Integrin $\alpha_v\beta_3$ Differentially Regulates Adhesive and Phagocytic Functions of the Fibronectin Receptor $\alpha_5\beta_1$

Scott D. Blystone, Irene L. Graham, Frederik P. Lindberg, and Eric J. Brown

Department of Medicine, Infectious Diseases Division, Washington University School of Medicine, St. Louis, Missouri 63110

Abstract. The plasma protein fibronectin is an important opsonin in wound repair and host defense. To better understand the process of fibronectin-mediated phagocytosis, we have transfected K562 cells, which endogenously express $\alpha_5\beta_1$, with $\alpha_v\beta_3$. In these transfectants, antibodies to $\alpha_v\beta_3$ block phagocytosis of fibronectin-opsonized beads completely, even though half the ingestion occurs through endogenous $\alpha_5\beta_1$ receptors. $\alpha_5\beta_1$-mediated adhesion to fibronectin-coated surfaces is unaffected by $\alpha_v\beta_3$ ligation. Neither $\alpha_v\beta_5$ nor $\alpha_M\beta_2$ ligation affects $\alpha_5\beta_1$ phagocytic function in transfectants expressing these receptors. Pharmacologic data suggest that $\alpha_v\beta_3$ ligation suppresses the phagocytic competence of high affinity $\alpha_5\beta_1$ receptors through a signal transduction pathway, perhaps involving protein kinase C. In addition to its significance for phagocytosis, $\alpha_v\beta_3$ regulation of $\alpha_5\beta_1$ function may be significant for its roles in cell migration, metastasis, and angiogenesis.

MACROPHAGE interaction with fibronectin (Fn)[1] is recognized as an important aspect of host defense and wound repair. Fibronectin opsonization is necessary for macrophage recognition and phagocytosis of particulate debris released from tissues after burn and trauma (23, 41, 53, 58), resolution of bacteremia during sepsis (48, 53), and clearance of fibrin during disseminated intravascular coagulation (9, 11, 60). In addition, macrophage adhesion to fibronectin-coated surfaces affects a variety of macrophage functions including chemotaxis (25, 50), differentiation (8), secretion (7, 45), and phagocytosis via immunologic receptors (12, 52, 66). Nonetheless, the molecular nature of the interactions leading to these critical macrophage functions is unknown.

Studies which have examined fibronectin receptors on macrophages in detail have demonstrated an unexpectedly large number of integrin and non-integrin fibronectin-binding proteins (10, 13, 16, 17, 29, 42, 59, 63). Four integrin receptors with fibronectin binding capability have been identified on mononuclear phagocytes. Fibronectin binding to VLA-5 ($\alpha_5\beta_1$), the vitronectin receptor ($\alpha_v\beta_3$) and the Leukocyte Response Integrin (LRI) appears dependent on the RGD adhesion sequence (13, 16, 29); (VLA-4 ($\alpha_4\beta_1$) binds fibronectin independent of this sequence (28). Additional macrophage integrins $\alpha_v\beta_5$, VLA-3 ($\alpha_3\beta_1$), and $\alpha_M\beta_2$, may also bind fibronectin (17,42,59,63). To add to the complexity of fibronectin binding by macrophages, several of these receptors can recognize alternative, potentially competing ligands. Moreover, $\alpha_5\beta_1$ and perhaps other of these integrins can assume two affinity states for fibronectin (26). The existence of these distinct but related receptors for the same ligand suggest that they may have different roles in macrophage function. The purpose of the present work was to begin to determine how these various receptors contributed to adhesion and phagocytosis. Because of the complexity of fibronectin receptor expression on macrophages, we have examined fibronectin-mediated adhesion and phagocytosis in K562 cells, which express only $\alpha_5\beta_1$. We have found this integrin capable of both functions. When K562 were transfected with $\alpha_v\beta_3$, ligation of $\alpha_v\beta_3$ with antibody or vitronectin inhibited both $\alpha_v\beta_3$- and $\alpha_5\beta_1$-mediated phagocytosis but had no effect on $\alpha_5\beta_1$-mediated adhesion. Pharmacologic data support the hypothesis that the effect of $\alpha_v\beta_3$ ligation on $\alpha_5\beta_1$ function requires a signal transduction pathway involving a serine/threonine kinase. These data show that there is crosstalk among integrin receptors. This previously unrecognized role for the $\alpha_v\beta_3$ integrin in differential regulation of functions of $\alpha_5\beta_1$ may be important not only in macrophage biology, but also in the function of $\alpha_v\beta_3$ in cell migration, angiogenesis, and metastasis (21, 42, 61).

Please address all correspondence to Dr. Eric J. Brown, Infectious Diseases, Campus Box 8051, Washington University School of Medicine, St. Louis, MO 63110.

1. *Abbreviations used in this paper*: AI, attachment index; Fn, fibronectin; H7, 1-(5)Isoquinolinesulfonyl)-2-methylpiperazine dihydrocholoride; PI, phagocytic index.

Materials and Methods

Cells

The human erythroleukemia cell line K562 was maintained in Iscove's Modified Dulbecco's Medium (GIBCO BRL, Gaithersburg, MD), contain-

ing 10% FBS (Hyclone, Logan, UT) and 1.0 μg/ml Gentamicin (Sigma Chem. Co., St. Louis, MO) and propagated in a 37°C, 5% CO_2, humidified incubator. Human monocytes were collected from healthy, normal volunteers by leukopheresis and purified to >95% by elutriation as described (69). Monocytes were cultured for 5 d in RPMI with 10% FBS on bacteriologic plastic and treated with 50 ng/ml GM-CSF for the last 16 h, as described (22). For experiments, monocytes were washed with PBS and removed from the plate after incubation with PBS containing 5.0 mM EDTA for 2 h at 4°C.

cDNA Clones and Cell Transfection

The human β_5 integrin subunit cDNA was the gift of Dr. Sarah Bodary, Genentech, Inc. (San Francisco, CA) (46). Human integrin α_v cDNA (44) was a gift of Dr. Joseph C. Loftus, Scripps Research Institute (La Jolla, CA). Human β_3 integrin cDNA was the gift of Dr. Jeffrey Smith (Scripps Research Institute). For transfection, the β_5 and β_3 cDNA were cloned into the stable expression vector pRc/RSV (Invitrogen, San Diego, CA) (β_5) or a derivative of pRc/RSV with the multicloning sites HindIII and XbaI in reverse orientation, (β_3) (43). The α_v cDNA was in pCDM8 (Invitrogen). K562 cells were cotransfected with either α_v and β_3 or α_v and β_5 by electroporation at 200 V, 600 uF using a Gene Pulser (Biorad Labs., Richmond, CA). Transfected cells were selected in media containing 1,200 μg/ml G418 (Genticin, Gibco), and populations of transfectants expressing the transfected integrin were obtained by fluorescence cell sorting. K562 were stably transfected with $\alpha_M\beta_2$ were as described (Graham, I. L., and E. J. Brown, manuscript submitted for publication). Control K562 were transfected with pRc/RSV vector alone.

