CD56⁺ that mediate major histocompatibility complex unrestricted cytotoxicity against certain malignant and virus-infected cells, and produce numerous cytokines following immune stimulation (6). The cell surface receptors on NK cells that are involved in target cell recognition have not been identified. There is considerable debate whether NK cells possess “specific” receptors for targets or alternatively whether the cytotoxic function can be initiated simply by appropriate engagement of cell adhesion molecules (7). Nonetheless, as with T cells, cell adhesion molecules are certainly involved in the interaction between NK cells and their targets. Although $\beta 2$ integrin (CD11/18) and CD2 have been implicated in certain NK/target cell interactions (8–10), in general the cell surface molecules involved in NK cell interactions with target cells have not been identified.

NK cells constitutively express intermediate affinity receptors for IL-2 and are able to kill a broad spectrum of tumor cell targets after activation by IL-2 (11–13). Colo-205 is a human colon adenocarcinoma cell line that is resistant

to killing by resting peripheral blood NK cells, but is efficiently lysed by IL-2-activated NK cells (12). mAbs were generated against this tumor in order to identify the membrane structures involved in the interaction between NK cells and carcinoma targets. In this report, we describe mAbs that react with normal secretory epithelial tissues and recognize a $\beta 4$, $\alpha 6$ integrin-associated antigen on the membrane of Colo-205. The role of this antigen in NK and CTL cytotoxicity is examined.

Materials and Methods

Hybridomas and Tumor Cell Lines. Colo-205, Colo-320, LoVo, and HT-29 are human colon carcinoma cell lines, ZR 75-1 is a breast carcinoma cell line, U937 is a myeloid leukemia cell line, K562 is an erythroleukemia cell line, HeLa is an epitheloid carcinoma cell line, PA-1 is an ovarian teratocarcinoma cell line, Jurkat and HPB-ALL are T leukemia cell lines, Daudi and Raji are Burkitt's lymphoma cell lines, HL-60 is a myeloid leukemia cell line, KG1a is an early hematopoietic leukemia cell line, SK-MEL-1 is a melanoma cell line, and JY is an EBV-transformed lymphoblastoid cell line (American Type Culture Collection, Rockville, MD). BDMEL-1 and BDMEL-2 are melanoma cell lines established from primary melanoma tumors generously provided by Drs. Brett Gemlo and Anthony Rayner (University of California, San Francisco, CA). SK-N-SH is a neuroblastoma cell line generously provided by Drs. Roxanne Duan and Wolfgang Sadee (University of California, San Francisco, CA). Colo-205 was determined to express HLA-A2 (Dr. Fran Ward, Duke University, Durham, NC personal communication). L279, L280, L281 mAb were generated by immunizing BALB/c mice with Colo-205. Immune splenocytes were fused with Sp2/0 and hybridomas selected by growth in azaserine. Hybridomas were cloned using a FACStar (Becton Dickinson Immunocytometry Systems, San Jose, CA). Hybridoma isotype was determined using rat anti-mouse Ig isotype-specific mAb, generously provided by Mr. David Buck (Becton Dickinson Immunocytometry Systems).

Peripheral Blood Natural Killer (NK) Cells and NK Clones. PBMCs (Stanford Blood Center, Stanford, CA) were isolated by Ficoll/Hypaque centrifugation. After plastic adherence and passage through nylon wool, NK cells were enriched by centrifugation on discontinuous gradients of Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) (12). These NK cell enriched peripheral blood preparations were ~ 30 –50% CD3⁺CD16⁺, CD56⁺ NK cells and 50–70% CD3⁺ T cells. Cells were cultured overnight in RPMI 1640 media (M. A. Bioproducts, Walkersville, MD) supplemented with 10% FCS (J. R. Scientific, Woodland, CA), 1 mM L-glutamine (Gibco Laboratories, Chagrin Falls, OH), antibiotics, and 200 IU/ml recombinant IL-2 (generously provided by Cetus Corp., Emeryville, CA) (12). CD3 ϵ ⁻, CD16⁺, CD56⁺ NK clones were established using culture conditions described previously (14), with 50 IU/ml recombinant IL-2 as growth factor.

Cytotoxic T Lymphocyte (CTL) Lines and Clones. For generation of anti-Colo-205 CTL, PMBCs (10^6 /ml) were cocultured with irradiated (7,000 rad) Colo-205 cells (10^5 /ml) and irradiated (2,500 rad) allogeneic PMBC feeders (4×10^5 /ml) in culture medium (RPMI-1640; M. A. Bioproducts) + 10% FCS (JR Scientific); 1 mM sodium pyruvate, 1 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin in a 24-well tissue culture plate (Falcon Plastics, Lincoln Park, NJ). CTL cell lines were restimulated three times by coculture with irradiated Colo-205 cells and assayed for cytotoxicity. These anti-Colo-205 CTL lines were

subsequently cloned by limiting dilution. The HLA-A2 specific $\alpha\beta$ -TCR⁺ JS86 (15) and $\gamma\delta$ -TCR⁺ ES204 (16) CTL clones have been described previously. CTL Q66.40 is a CD8⁺, $\alpha\beta$ -TCR⁺ CTL clone that was derived from a polyclonal culture described previously (17). This clone recognizes a peptide encompassing positions 59 to 62 of the influenza virus matrix protein in conjunction with HLA-A2 (S. Verma, H. Spits, and R. de Waal Malefyt, manuscript in preparation). CTL used in these studies do not express Fc receptors for IgG.

mAbs and Reagents. Control Ig, anti-CD3 (Leu-4), anti-CD16 (Leu 11a8c), and anti-CD56 (Leu 19) mAb were generously provided by Becton Dickinson Immunocytometry Systems. Anti-HLA-A,B,C (W6/32) hybridoma was obtained from American Type Culture Collection. GRGDSP, GRGESP, human laminin, and 3E1 mAb were purchased from Telios Pharmaceutical (San Diego, CA). GoH3 (18), AIIIB2 (19), and J1B5 mAb were generous gifts from Dr. A. Sonnenberg (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam) and Dr. C. Damsky (University of California, San Francisco). Anti- $\beta 4$ mAb 439-9B (20) was generously provided by Dr. S. Kennel (Oak Ridge, TN). The peptide of the influenza virus matrix protein (MAT 59-68) was a generous gift of Dr. Jonathan Rothbard (Immunology, Palo Alto, CA).

Cytotoxicity Assay. Tumor cell lines ($\sim 1.5 \times 10^6$ cells) were labeled with 100 μ Ci 51 Cr for 3 h, washed, and used as targets in a 4-h radioisotope release cytotoxicity assay (21). For antigen-pulsing, specific influenza peptide was added at 20 μ M during the 3-h incubation for 51 Cr labeling.

Immunohistology. Frozen sections of human colon, breast, lung, and stomach tissue obtained from normal autopsy tissue were stained with control Ig or L280 mAb at saturating concentrations, followed by horseradish peroxidase conjugated goat anti-mouse Ig as a second step.

Flow Cytometry. Methods of immunofluorescence and flow cytometry have been described previously (21). Flow cytometry was performed using a FACScan[®] or FACStar[®] (Becton Dickinson Immunocytometry Systems).

