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An anti-αv-integrin antibody that blocks integrin function inhibits the development of a human melanoma in nude mice

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SUMMARY

A series of murine monoclonal antibodies were raised against purified human αvβ3 integrin and against M21 human melanoma cells. Five notable hybridomas were identified by ELISA on purified integrins, and the isolated antibodies bound the αv-chain. These antibodies, 17E6, 20A9, 23G5, 14D9.F8 and 10G2, recognised the extracellular domains of the integrin, and were shown to be reactive in FACS, immunoprecipitation, ELISA, and ELISA on fixed cells with M21, M21-L4, and UCLA-P3, but not with the αv-deficient M21-L or M21-L-IIb (M21-L transfected with GpIIb integrin).

One antibody, 17E6, strongly perturbed cell attachment mediated by αv integrins, reacting at least with αvβ3, αvβ5, and αvβ1, and strongly inhibiting cell attachment to αv-ligands vitronectin and fibronectin with an IC_{50} of ~0.1 μg ml^{-1}. Furthermore, 17E6 at this concentration could induce cell retraction from the substrate, while LM609 (anti-αvβ3) and control antibody 14E2 (anti-200 kDa melanoma surface protein) at 1,000-fold higher concentrations had minimal effects on cell morphology. The action of 17E6 was reversible and was not due to toxic effects: in vitro 17E6 at 0.1 mg ml^{-1} did not affect either cell proliferation or DNA synthesis.

In two nude-mouse tumour models, subcutaneous tumour development and a lung colonisation (‘experimental metastasis’) assay, injection of 17E6 strongly inhibited tumour development, while isotype-matched controls had no effect. There was no obvious mechanism of cell or of complement-mediated tumour cytotoxicity; the antibody did not mediate ADCC or AECDC, or complement fixation. The data strongly support previous studies which have indicated the importance of αv-integrins, and especially αvβ3, in the tumour progression of human melanoma.

Key words: integrin, melanoma, vitronectin, M21, cell-adhesion, tumour, αvβ3

INTRODUCTION

New strategies for cancer treatment demand an understanding of the biology of the malignant phenotype. One crucial factor is an aberrant regulation of cell adhesion during tumour progression. The last decade has shown that integrins are major receptors involved in cell adhesion (Hynes, 1992; Sonnenberg, 1993) and so may be suitable targets for therapeutic intervention. In this study we have characterized antibody blockers of αv-series integrins and reassessed the role of these integrins in melanoma progression.

With the exception of erythrocytes, all human cells express one or more integrin heterodimers. Their functions are regulated at many levels, but primarily their ligand specificity depends on which α-chain associates with which β-chain in the heterodimer and on their activation state (Hynes, 1992; Diamond and Springer, 1994). The cellular background in which the integrins operate (Chan and Hemler, 1993) and the splice-variant form of the integrin which is used (Delwel et al., 1993) may also affect specificity. Given these complexities, one of the few reliable indications of integrin specificity is to directly perturb integrin function and analyse resulting cellular behaviour. Thus, reagents that can specifically block integrin function have proved crucial, from the CSAT-antibody, which first defined an integrin β1-chain (Neff et al., 1982), onward.

The αv-series integrins are now seen to be a major subfamily, with both classical and novel functions. As well as classically mediating cell attachment and spreading (Pytela et al., 1985; Cheresh, 1991), αv integrins are implicated in cell locomotion (Seftor et al., 1992), in ligand-receptor internalisation (Panetti and McKeown Longo, 1993a,b), as virus co-receptors (Wickham et al., 1993), in management of the extracellular protease cascades (de Boer et al., 1993), and as regulators of tumour progression (Felding-Habermann et al., 1992), angiogenesis (Brooks et al., 1994a) and apoptosis (Brooks et al., 1994b). The specificities of the five known αv-series integrins, αvβ1 (Zhang et al., 1993; Marshall et al., 1995), αvβ3 (Pytela et al., 1985; Cheresh et al., 1987), αvβ5 (Cheresh et al., 1989), αvβ6 (Busk et al., 1992) and αvβ8 (Moyle et al., 1991), have been defined and they exclusively recognise ligands via the tripeptide sequence RGD- (NH_2-arginine-glycine-aspartic acid-COOH), including vitronectin

($\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$), fibronectin ($\alpha v\beta 1$, $\alpha v\beta 5$, $\alpha v\beta 6$), and von Willebrand factor, fibrinogen and osteopontin ($\alpha v\beta 3$) (Smith et al., 1990; Charo et al., 1990; Cheng et al., 1991; Busk et al., 1992; Zhang et al., 1993; Denhardt and Guo, 1993; Smith and Cheresh, 1990; Pytela et al., 1985). Dimers with related specificities may be co-expressed on the same cell, but may control independent functions (e.g. $\alpha v\beta 3$ and $\alpha v\beta 5$ - for vitronectin on melanoma cells) (Wayner et al., 1991). However, the overlapping ligand specificities within the αv-family itself and also between αv- and β1-series integrins, means that the assignment of function to a defined receptor has to occur within a particular cellular environment. Function blocking antibodies have been vital in clarifying the function of $\alpha v\beta 3$ (Cheresh and Spiro, 1987; Chuntharapai et al., 1993) and $\alpha v\beta 5$ (Wayner et al., 1991). However, for the other αv-integrins, no antibodies which specify the complex and perturb function are known. In particular, few reagents that specify the αv-chain, and which strongly perturb integrin function of the αv-family (as antibodies AIIB2 and P4C10 perturb all β1-series integrins) are available.

We have been interested in the function of αv-series integrins in tumour development. Human malignant melanoma is an increasingly prevalent and aggressive skin cancer. Elevated levels of integrins $\alpha 2\beta 1$ (Danen et al., 1993; Etoh et al., 1992), $\alpha 3\beta 1$ (Natali et al., 1993; Yoshinaga et al., 1993), $\alpha 4\beta 1$ (Hart et al., 1991), and $\alpha 6\beta 1$ (Hart et al., 1991) have been correlated with melanoma progression, but the integrins most consistently implicated are those of the αv-series. In particular, both vertical progression of the primary melanoma and distant metastases are characterized histologically by an increased expression of $\alpha v\beta 3$ integrin. Primary non-invasive tumours and non-malignant melanotic nevi express little $\alpha v\beta 3$, and it is also rare in healthy adult tissue (Buck et al., 1990; Korhonen et al., 1991; Lessey et al., 1992; Pignatelli et al., 1992; Nesbitt et al., 1993; Brooks et al., 1994a). Immunohistochemistry of staged tumours and metastases showed a progressive increase in $\alpha v\beta 3$ with invasive stage (Albelda et al., 1990; Si and Hersey, 1994). Screening of melanoma cell lines uniformly reveals a high expression of αv-series integrins (Marshall et al., 1991; Sanders et al., 1992; Gehlsen et al., 1992) (and our unpublished observation).

Studies in vivo also implicate $\alpha v\beta 3$ in melanoma development. In the murine B16F10 melanoma system, experimental lung metastasis could be suppressed by high levels of RGD-peptides, blockers of αv-integrin function (Humphries et al., 1986; Hardan et al., 1993). More recently, Felding-Habermann and colleagues have shown that the presence of αv integrins correlates with enhanced subcutaneous tumour growth of M21 human melanoma in immune-deficient mice (Felding-Habermann et al., 1992). The M21 suite of cells express various αv-series integrins (Cheresh and Spiro, 1987; Kieffer et al., 1991; Felding-Habermann et al., 1992). The parent, M21, expresses $\alpha v\beta 3$ and $\alpha v\beta 5$ (Wayner et al., 1991): it attaches to vitronectin and grows as a subcutaneous tumour. M21-L, a somatic variant of M21, has no detectable αv (Cheresh and Spiro, 1987): it cannot bind vitronectin and develops slow growing tumours. M21-L4 is a transfectant of M21-L, stably re-expressing a full length αv-chain: it binds vitronectin and grows rapidly as a subcutaneous tumour (Felding-Habermann et al., 1992). Thus, the presence of αv integrins is correlated with M21 subcutaneous growth. However, interpretations of these data must also consider the selection pressures to which M21 was subjected during the establishment of the variant lines M21-L and M21-L4.

Therefore, in this study, we have attempted to directly block the function of αv-integrins on the native M21 population and investigated the effect on cell behaviour and tumour development. We chose not to use peptidic antagonists because of their poor bioavailability, short half-life in vivo, and rapid clearance (Humphries et al., 1988). Syngeneic antibodies offer an attractive experimental alternative to peptides. They have a long half-life in vivo (Tao and Morrison, 1989; Haba et al., 1985) and their binding specificities can be demonstrated by standard techniques. Unfortunately, although there are excellent αv-specific antibodies, there are few that block αv-integrin functions (Cheresh and Spiro, 1987; Lehmann et al., 1994).

Here we characterise a group of antibodies against αv, including an effective class-specific blocking antibody, 17E6. Amongst its other interesting properties, 17E6 has the ability to disrupt stable interaction between vitronectin and $\alpha v\beta 3$, and blocks the growth of M21 tumours in nude mice. This is to our knowledge the first report of an antibody that reverses αv-integrin-ligand interaction. The data support earlier work in the M21 system, and confirm that αv-blockade can severely disrupt the development of a tumour. The implications for therapy are discussed.