Protein Purifications

Fibronectin was provided by Dr. John E. Kaplan, Albany Medical College (Albany, NY), and was prepared as described by gelatin affinity chromatography (11). Vitronectin was prepared as described (68) by denaturation and adsorption on heparin. Casein was purchased in solution from Pierce (Rockford, IL). Purity of all protein reagents was determined to be greater than 99% by SDS-PAGE.

Antibodies

Polyclonal antibody against human $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins were purchased from Gibco Laboratories. The $\alpha_v\beta_5$-specific monoclonal antibody (mAb) P1F6 (63) was the gift of Dr. Dean Sheppard (University of California at San Francisco). The $\alpha_v\beta_3$-reactive mAb LM609 (15) was a gift of Dr. David Cheresh (Scripps). The β_1-reactive mAb P5D2 (14) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA). The mAb BIIG2 and AIIB2, reactive with human integrin subunits α_5 and β_1, respectively (33, 64), were the kind gift of Dr. Caroline Damsky (University of California at San Francisco). The human integrin α_5-reactive mAb 16 (20) was the gift of Dr. Kenneth Yamada, NIDH/NIH (Rockville, MD). mAb IB4 (67) was the gift of Dr. Sam Wright, Rockefeller University (New York, NY). The β_1 integrin-activating antibodies A1A5 (34) and 8A2 (39) were generously provided by Dr. Martin Hemler, Dana-Farber Cancer Institute (Boston, MA), and Drs. Nick Kovach and John Harlan, University of Washington (Seattle, WA), respectively. The HLA reactive mAb W6/32 (6) was purchased from the American Type Culture Collection (Rockville, MD). mAbs 7G2 specific for human integrin β_3, and 3F12 and 1C12 specific for human integrin α_v were made as described (31). Fluorescein-labeled antibodies directed against rat or mouse IgG were purchased from Sigma Chem. Co. All antibodies were used as purified IgG.

FACS Analysis of Receptor Expression

Receptor expression on macrophages and K562 cells was analyzed by fluorescent flow cytometry as described (13, 43).

Flow Cytometric Particulate Phagocytosis Assay

Fluoresceinated ligand (40) was covalently coupled to 3.0 micron amino polybeads (Polysciences, Warrington, PA) via an 8 carbon bridge according to manufacturer's instructions, and ligand-opsonized beads were presented to cells in suspension. Bead ingestion was determined by a modification of previously described assays (3, 30). Briefly, after washing in $HBSS^{--}$ (GIBCO), cells were resuspended at 3×10^6/ml in HBSS with 1.0 mM each of Mg^{++}, Mn^{++}, and Ca^{++} with 0.5% Human Serum Albumin (HSA) (Assay Buffer) containing inhibitors at given concentrations with vehicle controls or purified polyclonal or monoclonal antibodies at 5.0 μg/ml with preimmune sera or isotype matched controls, respectively. Ligand-coated beads were vortexed to resuspend, sonicated for 10 s to disperse aggregates, counted on a hemocytometer and added to cells at a target: cell ratio of 1,000:1. Samples were incubated for 2 h with gentle rocking at 37°C unless otherwise stated. Cell-associated fluorescence was determined using a flow cytometer (EPICS, Coulter Diagnostics, Miami, FL) set to measure forward and side scatter as well as fluorescence, with and without the addition of 0.05% trypan blue, which was sufficient to quench fluorescence of extracellular beads. Phagocytosis was quantitated by determining the mean fluorescence of 10,000 cells in the presence of trypan blue, normalized to the fluorescence of a single bead, and expressed as a Phagocytic Index (PI), the number of beads internalized per 100 cells PI (31). The assay was validated by comparing data obtained by flow cytometry with direct observation by fluorescence microscopy. As expected, pretreatment of phagocytic cells with 5 μg/ml cytochalasin D completely abolished cell-associated fluorescence after trypan blue in this assay, confirming that trypan blue–resistant fluorescent beads were ingested.

Adherent Cell Bead Attachment Assay

Multiwell Lab-Tek Chamber Slides (Thomas Scientific, Swedesboro, NJ) were coated with either fibronectin, vitronectin, or casein at 50 μg/ml in PBS or mAb at 5 μg/ml in PBS overnight at 4°C. Wells were washed twice with PBS and postcoated with 1.0% casein in PBS for 30 min at 37°C. Cells were added to the wells at 2×10^6/ml in HBSS containing 1.0 mM Ca^{++} and Mg^{++} and adhered for 2 h at 37°C resulting in sparsely distributed cells. Wells were rinsed and ligand-opsonized beads prepared exactly as above were added to the cells in Assay Buffer at a 500:1 target to cell ratio. After an additional 2 h at 37°C, unbound beads were removed by three washes with PBS, the chambers removed, and slides wet mounted. Beads associated with cells were counted manually by light microscopy and the number of beads attached per 100 cells was determined (Attachment Index, AI) as previously described (31).

Cell Adhesion

96-well microtiter plates (Dynatech, Chantilly, VA) were coated with ligand as described above. Cells were washed as above, labeled with Calcein AM fluorescent dye (Molecular Probes, Eugene, OR) according to manufacturer's instructions and suspended in Assay Buffer. Cells and inhibitors were added to wells in 200 μl at 10^6/ml and incubated for 1.5 h at 37°C. Plates were rinsed twice gently with PBS and adhesion measured in a fluorescence plate reader (Cytofluor 2300, Millipore, Marlborough, MA). Adhesion data is presented in arbitrary fluorescence units with internal controls.