Biochemistry. Colo-205 and HT-29 cells were surface labeled with 125 I (Amersham Corp., Arlington Heights, IL) and were solubilized in Tris buffered saline (TBS, 50 mM Tris, 150 mM NaCl, pH 8.0) containing 1% NP-40, 20 Kallikrein inhibitor U/ml aprotinin (Sigma Chemical Co., St. Louis, MO), and 1 mM PMSF (Sigma Chemical Co.) (22). Cell lysates were passed through a 0.2 ml (bed volume) column of Dowex 1 \times 8-400 (chloride form; Sigma Chemical Co.) equilibrated in TBS to remove unbound 125 I, and were precleared three times with 10 mg Pansorbin (Calbiochem-Behring Corp., San Diego, CA) coated with saturating amounts of rabbit anti-mouse Ig serum (22). Lysates were passed through lentil lectin-Sepharose 4B (Pharmacia Fine Chemicals) equilibrated in loading buffer (10 mM Tris, 150 mM NaCl, 0.25% NP-40, 0.05% NaN₃, pH 7.4) and glycoproteins eluted in loading buffer containing 300 mM 1-O-methyl α -D-glucopyranoside (Sigma Chemical Co.). Antigens were immunoprecipitated using Pansorbin coated with saturating amounts of rabbit anti-mouse Ig and mAb (22). Immunoprecipitates were washed five times in TBS containing 1% NP-40. For removal of N-linked oligosaccharides, immunoprecipitates were treated with 65 U/ml endo-F (New England Nuclear, Boston, MA) for 24 h at 37°C in the presence of 1 mM PMSF and 50 mM EDTA (23). For reprecipitation experiments, 125 I-labeled lentil lectin purified Colo-205 glycoproteins were incubated for 2 h with 25 μ l Sepharose 4B coupled by CNBr to L280 mAb (~ 3 mg Ig/ml beads) or 3E1 mAb (24). Beads were washed five times in 1% NP-40 TBS, and antigen was eluted in 50 μ l 50 mM

diethylamine (pH 12) and immediately neutralized with 50 μ l 1 M Tris, pH 7.6. Eluted antigen was diluted to 1 ml in cold 1% NP-40 TBS containing 10 mg/ml BSA and 1 mM PMSF (Sigma Chemical Co.) and used for reimmunoprecipitation experiments. Samples were analyzed by SDS-PAGE under reducing conditions, or by two-dimensional diagonal SDS-PAGE (first dimension non-reducing, second dimension reducing) using 7.5% acrylamide gels (22).

Results and Discussion

Generation of mAbs Inhibiting Interleukin (IL)-2 Activated Natural Killer (NK) Cell Cytotoxicity. To identify the membrane structures involved in the killing of carcinomas by IL-2 activated NK cells, mAbs were generated against Colo-205 and selected for their ability to inhibit cytotoxicity mediated by IL-2 activated NK cells. mAbs, designated L279 (IgG3, κ isotype), L280 (IgG1, κ) and L281 (IgM, κ), were identified that efficiently blocked NK cell-mediated cytotoxicity against Colo-205 (Fig. 1 A), but not other tumor cell targets, including K562, JY, LoVo, HT-29, and BDMEL-1 (not shown). Potent

inhibition of cytotoxicity was achieved with relatively low concentrations of purified L280 mAb (Fig. 1 B). These mAbs inhibited the cytolytic activity of polyclonal populations of peripheral blood NK cells cultured in IL-2, as well as IL-2-dependent NK clones, established from more than 20 individuals. All NK cells lines that mediated cytotoxicity against Colo-205 were efficiently inhibited with L280 mAb. Since L279, L280, and L280 mAbs were all determined to immunoprecipitate the same structure, subsequent experiments were performed with only L280 mAb.

Distribution of the Antigen Recognized by L280 mAb A panel of in vitro cultured cell lines and normal hematopoietic cells were tested for reactivity with L280 mAb by flow cytometry. As shown in Fig. 2, L280 mAb was strongly reactive with Colo-205. However, we failed to detect L280 mAb binding to any normal hematopoietic cells (including peripheral blood lymphocytes, monocytes, granulocytes, erythrocytes, platelets, neutrophils, thymocytes, or bone marrow cells). L280 mAb also failed to react with PHA lymphoblasts, IL-2-dependent CTL and NK clones, or any transformed hematopoietic cell lines (including Jurkat, HL-60, K562, U937, KG1a, HPB-ALL, Daudi, Raji, and several EBV lymphoblastoid cell lines). Other nonhematopoietic cell lines examined, including several colon carcinoma cell lines (HT-29, Colo-320, and LoVo), a neuroblastoma cell line (SK-N-SH), a breast carcinoma (ZR 75-1), an epitheloid carcinoma cell line (HeLA), an ovarian

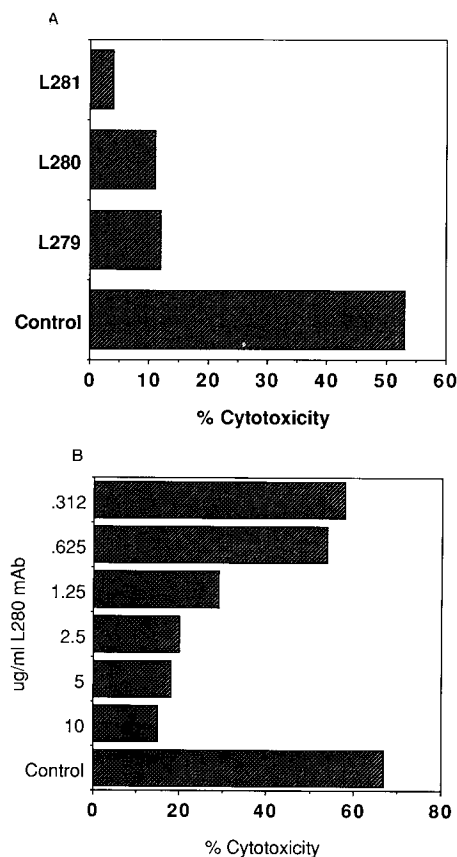


Figure 1. Inhibition of NK cell-mediated cytotoxicity. (A) IL-2-activated peripheral blood NK cell preparations were assayed for cytotoxicity against Colo-205 cells in the presence of mAb L279 (IgG3), L280 (IgG1), L281 (IgM) and control Ig. Effector-to-target (E/T) ratio was 25:1. Note that NK cells do not express Fc receptors that bind murine IgG1 or IgM mAb (33). (B) L280 mAb dose-dependent inhibition rIL-2-activated peripheral blood NK cell-mediated cytotoxicity against Colo-205. Control murine IgG1 mAb was used at 10 μ g/ml. E/T was 25:1.