MATERIALS AND METHODS

Miscellaneous methods

Animals

Mice for antibody production (female Balb/c; 8 weeks old) and for tumour models ('nude mice': female homozygotic athymic Balb/c nu/nu; 4-5 weeks old) were from Criffa (Barcelona, Spain). Newborn Balb/c mice for microglial preparations were from our own colony. Nude mice were maintained in a sterile room in micro-isolator cages, and were given sterilised food and water ad libitum. All manipulations were performed in a laminar flow hood.

Proteins

Fibronectin (Ruoslahti et al., 1982) and vitronectin (Yatohgo et al., 1988) were purified from fresh frozen human plasma, and fibrinogen (Kazal et al., 1963) from whole blood. Murine laminin was purified from Engelbreth-Holm-Swarm tumours (Paulsson et al., 1987).

Where not otherwise stated, all manipulations were done at 20°C, and all washings were with calcium-magnesium-free PBS. 'PBS++' is PBS with added 1 mM $MgCl_2$ and 1 mM $CaCl_2$.

Cell lines and cultures

American Type Culture Collection (ATCC) supplied SW1116, HT-29 human carcinomas, A375 human melanoma and Malme-3 human fibroblasts, while other human cell lines were the generous gift of colleagues: M21 melanoma, M21-L, M21-L12 and M21-L4 (Cheresh and Spiro, 1987), M21-L-IIb, prepared according to the method of Kieffer et al. (1991), and the UCLA-P3 human lung adenocarcinoma (Cheresh et al., 1989) (Dr D. A. Cheresh; Scripps), WM793 and WM164 melanoma (Herlyn et al., 1990) (Dr M. Herlyn; Wistar). NP18, pancreatic carcinoma (Dr G. Capellà; Hospital Sant Pau, Barcelona). B16F10 murine melanoma originally from Dr I. Fidler (Poste et al., 1980) (Dr S. Aliño; University of Valencia). EMM31 was established in our group from a tumour specimen defined by standard histological criteria as a primary melanoma (Jäggle et al.,

unpublished data). All cells were cultured at 37°C in 7.5% CO_2 92.5% air in 90% RPMI 1640, 10% fetal calf serum (FCS) plus 2 mM L-glutamine and antibiotics. Unless otherwise stated, cells were routinely passaged and harvested by treatment with EDTA (0.02% in PBS). Cell lines were consistently free of mycoplasma as evaluated by Mycotect (Gibco). Microglial cells were prepared from Balb/c mice (Sutter et al., 1991).

Cell attachment assays

As previously described, using hexosaminidase activity to detect attached cells (Landegren, 1984; Goodman et al., 1991). Dilutions and cell suspensions were made in attachment buffer (RPMI, 1% BSA, 25 mM HEPES, pH 7.4). Matrix proteins were coated onto 96- or 48-well plates, the wells were blocked with BSA and serially diluted antibodies were added followed by cells (2.5×10^4-5×10^4). After 1 hour at 37°C, non-adherent cells were washed away and attached cells counted against a standard curve run in parallel. Inhibition of attachment was calculated by using wells with no added antibodies as reference. Typically, over 70% of added cells had attached to vitronectin after 1 hour. Cell attachment to wells coated with BSA alone was routinely less than 5% of specifically attached cells.

Attachment reversal assay

Cells were plated in attachment buffer on wells coated and blocked as for cell attachment assays, and incubated at 37°C. After 1 hour, serially diluted antibodies were added. Alternatively, the cells were allowed to attach for 24 hours before addition of antibodies. After 2-3 hours at 37°C, the supernatants were removed and replaced 5 times by fresh, pre-warmed attachment buffer - resulting in a dilution factor of $>1\times10^5$ - and incubation continued.

Antibodies

Monoclonal antibody (mAb) fusions, ELISA screening, subcloning and maintenance of cultures were all performed using standard technologies (Harlow and Lane, 1988) unless otherwise specified.

Immunization

mAbs against the αvβ3 were produced by intraperitoneal (ip) injection of purified placental αvβ3 immobilised on Sepharose (80 μg αvβ3 on 80 μl Sepharose in 200 μl PBS) or of live M21 cells (1×10^6 cells in 0.5 ml PBS) every two weeks over twelve weeks. Four days after the last injection, PEG-induced fusion was performed using Friendly Myeloma (Ventrex) as partner. Antibodies to a 200 kDa melanoma-associated surface protein were produced by immunisation with intact M21 cells (1×10^6 cells in 0.5 ml PBS) and served as controls.

Screening

ELISA on receptors and on fixed M21 cells was used. For receptor ELISA, 96-well ELISA plates (Dynatech) were coated with purified αvβ3 (1 μg ml^{-1} in PBS, 16 hours, 4°C), blocked (1.5% skimmed milk in PBS, 1 hour, 37°C) and incubated with hybridoma supernatants. Bound immunoglobulins were detected with alkaline-phosphatase conjugated anti-mouse Ig (Dako) using *p*-nitrophenyl-phosphate as substrate. For cellular ELISA, M21 or M21-L, M21-L-IIb or UCLA-P3 cells on 96-well tissue culture plates were fixed (4% paraformaldehyde in PBS, 15 minutes, 20°C) and blocked (3% BSA, PBS, 1 hour, 4°C) before incubation with hybridoma supernatants and detection as in receptor ELISA. Positive hybridomas were subcloned three times by limiting dilution and adapted to RPMI medium. Immunoglobulin isotype was determined using isotype-specific heavy-chain antibodies (Zymed) or light-chain antibodies (Promega).

Other murine mAbs used in the studies were the gift of our colleagues: LM609 to αvβ3 and LM142 to αv (Cheresh and Spiro, 1987) (Dr D. A. Cheresh; Scripps), and P5H9 to αvβ5 integrin complex (Wayner et al., 1991) (Dr E. Wayner, University of Minnesota), B5-1A9 to β5 (Pasqualini and Hemler, 1994) (Dr M. Hemler; Dana Farber), CP8 to αIIbβ3 complex (Dr Ruggieri; Scripps), 9.2.27 against a melanoma cell surface proteoglycan (Harel et al., 1990) and 14.18G2a against ganglioside GD2 (Mujoo et al., 1989) (Dr R. Reisfeld; Scripps). In addition, AP3 to the β3 chain (Furihata et al., 1987) (ATCC) and P4C10 to β1 integrin (Carter et al., 1990) (Telios) were used.

Antibody purification and scaling up

For large scale purification, hybridoma supernatants were harvested from exponential phase cultures grown in roller bottles. The antibodies purified on Protein A-Sepharose CL-4B (Pharmacia) were dialysed against PBS before sterile filtration and storage at −70°C (Harlow and Lane, 1988). Purified antibodies were freed of endotoxins by passage over Kurimover-II columns (Kurita-Vater; Tokyo). This reduced the endotoxin levels to <0.2 i.u. mg^{-1} in the *Limulus* assay (Melvaer and Fystro, 1982).

$\alpha v\beta_3$ and αIIbβ3 integrin purifications

αvβ3 was purified from human placenta (Smith and Cheresh, 1988). Term placenta was minced in ~2 vols ice cold solution A (0.05% (w/v) digitonin, 2 mM $CaCl_2$, 2 mM PMSF, pH 7.4), then filtered. The retained material was extracted in ~4 vols ice cold buffer B (100 mM octyl-β-D-glucopyranoside (OG), 1 mM $CaCl_2$, 2 mM PMSF, in PBS) and centrifuged (12,000 ***g***max, 45 minutes, 4°C). The supernatant was re-circulated over an LM609 antibody column (16 hours, 4°C). After washing with buffer C (0.1% NP-40 in PBS, ~10 column volumes (cv)) and buffer D (0.1% NP-40, 2 mM $CaCl_2$, 10 mM Na-acetate, pH 4.5, ~10 cv), bound material was eluted with buffer E (buffer D adjusted to pH 3.1). The eluant was neutralized with 3 M Tris (pH 8.8), dialysed against buffer C, and concentrated ~10× using Aquacide II (Calbiochem). The purified receptor was stored at −70°C.

αIIbβ3 was prepared from human platelets (Pytela et al., 1986). Outdated platelet concentrates were mixed with one volume of Tyrodes buffer, pelleted (1,200 ***g***max) and the pellet extracted (1 hour, 20°C) with lysis buffer (50 mM OG, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 2 mM PMSF, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4). After centrifugation (32,000 ***g***max, 30 minutes, 4°C) the supernatant was re-circulated (16 hours, 4°C) over a GRGDSPK-conjugated CL-4B Sepharose column. The column was washed with lysis buffer (~10 cv) and eluted with the GRGDSPK (3 mg ml^{-1} in 90% lysis buffer, 10% DMSO). The peak was concentrated ~5-fold, dialysed against modified lysis buffer (0.1% NP-40 substituted for OG) and stored at −70°C.

The integrin preparations were ~95% pure as judged by anti-integrin ELISA using α-and β-chain specific monoclonal antibodies and by SDS-PAGE.

Cell surface labelling and immune-characterisations

Cell surface biotinylation and extraction

Cells in exponential growth were harvested, washed and 5×10^6 cells in PBS (1 ml) were surface-labelled with biotin-*N*-hydroxysuccinimido ester (10 μg ml^{-1}; Sigma) on an end-over-end rotator (2 hours, 20°C), washed, and 5×10^6 cells per millilitre were lysed for 1 hour at 20°C in extraction buffer (100 mM OG, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM PMSF in PBS). After centrifugation (12,000 ***g***, 20 minutes) the supernatant was used for immunoprecipitation.