Reagents

H7, HA-1004, calphostin C, genestein, herbimycin, PMA, and phorbol dibutyrate (PDBu) were all purchased from LC Laboratories (Woburn, MA). All other reagents were purchased from Fisher America (Waukesha, WI).

Data Presentation

Data are presented as either the mean ± SEM or as a representative study from at least three yielding equivalent results. Significance was determined by analysis of variance followed by Duncan's comparison testing. A minimum confidence interval of 95% was used in all analyses.

Results

Fibronectin Bead Phagocytosis by Human Macrophages

To investigate which macrophage integrin receptors were responsible for attachment and ingestion of fibronectin-opsinized particles, we tested the effect of mAb against various macrophage integrins (or HLA class I as a control) on Fn bead binding to adherent cultured human monocytes (Fig. 1). In vitro culture of monocytes induces expression of $\alpha_v\beta_5$ and $\alpha_v\beta_3$ (Fig. 2 *A* and [22]); $\alpha_5\beta_1$ is present on peripheral blood monocytes and is maintained during in vitro

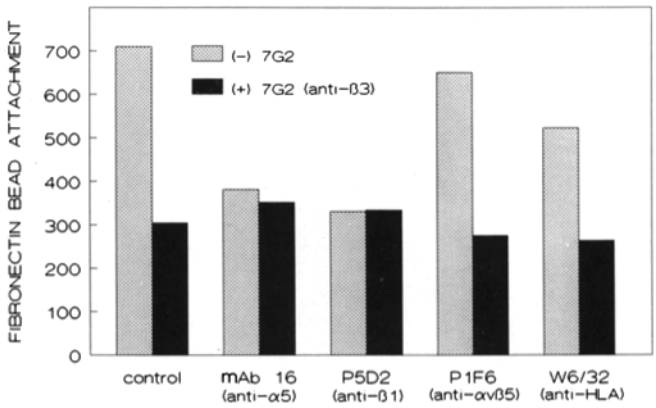


Figure 1. Inhibition of fibronectin bead binding by macrophages. Cultured human macrophages adherent to plastic were incubated with fibronectin-coated beads and various mAb in the presence or absence of mAb 7G2 (anti-β_3). Fibronectin bead attachment was assessed as described in Materials and Methods. More than 90% of attached beads were ingested (not shown). Data are expressed as the percent of casein-coated bead binding under identical conditions. Shown is a representative of three equivalent studies. Monoclonal antibody reactivities are shown in parentheses.

culture. Attachment of fibronectin-coated beads was decreased almost equivalently by mAb 7G2 (anti-β_3), mAb 16 (anti-α_5), and P5D2 (anti-β_1). Neither P1F6 (anti-$\alpha_v\beta_5$) nor W6/32 (anti-HLA class I) had any effect on fibronectin bead phagocytosis. Surprisingly, the effects of the inhibitory mAb were not additive, since the combination of anti-β_3 with either anti-α_5 or anti-β_1 resulted in no additional inhibition. These data suggested that β_3 integrins and $\alpha_5\beta_1$ did not act independently in fibronectin bead binding by macrophages. This suggested that binding of fibronectin to one integrin might affect the function of other potential fibronectin receptors. However, the complexity of expression of fibronectin receptors in macrophages precluded a more detailed analysis of this possibility.

Fibronectin Bead Phagocytosis by Transfected K562

To pursue the question of interaction between $\alpha_v\beta_3$ and $\alpha_5\beta_1$, a cell with a simpler pattern of integrin expression than macrophages was desirable. Thus, the erythroleukemia cell line K562 was stably transfected with $\alpha_v\beta_5$ or $\alpha_v\beta_3$ (Fig. 2 *A*). K562 were chosen because they can be phagocytic (Graham, I. L., and E. Brown, unpublished) and because, unlike macrophages, they express a very limited number of integrins, predominantly $\alpha_5\beta_1$. K562 expressed no detectable α_v integrins in the absence of transfection (data not shown), or when transfected with vector alone (Fig. 2 *A*). Transfection with $\alpha_v\beta_3$ and $\alpha_v\beta_5$ led to approximately equivalent levels of antigenic and functional receptors, as demonstrated by fluorescence with receptor-specific antibody (Fig. 2 *A*) and by ingestion of vitronectin beads (Fig. 2 *B*). Further, fluorescence of α_v expression in transfected K562 cells expressing $\alpha_v\beta_3$ or $\alpha_v\beta_5$ was equivalent as assessed by flow cytometry using an α_v-specific monoclonal antibody IC12 (data not shown). Transfection did not affect expression of α_5 (data not shown) or β_1 (Fig. 2 *A*).

$\alpha_v\beta_3$ and $\alpha_v\beta_5$ transfectants and the vector controls phagocytosed fibronectin beads (Fig. 2 *B*). Ingestion of fibronectin-opsonized beads was greater in cells transfected