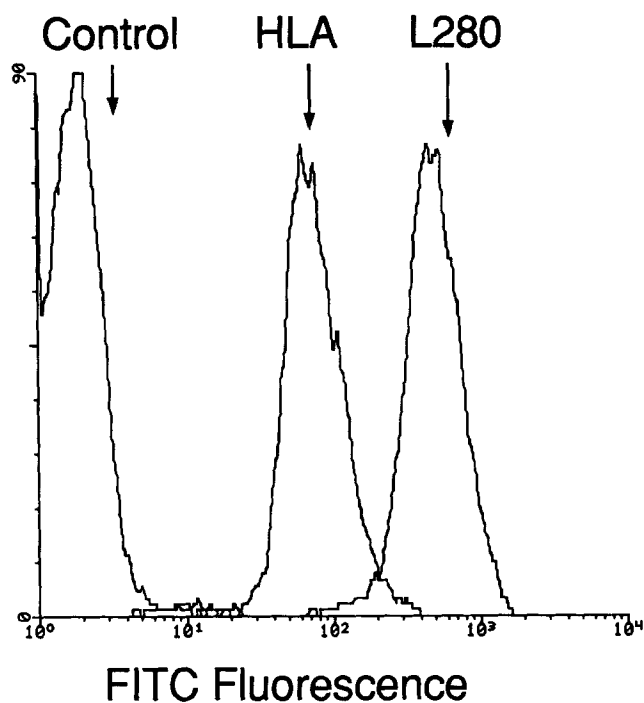
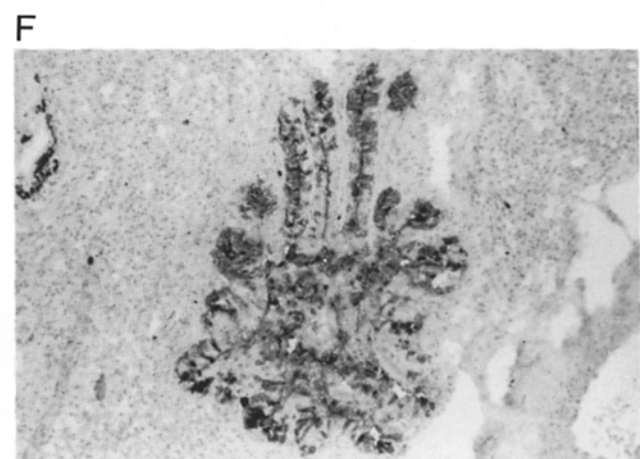
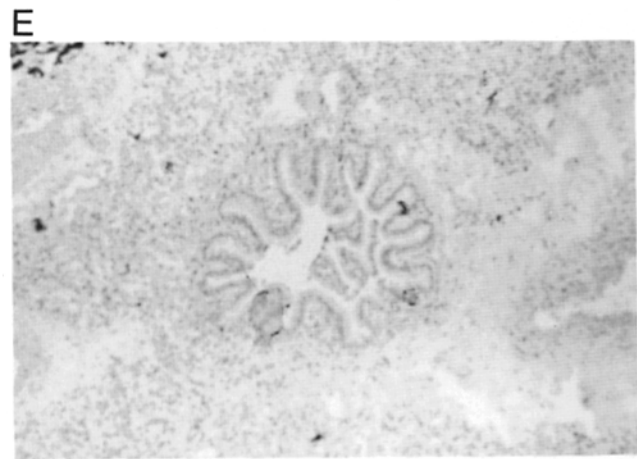
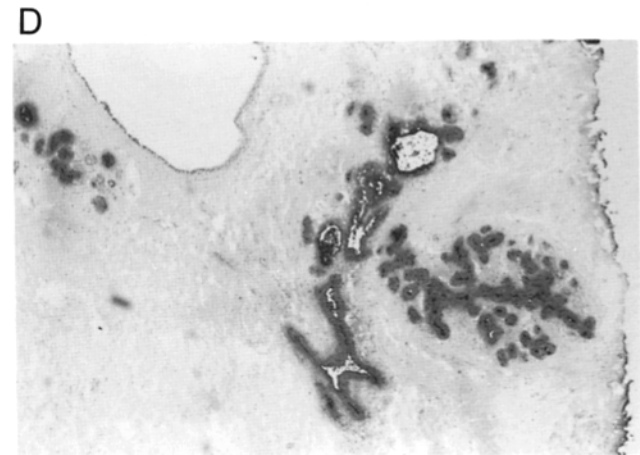
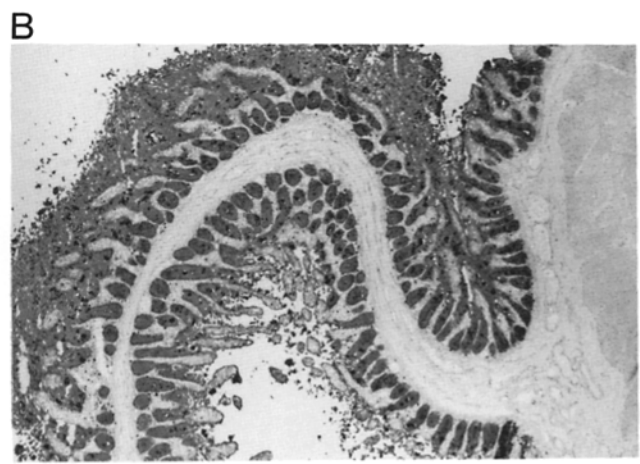
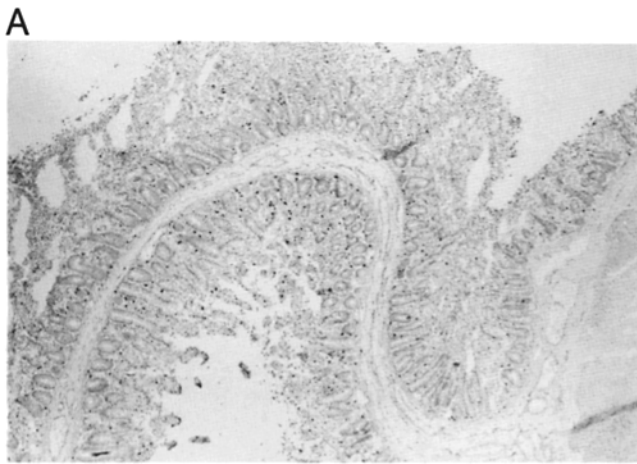


Figure 2. Binding of L280 mAb to Colo-205 cells. Colo-205 cells were stained with FITC-conjugated IgG1 control, FITC-conjugated L280 mAb, or FITC-conjugated anti-HLA-A,B,C. The x axis represents fluorescence (four decade log scale) and the y axis the relative cell number. Histograms from cells stained with control mAb (nearest the ordinate) are superimposed over the histogram of cells stained with L280 or anti-HLA mAb, as indicated.



(continued)

teratocarcinoma cell line (PA-1) and melanoma cell lines (SK-MEL-1, BDMEL-1, BDMEL-2), were also unreactive.

However, immunohistochemical staining of normal tissues indicated that the antigen recognized by L280 mAb is abundantly expressed in vivo. As shown in Fig. 3, L280 mAb specifically reacted with secretory epithelial cells in colon, breast, lung and stomach. Secretory epithelial cells of the duodenum and jejunal mucosa, salivary glands, and skin were also stained with this mAb (not shown). In lymphoid tissues,

L280 mAb prominently stained the epithelial crypts of the palatine tonsil and thymic Hassall's corpuscles (not shown), but did not react with hematopoietic cells. Given the broad distribution in normal tissues, it was surprising that the carcinoma cell lines other than Colo-205 that were examined failed to react with L280 mAb. It is possible that expression of the antigen is lost during transformation or during in vitro culture. A more extensive examination of primary carcinomas before and after culture will be necessary to resolve this issue.