Immunoprecipitation

Extracts from biotinylated cells were immunoprecipitated with anti-integrin mAbs purified over Protein A-Sepharose and coupled to Affigel-10 beads (Bio-Rad), or bound to Protein G-Sepharose (Pharmacia). Coupled mAb was incubated with 5×10^6 cell equivalents of biotinylated cell extracts overnight at 4°C. Washed beads were boiled in non-reducing SDS-sample buffer, centrifuged and resolved on 7.5% SDS-PAGE. After electrophoretic transfer to nitrocellulose (Towbin et al., 1979), bands were visualized with goat anti-biotin

Table 1. Reaction pattern of monoclonal antibodies in ELISAs and CELISAs

Antibody	Immunogen	Isotype	αvβ3	αIIbβ3	M21	M21-L	M21-L-IIb	UCLA-P3	Specificity
17E6	αvβ3	IgG1/κ	+	−	+	−	−	+	αv
20A9	M21	IgG1/κ	+	−	+	−	−	+	αv
23G5	M21	IgG1/κ	+	−	+	−	−	+	αv
14D9.F8	M21	IgG1/κ	+	−	+	−	−	+	αv
10G2	M21	IgG1/κ	+	−	+	−	−	+	αv
14E2	M21	IgG1/κ	−	−	+	+	+	−	200 kDa
21H6	M21	IgG2a/κ	−	−	+	+	+	−	200 kDa
LM609		IgG1/κ	+	−	+	−	−	−	αvβ3
LM142		IgG1/κ	+	−	+	−	−	+	αv
P5H9		IgG1/κ	−	−	+	−	−	+	αvβ5
CP8		IgG1/κ	−	+	−	−	+	−	αIIbβ3
P4C10		IgG1/κ	−	−	+	+	+	+	β1
AP3		IgG1/κ	+	+	+	−	+	−	β3

Antibodies were compared in ELISA and cell ELISA (CELISA). Reagents and specificities are discussed fully in text. +, antibodies bind; −, antibodies do not bind. The antibodies characterized in this study are in bold, standard reagent antibodies are italicised. Immunogen: αvβ3 = immobilized human placental integrin αvβ3. M21= viable M21 cells. Purified αvβ3 and αIIbβ3 were immobilized on 96-well plates for ELISA. The cell lines M21, M21-L and M21-L-IIb, and UCLA-P3 were grown and fixed on the plates and tested for antibody reactivity in CELISA. Specificity: (See text) αv = αv chain of mammalian integrins. 200 kDa = unknown 200,000 Da surface protein of melanoma cells. αvβ5 = integrin αvβ5. β1 = integrin β1 chain. β3 = integrin β3 chain.

alkaline phosphatase conjugate (Dako) using NBT-BCIP (Bio-Rad) as substrate.

Flow cytometry

Cells were harvested, washed, and 10^6 cells in PBS-1% BSA were incubated with mAb (10 μg ml^{-1}, 15 minutes, 4°C). After washing and labelling (15 minutes, 4°C) with goat anti-mouse Ig-FITC (Becton-Dickinson), cells were resuspended in propidium iodide (5 μg ml^{-1} in PBS) before flow cytometric analysis (FACScan, Becton-Dickison). Living cells were gated on the basis of their side and forward scatter and of propidium iodide fluorescence. Fluorescein was excited with an argon ion laser (488 nm) and the emitted fluorescence (525 nm) was recorded. In some experiments, M21-L cells were stained following fixation and permeabilization (70% ethanol, 5 minutes, −20°C).

Complement dependent cytotoxicity (CDC)

M21 cells (10,000) were plated in a 96-well plate in 50 μl of complete medium and 20 μl of antibodies. 50 μl rabbit serum (Behring) diluted 1:5 in medium was added as source of complement and the plate was incubated (60 minutes, 37°C,). Lysis was quantified by the MTT technique (Mosmann, 1983). The percentage of lysis was calculated using wells with 1% Tween-20 as 100% lysed controls, and without antibody as 0% lysed control.

Cell growth, viability and activation

Proliferation assays

In order to test for toxic antibody effects, 10^6 cells were incubated in the presence of mAbs (70 μg ml^{-1} in PBS, 1 hour, 20°C) on an end-over-end rotator, washed, then resuspended and further cultured in RPMI-medium. Growth and viability were estimated by trypan blue dye exclusion. Activation was measured by the MTT assay (above) or by [^{3}H]thymidine incorporation (below) and cell numbers were counted daily using a cell counter (Coulter).

Melanoma cells were seeded into 96-well flat-bottom microtest plates (1×10^4 cells per well) and cultured without (control) or in the presence of serially diluted antibodies in RPMI medium with 10% FCS. After 48 hours, cells were pulse labelled for 18 hours (18.5 kBq [^{3}H]thymidine per well), harvested, and incorporated nuclear [^{3}H]label was measured.

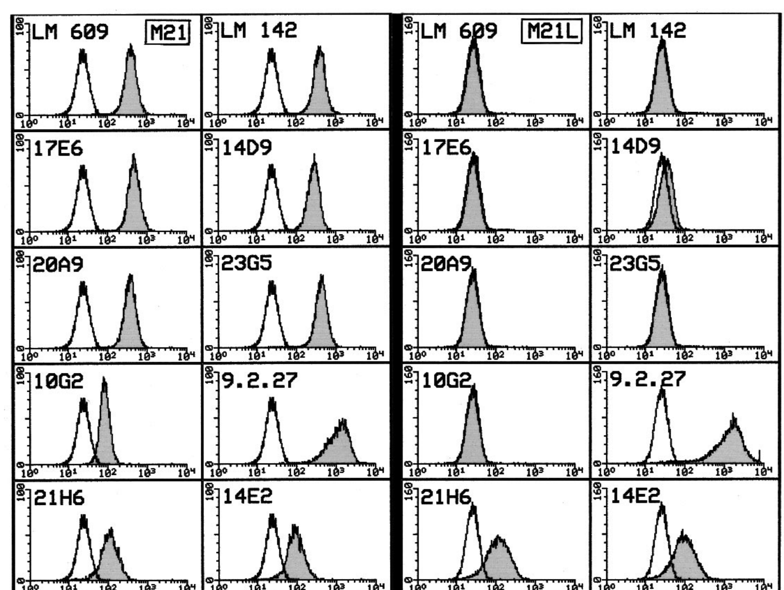

Fig. 1. FACS analysis of the alpha-v group antibody binding on M21 and M21-L human melanoma cells. Cells were indirectly labelled with antibodies. Propidium iodide was added and 15,000 cells per sample were acquired and analysed after gating. The open peak represents the binding of the fluorescently-labelled second layer antibody alone. The closed peak, the intensity of the primary and secondary together. Vertical axis shows cell number, horizontal axis shows log fluorescence intensity. M21 express surface αv integrin, whereas M21-L does not. The pattern of staining for the alpha-v group antibodies (17E6, 20A9, 23G5, 14D9.F8) closely matches the LM142 (αv-specific) and LM609 (αvβ3-specific) staining. They bind M21 but not M21-L cells. Antibody 9.2.27 reacts with a melanoma proteoglycan. 14E2 and 21H6 recognise a 200 kDa melanoma surface protein. Their staining patterns are discrete from those of the alpha-v group and similar for both cell lines.

Table 2. Summary of monoclonal antibody reactivity in flow cytometry

		Test cell ⇒	M21	M21-L	M21-L (p)	M21-L-IIb	M21-L4	M21-L12	UCLA-P3	WM793
Antibody	Specificity	αv-Integrin pattern	αvβ3; αvβ5	(β3)*	(β3)*	αIIbβ3	αvβ3; αvβ5	(β3)*	αvβ5	αvβ3; αvβ5
17E6	αv		++	–	–	–	++	–	+++	+++
20A9	αv		++	–	–	–	++	–	+++	+++
23G5	αv		++	–	–	–	++	–	+++	+++
14D9.F8	αv		++	–	–	–	++	–	+++	+++
14E2	200 kDa		++	++	++	++	++	++	–	+++
LM609	αvβ3		++	–	–	–	+	–	–	++
LM142	αv		+++	–	–	–	++	–	++	+++
P5H9	αvβ5		+	–	–	–	+	–	+++	+
P4C10	β1		++	++	++	++	++	++	++	++
AP3	β3		++	–	++	++	+	+	–	+
CP8	αIIbβ3		–	–	–	++	–	–	–	–

Levels of reactivity relative to control (secondary antibody only) were graded as follows: 1-2 (–), 2-4 (+), 4-9 (++), >9 (+++). For example, on M21, the relative fluorescent intensity of the control was typically 30-50 units (–), and that of LM142 binding was 300-500 units, giving mean relative reactivity around 10 (400/40) (+++). (p) M21-L was permeabilized with 70% ethanol at –20°C.

*M21-L has intracellular pools of β3 chain of αvβ3 integrin.

Antibody-dependent cellular cytotoxicity assays (ADCC)

Conditions for ADCC were essentially as described (Sutter et al., 1991). M21 cells in logarithmic growth were pulsed for 24 hours with [^{3}H]thymidine (73 kBq ml^{-1}), purged with EDTA (0.1% in PBS, 7 minutes), and incubated on 96-well plates together with microglia (5×10^4 cells per well) in 200 μl DMEM/FCS (10%) in the presence of antibodies. After 48 hours [^{3}H]label retained in the nuclear compartment of target cells was measured. mAb dependent cytotoxicity was calculated using the formula:

$$\% \text{ cytotoxicity} = 100\times[(A-B)/A],$$

where A = counts per minute in M21 cells cultured alone; B = counts per minute in M21 cells cultured with microglia and/or antibodies.