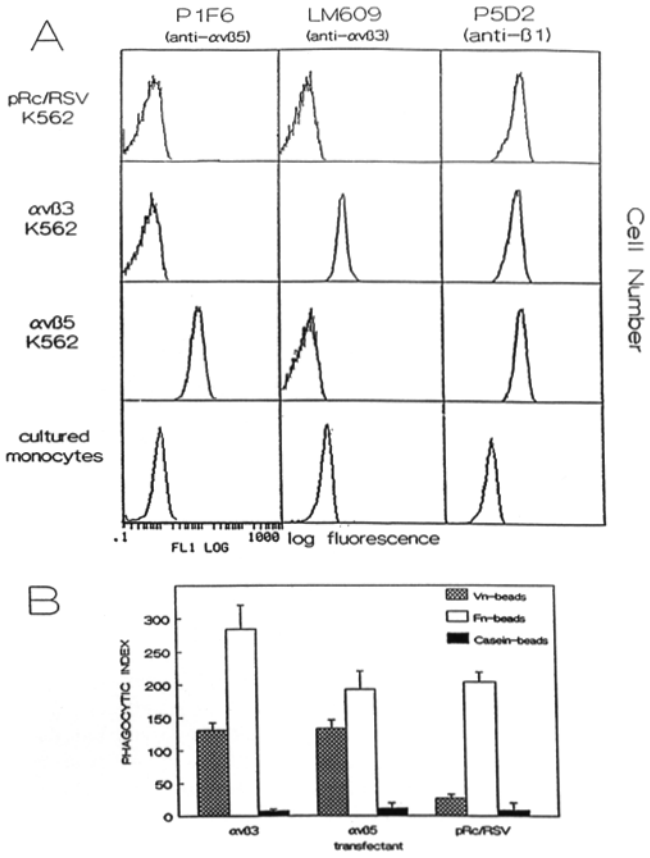


Figure 2. Expression and function of α_v-integrin receptors in transfected K562 and human macrophages. K562 cells were transfected with $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins, and human monocytes were cultured to induce expression of α_v integrins as described in Materials and Methods. (*A*) FACS analysis of α_v integrin receptor expression. (*B*) The ability of transfected K562 integrins to bind and internalize opsonized particles was determined using the flow cytometric phagocytosis assay described in Materials and Methods. Data are the summary of three independent experiments. Monoclonal antibody reactivities are shown in parentheses.

with $\alpha_v\beta_3$ than those transfected with $\alpha_v\beta_5$ or vector, suggesting that, as in macrophages, $\alpha_v\beta_3$ can contribute to the ingestion of fibronectin beads in transfected K562. Treatment with phorbol esters (1–100 ng/ml) for up to 2 h had no effect on the phagocytosis of fibronectin or vitronectin beads.

The effects of anti-integrin antibodies on fibronectin bead phagocytosis by vector- and $\alpha_v\beta_3$-transfected K562 were determined (Fig. 3). Phagocytosis by vector-transfected cells was inhibited by mAb's BIIG2 (anti-α_5) or AIIB2 (anti-β_1) to the same extent as by chelation of all divalent cations with EDTA. This very low level of ingestion was equivalent to the nonspecific ingestion of casein opsonized beads (not shown). mAb 16 (anti-α_5) and P5D2 (anti-β_1) also inhibited fibronectin bead phagocytosis (not shown). Inhibition of $\alpha_5\beta_1$ function with the same mAb in the $\alpha_v\beta_3$ transfectants resulted in an ~50% decrease in phagocytosis. Monoclonal and polyclonal antibodies at 5.0 μg/ml against $\alpha_v\beta_3$ had no effect on phagocytosis by control K562, but completely abolished phagocytosis in the $\alpha_v\beta_3$ transfectants at the same concentration. mAb 7G2 (anti-β_3) and LM609 (anti-$\alpha_v\beta_3$) inhibited equivalently at 5.0 μg/ml. This effect

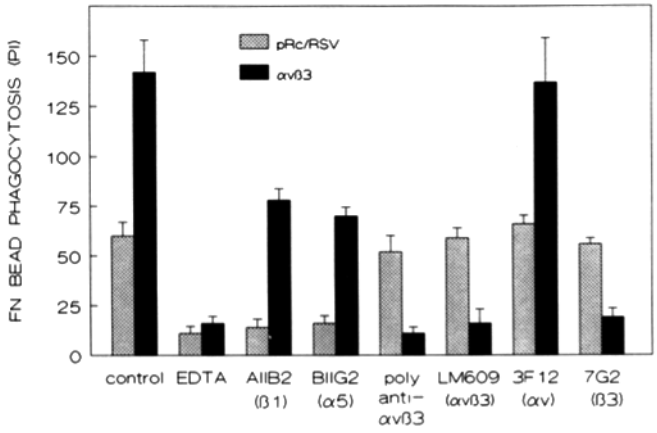


Figure 3. Antibody inhibition of fibronectin bead phagocytosis by transfected K562 cells. K562 cells transfected with vector (pRc/RSV) or with $\alpha_v\beta_3$ were presented with fibronectin-opsonized beads in the fluid phase, with and without polyclonal antibodies to $\alpha_v\beta_3$ or various monoclonal antibodies against integrin subunits. Antibodies were included as purified IgG at 5.0 μg/ml. Phagocytosis was assessed as in Fig. 2. Bars labeled EDTA were performed in a buffer identical to standard assay buffer, except that EDTA (5 mM) was included in place of divalent cations. Nonimmune rabbit IgG, a control for the polyclonal anti-$\alpha_v\beta_3$, had no effect on ingestion (not shown). Data, expressed as the phagocytic index (PI, number of beads ingested/100 cells), are the summary of eight studies. For all studies, the PI for casein-opsonized beads averaged less than 12 (not shown). Monoclonal antibody reactivities are shown in parentheses.

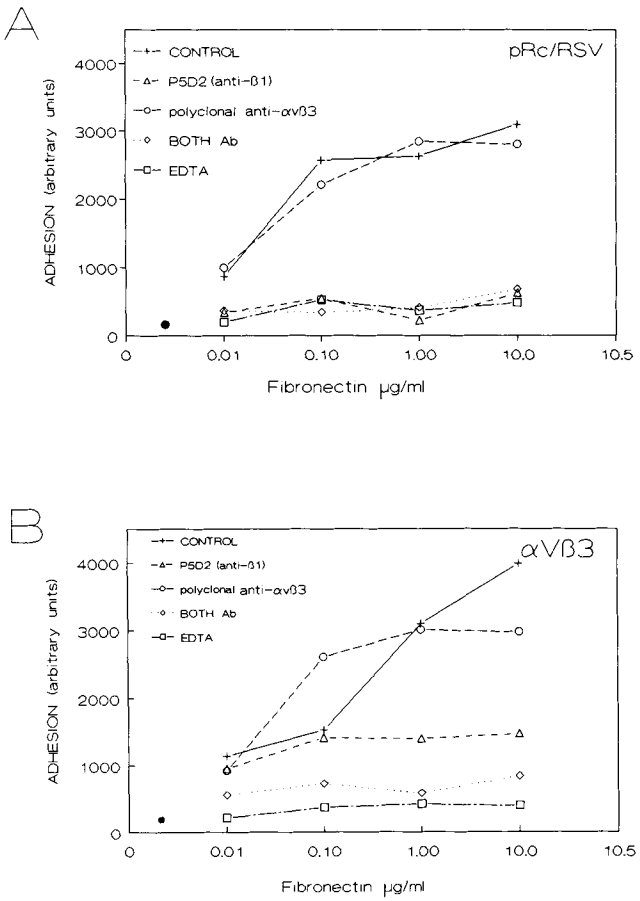


Figure 4. Adhesion of transfected K562 cells to fibronectin. K562 cells transfected with vector (*A*) or with $\alpha_v\beta_3$ (*B*) were adhered to substrate coated with varied concentrations of fibronectin in the presence and absence of P5D2 (anti-β_1) or polyclonal antibody to $\alpha_v\beta_3$. Binding was performed in assay buffer or in buffer containing 5 mM EDTA rather than divalent cations (*EDTA*). In both panels, the filled circle represents binding to casein-coated (50 μg/ml) wells. Data are expressed in arbitrary fluorescence units. Shown is a representative of three studies yielding comparable results.