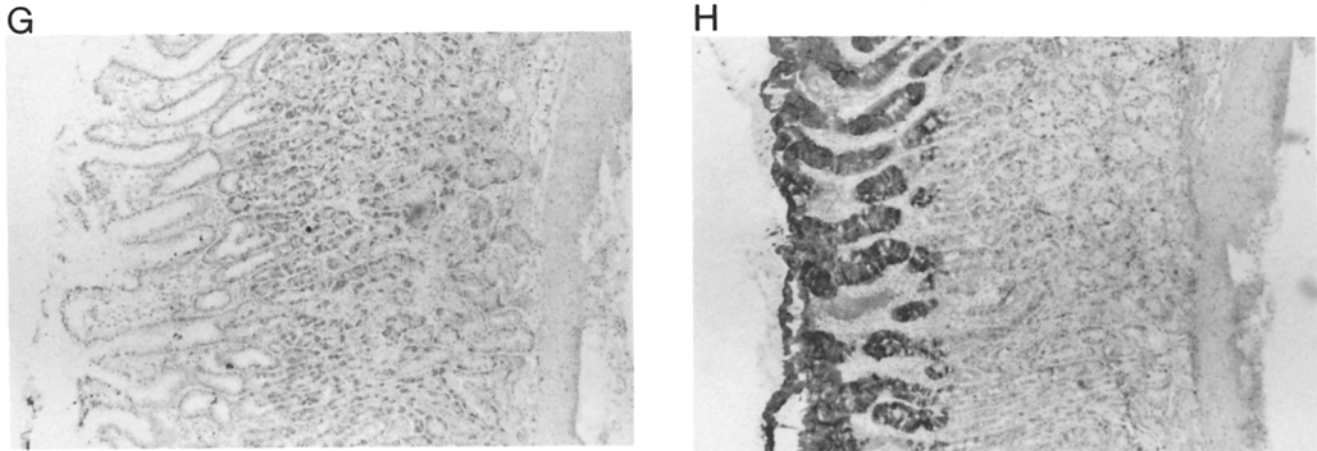


Figure 3. Reactivity of L280 mAb with tissue. Frozen structures of human colon (A and B), breast (C and D), lung (E and F), and stomach (G and H) tissue were stained with control Ig (A, C, E, and G) or L280 mAb (B, D, F, and H), followed by horseradish peroxidase conjugated goat anti-mouse Ig as a second step.

Nonetheless, the antigen recognized by L280 mAb is abundantly distributed on normal epithelial tissues *in vivo*, demonstrating that the antigen is not "tumor-associated" or restricted to expression on the Colo-205 cell line.

Biochemical Characterization. Colo-205 cells were surface-labeled with ^{125}I , detergent solubilized and glycoproteins isolated by lentil lectin affinity chromatography. At least 5 proteins (170, 150, 135, 118, and 98 kD) were immunoprecipitated using the L280 mAb (Fig. 4 A), L279, and L281 mAb (not shown). Direct comparison of the unfractionated, lentil-lectin bound, and nonbinding antigens revealed no substantial qualitative differences in the structures immunoprecipitated with L280 mAb and indicated the L280 mAb reactive glycoproteins were essentially exclusively in the lentil-lectin bound fraction (not shown). Since an initial lentil-lectin chromatography step resulted in substantially lower backgrounds in the immunoprecipitation experiments, we routinely incorporated this step in subsequent experiments. Two-dimensional diagonal gel electrophoresis (first dimension, non-reduced; second dimension, reduced) revealed that the 170-, 150-, 118-, and 98-kD proteins migrated either on or slightly above the diagonal, indicating the presence of possible intrachain disulfide bonds, but lack of interchain disulfide linkages to other proteins (Fig. 4 B). However, the 135-kD subunit migrated below the diagonal due to the cleavage of a small disulfide linked component of ~ 25 kD, that was only revealed in subsequent experiments using gradient gels (not shown).

The 170-, 150-, 135-, 118-kD proteins appeared very similar to the $\beta 4, \alpha 6$ integrin that is preferentially expressed on colon carcinomas (20, 25–27). Location of the 135-kD subunit below the diagonal was consistent with the migration of $\alpha 6$ integrin on diagonal gels, as reported by Hemler and colleagues (26). Moreover, as with prior studies describing the $\beta 4, \alpha 6$ integrin, the relative labeling intensity of the different chains varied between experiments, and in some experiments an ad-

ditional band of ~ 190 kD was observed. Recent studies have indicated that $\beta 4$ exists in isoforms of several sizes, based on proteolytic cleavage of the cytoplasmic segment (28).

Direct comparative analysis indicated that while two mAb against $\beta 4$ (3E1 and 439-9B) and two mAbs against $\alpha 6$ (GoH3 and J1B5) immunoprecipitated the 170-, 150-, 135-, and 118-kD proteins, only L280 immunoprecipitated an additional gp98 subunit (Fig. 4 A). Although $\beta 1$ integrin is expressed on Colo-205 cells, anti- $\beta 1$ mAb did not coimmunoprecipitate $\beta 4$, $\alpha 6$, or gp98 subunits (not shown). Since a proteolytic fragment of $\beta 4$ has been observed to migrate at 85 kD (26), further studies were undertaken to provide evidence that gp98 is a distinct polypeptide. As shown in Fig. 5, removal of N-linked oligosaccharides with endo-F reduced the mobility of the $\beta 4$ and $\alpha 6$ subunits by 5–10 kD consistent with prior studies (25); however, gp98 was diminished to 55 kD. This high degree of N-linked glycosylation clearly distinguishes gp98 from $\beta 4$ and $\alpha 6$. Peptide mapping experiments further indicated that gp98 is distinct from the $\beta 4$ glycoprotein (not shown). Treatment with neuraminidase to remove sialic acid and O-glycanase to remove O-linked oligosaccharides resulted in only minor changes in mobility of the $\beta 4$, $\alpha 6$, or gp98 subunits (not shown).

That $\beta 4$ and $\alpha 6$ are components of the antigen immunoprecipitated by L280 mAb was demonstrated by the ability to affinity-purify the antigen using L280 mAb-Sepharose, elute the proteins and then reimmunoprecipitate the eluted proteins with anti- $\beta 4$, anti- $\alpha 6$ and L280 mAbs (Fig. 6 A). Note that while L280 mAb reimmunoprecipitated all of the original components of the complex, anti- $\beta 4$ and anti- $\alpha 6$ only immunoprecipitated the higher mol wt structures and not gp98. This suggests that anti- $\beta 4$ and anti- $\alpha 6$ are unable to react with gp98. L280 mAb directly binds to the $\alpha 6, \beta 4$ integrin, in the absence of the gp98 glycoprotein, based on the ability of L280 mAb to reimmunoprecipitate $\beta 4, \alpha 6$ integrin affinity-purified from Colo-205 using anti- $\beta 4$ Sepharose (Fig. 6 B).

