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A Novel Mechanism of Colon Carcinoma Cell Adhesion to the Endothelium Triggered by β_1 Integrin Chain*

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We have found a monoclonal antibody, called BV7, that rapidly stimulated by 6–10-folds HT-29 colon carcinoma cell adhesion to resting human umbilical vein endothelial cells. This effect was directed to tumor cells and not to endothelial cells and was cell-specific. BV7 was also active on the HCCP-2998 but did not change adhesion to endothelial cells of other tumor cells (MG63 osteosarcoma, A375 melanoma, MHCC-1410 and Lovo colon carcinoma) even if, by flow cytometry, this monoclonal antibody could bind to them. Additionally, BV7 effect was substratum-specific, since it did not increase but rather blocked HT-29 adhesion to matrix proteins. Immunoprecipitation analysis and binding to specific transfectants revealed that BV7 recognizes β_1-subunit of integrin receptors and antibody blocking experiments showed that $\alpha_2\beta_1$ antibodies were able to counteract BV7 effect on HT-29 adhesion to endothelial cells. In contrast, antibodies directed to other integrins or endothelial adhesive receptors (E- and P-selectin, VCAM-1, ICAM-1, ICAM-2) were ineffective. Induction of HT-29 adhesion to endothelial cells by BV7 was Fc- and protein synthesis-independent but required metabolically active cells. The presence of physiological concentrations of divalent cations and of cytoskeletal integrity was not needed. Comparative studies with eight different prototypic β_1 antibodies showed that five of them induced HT-29 adhesion to endothelial cells in a way unrelated to their ability to interfere with HT-29 adhesion to matrix proteins. Cross-blocking binding assays demonstrated that all the five antibodies recognized a topographically related epitope.

Taken together these results provide evidence that β_1 antibodies may trigger a novel pathway of HT-29 colon carcinoma adhesion to endothelial cells with different features in respect to other described mechanisms of tumor cell interaction with the endothelium.

In the metastatic process, tumor cells (TC)[1] first detach from the primary tumor, then enter the circulation, adhere to the microvascular endothelium, and finally extravasate and proliferate in the target organ (1). In this sequence of events, TC adhesion to endothelial cells (EC) plays a relevant role, and it is probably responsible for the organ specificity of metastatic dissemination (for review see Refs. 2–4). A wealth of experimental evidence indicates that specific recognition mechanisms exist between TC and the endothelium (1–4). It has been reported that upon activation by inflammatory cytokines (such as interleukin-1 (IL-1) and tumor necrosis factor), EC express adhesive molecules that promote TC adhesion to them (5, 6). *In vivo*, this process is accompanied by an increased number of experimental metastasis in animals treated with cytokines (7). Further studies have shown that TC interact with the same adhesive proteins that mediate attachment of leukocytes to the inflamed endothelium. This process occurs in a cell-specific way, for instance colon carcinoma cells, similar to granulocytes and monocytes, selectively bind E-selectin (6, 8, 9), whereas melanoma cells, similar to lymphocytes and monocytes, bind VCAM-1 through the integrin $\alpha_4\beta_1$ (10, 11).

In addition, TC are also able to recognize and bind to resting endothelium. This is particularly relevant since the metastatic process frequently occurs in the absence of overt inflammatory conditions. Most probably TC adhesion to resting EC follows mechanisms that are cell- and substratum-specific. For instance, an adhesion molecule (12) in pulmonary EC able to selectively promote adhesion and lung colony formation of B16 melanoma cells (but not of KLN-205 squamous carcinoma) has been described recently.

In general, little is known about the molecules responsible for TC adhesion to EC in resting conditions. In this study, we screened a series of monoclonal antibodies (mAbs) for their capacity to modify adhesion of colon carcinoma, osteosarcoma, and melanoma cell lines to human EC *in vitro*. Through the screening we found a mAb (BV7) that was able to markedly increase HT-29 cell adhesion to the endothelium. This effect was: (i) directed to TC and not to the endothelium; (ii) cell-specific, the antibody did not affect adhesion of other TC types tested; (iii) substratum-specific, BV7 did not increase but rather inhibited cell adhesion to matrix proteins. We found that BV7 recognizes integrin β_1 chain and that antibodies to α_2 and β_1 could counteract its effect on HT-29 cell adhesion to the endothelium. These data describe a novel activity triggered by β_1 integrin chain involved in heterotypic cell to cell adhesion.

EXPERIMENTAL PROCEDURES

Reagents—Chemical reagents were purchased from the following sources: bovine serum albumin (fraction V), pristane (2,6,10,14-tetra-

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[1] The abbreviations used are: TC, tumor cells; EC, endothelial cells; VCAM-1, vascular cell adhesion molecule-1; ICAM, intercellular cell adhesion molecule; NCS, newborn calf serum; CHO, Chinese hamster ovary; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DPBS, Dulbecco's PBS; EIPA, *N*-ethyl-*N*-isopropyl amiloride.

methylpentadecane), 2-deoxy-D-glucose, cycloheximide, tunicamycin, monensin, staurosporin, cytochalasin B, Tris (tris(hydroxymethyl)aminomethane), glucose oxidase type VII from *Aspergillus niger*, phenylmethylsulfonyl fluoride, heparin (sodium salt, grade I), L-cysteine, and leupeptin were from Sigma; α-iodoacetamide and lactoperoxidase were from Calbiochem; carrier free $Na^{125}I$ was from DuPont NEN; culture reagents were from Gibco (Gibco-Europe, Paisley, United Kingdom); tissue culture plates and flasks were obtained from Falcon (Becton Dickinson Labware, Lincoln Park, NJ); paraformaldehyde and sodium azide were from Merck (Merck Inc., Darmstadt, Germany); human serum albumin was purchased from Istituto Serologico Milanese (Milan, Italy); Chelex 100, Triton X-100, and all electrophoretic reagents were from Bio-Rad; protein A-Sepharose CL-4B, Sepharose G-100, and column PD10 were from Pharmacia (Pharmacia LKB Biotechnology, Uppsala, Sweden); [^{125}I]iododeoxyuridine was from Amersham (Amersham International, Buckinghamshire, United Kingdom); IODO-GEN was from Pierce Chemical Company; Trasylol was from Bayer (Bayer, Leverkusen, Germany); affinity-isolated rabbit immunoglobulins to mouse immunoglobulins were from Dakopatts (Glostrup, Denmark). EIPA (*N*-ethyl-*N*-isopropyl amiloride) was kindly provided by Dr. E. Cragoe. Human recombinant interleukin-1 β (specific activity 10^3 units/μg) was from Sclavo (Siena, Italy).

Cells and Cell Cultures—EC were isolated from umbilical vein and cultured as described previously (13). Cells were grown to confluence in medium 199 (M199) supplemented with 20% newborn calf serum (NCS), 50 μg/ml endothelial cell growth supplement (prepared from bovine brain), 100 μg/ml heparin (from bovine intestinal mucosa), 50 units/ml of penicillin, and 50 μg/ml of streptomycin. EC were used within three passages.