required recognition of specific $\alpha_v\beta_3$ epitopes, since fibronectin bead phagocytosis was not inhibited by 3F12, an immunoprecipitating antibody against α_v (31) which has no known inhibitory properties although it has greater affinity for $\alpha_v\beta_3$ than LM609 or 7G2 as assessed by flow cytometry. Thus, ligation of $\alpha_v\beta_3$ with antibody resulted in inhibition of the phagocytic capability of $\alpha_5\beta_1$ as well as $\alpha_v\beta_3$; this conclusion can explain the non-additive inhibitory effects of the mAbs on fibronectin bead attachment to macrophages (Fig. 1).

Anti-$\alpha_v\beta_3$ Fails to Inhibit Adhesion of K562 Transfectants to Fibronectin

Since mAb against $\alpha_v\beta_3$ inhibited phagocytosis by $\alpha_5\beta_1$, we evaluated the effect of these antibodies on $\alpha_5\beta_1$-mediated adhesion to fibronectin-coated substrate (Fig. 4). Vector transfectants showed fibronectin dose-dependent adhesion which was inhibited by P5D2 (anti-β_1) at all coating concentrations of fibronectin (Fig. 4 *A*). As expected, neither polyclonal antibody to $\alpha_v\beta_3$ or mAbs 7G2, 3F12, or LM609 had any effect on the adhesion of the vector-transfected cells (Fig. 4 *A* and data not shown). In contrast to fibronectin bead phagocytosis, adhesion of the $\alpha_v\beta_3$ transfectants to the fibronectin substrate was almost entirely inhibited by the anti-β_1 mAb (Fig. 4 *B*). In the absence of anti-β_1, neither polyclonal antibody against $\alpha_v\beta_3$ nor any of the anti-$\alpha_v\beta_3$ mAb significantly affected adhesion by these cells. This was true at multiple coating densities of fibronectin, suggesting that the difference between adhesion and phagocytosis could not be attributed to differences in ligand density between coated substrate and opsonized bead. The limited fibronectin adhesion of the $\alpha_v\beta_3$ transfectants in the presence of anti-β_1 was inhibited to baseline by the addition of polyclonal antibody against $\alpha_v\beta_3$, demonstrating that these are the only functional fibronectin adhesive receptors on these cells.

Adhesion to $\alpha_v\beta_3$ Ligands Blocks Fibronectin Bead Binding

One explanation for the effect of the anti-$\alpha_v\beta_3$ on $\alpha_5\beta_1$ function is that these antibodies somehow sterically interfere with $\alpha_5\beta_1$ ligand binding. The failure of anti-$\alpha_v\beta_3$ to inhibit $\alpha_5\beta_1$-mediated adhesion (Fig. 4) is evidence against this hypothesis. To further explore this possibility, we examined fibronectin bead binding on cells adherent to various monoclonal antibodies or protein substrates (Fig. 5). Adhesion to anti-α_5 (BIIG2) or to anti-β_1 (AIIB2) decreased the fibronectin bead binding of controls and $\alpha_v\beta_3$ transfectants 37% compared to adhesion to casein or the control mAb W6/32 (anti-HLA class I). Adhesion to fibronectin also had mini-

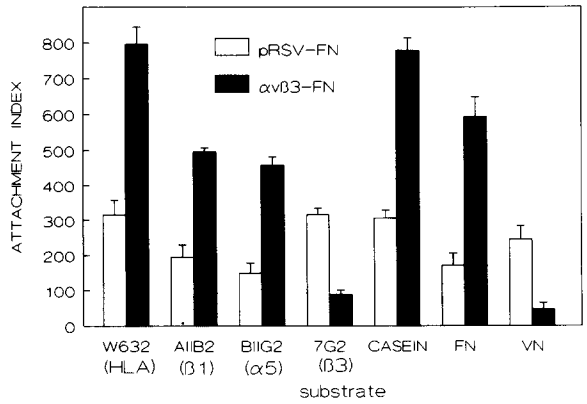
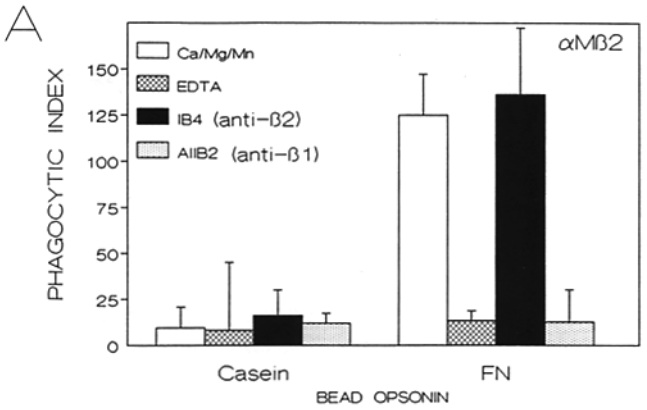


Figure 5. Fibronectin bead binding by transfected K562 cells adherent to antibody or ligand. K562 cells transfected with vector or $\alpha_v\beta_3$ were adhered to wells coated with mAb, integrin ligands, or casein. Fibronectin-opsonized beads were presented to these cells and bead attachment determined as described in Materials and Methods. Data are presented as the Attachment Index (AI) and are the summary of three independent studies. AI of casein-opsonized beads in parallel samples averaged less than 25 (not shown). mAb reactivities are shown in parentheses.