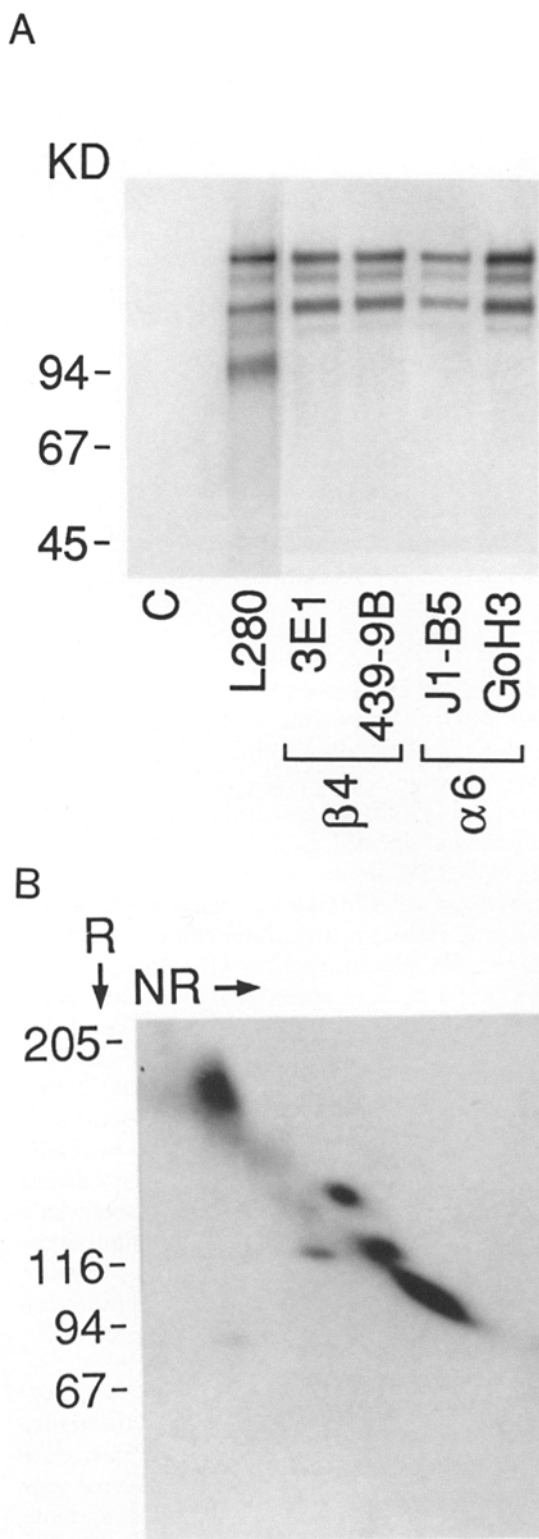


Figure 4. Biochemical analysis. (A) Antigens were immunoprecipitated from ^{125}I Colo-205 cells using control Ig (C), L280 mAb, anti- $\beta 4$ (3E1 and 439-9B) and anti- $\alpha 6$ (GoH3 and J1B5) and analyzed by SDS-PAGE. (B) L280 mAb immunoprecipitate from ^{125}I -labeled Colo-205 was analyzed by two-dimensional diagonal SDS-PAGE (NR, nonreducing; R, reducing).

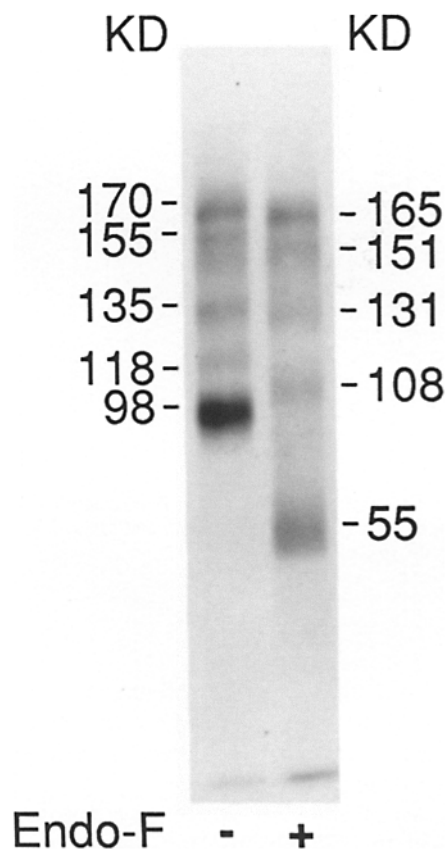


Figure 5. Deglycosylation. L280 mAb immunoprecipitate from ^{125}I -labeled Colo-205 was treated with endo-F and analyzed by SDS-PAGE.

However, L280 mAb fails to react with $\beta 4, \alpha 6$ integrin expressed on HT-29, another colon carcinoma cell line that expresses the $\beta 4, \alpha 6$ integrin (Fig. 7).

Sequential immunoprecipitation was undertaken to further explore the relationship between reactivity of L280 and the $\beta 4, \alpha 6$ integrin (Fig. 8). ^{125}I lysates from Colo-205 were immunoprecipitated with L280 mAb, anti- $\beta 4$ mAb, or anti- $\alpha 6$ mAb to remove all reactive structures with the individual antibodies. Depletion with anti- $\beta 4$ removed all $\alpha 6$ antigen. L280 mAb immunoprecipitated gp98 (and also small amounts of several higher mol wt proteins) from this $\beta 4$ depleted lysate. After depletion of $\alpha 6$, residual $\beta 4$ was immunoprecipitated with anti- $\beta 4$, and $\beta 4$ and gp98 were detected with L280 mAb. Similarly, after depletion of antigen with L280, detectable amounts of $\beta 4$ and $\alpha 6$ remained. These results indicate that a subset of the $\beta 4, \alpha 6$ integrin molecules are reactive with L280 mAb, and that anti- $\beta 4$ and anti- $\alpha 6$ mAb are unreactive with gp98. Moreover, these preclearing experiments demonstrate that $\beta 4, \alpha 6$ integrin is a major component of the ^{125}I -labeled membrane glycoproteins reactive with L280 mAb.

Collectively, these results suggest that L280 mAb recognizes an epitope that is expressed both on the $\beta 4, \alpha 6$ integrin and the gp98 glycoprotein on the surface of Colo-205. However, since L280 mAb failed to react with $\beta 4, \alpha 6$ integrin on the HT-29 cell line, the most plausible interpretation is that

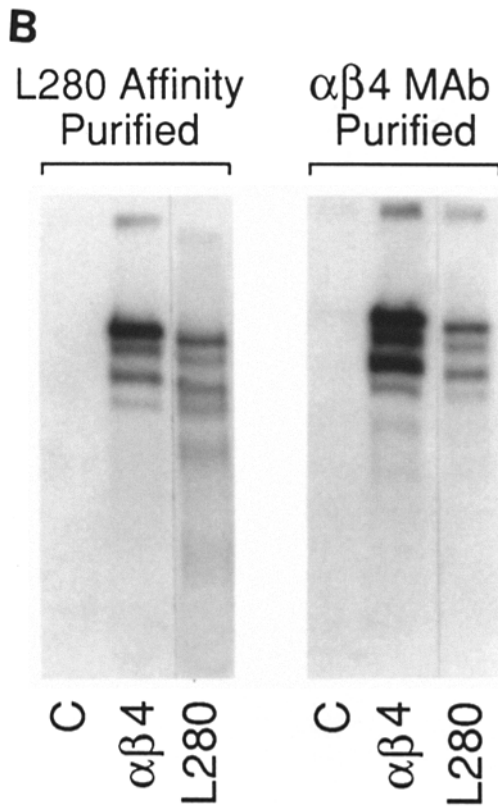
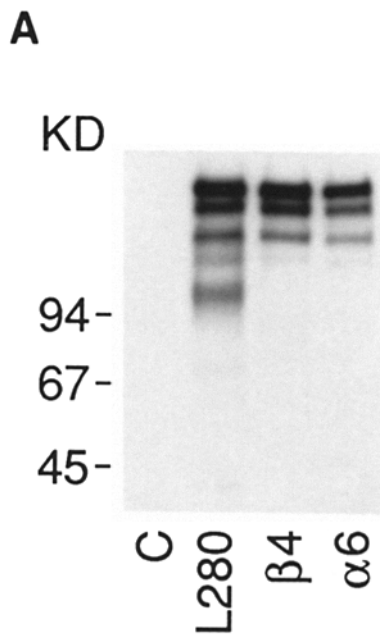