The following human TC lines were used, obtained from the American Type Culture Collection (Rockville, MD) and cultured as described previously (8, 14): HT-29 and Lovo colon carcinoma, MG63 osteosarcoma, and A375 melanoma. HCCP-2998 and MHCC-1410 colon carcinoma were provided by Dr. R. Giavazzi (Istituto Mario Negri, Bergamo, Italy) and grown as described (15). Human skin fibroblasts were isolated from skin biopsies and cultured as described (16).

CHO cells transfected with either the integrin β1 subunit or the vector pECE alone were kindly provided by Dr. G. Tarone (University of Torino, Torino, Italy). These cells were characterized previously in detail (17).

Antibodies and Proteins—The mAbs to integrin β_1 subunit were kindly donated by the following investigators: Lia 1/2, Lia 1/5 (18), and TS 2/16 (19) by Dr. F. Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain); AIIB2, by Dr. C.H. Damsky (University of California, San Francisco, CA) (20); JM 103 and P4C10 by Dr. M. J. Elices (Cytel Co., San Diego, CA) (21); MAR-4 by Dr. S. Menard (Istituto dei Tumori, Milano, Italy) (22); mAb K20 was from Immunotech (Marseille, France) (23).

The following mAbs against integrin α chains were used: anti-α_1 TS 2/7 kindly donated by Dr. M. E. Hemler (Dana-Farber Cancer Institute, Boston, MA) (19); anti-α_2 P1E6, anti-α_3 P1B5, and anti-α_5 P1D6 from Telios (Telios Pharmaceuticals Inc., San Diego, CA) (21); anti-α_6 GoH3, gift of Dr. A. Sonnenberg (The Netherlands Cancer Institute, Amsterdam, The Netherlands) (24); anti-α_4 HP 1/2 (25) kindly provided by Dr. F. Sanchez-Madrid; anti-α_2 5E8 obtained through the courtesy of Dr. R. B. Bankert (T and B Bioclone, Inc., Buffalo, NY) (26).

Polyclonal antisera directed to β_1 (27) and α_2 (28) cytoplasmic domains were provided by Dr. G. Tarone. A rabbit antiserum raised by injecting placenta purified $\alpha_5\beta_1$ was prepared in our laboratory (29), this antiserum is able to bind all β_1 integrins; a rabbit antiserum raised against placenta-purified $\alpha_v\beta_3$ was obtained from Telios.

mAbs directed to endothelial adhesive molecules were generously provided by the following investigators: 6.5B5 to ICAM-1 by Dr. D. O. Haskard (Hammersmith Hospital, London, United Kingdom) (30); 4B9 to VCAM-1 by Dr. J. M. Harlan (University of Washington, Seattle, WA) (31); RUU.SP.2.17 to P-selectin by Dr. M. J. Metzelaar (University Hospital, Utrecht, The Netherlands) (32); BBG-E6 to E-selectin and BBIG 7 to PECAM-1 by Dr. A. Gearing (British Biotechnology Products, Oxford, United Kingdom). 6D5 to ICAM-2 by Dr. C. G. Gahmberg (University of Helsinki, Finland) (33); HP2/9 to CD44 by F. Sanchez-Madrid (34).

The synthetic peptides Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) and Gly-Arg-Gly-Glu-Ser-Pro (GRGESP) were synthesized using an automated peptide synthesizer (model n 430, Applied Biosystems, Foster City, CA), as described (35).

Human plasma vitronectin and fibronectin were prepared as described previously (36); laminin was a gift of Dr. G. Taraboletti (Istituto Mario Negri, Bergamo, Italy) (37), and bovine collagen type I was purchased from Sigma.

Generation of mAb BV7—BALB/c mice were immunized with human umbilical vein EC. Hybridomas were produced by fusion of immunized mouse splenocytes with NS-0 cell line using standard techniques (38). Hybridoma supernatants were screened by standard enzyme-linked immunoassay for binding to EC (39). Positive hybridomas were then evaluated on adherence of TC lines to EC (see below). mAb BV7 was selected after the first screening, and the corresponding hybridoma was serially cloned twice by the method of limiting dilutions. mAb BV7 was determined to be IgG1 isotype using a mouse mAb isotyping kit (Sigma).

Ascites were produced by a standard technique (40). Briefly, BALB/c mice were primed with intraperitoneal injections of 0.5 ml of pristane 6 days before intraperitoneal injection of 6×10^6 BV7 hybridoma cells. Ascites was collected after 2–3 weeks. mAb BV7 was purified from ascites by binding to immobilized protein A (Pharmacia), as described (41). $F(ab')_2$ fragments were obtained from purified mAb BV7 by digestion with immobilized pepsin (ImmunoPure $F(ab')_2$ preparation Kit, Pierce) for 8 h (42). $F(ab')_2$ fragments were separated from Fc fragments and undigested IgG by binding to immobilized protein A and by dialyzing overnight using dialysis tubing with M_r cutoff of 50,000. The purity of the preparation was checked by flow cytometry analysis (see below) with Fc fragment-specific Ab (Sigma). Fab' fragments were prepared by reduction and alkylation of $F(ab')_2$ by a standard procedure described elsewhere (43). Briefly, $F(ab')_2$ were incubated with 0,01 M cysteine at 37 °C for 2 h and then incubated with 0,025 M iodoacetamide at room temperature for 30 min. After a separation in a Sephadex G-100 column, the purity of the preparation was tested by SDS-PAGE under nonreducing conditions.

Fluorescence Flow Cytometric Analysis—Fluorescence flow cytometric analysis was performed by an FACStar Plus* apparatus (Becton Dickinson & Co., Mountainview, CA). Cells were detached by incubation with 5 mM EGTA in phosphate-buffered saline (PBS). 200 μl of cell suspension (1.6×10^6/ml) in PBS supplemented with 2% human serum were incubated with mAbs to be tested, for 30 min at 4 °C. Cells were then washed twice in PBS, 2% human serum. The second incubation was carried out at 4 °C using fluorescein isothiocyanate-conjugated goat $F(ab')_2$ anti-mouse IgG (Technogenetics, New England Corp., Boston, MA) or, for the same experiments, fluorescein isothiocyanate-conjugated goat anti-mouse Fc fragment of IgG. After 30 min cells were washed three times in PBS, 2% human serum and then analyzed.

Adhesion Assay—EC, grown to confluence in 96-well plates (3×10^3 cells/well), were washed twice with fresh medium before refilling the wells with 50 μl of culture medium. [I^{125}]Iododeoxyuridine (1 μCi/ml for 18 h at 37 °C)-labeled TC were detached by incubation in 5 mM EGTA in PBS, washed twice in complete medium by centrifugation ($250 \times g$ for 5 min), and resuspended in M199, 20% NCS at 6×10^5 cells/ml as described previously (14). An aliquot (50 μl) of radiolabeled TC suspensions was added to each EC well and incubated for 30 min at 37 °C. Nonadherent TC were removed by washing plates three times with PBS, 2% NCS. The content of each well was solubilized with 50 μl of NaOH, 0.1% SDS and then the lysates counted in a ^{125}iodine γ counter (Beckman, Fullerton, CA). In some experiments, before the adhesion assay, radiolabeled TC suspensions were centrifuged and resuspended in 2 mM EDTA in PBS, maintained for 10 min, and then centrifuged again and resuspended in TBS treated with Chelex 100 supplemented or not with 1 mM Ca^{2+} and 1 mM Mg^{2+}.

mAb BV7 and other mAbs to β_1 integrin subunit were added to TC suspensions 20 min before the adhesion assay and then maintained for the duration of the experiment. In blocking studies (Fig. 8), HT-29 cells were pretreated with mAb BV7 for 20 min, washed, and then incubated with the antisera or anti-integrin subunit mAbs, which were maintained throughout the adhesion assay. mAbs directed to endothelial adhesive receptors were added to EC monolayers for 20 min and left for the adhesion assay.