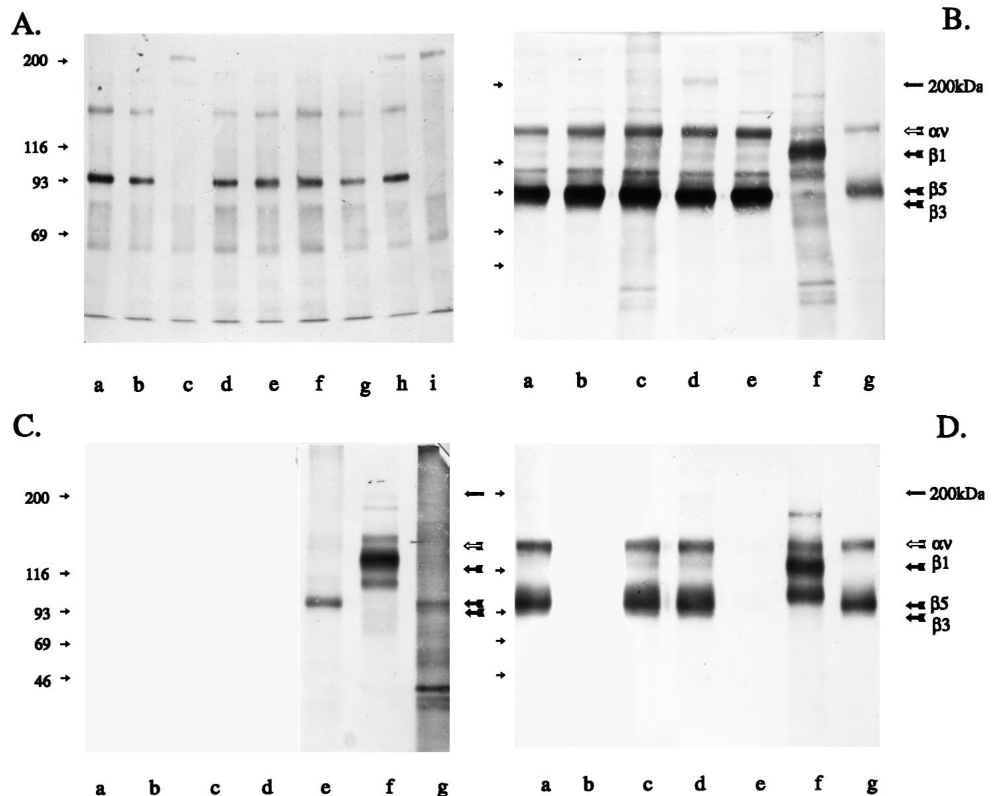

Fig. 2. The alpha-v group antibodies immunoprecipitate similar proteins. Viable M21 melanoma (A) were surface labelled, extracted with detergent, and immunoprecipitated and resolved on a non-reducing 7.5% SDS-PAGE gel. Migration of standards (weights in kDa) are indicated to the left. Precipitation was with the control antibodies LM142 (anti-αv: lane a) and LM609 (anti-αvβ3: lane b), and 21H6 (lane c), 10G2 (lane d), 20A9 (lane e), 23G5 (lane f), 17E6 (lane g), 14D9 (lane h), and 14E2 (lane i). LM142 and LM609 precipitate a similar pattern of proteins to 10G2, 20A9, 23G5, and 17E6, while 21H6 and 14E2 precipitate a band at ~200 kDa. 14D9 precipitates both patterns. M21 melanoma (B), M21-L αv-deficient melanoma (C), and UCLA-P3 cells (D) were cell-surface labelled and immunoprecipitated. Precipitation was with antibodies LM142 (anti-αv: lane a), LM609 (anti-αvβ3: lane b), 17E6 (lane c), 20A9 (lane d), AP3 (anti-β3: lane e), P4C10 (anti-β1: lane f), and B5-1A9 (anti-β5: lane g). Positions of molecular weight markers are shown as small arrows to left of the panel, with weights in kDa (shown in C). The position of αv (open arrow), β1, β5, β3, (closed arrows) and ~200 kDa chains is indicated to the right of each panel. Note the β3 chain precipitated from M21-L cells (lane e) permeabilized during surface labelling.

Antibody and effector cell-dependent cytostasis (AECDC)

As for ADCC, but instead of pulsed cells, freshly passaged unlabelled M21 cells were used. After 24 hours the effector-target cell co-cultures were pulsed with [^{3}H]thymidine (18 kBq per well) for an additional 24 hours and the incorporated nuclear [^{3}H] label measured.

In vivo tumour development

Tumour cells in logarithmic growth were harvested, washed and examined for viability by trypan blue dye exclusion. Viability was between 96-99%. For primary tumour growth, cells (0.1-1.0×10^6 in 0.2 ml PBS) were injected subcutaneously into the flanks of nude mice. A minimum of eight animals were used per group. Tumour growth was followed by measuring tumour diameters with callipers and the tumour volume was calculated using an approximated formula for a prolate ellipsoid:

$$\text{volume} = [(a{\cdot}b^2)/2],$$

where a is the longest axis of the tumour and b the shortest.

For lung colonisation assays (‘experimental metastasis’), cells were harvested and injected into the tail vein of nude mice (0.1-1.0×10^6 in 0.2 ml PBS). After 7 weeks the animals were killed, the lungs removed and fixed in a Bouins’ solution, and the tumour foci on the surface of the lungs counted.

For antibody treatment, harvested and washed cells were incubated with purified endotoxin-free mAbs (175 μg ml^{-1} in 0.4 ml PBS) for 30 minutes at 20°C in an end-over-end rotator before injection (in 0.2 ml PBS). In some experiments, the antibodies were injected intraperitoneally shortly before or after cell injection. This did not affect the results. For consistency, only data from co-incubation experiments is reported. There was no significant difference in cell viability, assessed by trypan blue dye exclusion, before and after completing the injection schedule (viability pre-injection = viability post injection ±5%). The tumour inhibition data was analysed using the 2-tailed Student’s t-test.

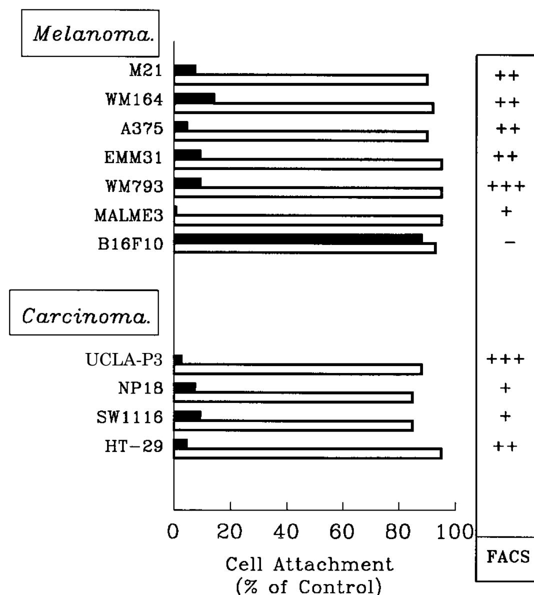

Fig. 3. 17E6 inhibits initial cell attachment to vitronectin. A series of melanoma cell lines and carcinoma cell lines were screened by FACS for reactivity with 17E6 mAb. FACS intensities were determined as described in Table 2. The cells were also allowed to attach to vitronectin-coated substrates (0.5 μg ml^{-1} coating) in the presence of hybridoma supernatants from 14E2 (open bars) or 17E6 (filled bars). Attached cells were counted and the data normalised to attachment in the absence of antibodies (100%). All cells were of human origin except B16F10 (murine melanoma). Malme-3 is a human fibroblast line. Absolute percentage of cells attached in the absence of antibodies was for M21 (70%), WM164 (68%), A375 (75%), EMM31 (67%), WM793 (65%), Malme-3 (67%), B16F10 (70%), UCLA-P3 (76%), NP-18 (65%), SW1116 (68%), HT-29 (65%).