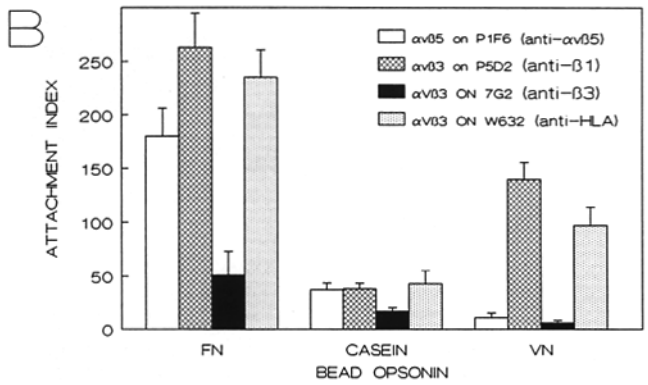


Figure 6. Opsonized bead attachment and phagocytosis by K562 cells transfected with $\alpha_v\beta_5$ or $\alpha_M\beta_2$. (*A*) K562 transfected with $\alpha_M\beta_2$ were presented with fibronectin beads in the presence and absence of monoclonal antibody specific for $\alpha_5\beta_1$ (AIIB2) or $\alpha_M\beta_2$ (IB4) and phagocytosis assessed by flow cytometry as in Fig. 2. Antibodies were included as purified IgG at 5.0 μg/ml. (*B*) Attachment of fibronectin-, casein-, and vitronectin-opsonized beads was assessed in K562 transfected with $\alpha_v\beta_3$ or $\alpha_v\beta_5$ adherent to mAb specific for K562 integrins or HLA. Bead attachment to adherent cells was determined as in Fig. 5. Data in each panel are presented as the summary of three independent experiments. mAb reactivities are shown in parentheses.

mal effect on fibronectin bead binding. This minimal effect is consistent with previous data (32). Residual fibronectin bead binding in cells adherent to fibronectin was completely (controls) or partially (transfectants) inhibited by antibody to α_5 or β_1 in solution (data not shown), and is consistent with redistribution of a small proportion of fibronectin receptors from the apical to the basal adherent surface (47). In contrast, adhesion to vitronectin or to the anti-β_3 mAb 7G2 blocked fibronectin bead binding to the $\alpha_v\beta_3$ transfectants completely despite the observation that significantly more cells were adherent to fibronectin or anti-$\alpha_5\beta_1$ antibody-coated substrates (data not shown). Thus variation in the number of cells adherent to each substrate cannot account for the observed differences in fibronectin bead binding. Adhesion to 7G2 or vitronectin had no effect on $\alpha_5\beta_1$ expression or distribution on the transfected cells, as judged by fluorescence microscopy (not shown). These data show that ligation of $\alpha_v\beta_3$ on the basal adherent cell surface inhibits the binding and phagocytosis of fibronectin beads by $\alpha_5\beta_1$ on the apical cell surface. In contrast, ligation of $\alpha_5\beta_1$ did not block fibronectin bead binding by $\alpha_v\beta_3$. Cell adhesion to anti-β_1 (Fig. 6) or to fibronectin (not shown) did not affect vitronectin bead binding by the transfectants.

Ligation of $\alpha_v\beta_3$ but Not $\alpha_v\beta_5$ or $\alpha_M\beta_2$ Inhibits $\alpha_5\beta_1$-mediated Phagocytosis

To determine whether integrins other than $\alpha_v\beta_3$ could affect $\alpha_5\beta_1$ phagocytosis, we examined K562 transfected with $\alpha_M\beta_2$ and with $\alpha_v\beta_5$ (Fig. 6). In K562 cells transfected with $\alpha_M\beta_2$, ligation of $\alpha_M\beta_2$ with mAb IB4 did not affect fibronectin phagocytosis, whereas anti-β_1 mAb AIIB2 inhibited completely (Fig. 6 *A*). These cells expressed $\alpha_M\beta_2$ at levels greater than activated neutrophils, as assessed by flow cytometry using antibody IB4 (data not shown). Complete inhibition by AIIB2 suggests that $\alpha_M\beta_2$ does not contribute to fibronectin bead binding, and these transfectants are equivalent to the control, vector-transfected K562 with respect to fibronectin bead phagocytosis. Additionally, in studies similar to that shown in Fig. 5, adhesion of $\alpha_M\beta_2$ transfected K562 to substrate coated with IB4 or C3B, a $\alpha_M\beta_2$ ligand, had no effect on fibronectin bead binding (data not shown).

In the $\alpha_v\beta_5$ transfectants, adhesion to mAb P1F6 (anti-$\alpha_v\beta_5$) (Fig. 6 *B*) or vitronectin (data not shown) had no effect on fibronectin bead phagocytosis. Nonetheless, it did eliminate vitronectin bead binding. As with the $\alpha_M\beta_2$ transfectants, fibronectin bead binding was completely abolished by addition of mAb against $\alpha_5\beta_1$ in solution (not shown). Thus, ligation of integrins other than $\alpha_v\beta_3$ does not affect fibronectin bead binding by $\alpha_5\beta_1$.

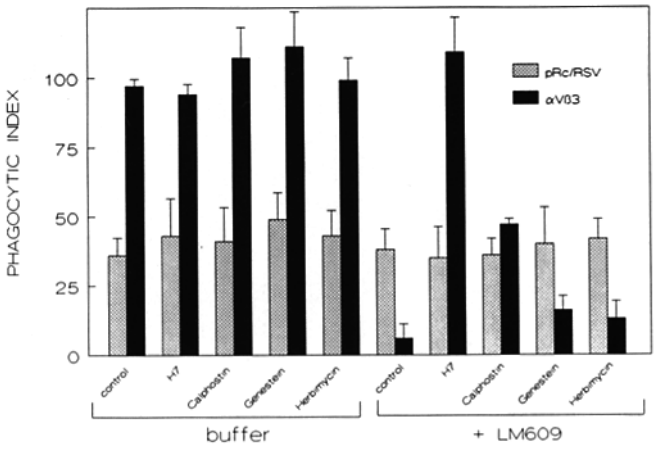


Figure 7. Effects of pharmacologic inhibitors on fibronectin bead phagocytosis. Phagocytosis of fibronectin-opsonized beads was assessed in K562 cells transfected with vector or $\alpha_v\beta_3$ in the presence or absence of purified mAb anti-$\alpha_v\beta_3$ (LM609) at 5.0 μg/ml and various inhibitors of signaling pathways; H7 (50 nM), calphostin C (100 nM), Genestein (50 μg/ml), and Herbimycin (10 μg/ml). Data, expressed as the phagocytic index, are the summary of three studies.