Experiments of HT-29 adhesion to extracellular matrix components were performed as described (14). Briefly, 50 μl of purified protein solution in PBS of collagen (10 μg/ml), laminin (15 μg/ml), fibronectin (3 μg/ml), or vitronectin (3 μg/ml) were used for coating each single plate well overnight at 4 °C. Wells were then washed once with PBS and incubated with Dulbecco's PBS (DPBS) plus 1% BSA for 1 h at 37 °C in a humidified atmosphere. Plates were then washed once with DPBS before addition of 100 μl of labeled TC suspension. mAbs were added to TC 20 min before the adhesion assay and then maintained throughout the duration of the test.

Cell Radiolabeling and Immunoprecipitation—Cells grown to confluence in 10-cm^2 well plates were washed two times with cold DPBS. Cells were surface labeled using the glucose oxidase-lactoperoxidase method as described (16). Briefly, one well was incubated with 20 μg/ml lactoperoxidase, 300 μCi/ml ^{125}Iodine and 80 microunits/ml glucose oxidase

in 1.5 ml of 20 mM dextrose in DPBS for 30 min on ice. Reaction was stopped with DPBS, 0.02% NaN_3. The cell layer was then washed three times with DPBS-0.02 % NaN_3, two times with 0.1 M NaI in DPBS, and three times with DPBS. Radioiodinated samples were lysed by scraping with 0.5% Triton X-100 in 150 mM NaCl, 10 mM Tris, pH 7.4, containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 15 μg/ml leupeptin, 20 KIU/ml Trasylol) and spinning down insoluble material in a microcentrifuge for 20 min at 4 °C. Labeled cell lysates were immunoprecipitated with the following polyclonal Abs or mAbs: polyclonal antisera to α_2 cytoplasmatic (20 μl/ml), to β_1 cytoplasmatic (20 μl/ml), and nonimmune rabbit serum (40 μl/ml) were coupled to protein A-Sepharose CL-4B (30 μl); mAbs BV7 (600 μl/ml culture supernatant or 40 μg/ml purified IgG), P4C10 (600 μl/ml culture supernatant), and an irrelevant mAb (40 μg/ml purified IgG) were coupled to protein A-Sepharose CL-4B (30 μl) that was incubated previously with rabbit anti-mouse IgG (5 μg/ml). Immunoprecipitation was performed as described previously (29). Immunocomplexes were subjected to SDS-PAGE, and the electrophoresed gels were fixed, dried, and analyzed by autoradiography.

mAb Radiolabeling and Cross-competitive mAb Cell Binding Assay—BV7-purified IgG was labeled with $Na^{125}I$ by the IODO-GEN procedure (44) for 15 min at room temperature. The reaction was stopped with 1% KI and then the mixture was applied to a column PD10 to separate free $Na^{125}I$ from the iodinated protein (specific activity, 0.84 μCi/μg).

For cross-competitive mAb binding assays (Fig. 9), HT-29 cells grown to confluence in 96-well plates were preicubated with 4% human serum albumin in 100 μl of complete medium overnight at 37 °C. Then cell monolayers were washed with 0.02% NaN_3 in complete medium and preincubated with an excess of unlabeled mAbs in 200 μl (final dilutions: 1:5 of culture supernatant, 1:100 of ascites, or 10 μg/ml of purified mAb) which were maintained throughout the assay. After 30 min, trace amounts of ^{125}I-BV7 (300,000 cpm/well; specific activity, 0.84 μCi/μg) were added to each well and incubated for 30 min a 37 °C. Unbound mAbs were removed by washing plates three times with PBS, 2% NCS. Each well was solubilized and the lysates counted in a ^{125}iodine γ counter.

RESULTS

Development and Characterization of mAb BV7—In order to identify new molecules in EC able to modulate TC adhesion to resting endothelium, we immunized mice with cultured human umbilical vein EC. The hybridoma supernatants were then screened for their capacity to change the adhesion of three different TC lines (colon carcinoma HT-29, osteosarcoma MG63, and melanoma A375). One mAb called BV7, out of 119 mAbs tested, had a particularly remarkable effect: as reported in Fig. 1, *panel A*, it markedly increased basal adhesion of HT-29 colon carcinoma to the endothelium, leaving unchanged the adhesion of MG63 osteosarcoma and A375 melanoma cells. In 92 experiments performed during 1 year, basal HT29 adhesion ranged from 2 to 6% of total cell added (3×10^4 cells/well for 30 min at 37 °C) and adhesion increased to 22–33% when BV7 (1 μg/ml) was added during the assay. A series of colon carcinoma cell lines were further examined: mAb BV7 increased adhesion of HCCP-2998, although to a lesser extent than HT-29, whereas it did not affect adhesion of MHCC-1410 and Lovo cells (Fig. 1, *panel B*). By flow cytometry, mAb BV7 could bind HT29, MG63, A375, and EC (Fig. 2) and the colon carcinoma cell lines tested (in a typical experiment HT29 presented 97.6%; HCCP-2998, 98.3%; Lovo, 97.2%; and MHCC-1410, 85.55% cell-positive to BV7 with mean channel of fluorescence intensity on a linear scale: 653 for HT29, 475 for HCCP-2998, 316 for Lovo, and 143 for MHCC-1410).

Surprisingly the effect of the mAb was directed to TC and not to EC. HT-29 pretreatment with mAb BV7, followed by washing, increased adhesion to an extent comparable with that observed when the mAb was present during the adhesion assay; in contrast, EC pretreatment was ineffective (Fig. 3, *panel A*). The effect of BV7 occurred rapidly and was not reversible: when HT-29 were preincubated with BV7 (1 μg/ml) for different times (5–60 min), the maximal effect on adhesion was observed at 10 min, with no further enhancement or decrease up to 60 min

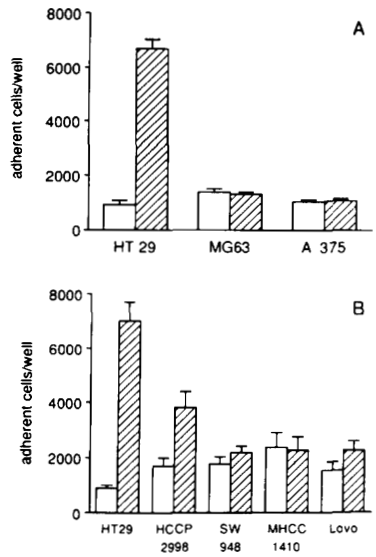

FIG. 1. *Panel A*, mAb BV7 increases adhesion of HT-29 colon carcinoma, but not of MG63 osteosarcoma or A375 melanoma cells, to EC. *Panel B*, mAb BV7 increases adhesion of HCCP-2998, but not of other colon carcinoma cell lines (MHCC-1410, Lovo). Suspensions (6×10^5/ml) of TC were preincubated for 20 min at 37 °C with either control buffer (*open bars*) or mAb BV7 (1 μg/ml; *dashed bars*). Cells were then layered onto EC monolayers and adhesion performed as described under "Experimental Procedures." Data are expressed as number of adherent cells/well and are means ± S.D. of three different experiments each performed in six replicates.