Figure 6. Re-immunoprecipitation. (A) Affinity-purified L280 antigen was reimmunoprecipitated with control Ig (C), L280 mAb, anti- $\beta 4$ (439-9B), or anti- $\alpha 6$ (J1B5) and analyzed by SDS-PAGE. (B) Affinity-purified L280 antigen (*left panel*) and affinity purified $\beta 4, \alpha 6$ integrin (*right panel*) were reimmunoprecipitated with control Ig (C), L280 mAb or anti- $\beta 4$ (439-9B) and analyzed by SDS-PAGE.

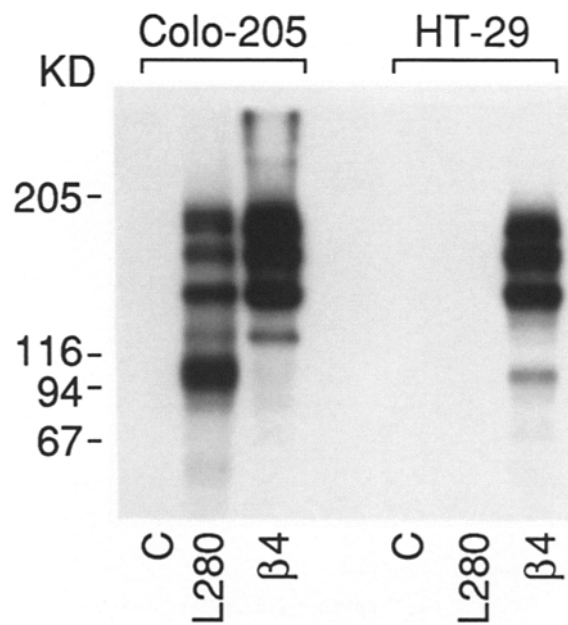


Figure 7. Immunoprecipitation from HT-29. Antigens were immunoprecipitated from ^{125}I HT-29 and Colo-205 cells using control Ig (C), L280 mAb and anti- $\beta 4$ (439-9B) and analyzed by SDS-PAGE. Immunoprecipitation of HT-29 with anti- $\alpha 6$ mAb demonstrated an identical profile of bands as shown for anti- $\beta 4$ (not shown). The minor ~ 100 -kD band in the anti- $\beta 4$ immunoprecipitate of HT-29 likely represents a proteolytic fragment of $\beta 4$ or $\alpha 6$, and was not observed in other experiments.

L280 mAb reacts with a carbohydrate antigen shared between $\beta 4, \alpha 6$ and gp98. Preliminary attempts to deglycosylate $\beta 4, \alpha 6$ and gp98 before binding with L280 mAb provided inconclusive results. However, a common feature of carbohydrate antigens is their resistance to denaturation by boiling, whereas polypeptide antigens are often destroyed by this treatment. As shown in Fig. 9, the antigenic determinants recognized by L280 mAb in fact were resistant to boiling. By contrast, reactivity of $\beta 4, \alpha 6$ with anti- $\beta 4$ and anti- $\alpha 6$ mAbs was labile to this treatment. However, it should be appreciated that if the antigen recognized by L280 is actually carbohydrate, this structure apparently is restricted in expression to secretory epithelial tissues.

Effect of mAb against $\beta 4$ and $\alpha 6$ on Natural Killer (NK) Cell-mediated Cytotoxicity. MAb against $\alpha 6$ or $\beta 4$ failed to affect the cytotoxicity mediated by NK clones (Fig. 10) or polyclonal IL-2-activated NK cell cultures from several donors (not shown), demonstrating the specificity of the inhibition observed with the L279, L280, and L281 mAb. The mechanism whereby L280 mAb inhibits NK cell-mediated cytotoxicity has not as yet been determined. Preliminary studies to examine the ability of L280 mAb to affect NK cell conjugate formation with Colo-205 target cells have proven inconclusive. L280 mAb does not modulate expression of the antigen from the surface of Colo-205 even after overnight culture in saturating concentrations of mAb (not shown).

As yet, ligands for the $\beta 4, \alpha 6$ integrin have not yet been identified, although it has been suggested that laminin may bind to $\beta 4, \alpha 6$ on certain cell lines (29). Laminin fails to affect

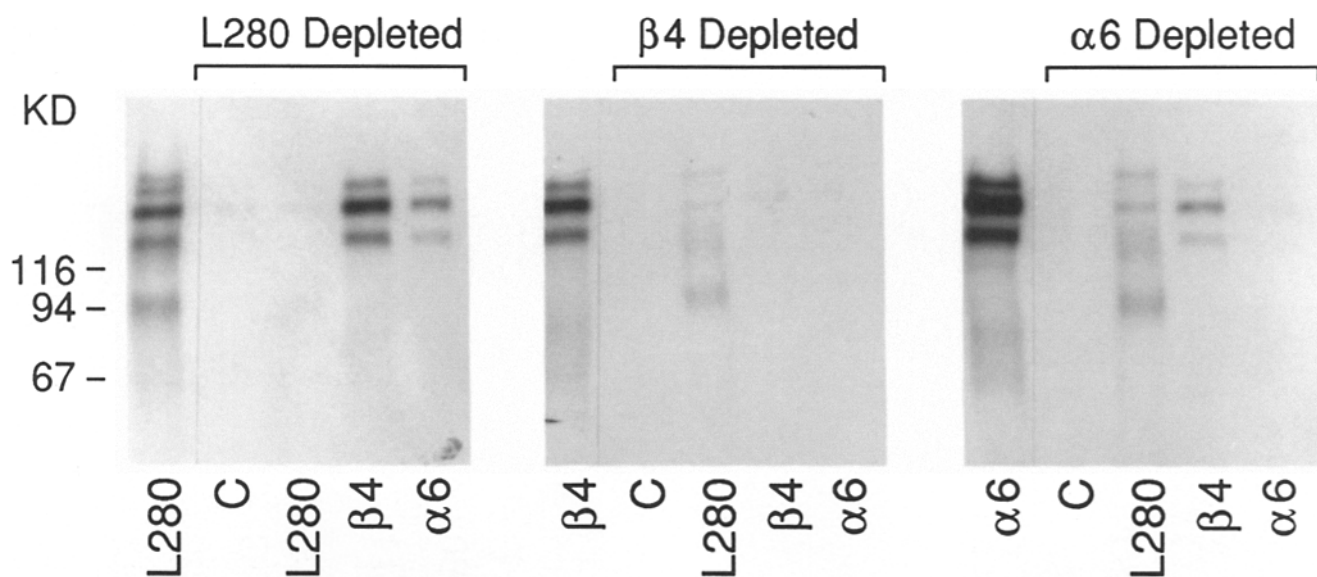


Figure 8. Sequential immunoprecipitation. Antigens were depleted from ^{125}I Colo-205 lysates by repeated immunoprecipitation with L280 mAb (*left*), anti- $\beta 4$ mAb (439-9B) (*center*) or anti- $\alpha 6$ mAb (J1-B5) (*right*). Depleted lysates were then immunoprecipitated with control Ig (C), L280 mAb, anti- $\beta 4$, or anti- $\alpha 6$, as indicated.