RESULTS

The alpha-v group monoclonal antibodies react with integrin αv-chain

Antibody screening by ELISA on purified αvβ3 and αIIbβ3 revealed five clones, 17E6, 20A9, 23G5, 14D9.F8, 10G2 which reacted specifically with αvβ3 (Table 1). We term these mAbs ‘the alpha-v group’. All were IgG1/κ isotype. In the same ELISA assay, anti-integrin antibodies of known specificity against the αvβ3 complex (LM609), the αv chains (LM142), the αvβ5 complex (P5H9), the αIIbβ3 complex (CP8), the β3 chains (AP3), and the β1 chains (P4C10), reacted as predicted from the literature (Table 1). In ELISA on fixed cells (‘CELISA’), with cells expressing αvβ3 and αvβ5 (M21), αvβ5 but no αvβ3 (UCLA-P3), neither αvβ3 nor αvβ5 (M21-L), and αIIbβ3 (M21-L-IIb), the alpha-v group showed a reaction pattern consistent with their recognition of the αv-integrin chain, and clearly distinct from a reaction with β3, β5,

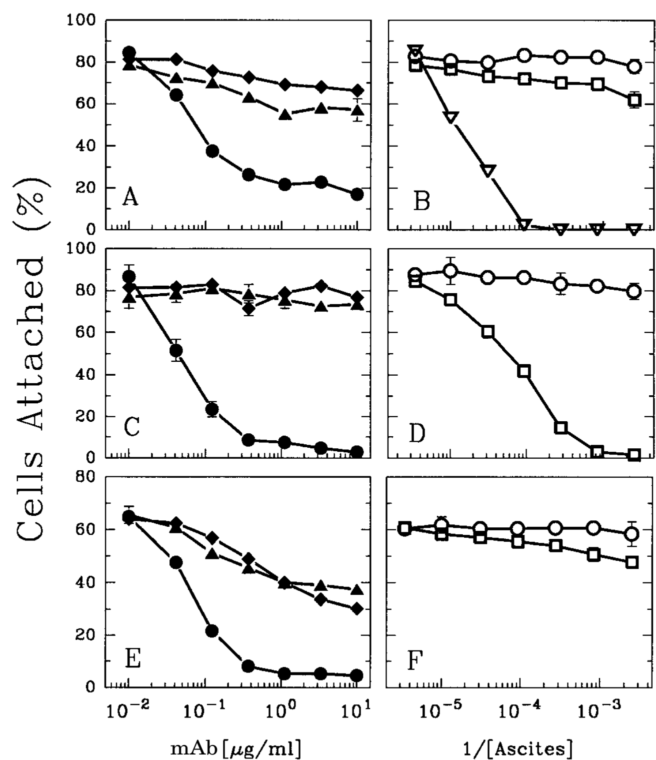

Fig. 4. 17E6 perturbs adhesion mediated by both αvβ3 and αvβ5 integrins. Purified monoclonal antibodies (mAb) or mouse ascites were co-incubated during cell attachment to vitronectin-coated substrates (5 μg ml^{-1}). Cell lines (A,B) M21; (C,D) UCLA-P3; (E,F) WM793. Symbols represent the following antibodies (specificities): (●) 17E6 (αv); (▲) LM609 (αvβ3); (◆) 14D9.F8 (αv); (○) P4C10 (β1); (□) P5H9 (αvβ5); (▽) P5H9 + LM609 (dilution starting at 10 μg ml^{-1}) (αvβ3+αvβ5).

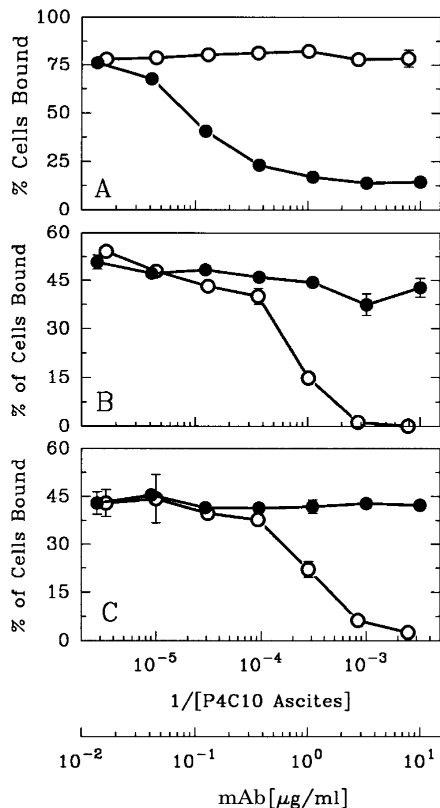

Fig. 5. 17E6 perturbs M21 adhesion to vitronectin but not to other matrix components. The inhibitory effect of 17E6 (αv, ●) and P4C10 (β1, ○) on M21 cell attachment to vitronectin (A), laminin (B) or collagen type I (C) was studied. 17E6 only blocked cell attachment on vitronectin-coated surfaces. Whilst P4C10 only blocked cell attachment on collagen type I and laminin.

β1, or other α-chains (Table 1). The results corroborated the ELISA data with purified receptors. mAbs with specificities for β3 and αIIbβ3 clearly reacted differently from the alpha-v group. 17E6, 14D9.F8, 20A9 and 23G5 bound αvβ3 with similar apparent affinity, assessed by ELISA. 50% binding was achieved at ~10-20 ng ml^{-1} (~50-100 pM - similar to LM609). 10G2 binding was similar to that of LM142, with about 10 times lower affinity. CP8 against αIIbβ3 and control antibody 14E2 (see below), showed minimal binding to αvβ3 at concentrations up to 100 nM (data not shown).

The ability of the alpha-v group to recognise native αv-integrins was tested by FACS (Fig. 1; Table 2) and by immunoprecipitation from surface labelled cells (Fig. 2). In FACS analysis (Fig. 1), the αv-expressing cells (M21) reacted strongly with 17E6, 14D9.F8, 20A9, 23G5, and with the αv-defining antibodies LM142 and LM609, moderately with 10G2, and also with the control mAbs 14E2 and 21H6 and mAb 9.2.27. By contrast, αv-deficient variant (M21-L) did not react with the alpha-v group, or with LM142 and LM609, but showed similar reactivity to that of M21 with 14E2, 21H6 and 9.2.27. M21-L has an intracellular pool of β3 subunits which were detected in FACS only when the cells were permeabilized (Table 2). In FACS analysis of M21-L4 (αv-retransfected M21-L cells; Felding-Habermann et al., 1992), the alpha-v group gave reaction patterns as seen on M21. The control vector transfectants, M21-L12 and the GpIIb transfectants, M21-L-IIb (Kieffer et al., 1991), showed no reactions with the alpha-v group (Table 1). UCLA-P3 adenocarcinoma reacted with the alpha-v group, with LM142 and P5H9, but not with LM609. UCLA-P3 does not express β3 (see Introduction). The melanoma WM793 had the same reaction pattern as M21. In immunoprecipitation screening of M21 cells, the alpha-v group gave the same immunoprecipitation patterns as LM142 (anti-αv), and LM609 (anti-αvβ3) (Fig. 2a). Under non-reducing conditions, a strong broad band was seen at ~95 kDa and a weaker band at ~145 kDa, with weak accompanying bands at ~100 kDa, a pattern characteristic of surface labelled αvβ3 and αvβ5 integrins (Wayner et al., 1991). In comparison, on M21-L, none of the alpha-v group precipitated proteins (data from 17E6 and 20A9 are shown), and neither did LM142 or LM609 (Fig. 2c). β1-specific antibodies gave similar precipitation patterns from M21 and M21-L. In M21-L, precipitation with anti-β3 antibodies gave a band at ~95 kDa, due to intracellular β3 labelled in permeable (possibly necrotic) cells. UCLA-P3 (Fig. 2d) gave no precipitate with LM609, but a ~95 kDa/145 kDa complex was precipitated, by the alpha-v group, by LM142, and by B5-1A9 (anti-β5) (Fig. 2d). In summary, ELISA, CELISA, FACS analyses and immunoprecipitations gave consistent reaction patterns and strongly suggested that mAbs of the alpha-v group react with extracellular domains on human αv-integrin chains.

mAb 17E6 is a function blocking antibody

17E6 can modify initial cell attachment to αv-ligands

αv-integrins can function as receptors for vitronectin, so we screened the alpha-v group antibodies for their possible effects on cell attachment to vitronectin substrates. First, cells were analysed by FACS. Human melanoma and carcinoma cell lines reacted similarly with the alpha-v group. The reaction with 17E6 is summarised in Fig. 3. The attachment to vitronectin of cells reacting in FACS with 17E6 was strongly blocked by that antibody; other members of the alpha-v group were weaker (Fig. 4.) and the control antibody 14E2 was inactive (Fig. 3). The attachment of murine cell B16F10 on vitronectin was not affected by 17E6, and B16F10 did not react with 17E6 in FACS. As predicted (Cheresh and Harper, 1987), B16F10 attachment to human vitronectin was sensitive to micromolar concentrations of RGD-peptides, suggesting the presence of functional surface αv-integrins, not recognized by 17E6 and the alpha-v group.