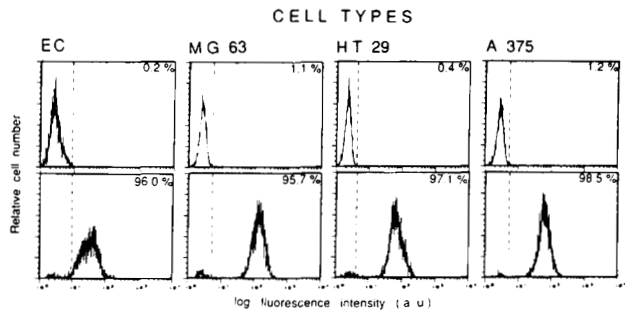

FIG. 2. **Fluorescence flow cytometric analysis of BV7 antigen expression on different cell types: EC, MG63, osteosarcoma, HT-29 colon carcinoma, and A375 melanoma.** The *upper panels* represent cell staining with an irrelevant antibody. Data were collected in a logarithmic scale in arbitrary units, and the numbers inside the panels represent the percentage of positive cells to staining with the antibody.

(Fig. 3, *panel B*).

The effect of BV7 was not specific for HT-29 adhesion to EC. Addition of 1 μg/ml of the mAb to HT-29 (6×10^5/ml) for 20 min at 37 °C caused a 3-fold increase in their adhesion to confluent human skin fibroblasts (basal adhesion after 30 min at 37 °C in absence of BV7 was 2945 ± 492 cells/well, whereas in the presence of BV7, it was 8577 ± 1321 cells/well, mean ± S.D. of five replicates from a typical experiment out of two performed).

mAb BV7 Is Directed to β_1 Integrin Chain—In order to characterize BV7 antigen, we performed immunoprecipitation analysis of detergent lysates of ^{125}I-labeled HT-29, EC, and A375 cells. As reported in Fig. 4, in all three cell types, mAb BV7 immunoprecipitated two bands of apparent M_r 165,000 and 135,000, respectively, under reduction. A similar pattern was obtained when the same cell extracts were immunoprecipitated by either a β_1 or an α_2 Ab, thus suggesting that BV7 could interact with either β_1 or α_2 integrin chains. To directly investigate this possibility, ^{125}I-labeled HT-29 and EC lysates were precleared by sequential immunoprecipitations with α_2 (Fig. 5) and then precipitated with mAb BV7. After sequential immu-

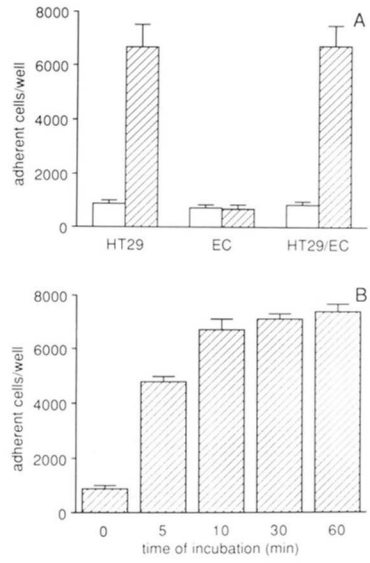

FIG. 3. *Panel A*, the effect of mAb BV7 is directed to HT-29 cells. HT-29 suspensions (*HT29*) or EC monolayers (*EC*) were preincubated for 20 min at 37 °C with either control buffer (*open bars*) or mAb BV7 (μg/ml; *dashed bars*). Preincubation was then followed by three PBS washes and then HT-29 adhesion to EC was performed as described under "Experimental Procedures." In coincubation experiments, mAb BV7 was added to TC suspension and maintained throughout the assay (*HT29/EC*). *Panel B*, the effect of BV7 occurred rapidly and is maintained up to 6O min. HT-29 was preincubated at 37 °C with either control buffer (*open bars*) or mAb BV7 (μg/ml) for different times (5–60 min; *dashed bars*). Cells were then washed once, HT-29 were layered onto EC monolayers, and adhesion performed as described under "Experimental Procedures." Data are expressed as number of adherent cells/well and are means ± S.D. of three different experiments each performed in six replicates.

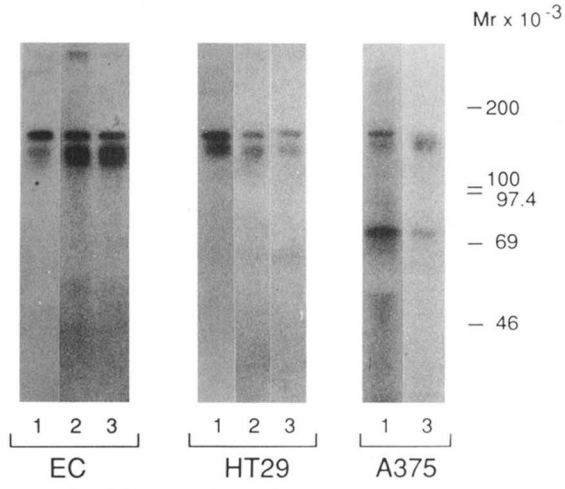

FIG. 4. **mAb BV7 precipitates bands that comigrate with bands precipitated by anti-β_1 and -α_2 Abs.** Detergent lysates of ^{125}I-radiolabeled EC, HT-29, and A375 (150,000 cpm of trichloroacetic acid-precipitable material) were immunoprecipitated by rabbit antisera to the cytoplasmic domain of α_2 (*lane 1*) and β_1 chain (*lane 2*) and by mAb BV7 (*lane 3*). Immunocomplexes were run in a 7.5% SDS-PAGE gel under reducing conditions and revealed by autoradiography.

noprecipitation with anti-α_2 Ab, mAb BV7 could not immunoprecipitate the band at 165,000 but only a single band of M_r 135,000 (Fig. 5) which could correspond to β_1 chain comigrating with other α chains such as α_5 and α_3. Since these data strongly suggested that BV7 recognizes the β_1 integrin chain, to get a direct evidence of this possibility, we analyzed BV7 binding to human β_1-transfected CHO cells. As shown in Fig. 6, by flow cytometry mAb BV7 selectively recognized β_1 and not control CHO transfectants, indicating that this mAb was indeed directed to this integrin subunit.