NK cell-mediated lysis of Colo-205 (Fig. 10) and L280 mAb did not prevent the binding of Colo-205 cells to laminin coated plates (J. Phillips, unpublished observation). Since many integrin molecules have been shown to recognize cellular ligands via an RGD peptide sequence (30), GRGDSP and an inac-

tive analogue GRGESP were assayed for inhibition of NK cytotoxicity against Colo-205, but were ineffective (Fig. 10).

L280 MAb Inhibition of Antigen-specific T Cell Cytotoxicity. The ability of L280 mAb to essentially completely inhibit NK cell-mediated cytotoxicity against Colo-205 indi-

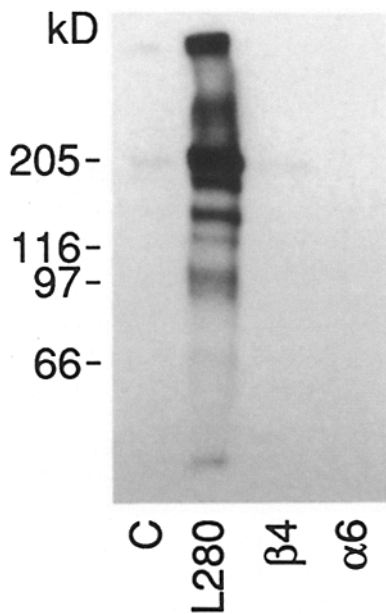


Figure 9. Effect of boiling on L280 mAb reactivity. ^{125}I -labeled Colo-205 lysates were placed in a boiling water bath for 5 min before immunoprecipitation with control Ig (C), L280 mAb, anti- $\beta 4$ (439-9B), or anti- $\alpha 6$ (J1-B5) mAb.

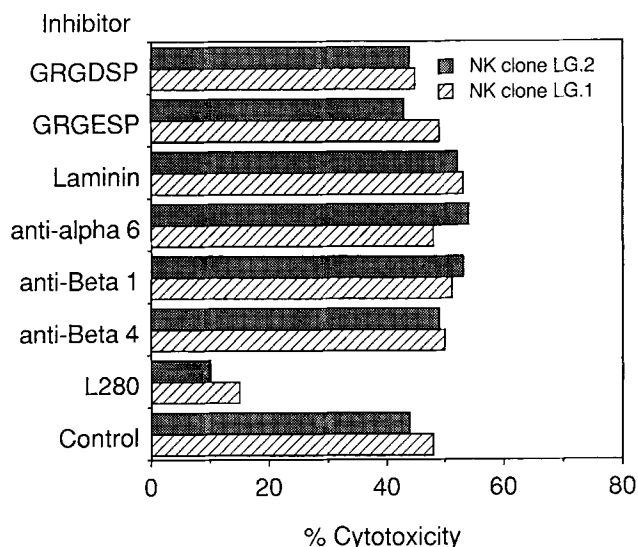


Figure 10. Effect of anti- $\beta 4$ and anti- $\alpha 6$ mAb on NK-cell mediated cytotoxicity. NK clones NKL.G.1 (▨) and NKL.G.2 (■) were assayed for cytotoxicity against Colo-205 cells in the presence of control Ig, L280 mAb, GRGDSP peptide, GRGESP control peptide, human laminin, and mAb against $\beta 1$ (A1IB2), $\beta 4$ (3E1), $\alpha 6$ (GoH3) E/T ratio was 3:1. For inhibition assays, human laminin, GRGDSP, and GRGESP were used at $100 \mu\text{g}/\text{ml}$ and mAb were used at $10 \mu\text{g}/\text{ml}$ or at saturating concentrations of hybridoma supernatant and were added to the targets 30 min before effector cells.

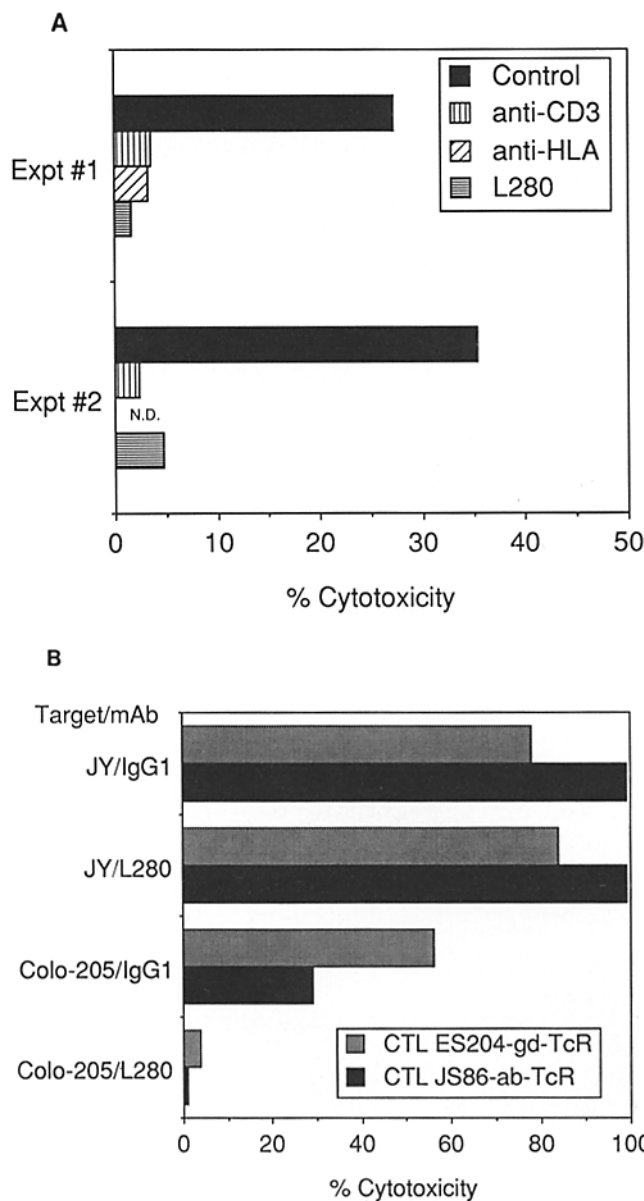


Figure 11. Effect of L280 mAb on alloantigen specific CTL. (A) CTL lines generated from mixed lymphocyte response cultures of PBMC with irradiated Colo-205 cells were assayed for cytotoxicity against ^{51}Cr -labeled Colo-205 cells in the presence or absence of control Ig (■), L280 mAb (▨), anti-HLA-A,B,C (▧), or anti-CD3 (▩). All mAbs were used at $5 \mu\text{g}/\text{ml}$. E/T ratio was 25:1. Two experiments are shown. (B) HLA-A2 alloantigen-specific CTL clones generated against an EBV-transformed B lymphoblastoid cell line were assayed for cytotoxicity against ^{51}Cr -labeled JY B lymphoblastoid cells and Colo-205 in the presence of control Ig or L280 mAb ($5 \mu\text{g}/\text{ml}$). JS86 (■) expresses an $\alpha\beta$ -TCR, whereas ES204 (▨) expresses a $\gamma\delta$ -TCR. E/T ratio was 5:1.