We investigated more closely the effect of 17E6 on cell attachment. 17E6 blocked M21 attachment (~90%) to vitronectin with an IC_{50} of ~0.1 μg ml^{-1} (Fig. 4a). We confirmed previous studies (Wayner et al., 1991) by showing that M21 attachment was poorly blocked by antibodies to αvβ3 (LM609) or αvβ5 (P5H9) alone, but was strongly blocked when both were added together (Fig. 4a,b). Anti-β1 integrin antibodies (P4C10) had no effect on M21 attachment to vitronectin (Fig. 4b). The lines UCLA-P3 and WM793 attached to vitronectin and this attachment was blocked by 17E6, and also by the αvβ5-specific (P5H9 on UCLA-P3) or αvβ3-specific antibodies (LM609 on WM793) (Fig. 4c-f). This pattern of blocking corresponds to the surface patterns of integrin expression seen in FACS and by immunoprecipitation (e.g. Fig. 2D). UCLA-P3 expresses αvβ5 but no αvβ3

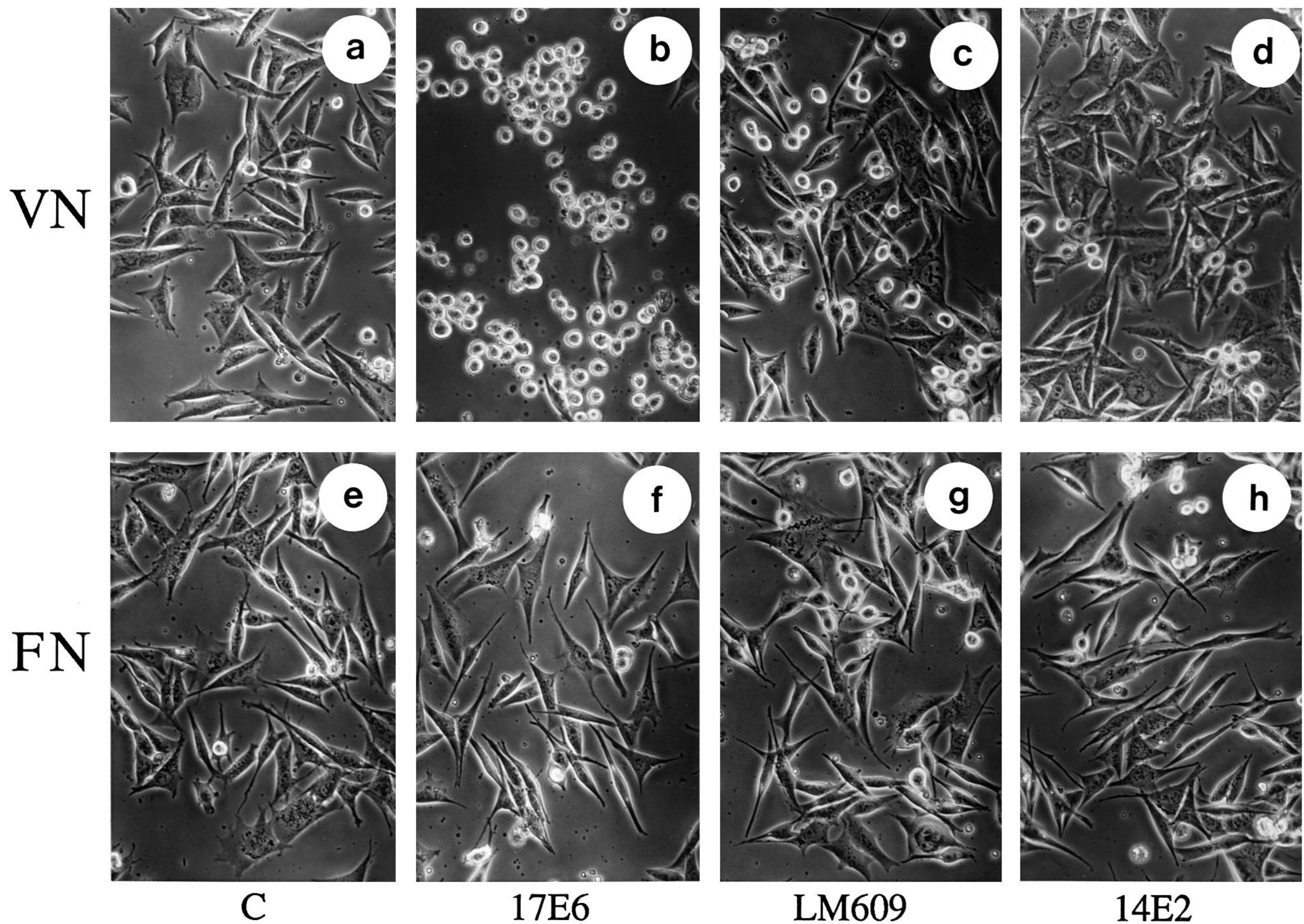

Fig. 6. 17E6 reverses established cell-vitronectin contacts. M21 melanoma were allowed to attach for 24 hours to surfaces coated with vitronectin (VN - upper row) or fibronectin (FN - lower row). The cells attached within 30 minutes and were well spread, and proliferating after 24 hours (a,e) when antibodies were added to the culture medium. With 17E6 (b,f), after 30 minutes on vitronectin cells rounded (b), while on fibronectin they remained spread (f). LM609 (c,g) and 14E2 (d,h) had little effect on either substrate. Antibody concentrations: (b) 0.1 μg ml^{-1}; (c,d,f-h) 100 μg ml^{-1}.

(Wayner et al., 1991), while WM793 expresses high levels of $\alpha v\beta 3$ (Table 2). On UCLA-P3 17E6 had an IC_{50} of ~30 ng ml^{-1}. On WM793 it had an IC_{50} of ~60 ng ml^{-1}, while for LM609 the IC_{50} was ~600 ng ml^{-1}. The isotype-matched anti-αv antibody 14D9.F8 only weakly affected cell adhesion. The specificity of 17E6 was confirmed by its lack of effect on cell attachment to other matrix substrates. P4C10 (anti-β1) abolished M21 attachment to laminin and collagen type I (Fig. 5). Cell adhesion to these substrates can be mediated by β1-series integrins (Sonnenberg et al., 1988; Takada et al., 1987).

Taken together with the biochemical data, these results are consistent with the theory that 17E6 binds the αv-chain of various integrin complexes and disturbs their interaction with their ligands.

17E6 triggers reversal of established cell matrix interactions mediated by αv-integrins

We next investigated whether 17E6 could affect established cell-matrix interactions. When 17E6 was added to attached and spread M21 cells at low concentrations (~0.1 μg ml^{-1}), it induced cell rounding after 0.5-1 hour at 37°C even after the cells had first attached for 24 hours (Fig. 6). The effect was reversible and after antibody removal the cells spread. By contrast, the antibodies LM609 and 14E2 affected morphology only slightly even at high concentrations (100 μg ml^{-1}). The antibodies had no morphological effects even at 100 μg ml^{-1} on fibronectin coated substrates (Fig. 6). M21-L4 (and other cells attached via αv) were similarly affected by 17E6 (not shown).

17E6 blocks M21 tumour development in nude mice

We investigated the effect of the αv-blocking antibody 17E6 on the subcutaneous development of M21 tumours in Balb/c nu/nu mice, which has been correlated with the cell surface expression of αv-series integrins (see Introduction). We subcutaneously injected M21 cells with antibodies. 17E6 inhibited the development of M21 tumours (10/10 experiments: Fig. 7a; Table 3). At high doses (1×10^6 cells per animal: Fig. 7a), tumours took (10/16 animals) but tumour development was strongly inhibited. Similar inhibition was seen when 17E6 was

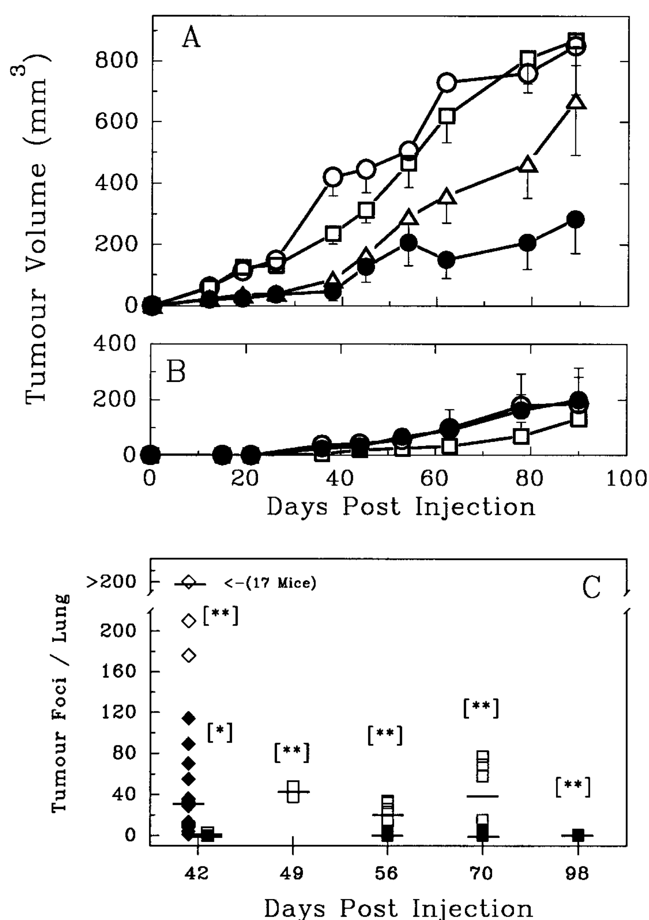

Fig. 7. M21 human melanoma development in Balb/c nude mice is modulated by αv-integrins. (A,B) Effect of 17E6 on subcutaneous M21 development. M21 (A) or M21-L (B) were incubated with buffer (○), antibodies 17E6 (●), 14D9.F8 (△) or 14E2 (□) and injected subcutaneously into nude mice. With time, the tumour dimensions were measured and the volume plotted. Error bars show the s.e.m. (n=8). (See also Table 4.) (C) Effect of αv on M21 experimental metastasis. 0.32×10^6 (◆,■) or 1×10^6 (◇,□) M21 (◆,◇) or M21-L (■,□) cells were injected into the tail vein of nude mice. At the time shown, groups of animals were killed and the lungs examined for tumour nodules. All the M21 mice were killed on day 42. For the M21-L mice, 3-6 mice were killed at each point. The tumour burden after 6 weeks in the M21 mice was too high to count (>>250 per lung), so t-statistics are shown for the hypothesis that an M21-L group is from the same population as the control M21 group at 42 days at the <0.001(**) or <0.02 (*) level. Where both 0.32×10^6 and 1×10^6 injected groups have the same level of significance, only one is shown. The bars show the mean tumour number for the groups.

injected intraperitoneally within 24 hours of the subcutaneous M21 implant. At low and intermediate cell doses (0.1-0.5×10^6 per animal), no tumour take was seen in the presence of 17E6 (0/32 animals). Weakly blocking antibodies against the αv-chain (14D9.F8) and other control antibodies (14E2) showed effects on tumour development not significantly different from the buffer treated control after 6 weeks (Table 3). In 14E2 treated controls, take of tumours varied between experiments from 100-60%, but established tumours grew at the same rate as the controls and, like them, the tumour-bearing animals had micro-metastases which grew into colonies when the lungs were brought into tissue culture. By contrast, 17E6 treated animals without primary tumours had no metastases in lungs, liver, kidney, spleen, colon, stomach, nor in thoracic or abdominal body cavities when killed at 6 months. The αv-deficient cell line M21-L grew more slowly than M21 after subcutaneous injection, and was unaffected by 17E6 (0.1-1.0×10^6 cells per animal were tested: 0.5×10^6 shown in Fig. 7b).