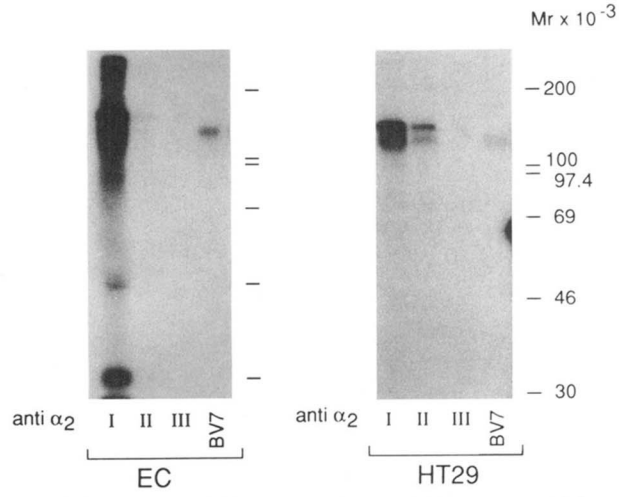

FIG. 5. **Immunodepletion of $\alpha_2\beta_1$ integrin by sequential immunoprecipitation of ^{125}I-labeled EC and HT-29 cell membrane extracts.** 300,000 cpm of EC and 200,000 cpm of HT-29 trichloroacetic acid-precipitable material were precleared by three sequential immunoprecipitations (*lanes I, II,* and *III*) with α_2 cytoplasmatic domain antiserum. The remaining supernatants were then immunoprecipitated a fourth time with mAb BV7. The obtained immunocomplexes were fractionated by 7.5% SDS-PAGE under reducing conditions.

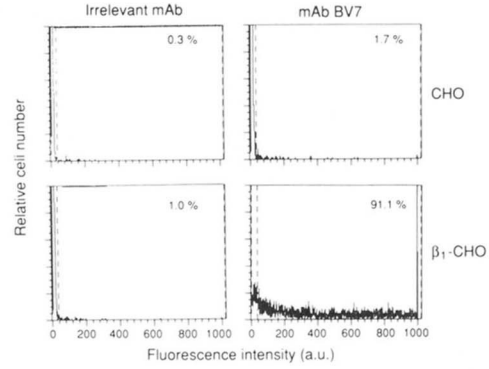

FIG. 6. **mAb BV7 binds β_1-CHO-transfected cells.** Fluorescence flow cytometric analysis of control and β_1-CHO-transfected cells were performed with an irrelevant antibody (*left panels*) or mAb BV7 (*right panels*). Data were collected in a linear scale in arbitrary units, and the number inside the panels represents positive cells stained with the antibody.

mAb BV7 Inhibits Cell Adhesion to Extracellular Matrix Proteins—The increase in adhesion of HT-29 induced by mAb BV7 was substratum-specific. HT-29 adhesion to collagen, laminin, and fibronectin (but not vitronectin) was rather reduced by mAb BV7 (Fig. 7, *panel A*). This effect was concentration-dependent (Fig. 7, *panel B*). The range of concentrations of the antibody (1–10 μg/ml) able to maximally increase adhesion to the endothelium maximally inhibited cell adhesion to collagen.

The effect of mAb BV7 on HT-29 adhesion to the endothelium requires active cell metabolism and is ion-independent. As reported in Table I, the effect of BV7 on HT-29 adhesion to EC was blocked by cell chilling at 4 °C, by cell fixation with paraformaldehyde, and by a combination of 2-deoxy-D-glucose and sodium azide, which inhibits glucose metabolism and electron transport (45).

In contrast, the effect of BV7 did not require protein synthesis, glycosylation, or secretion, since adhesion was unaffected by treatment with cycloheximide, tunicamycin, or monensin (Table I). The three drugs were used at concentrations described previously as effective for this cell line (46). Cytochalasin B treatment of HT-29 also left BV7 enhancement of adhesion unchanged. The effect of cytochalasin B on blocking HT-29 actin organization was controlled by immunofluorescence mi-

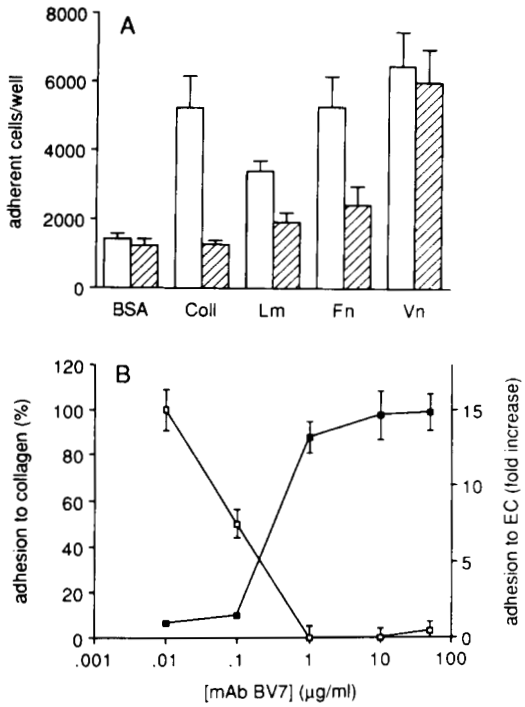

FIG. 7. *Panel A*, mAb BV7 inhibits HT-29 adhesion to purified extracellular matrix proteins. HT-29 suspensions (3×10^5/ml) were preincubated for 20 min at 37 °C with either control buffer (*open bars*) or mAb BV7 (1 μg/ml; *dashed bars*). Cells were then layered onto 96-well plates coated with bovine serum albumin (*BSA*; 1%), collagen type I (*Coll*; 10 μg/ml), laminin (*Lm*; 15 μg/ml), fibronectin (*Fn*; 3 μg/ml) or vitronectin (*Vn*; 3 μg/ml). Adhesion was performed as described under "Experimental Procedures." Data are means ± S.D. of three different experiments each performed in four replicates. *Panel B*, dose dependence of mAb BV7 effect on HT-29 adhesion to EC (■) and to collagen (□). HT-29 suspensions were preincubated for 20 min at 37 °C with different concentrations of mAb BV7. Cells were then washed once, HT-29 were layered either onto EC monolayers or plates coated with collagen type I (10 mg/ml), and adhesion performed as described. Data for adhesion to collagen are expressed as percent of adhesion in absence of the antibody (subtracted with adhesion to BSA), and data for adhesion to EC are expressed as -fold increase in respect to adhesion in the absence of the antibody. In both cases data are means ± S.D. of three different experiments each performed in six replicates.

TABLE I

Functional characteristics of mAb BV7-induced adhesion to EC

HT-29 colon carcinoma cells were pretreated as indicated and then exposed to either control buffer or mAb BV7 (1 μg/ml) for 20 min. Cells were then layered on EC monolayers for the adhesion assay. HT-29 pretreatment was carried out for 30 min (or for 15 min on ice for treatment with paraformaldehyde or for 16 h for treatment with tunicamycin) at 37 °C. Different treatments did not affect cellular viability as assessed by trypan blue staining. Data are expressed as number of adherent HT-29/well and are means ± S.D. of six replicates from a representative experiment (out of six performed).