icates that the L280 antigen must interact with a receptor or cellular ligand present on the surface of NK cells. The "target-specific" receptors on NK cells are unknown; therefore, it is impossible to distinguish between the possibilities that the L280 antigen on Colo-205 serves as a "target-antigen" for NK cells or serves a more general role as an accessory cell molecule. Since the TcR is well characterized, we under-

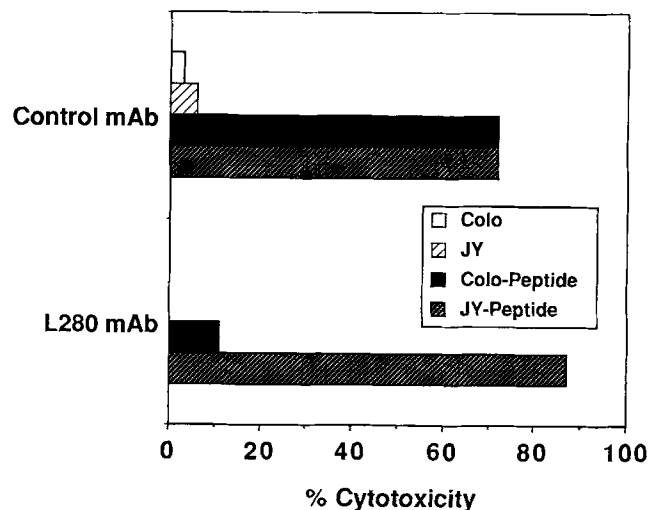


Figure 12. Effect of L280 mAb on influenza peptide-specific, HLA-A2 restricted CTL. Influenza peptide specific, HLA-A2 restricted CTL Q66.40 was assayed for cytotoxicity against ^{51}Cr -labeled JY B lymphoblastoid cells (▧, ▨) and Colo-205 cells (□, ■) that were preincubated in media (□, ▧) or media containing $20 \mu\text{M}$ influenza matrix peptide 59-68 (■, ▨). Control Ig or L280 mAb were added to the targets at $5 \mu\text{g}/\text{ml}$ 30 min before CTL effectors. E/T ratio was 10:1.

took experiments to determine whether L280 mAb affects specific immune interactions between T lymphocytes and Colo-205 cells.

CTL were generated against Colo-205 cells in a mixed lymphocyte/tumor cell culture. CTL lines were established that demonstrated cytotoxicity against Colo-205 (Fig. 11). Cytotoxicity mediated by these CTL cell lines was inhibited by anti-CD3 and anti-HLA-mAbs, demonstrating that the effector cells were CD3^+ CTL and likely recognized allogeneic HLA on the Colo-205 targets. L280 mAb efficiently blocked CTL-mediated cytotoxicity against Colo-205 targets. CD8^+ CTL clones established from these polyclonal cell lines also lysed Colo-205 cells and were blocked by anti-CD3, anti-HLA, anti-CD8 and L280 mAbs (not shown). These results clearly demonstrated that the antigen identified by L280 mAb participates in immune interactions between CTL and Colo-205. Since antigen-specific recognition is undoubtedly mediated by the TCR, these findings suggest that the L280 antigen serves an accessory role and that the receptor for L280 antigen is present on T lymphocytes in addition to NK cells.

While experiments using CTL generated against Colo-205 demonstrate that the L280 antigen can function in T cell interactions, it is possible that this is a rare event and that T cells capable of using this accessory molecule were selected in vitro during the MLR. Therefore, to obtain an unbiased assessment, CTL clones generated against B lymphoblastoid cell lines were tested for inhibition with L280 mAb (Fig. 11). Colo-205 expresses HLA-A2, therefore several well-characterized HLA-A2 alloantigen specific CTL clones were available for analysis. CTL clones JS86 (15) and ES204 (16) are HLA-A2 specific, and express $\alpha\beta$ -TCR and $\gamma\delta$ -TCR, respectively. These effectors killed Colo-205, as well as JY, an HLA-A2 bearing B lymphoblastoid cell line. Cytotoxicity

against Colo-205, but not JY, was essentially completely inhibited by L280 mAb. Since these alloantigen-specific CTL lines were selected and re-stimulated using B lymphoblastoid cells, there was no preselection for use of the L280 accessory cell molecule. Therefore, we conclude that the receptor for L280 antigen is expressed on CTL without deliberate induction or selection.

Since the physiological role of alloantigen recognition is unclear, further studies were undertaken to determine whether the L280 antigen is also involved in HLA-restricted, peptide-specific T cell recognition. CTL Q66.40 is a CD8⁺, $\alpha\beta$ -TCR⁺ T cell clone that specifically recognizes influenza virus matrix peptide 59-68 in conjunction with HLA-A2 (S. Verma, H. Spits, and R. de Waal Malefyt, manuscript in preparation). As shown in Fig. 12, CTL Q66.40 did not kill JY or Colo-205. However, strong cytolytic activity was observed against these targets after pre-incubated with specific influenza peptide. L280 mAb efficiently blocked HLA-A2 restricted, peptide-specific lysis of Colo-205, but not JY targets.

In summary, we have identified a novel accessory cell molecule that is involved in both NK and T cell-mediated cyto-

toxicity. Given the distribution of staining with L280 mAb, this molecule may participate in the interaction between lymphocytes and epithelial cells in numerous tissues and may be of relevance in CTL or NK cell-mediated immunity against virus-infected epithelial cells. Biochemical analysis suggests that the epitope recognized by L280 mAb is possibly carbohydrate in nature and is expressed on both the $\beta 4, \alpha 6$ integrin, as well as another previously unidentified glycoprotein, designated gp98. The L280 antigen may interact with an as yet unknown counter-receptor (or ligand) on the membrane of NK and $\alpha\beta$ -TCR⁺ and $\gamma\delta$ -TCR⁺ T cells. If the L280 antigen is a carbohydrate, its receptor on lymphocytes could conceivably possess lectin-binding domains, similar to the recently described lectin cell adhesion molecule family of cell adhesion molecules (31, 32). Alternatively, it is possible that while L280 mAb reacts with a carbohydrate epitope, the protein attached to this oligosaccharide may in fact be responsible for the interaction. Therefore, further studies are necessary to explore the role of $\beta 4, \alpha 6$ integrin in possible epithelial cell/lymphocyte interactions, as well as to elucidate the structure and function of the gp98 glycoprotein.

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