We also compared the growth of M21 and M21-L and the effect of the antibody 17E6 in a lung colonisation tail-vein injection model. M21 formed lung colonies in a dose dependent manner, while M21-L formed significantly fewer colonies which developed slowly (Fig. 7c; Table 4). Thus, tumour growth in the lungs was enhanced by the presence of cell surface αv-integrins, and pre-incubation of M21 (but not M21-L) with 17E6 reduced the numbers of tumour foci that formed, while control antibodies had no significant effect (Table 4). The amount of foci formed when M21 was treated with 17E6 was similar to that achieved by M21-L cells in the same experiment.

In summary, the presence of αv at the cell surface correlated with vigourous formation of M21 tumours both in subcutaneous and in experimental metastatic models. Antibody 17E6 suppressed tumour formation while the αv-binding control, 14D9.F8, had little effect. In a lung colonisation model, the growth of M21 and the poor growth of M21-L corresponded to the subcutaneous growth pattern, and here too M21 growth was suppressed by 17E6, but not by control antibody. These data strengthen the link between αv-integrins and the development of M21 human melanoma.

Anti-tumour activity of 17E6 is not due to antibody-mediated cytotoxicity

After observing its strong effects on attachment, morphology, and tumour development, we attempted to find the mechanism of 17E6-mediated tumour suppression. We tested 17E6 for cytotoxicity (Fig. 8). The kinetic of cell growth and the final cell densities achieved in culture were not greatly influenced by the presence of 17E6 or control antibody (Fig. 8a).

Nude mice are immune deficient. Of the immune competent cells which remain, macrophages and NK cells are the most likely to direct a cytotoxic, antibody-mediated response. To test the possibility that the antibodies were directing cellular cytotoxicity (ADCC), murine macrophages syngeneic with the mouse strain used to generate the alpha-v group were tested in ADCC against M21 cells. As effector cells murine brain macrophages (microglia) are especially effective mediators of ADCC (Sutter et al., 1991). The positive control, anti-ganglioside mAb 14.18G2a caused nearly complete lysis of M21 at $<10^{-9}$ M, while 17E6 at up to 10^{-7} M did not mediate ADCC (Fig. 8b).

To evaluate whether 17E6 might exert a cytostatic activity in the presence of effector cells, DNA synthesis of M21 microglial co-cultures was measured in the presence of the antibodies (Fig. 8c). At 10^{-10} M 14.18G2a caused a ≥90% inhibition in [^{3}H]thymidine incorporation, while 17E6 had no effect at up to 10^{-7} M. 17E6 and 14E2 were IgG1/κ isotypes, and did not support complement mediated lysis on M21 cells (not shown).

Having tested the effects on cell proliferation, ADCC and

Table 3. Inhibition of development of M21 tumour development by 17E6 mAb in Balb/c nu/nu mice subcutaneous growth assay

		Tumour volume (mm^3)					
		30 Days			90 Days		
Cells and treatment	Tumour take	Mean	(Range)	*P*=	Mean	(Range)	*P*=
M21 + PBS	8/8	296	(124-758)	–	812	(613-1090)	–
M21 + 17E6	6/8	76	(0-232)	0.019*	275	(0-614)	0.056*
M21 + 14D9.F8	8/8	93	(32-238)	0.025*	687	(381-1548)	0.75**
M21 + 14E2	8/8	269	(51-506)	0.78**	843	(218-1065)	0.64**

The experiment of Fig. 7A was analysed with Student's *t*-test for unpaired data at 30 days and 90 days of growth. Mean tumour volume and range of volumes is shown (1×10^6 cells injected per animal). *P* = *t*-test probability for the hypothesis that 'antibody treated cells develop into tumours at the same rate as PBS-treated controls'.
*Significantly different from control.
**Not significantly different.

AECDC, we examined whether the levels of DNA synthesis in M21 cells were affected by the mAbs. 17E6, 14E2, and LM609 at 90 μg ml^{-1} (0.5 μM) had no effect on thymidine incorporation at serum concentrations of 10, 1, or 0.1% (Fig. 9 shows 10%). DNA synthesis in M21-L, M21-L4 and M21-L-IIb cells was also unaffected by the antibodies. M21-L and M21-L-IIb react neither with 17E6 nor LM609, but do react with 14E2 (Fig. 1).

We concluded that the effects of 17E6 were specific to αv-integrins, and not to toxic effects on the melanoma cells, nor to related antibody-mediated cellular cytotoxicity or growth inhibition.

DISCUSSION

Tumour progression and metastasis are classically conditions where cells escape normal growth and adhesion controls and invade, migrate, attach and grow at inappropriate sites. Integrins are now known to control many cell adhesion events, and adhesion can in turn regulate mechanistically interwoven events including growth, differentiation, cellular movement and the activity of protease networks, developmental events which are often reiterated in the metastatic cascade (Fidler, 1988; Liotta et al., 1991; Stetler Stevenson et al., 1993). In this study we describe antibodies directed against human αv-series integrins one of which, 17E6, perturbs initial cell attachment, disrupts stable αv-ligand interactions and interferes with human melanoma development in in vivo animal models. In biochemical analyses the alpha-v group antibodies showed reaction patterns closely related to LM142 a well defined antibody to human αv - but distinct from the reaction patterns of αvβ3-specific (LM609), αvβ5-specific (P5H9) and from other defined anti-integrin antibodies. Thus, the 'alpha-v group' antibodies recognise the human αv-integrin chain.

Table 4. Inhibition of development of M21 tumour foci by 17E6 mAb in Balb/c nu/nu mice lung colonisation 'experimental metastasis' assay

		Number of tumour foci		
Cells and treatment	Tumour take	Mean±s.e.m.	(Range)	*P*=
M21 (Control)	8/8	61±14	(3-124)	–
M21+17E6	7/8	8±2.9	(0-21)	0.003*
M21+14E2	5/5	35±15	(16-86)	0.3**
M21-L	5/6	19±9.9	(0-60)	0.045*

Cells were harvested, incubated with antibody, washed and injected into the tail vein of nude mice. 7 weeks later the lung surfaces were examined. The numbers of foci that developed were analysed with Student's *t*-test for unpaired data. Mean number of foci and range of foci numbers is shown. Pre treatment with 17E6 significantly lowered the numbers of foci that developed, control antibody 14E2 did not. Similar numbers of foci developed following injection of 0.5×10^6 cells per animal M21-L (lacking αv) or 17E6-treated M21 (where αv-function was blocked). s.e.m. = standard error of the mean; *P* = *t*-test probability for the hypothesis that 'antibody treated cells develop the same number of foci as PBS-treated controls'.
*Significantly different from control.
**Not significantly different.

17E6 strongly perturbs cell attachment and spreading mediated by αv. It blocks cell attachment with an IC_{50} of ~0.3-1 nM. By comparison, the peptidic blocker, GRGDSPK, has an IC_{50} of ~5 μM. Cell lines expressing mainly αvβ3 (WM793) or αvβ5 (UCLA-P3) are blocked by 17E6, as are cells expressing mainly αvβ1 (Marshall et al., 1995). We could confirm previous studies where initial attachment of M21 on vitronectin was blocked only by *mixtures* of anti-αvβ3 and anti-αvβ5 antibodies, implying that both αvβ3 and αvβ5 were involved (Wayner et al., 1991), yet 17E6 alone blocks M21 attachment to vitronectin. Thus, 17E6 powerfully inhibits several αv-integrins. Antibodies that perturb function have been vital to our understanding of integrin function, but the αv-field has lacked an effective class-specific blocking antibody. Only 13C2 (Davies et al., 1989), a weak blocker, had been described until recently, when 69-6-5 was reported, which blocks αv-dependent cell attachment with an IC_{50} similar to 17E6 (Lehmann et al., 1994). The effects of 69-6-5 on established cell contacts and on tumour development have not yet been reported and comparison with 17E6 will be interesting.