HT-29 pretreatment	Basal adhesion	BV7-stimulated adhesion
None	1505 ± 780	8695 ± 529
4 °C	141 ± 9	183 ± 25
Paraformaldehyde (3%)	231 ± 38	307 ± 89
Deoxy-D-glucose (50 mM) + NaN_3 (0.3%)	31 ± 23	242 ± 447
Cycloheximide (10 μg/ml)	1259 ± 186	7973 ± 934
Tunicamycin (10 μM)	539 ± 44	6088 ± 996
Monensin (1 μM)	1631 ± 54	7228 ± 979
Cytochalasin B (20 μM)	1236 ± 173	7982 ± 957
EIPA (50 μM)	1272 ± 88	8907 ± 909
Staurosporine (100 nM)	1410 ± 134	9103 ± 1376

croscopy seeding the cells on coverslips in the presence of 20% NCS for 2 h and then fixing and staining them with fluorescein-tagged falloidin as described previously (35, 36). Inhibition of

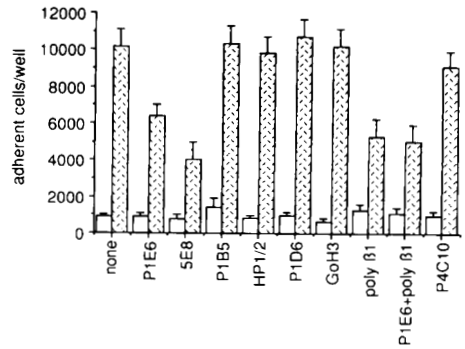

FIG. 8. **mAb BV7-induced HT-29 adhesion to EC is partly mediated by $\alpha_2\beta_1$ integrin.** Suspensions (6×10^5/ml) of HT-29 colon carcinoma cells were preincubated for 20 min at 37 °C with either control buffer (*open bars*) or mAb BV7 (1 μg/ml; *dotted bars*). Cells were then exposed for 20 min at 37 °C to the following mAbs: P1E6 and 5E8 (anti-α_2), P1B5 (anti-α_3), P1D6 (anti-α_5), GoH3 (anti-α_6), P4C10 (anti-β_1), or to the $\alpha_5\beta_1$ antiserum (poly-β_1). mAbs P1E6, 5E8, P1B5, and P1D6 were in the form of purified IgG and were used at 1 μg/ml; mAbs GoH3 and P4C10 were in the form of culture supernatant and used at 1:10 dilution; the antiserum was used at 1:100 dilution. The concentrations of the antibodies used were found to give maximal biological activity in preliminary experiments and in previously published work (see pertinent references under "Experimental Procedures"). Data are expressed as number of adherent cells/well and are means ± S.D. of three different experiments each performed in six replicates.

either Na^+/H^+ antiporter by high concentrations of EIPA, or protein kinase C by staurosporin, was also ineffective (45).

BV7-induced HT-29 adhesion to EC did not require a physiological concentration of divalent cations. HT-29 suspensions were washed with 2 mM EDTA in PBS and resuspended in Tris-buffered saline (pretreated with Chelex 100) supplemented or not with cations. HT-29 adhesion to EC in the presence of BV7 (1 μg/ml) was comparable in cation-depleted buffer, in the presence of 1 mM $CaCl_2$ or $MgCl_2$, or the combination of both. In contrast, as a positive control, adhesion of HT-29 cells to collagen was blocked (79.5% inhibition) in the absence of divalent cations (not shown).

Serum (NCS, 20%) was routinely present during the adhesion assay. However, when it was omitted (cells were resuspended in M199, 2% BSA) the effect of BV7 was unchanged (in a typical experiment out of three performed, BV7 (1 μg/ml) induced a 7.6- and 6.9-fold increase in adhesion with or without serum, respectively).

Finally BV7-mediated adhesion was not mediated by Fc receptors in EC, since $F(ab')_2$ and F(ab') fragments of the antibody were equally effective (8.2- and 7.9-fold increase in adhesion, respectively, in a representative experiment out of three performed).

BV7-induced HT-29 Adhesion to EC Is Inhibited by $\alpha_2\beta_1$ Antibodies—Flow cytometry analysis indicated that HT-29 cells express the integrin subunits β_1, α_1, α_2, α_3, and α_6 (>98% positive cells), but not α_4 and α_5 (4.8 and 0.5% positive cells, respectively). As reported in Fig. 8, BV7-induced HT-29 adhesion to EC was reduced only by the anti-α_2 mAbs P1E6 and 5E8, whereas anti-α_3 (P1B5), anti-α_4 (HP1/2), anti-α_5 (P1D6), anti-α_6 (GoH3), and anti-β_1 (P4C10) mAbs were ineffective.

A goat polyclonal antiserum to human β_1 (used at 1:100 dilution) (29), inhibited HT-29 adhesion induced by BV7, whereas neither a preimmune goat serum nor an $\alpha_v\beta_3$ rabbit antiserum (both used at 1:100 dilution) modified this parameter (not shown). The combination of α_2 mAb P1E6 and β_1 antiserum was not more effective than the antibodies alone.

In the experiments reported in Fig. 8, the inhibitory antibodies were added after cell preincubation with BV7 and left during the adhesion assay (see legend to the figure). In three experiments the antibodies were added to the cells (20 min at

TABLE II
Effect of anti-β_1 mAbs on HT-29 adhesion to EC and extracellular matrix proteins

Suspensions (6×10^5/ml) of HT-29 colon carcinoma cells were preincubated for 20 min at 37 °C with the indicated mAbs (1:10 final dilution, as hybridoma culture supernatant). The concentrations of the antibodies used were found to give maximal inhibition in preliminary experiments and in previously published work (see pertinent references under "Experimental Procedures"). Cells were then layered on EC monolayers or on wells coated with either collagen (10 μg/ml) or laminin (15 μg/ml). Adhesion assay was performed as described under "Experimental Procedures." Data are expressed as number of adherent HT-29/well and are mean ± S.D. of six replicates from a representative experiment (out of four performed).

mAb	Adhesion to		
	EC	Collagen	Laminin
	1127 ± 91	6946 ± 867	2094 ± 79
BV7	8928 ± 382	1687 ± 165	1203 ± 267
Lia 1/2	5640 ± 917	3024 ± 251	1324 ± 114
AIIB2	4729 ± 613	2911 ± 994	1628 ± 286
Lia 1/5	6438 ± 997	7225 ± 496	2215 ± 351
JM 103	8980 ± 800	17913 ± 1767	3409 ± 593
TS 2/16	3473 ± 737	28330 ± 2421	23467 ± 1776
P4C10	1255 ± 217	2946 ± 369	1072 ± 222
Mar-4	1093 ± 186	6932 ± 949	1925 ± 293
K20	1236 ± 260		

37 °C) before BV7 incubation (20 min at 37 °C) with comparable results (not shown).

When HT-29 were pretreated with α_2 mAb P1E6 (5 μg/ml, 20 min at 37 °C), followed by washing, inhibition of BV7-stimulated adhesion was comparable with that observed when the mAb was present during the adhesion assay. In contrast, EC pretreatment with P1E6, followed by washing, was ineffective (not shown).

Incubation of HT-29 cells with BV7 did not modify the expression of α_2 chain. Cells preincubated with $F(ab')_2$ BV7 for 30 min at 37 °C were then incubated with α_2 mAb P1E6 5 μg/ml for 30 min and analyzed by flow cytometry. No difference in the number of positive cells (99%), and fluorescence intensities were observed comparing cells preincubated or not with BV7 (not shown).

GRGDSP and GRGESP peptides up to a concentration of 400 μM were ineffective on HT-29 (6×10^5 cells/ml for 20 min at 37 °C) basal and BV7-stimulated adhesion to EC (not shown).