$\alpha_v\beta_3$-mediated Inhibition of $\alpha_5\beta_1$ Is Blocked by H7

Ligation of $\alpha_v\beta_3$ might affect $\alpha_5\beta_1$ phagocytosis through a second messenger signaling pathway. To investigate this possibility, we determined the effects of various pharmocologic inhibitors on the ability of anti-β_3 to block fibronectin bead phagocytosis (Fig. 7). While 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7), a serine/threonine kinase inhibitor, had no effect on phagocytosis by either controls or $\alpha_v\beta_3$ transfectants, it completely reversed the inhibition of phagocytosis resulting from LM609 ligation of $\alpha_v\beta_3$. H7 reversal of inhibition was dose dependent, with an IC_{50} of 5.0 nM (data not shown). HA-1004, which inhibits cAMP-dependent protein kinase similarly to H7, but is a poor inhibitor of protein kinase C (35), had no effect on the LM609 effect at concentrations up to 1 μM. Calphostin C, a more selective inhibitor of protein kinase C (37) also partially reversed the LM609 inhibition of fibronectin bead phagocytosis, with an IC_{50} 12.5 nM. The tyrosine kinase inhibitors genestein and herbimycin had no effect on fibronectin bead phagocytosis, whether or not LM609 was included in the assay. These data support the hypothesis that $\alpha_v\beta_3$ ligation affects $\alpha_5\beta_1$ phagocytic function through a signal transduction pathway, possibly involving protein kinase C.

Phagocytosis by $\alpha_5\beta_1$ in K652 Cells Requires Mn^{++} or Activating mAb

There was very little Fn-mediated phagocytosis in K562 in the absence of Mn^{++}, whether or not they had been transfected with $\alpha_v\beta_3$ (Fig. 8). Incubation of K562 with "activating" mAb which recognized the integrin β_1 chain (4, 5, 54) induced efficient Fn-mediated phagocytosis in both control and $\alpha_v\beta_3$-expressing K562 in the absence of Mn^{++} (Fig. 8 *A*). Addition of 750 nM (300 μg/ml) soluble fibronectin completely inhibited phagocytosis of fibronectin beads in

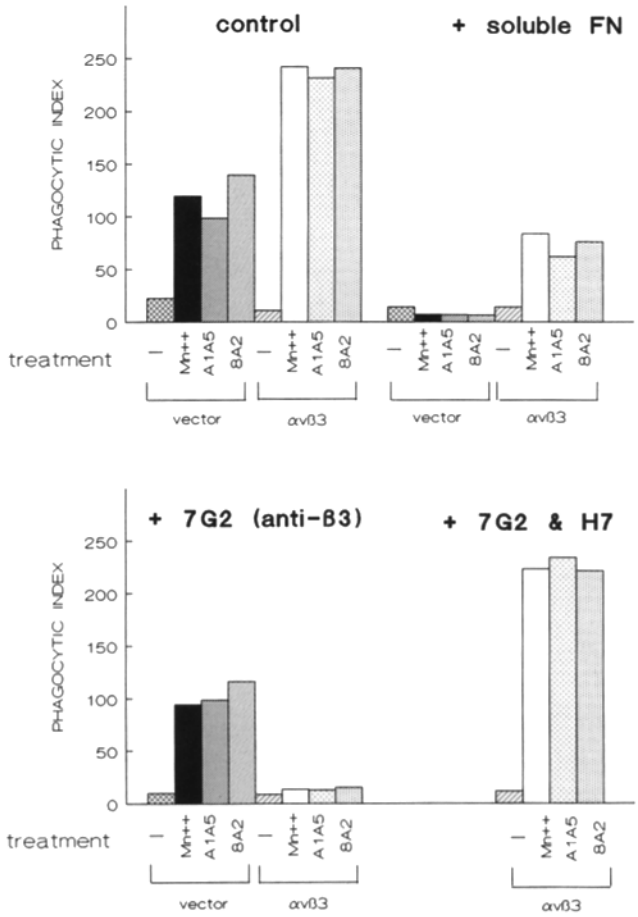


Figure 8. Effect of soluble fibronectin and of activating antibodies on fibronectin bead phagocytosis. Phagocytosis of fibronectin-opsonized beads was assessed in K562 transfected with vector or $\alpha_v\beta_3$ in assay buffer containing Ca^{++} and Mg^{++} alone (—), with the addition of Mn^{++} (Mn++), or in the presence of mAb A1A5 or 8A2 in assay buffer without Mn^{++} (control). The effects of 750 nM soluble fibronectin (+ soluble FN) (*upper panel*) and mAb 7G2 (anti-β_3) with (+ 7G2 & H7) and without 25 nM H7 (+ 7G2) (*lower panel*) on fibronectin phagocytosis was determined in $\alpha_v\beta_3$-expressing K562. All antibodies were used as purified IgG at 5.0 μg/ml. Data presented as the Phagocytic Index, are representatives of five studies yielding similar results. PI of casein-opsonized beads in parallel samples averaged less than 25 for all groups.

vector-transfected K562 cells. Soluble fibronectin inhibited the $\alpha_5\beta_1$ component of phagocytosis in the $\alpha_v\beta_3$ transfectants, since addition of P5D2 anti-β_1 did not further inhibit fibronectin bead phagocytosis in the presence of 300 μg/ml fibronectin. In the presence of Mn^{++}, A1A5, or 8A2, $\alpha_v\beta_3$ ligation inhibited phagocytosis, since 7G2 anti-β_3 inhibited ingestion in the $\alpha_v\beta_3$ transfectants completely in each of these cases (Fig. 8 *B*). For β_1-activating mAb as well as Mn^{++}, H7 overcame the inhibitory effect of 7G2.