Not only does 17E6 prevent the initial interaction of αv-integrins with their ligands, but it also reverses well established interactions, causing rapid retraction of cells attached on vitronectin. The interactions of αvβ3 with vitronectin have been described as 'non-dissociable', an initial blockable interaction being followed by an 'irreversible' stabilizing reaction (Orlando and Cheresh, 1991). By contrast, 17E6 rapidly caused retraction and partial detachment of M21 and other cells from vitronectin substrates even after they had formed stable contacts (e.g. after 24 hours). Earlier studies have found that the growth of M21 tumours in nude mice is strongly correlated

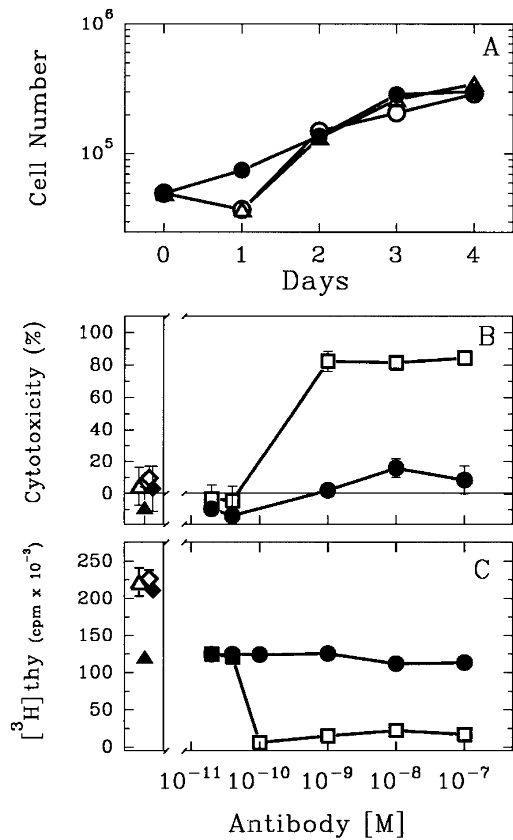

Fig. 8. 17E6 is not cytotoxic. (A) Effect of 17E6 on M21 cell proliferation. 5×10^4 M21 were seeded in DMEM/FCS with carrier (○), or in the presence of 50 μg ml^{-1} antibodies 17E6 (●) or 14E2 (△) and the cell numbers were counted daily. Kinetics and saturation density of proliferation are unaffected by the antibodies. (B) 17E6-dependent lysis of M21 cells by microglial cells. Thymidine labelled M21 cells were mixed with Balb/c-derived microglial brain macrophages, serially diluted antibodies were added, and following incubation, thymidine associated with the nuclei of M21 cells was measured. Controls: (△) M21 cells alone; (◆) M21 + 17E6 (100 nM); (◇) M21+14.18G2a; (▲) M21 + microglia. Mean ± s.d. (*n*=6). Experimental: (●) M21 + microglia + 17E6; (□) M21 + microglia + 14.18G2a·17E6 did not induce antibody-dependent cell lysis. Mean ± s.d. (*n*=3). (C) 17E6-dependent cytostasis of M21 cells by microglial cells. M21 cells were incubated with microglial cells and serially diluted antibody, and then pulsed with [^{3}H]thymidine to measure DNA synthesis. Thymidine incorporation ([^{3}H]thy) was measured. Symbols as in B. Note the cytostatic activity of the effector cells alone (▲). Microscopical observation of the assay showed that in regions of homogeneous distribution of microglial cells at 14.18G2a $>10^{-10}$ M no M21 cells survived.

with expression of αv-integrins (Felding-Habermann et al., 1992; Sanders et al., 1992). As 17E6 could modulate stable αv-ligand interactions and its action was long lasting we examined its effect on tumour development. 17E6 blocked the development of subcutaneous M21 tumours in nude mice. In addition, αv supported and 17E6 inhibited the development of M21 as lung colonies. We could also show that M21-L tumours developed more slowly than M21, and were unaffected by antibody 17E6. We have thus independently confirmed the studies of Felding-Habermann et al. and underlined the importance of αv-integrins in the development of the M21 tumour.

Is not the drastic effect of a single subcutaneous antibody injection on long-term tumour development a surprise? In vitro 17E6 effects continued for >24 hours following wash-out, suggesting that antibody endocytosis and destruction were slow. Given a mouse volume of 5-20 ml, the initial antibody concentration we used in vivo was 50-10 nM, an order of magnitude excess over the concentration necessary for reversing cell-ligand interactions in vitro. 17E6 is syngeneic for the treated animals and the half life of murine IgG1 is 20-200 hours (Tao and Morrison, 1989; Haba et al., 1985). Thus, the IC_{50} for reversing M21-ligand interaction could be exceeded for at least 100 hours in this model (i.e. 5 half-lives). RGD-peptides which block αv-integrins have also been used to modulate tumour development. In the B16F10-C57bl/6 murine melanoma model, coinjected peptides inhibited the development of pulmonary tumours (Humphries et al., 1986; Hardan et al., 1993). With the same assumptions as for 17E6, ~100 μM RGD-peptide was present (Hardan et al., 1993), some two orders of magnitude over the dose required to block cell attachment to vitronectin. However, RGDS has a short serum half-life of 8 minutes (Humphries et al., 1988). As a general therapeutic goal, it might be preferable to generate long lived blockers for suppressing tumour development.

How might 17E6 affect tumour development? As it does not react with murine cells, it acts on the tumour cells and we can exclude effects on tumour angiogenesis (Brooks et al., 1994a,b). The only effects of 17E6 we can identify are: (a) its binding to the extracellular domain of αv-integrins; and (b) its ability to perturb αv-mediated cellular interactions. We have excluded most sources of antibody-mediated cellular killing available to a nude mouse. And, most revealingly, 14D9.F8, a matched control antibody that binds αv but only weakly affects function of αvβ3 and αvβ5 integrins, does not affect tumour development. 17E6 itself did not chronically or acutely affect M21 cell growth and viability. In vitro it did not affect DNA synthesis, it did not mediate complement fixation, and it did not mediate syngeneic macrophage-mediated cytotoxicity or cytostasis. Nevertheless, M21 cells were sensitive to ADCC for they were effectively killed by microglial cells in the presence of control mAb 14.18G2a. Antibodies of the IgG1 isotype (like 17E6) can mediate murine macrophage ADCC (Herlyn et al., 1985a). It is possible that the number of αv sites expressed per M21 cell may be below the critical threshold for ADCC to occur (Herlyn et al., 1985b). Macrophages in contrast to other myeloid or lymphocytic effector cells express all three classes of Fc receptors. Therefore, the data also argue against ADCC by effector cells other than macrophages as the mechanism of inhibition of tumour growth. Non-specific killing can be excluded as the antibodies were endotoxin free. We cannot exclude the possibility that NK cell mediated killing is activated by 17E6, but if so it is far more weakly activated by the syngeneic isotype- and specificity-matched control antibodies we used.

Since the proliferation of M21 cells was not affected by anti-αv antibodies, we conclude that 17E6 probably acts by modulating the function of αv-integrins. Functions which 17E6 might block, and where αv might participate, include cell adhesion, interaction with soluble matrix components (Seftor et al., 1992), modulation of a protease/protease inhibitor network (Gehlsen et al., 1992; de Boer et al., 1993), stimulating cell movement (Seftor et al., 1992; Gehlsen et al., 1992),

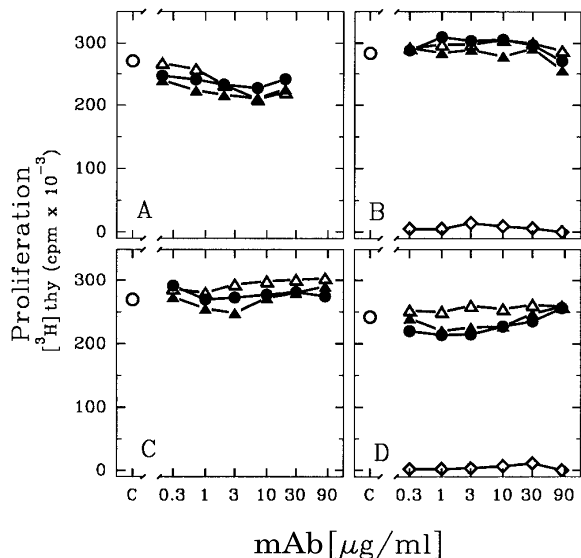

Fig. 9. 17E6 does not inhibit DNA synthesis in vitro by M21 cells. Cell lines (A) M21; (B) M21-L; (C) M21-L4; (D) M21-L-IIb were cultured in the presence of the antibodies 17E6 (●), LM609 (▲) or 14E2 (△) at the concentrations indicated, or in culture medium alone (○). In (B) and (D), the routine positive control, tumouricidic taxol (◇) is shown. After 48 hours, the cells were pulsed with [^{3}H]thymidine and the incorporated radioactivity was measured. The antibodies did not inhibit DNA synthesis. Taxol completely suppressed it (cf. Fig. 8: 90μg ml^{-1}, mAb = 600 nM).

affecting receptor internalisation (Wickham et al., 1993; Panetti and McKeown Longo, 1993b), but which if any is involved remains a matter for further experimentation.

In conclusion, a suite of monoclonal antibodies against the human αv-integrin chain was developed. One, 17E6, both blocks and reverses αv-mediated processes in vitro. It also blocks αv-dependent human melanoma development in nude mice. Brooks et al. (1994b) recently showed that blocking of αvβ3 on tumour-invasive blood vessels induces endothelial apoptosis; conceivably αv-bearing tumour cells might also be so affected. If so, αv-integrins would be especially interesting targets: reagents that block αv-integrins might act as double-edged weapons in tumour therapy, striking simultaneously at the developing tumour and at its blood supply.

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