The Activity of BV7 in Promoting HT-29 Adhesion to EC Is Shared by Other β_1 Antibodies—The effect of BV7 on HT-29 adhesion to EC and to matrix proteins was compared with that of other β_1 antibodies. As reported in Table II, five out of eight β_1 antibodies (Lia 1/2, Lia 1/5, AIIB2, JM 103, and TS 2/16) were able to significantly increase HT-29 adhesion to EC. This activity was unrelated to the effect on cell adhesion to collagen or laminin since two mAbs (Lia 1/2 and AIIB2) were inhibitory, two (JM 103 and TS 2/16) increased, and one (Lia 1/5) was ineffective. Of the other three mAbs tested (inactive on HT-29 adhesion to EC), one (Mar-4) was also inactive on cell adhesion to collagen and laminin, whereas one (P4C10) inhibited cell adhesion to these proteins.

We have then compared the epitope recognized by BV7 with that bound by the other β_1 antibodies studied by cross-blocking cell binding assays. As reported in Fig. 9, the five antibodies able to increase HT-29 adhesion to EC were also able to compete with BV7 binding, thus indicating that they bind to a topographically related epitope.

BV7-induced HT-29 Adhesion Is Not Mediated by Known EC Adhesion Receptors—We used a series of mAbs directed to different EC molecules known to promote leukocyte and TC adhesion (for review see Refs. 4 and 47): ICAM-1 (6.5B5), VCAM-1 (4B9), P-selectin (RUU.SP.2.17), E-selectin (BBG-E6), ICAM-2 (6B5), PECAM-1 (BBA 7), and CD44 (HP 2/9). None of these

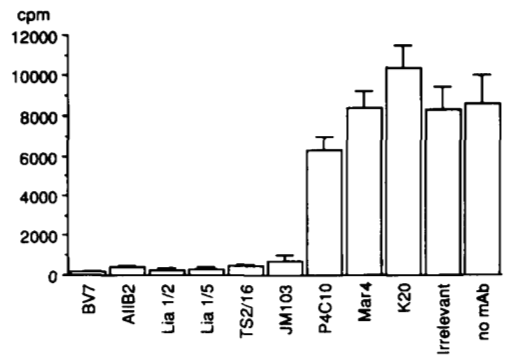

FIG. 9. **Effect of preincubation with different β_1 mAbs on BV7 binding to HT-29 cells.** ^{125}I-Labeled purified BV7 IgG was added (for 20 min a 37 °C) to HT-29 cells after cell preincubation with an excess of the indicated mAbs (for 20 min a 37 °C). Abs AIIB2, Lia 1/2, Lia 1/5, TS 2/16, JM 103, and P4C10 were in form of culture supernatants and were used at 1:5 final dilution; mAbs Mar-4 and K20 were in form ascitic fluid and were used at 1/100 dilution. Data are expressed as counts/min bound per well and are means ± S.D. of five different experiments each performed in six replicates.

mAbs, at blocking concentrations, affected mAb BV7-induced adhesion to EC when added during the adhesion assay.

When EC were activated by IL-1β (20 units/ml for 6 h) a 230 ± 30% increase in HT-29 adhesion was observed, as described previously (7). BV7 (1 μg/ml), preincubated with HT-29 for 20 min and then maintained during the adhesion assay, had only an additive effect (it caused a 437 ± 39% increase in adhesion to control EC and a 667 ± 72% increase to IL-1β-treated EC, means ± S.D. of five replicates from a typical experiment out of three performed).

DISCUSSION

In this paper we describe a novel mechanism of colon carcinoma cell adhesion to human EC triggered by a β_1 integrin antibody (BV7). The effect of BV7 mAb has specific features: 1) it is substratum-specific. BV7 markedly increased adhesion of HT-29 cells only to cultured EC, whereas it was inhibitory on HT-29 adhesion to matrix proteins (such as collagen, laminin, and fibronectin). This distinguishes BV7 from other described β_1 activating mAbs such as TS2/16 (45, 48), 8A2 (49, 50), and A/1A5 (45, 51). These mAbs have been shown to stimulate leukocyte adhesion to VCAM-1, but they were also effective in inducing cell adhesion to matrix. These effects were mediated by a general activation of β_1 integrins on the cells ($\alpha_4\beta_1$ for adhesion to endothelial VCAM-1 and $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_2\beta_1$ for adhesion to matrix proteins); 2) it is inhibited by $\alpha_2\beta_1$ antibodies but not by other α chain blocking mAbs; 3) it is cell-specific. Among six different TC lines tested, BV7 only increased adhesion to EC of two colon carcinoma lines (HT-29 and HCCP-2998), leaving unchanged adhesion of an osteosarcoma, melanoma, and two other colon carcinoma cells; 4) it is ion independent; 5) it is not mediated by the previously described endothelial adhesive molecules for leukocytes and TC (such as E- and P-selectins, VCAM-1, ICAM-1, and ICAM-2).

The contrasting effect of BV7 on cell adhesion to EC or to matrix proteins suggests that these β_1 activities are mediated by two closely related but different epitopes. BV7 might induce a change in the β_1 molecule that causes increase in affinity for the EC ligand while blocking its interaction with matrix. Five other mAbs (out of eight tested) directed to β_1 chain were able to increase HT-29 adhesion to EC. These antibodies could compete for BV7 binding to the cells, thus indicating that they bind to a closely related epitope. Interestingly these mAbs have disparate effect on HT-29 adhesion to matrix proteins (two being inhibitors, one increasing, and one being ineffective). A

recent report (52) identified a small region in the β_1 integrin subunit critical for the binding of both inhibiting and activating mAbs to β_1. This region most likely corresponds to the predicted β turn region connecting two putative ligand binding sites of β_1. The authors suggested that activating and inhibiting mAbs by binding to this flexible region can induce change in the conformation/availability of the two lignad binding domains and in this way activate or inactivate β_1 function. Interestingly two of the antibodies (AIIB2 and TS2/16) able to compete for BV7 binding were found to bind to this region. Indirectly, this suggest that BV7 too bind two a closely located epitope. Other mAbs directed to different integrin subunits may elicit both inductive and inhibitory effects on cell adhesive functions. For instance some mAbs directed to integrin α_4 induce homotypic aggregation of Ramos B cells but inhibit cell binding to both fibronectin and VCAM-1 (53). mAb P1B5 directed to $\alpha_3\beta_1$ induced homotypic aggregation of epidermal cells in suspension but inhibited cell adhesion to the matrix component epiligrin (54). An anti-chicken β_1 mAb TASC promotes retinal neuron adhesion to laminin and collagen, but inhibits β_1-dependent adhesion to vitronectin (55).

The inhibitory activity of $\alpha_2\beta_1$ antibodies (but not of the other integrin antibodies tested) on BV7-induced increase in HT-29 adhesion to EC strongly suggests that this effect is mediated, at least up to 60%, by this molecule.

Other reports showed that $\alpha_2\beta_1$ integrin may play a role in cell-to-cell adhesion. Anti β_1-induced homotypic aggregation is much more evident in α_2-transfected K562 cells than in parental cells (18). In cultured keratinocytes and EC, $\alpha_2\beta_1$ integrins are localized in areas of cell-cell contacts, where they contribute to the maintenance of intercellular contacts along with desmosomes and adherens-type junctions (21, 35, 56, 57). Interestingly, the role of β_1 integrins in keratinocyte cell-cell adhesion can be separated from their role in cell-matrix interactions, since intercellular adhesion can be inhibited without affecting cell spreading on extracellular matrix (56).

Symington *et al.* (54) reported that homotypic aggregation of keratinocytes is mediated by $\alpha_2\beta_1$ interaction with $\alpha_3\beta_1$. We cannot exclude that HT-29 adhesion to EC is mediated by an homophilic interaction between $\alpha_2\beta_1$ molecules present in the two cell types or through an $\alpha_2\beta_1$-$\alpha_3\beta_1$ type of recognition. However these possibilities seem unlikely, since selective incubation of EC (in contrast to HT-29) β_1 antibodies does not inhibit adhesion and since $\alpha_3\beta_1$ and other anti-α blocking mAbs were ineffective, even if present during the adhesion assay. The presence of other still unknown counter receptors for $\alpha_2\beta_1$ has been hypothesized in other cell types (18).

Other authors observed that α_4 (58) and β_1 (18, 59) antibodies could induce homotypic aggregation of T-lymphocytes and related leukemic cell lines. BV7 does not induce homotypic aggregation of HT-29 (at least in the time frame of the adhesion experiments performed in this study),[2] this effect therefore does not contribute to HT-29 increased adhesion to EC.

The cell specificity of BV7 effect on HT-29 adhesion to EC is quite intriguing. Comparison of BV7 binding to different colon carcinoma cells showed that there was some correlation between mAb binding efficiency (as indicated by the mean fluorescence intensity) and its capacity to increase cell adhesion to EC. However this was not the case comparing BV7 binding to HT29 and to MG63 and A375 (see Fig. 2). The difference in BV7 effect was not even due to a different expression of α_2 chain, since by flow cytometry MG63 and A375 express amounts of this molecule comparable with HT-29 (not shown). These data underline the cell specificity observed by other authors on the capacity of antibodies to induce cell activation. Caixa *et al.* (59) and Campanero *et al.* (58) have found that different cell lines have a different susceptibility to intercellular aggregation induced by integrin antibodies independently from the amount of the specific integrin expressed. In addition, it has been described that $\alpha_2\beta_1$ express multiple ligand binding specificity in different cell types (60, 61). Transfection of a single α_2 cDNA clone in different cells leads to different functional forms of the molecule (51). Similarly, transfection of α_3 cDNA in RD and K562 cells leads to different functional phenotypes (62), and anti-$\alpha_3\beta_1$ antibodies had a different effect, they were able to induce homotypic aggregation of RD but not of K5632 cells.

Overall these reports indicate that there are cell-specific differences in integrin function and susceptibility to activation by mAbs. One possible explanation is that the conformation and flexibility of the molecule might vary in the different cell types in function of small changes in the structure or in the membrane microenvironment in which the integrin is located. We have shown previously that membrane phospholipid composition could markedly change the ligand specificity of $\alpha_v\beta_3$ (63), a lipid molecule, and chondroitin sulfate proteoglycan appeared to modulate $\alpha_M\beta_2$ (64) β_1 (65) function, respectively.

An alternative explanation is that HT-29 cells express a β_1 chain variant, thus being responsible for cell specific activation. Two alternative spliced β_1 variants have been described (17, 66). Immunoprecipitation analysis of HT-29 and A375 membrane extracts with rabbit antisera specifically directed to these two β_1 variants, however, showed that these cells do not express detectable amounts of these molecules.[3]

We have put some effort in trying to clarify the mechanism whereby BV7 up-regulates adhesion of HT-29 to EC. The most surprising observation is that BV7 does not require physiological concentrations of ions or cytoskeletal integrity for inducing HT-29 adhesion to EC, whereas, as expected, millimolar concentrations of ions are need for HT-29 binding to collagen. Other integrins can trigger cell adhesion phenomena regardless of the presence of ions. Weitzman *et al.* (62) reported that α_3 antibodies were able to induce homotypic cell aggregation in presence of EDTA. In addition, adhesion of $\alpha_v\beta_5$ to the HIV tat protein and to vitronectin basic domains was ion-independent (67). Overall these data indicate that the same integrin can express ion-dependent and -independent binding activities.

We feel to exclude other nonspecific adhesion mechanisms. BV7 effect is not mediated by cell agglutination, since it requires active metabolism and is blocked at 4 °C. It is not even due to binding to Fc receptor, since it is unchanged using $F(ab')_2$ fragments of the antibody. Finally it cannot be mediated by bridging/cross-linking of β_1 molecules in EC and HT-29, since: 1) BV7 does not increase adhesion of other cell lines, even if can efficiently bind to them; 2) preincubation of BV7 with EC only does not change adhesion of TC; 3) antibodies of the same Ig isotype than BV7 (for instance, mAb Mar-4 and P4C10) do not increase HT-29 adhesion to EC and finally; and 4) Fab fragments of BV7 retain the biological activity.

It is possible that BV7 activity is mediated by triggering of intracellular signals as it has been described for other β_1 mAbs (68–70). BV7 effect is indeed energy-dependent and is completely lost in fixed cells that presumably lack any intracellular signaling mechanisms. However BV7-mediated cell adhesion is independent of either protein kinase C activation or Na^+/H^+ antiporter integrity. mAb BV7 effect does not require *de novo* protein synthesis and release, since it follows a very rapid kinetics and is unaffected by cycloheximide and monensin. In addition, we were unable to detect any increase in intracellular Ca^{2+} after HT-29 activation with the antibody.[4]

[2] I. Martìn-Padura, unpublished observation.

[3] A. Zanetti, unpublished observation.

[4] I. Martìn-Padura, G. Bazzoni, A. Zanetti, S. Bernasconi, M. J. Eli-

All these data suggest that the effect of mAb BV7 might be due to a conformational change of the receptor induced by its binding to β_1. This change might possibly induce modifications of the associated α chain, leading to an increased affinity of the receptor for the putative endothelial ligand. A similar mechanism has been proposed for the proaggregatory effect of mAbs to β_1, β_2, and β_3 integrins in leukocytes and platelets (71–73). Conformational changes of integrins due to antibody or ligand binding have been reported previously (50, 51, 74–79). The peculiar characteristic of mAb BV7 would be that its binding to β_1 would expose a binding site for EC ligand blocking the domain responsible for cell interaction with matrix proteins.

In conclusion, we describe a novel β_1-mediated pathway of TC adhesion to EC. This pathway presents features which make it stand apart from integrin-mediated cell adhesion to matrix. The endothelial counter-receptor for HT-29 adhesion is still unknown and does not correspond to any previously described endothelial adhesive molecule for TC. The described activity is inhibited by $\alpha_2\beta_1$ integrin antibodies. Taking into account the positive correlation existing between $\alpha_2\beta_1$ integrin expression and TC metastatic ability (80), these findings might be relevant to the understanding of cell-specific mechanisms whereby TC localize to the microcirculation of target organs.

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