In contrast, adhesion of both control and $\alpha_v\beta_3$-expressing K562 to fibronectin-coated substrates was unaffected by soluble fibronectin or by anti-β_3 (not shown). This was true for adhesion in the absence or presence of Mn^{++}, A1A5 and 8A2, all of which enhanced adhesion of K562 approximately threefold when included.

Discussion

In addition to its role as an adhesive ligand in extracellular matrix, fibronectin is a major opsonin in human serum which functions in both host defense and wound repair. While this aspect of fibronectin function has been known for many years, the molecular aspects of fibronectin's interaction with cells as an opsonin, rather than as a conventional adhesion molecule, are not known. Among the reasons for this lack of understanding is the large number of potential fibronectin receptors on macrophages, the major effector cells in recognition of fibronectin-opsonized bacteria and debris. In an attempt to develop a molecular understanding of fibronectin-macrophage interactions in host defense and wound repair, we tested the effects of monoclonal antibodies against specific fibronectin receptors on macrophage recognition of fibronectin-opsonized particles. We found that monoclonal antibodies recognizing both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ inhibited the uptake of fibronectin-opsonized particles significantly, but that, surprisingly, their effects were not additive.

To study the apparent lack of independence of these two macrophage integrin fibronectin receptors in more detail, we transfected $\alpha_v\beta_3$ into K562 cells, which express $\alpha_5\beta_1$ as their predominant integrin. In these cells $\alpha_v\beta_3$ ligation inhibited $\alpha_5\beta_1$ phagocytic function. This finding explained the lack of additive effects of the antibodies on macrophage phagocytosis of fibronectin beads. Because these antibodies did not inhibit fibronectin-mediated phagocytosis completely in macrophages, the possibility of a contribution from other fibronectin receptors remains. However, our transfectant data did not suggest any contribution from $\alpha_v\beta_5$ or $\alpha_M\beta_2$, and antibodies to $\alpha_4\beta_1$ did not inhibit macrophage fibronectin-mediated phagocytosis (Blystone, S. D., and E. J. Brown, unpublished), suggesting these integrins are not involved in fibronectin-mediated phagocytosis. Other non-integrin receptors may account for the residual binding and ingestion (10).

The most striking observation from the transfectants is that $\alpha_v\beta_3$ ligation inhibits $\alpha_5\beta_1$ phagocytic function. This effect results not from steric hindrance, as would occur if the two receptors were closely associated in the plasma membrane, but from signal transduction. The effect on $\alpha_5\beta_1$ function is quite specific for $\alpha_v\beta_3$ ligation, since neither the closely related integrin $\alpha_v\beta_5$ nor $\alpha_M\beta_2$ have the same effect. The failure of ligation of $\alpha_v\beta_5$ to affect $\alpha_5\beta_1$ phagocytic function suggests that there are β_3-specific sequences required for the signal transduction. Because both the isoquinolone sulfonamide H7 and calphostin C inhibit signal transduction from $\alpha_v\beta_3$, it is tempting to speculate that a protein kinase C is involved in the signaling pathway. The failure of PMA to affect fibronectin bead phagocytosis does not invalidate this hypothesis, since PMA is a nonspecific activator of many protein kinase C isoforms and may lead to activation of signal transduction pathways with varying effects on phagocytosis. The failure of protein tyrosine kinase inhibitors to affect $\alpha_v\beta_3$ signal transduction suggests that the recently elucidated pathway involving ppl25^{FAK} and *c-src* (18, 38, 56), which is engaged on integrin cross-linking or adhesion to ligand-coated substrate, is not involved in the regulation of $\alpha_5\beta_1$ phagocytosis by $\alpha_v\beta_3$.

Regulation of $\alpha_5\beta_1$ function by $\alpha_v\beta_3$ ligation is relatively subtle, since it affects phagocytosis but not adhesion by $\alpha_5\beta_1$. $\alpha_5\beta_1$ can adopt two conformations, which have two distinct affinities for fibronectin (4, 5, 26, 51, 54). Our data suggest that fibronectin-mediated phagocytosis requires the higher affinity conformation, since addition of Mn^{++} or activating anti-β_1 mAb is required in K562. The fact that phagocytosis can be inhibited by fibronectin in solution is support of this hypothesis. Adhesion to a fibronectin-coated surface does not require the high affinity conformation of $\alpha_5\beta_1$, since it is unaffected by fibronectin in solution, even in the presence of Mn^{++} or activating mAb. These data suggest the hypothesis that $\alpha_v\beta_3$ ligation specifically regulates $\alpha_5\beta_1$ functions which require the high affinity state of the receptor. Signal transduction from $\alpha_v\beta_3$ may inhibit induction of the high affinity state by Mn^{++} or activating antibodies, or may regulate a subsequent step in attachment of fibronectin-opsonized particles for which the high affinity receptor is necessary. Because the mAb and Mn^{++} induce the high affinity conformation even in detergent-solubilized receptors, we favor the hypothesis that the target of the $\alpha_v\beta_3$-dependent signal transduction is a process subsequent to development of the high affinity state.

The signal transduction between $\alpha_v\beta_3$ and $\alpha_5\beta_1$ is a unidirectional process, since ligation of $\alpha_5\beta_1$ does not affect the binding and phagocytic properties of $\alpha_v\beta_3$. It is possible that $\alpha_v\beta_3$ is not a high affinity receptor for fibronectin, since it is not inhibited by fibronectin in solution, and that, unlike $\alpha_5\beta_1$, the high affinity state of this receptor (if it exists) is not required for phagocytosis.

Little is known about the functions of the high affinity state of $\alpha_5\beta_1$ or any integrin. Macrophage phagocytosis of fibronectin-opsonized targets is one of the first identified cellular functions which clearly requires the high affinity integrin state. Platelet $\alpha_{IIb}\beta_3$ binding of fibrinogen requires induction of high affinity; this is the best understood integrin affinity regulation (36). Since $\alpha_{IIb}\beta_3$ binds fibrinogen on a surface without activation (55), the role of the high affinity state is to induce platelet aggregation rather than to mediate adhesion to a nascent thrombus. The leukocyte β_2 integrins bind ligand efficiently only after cell activation (1, 2). In particular, $\alpha_M\beta_2$ (Mac-1) function on phagocytes has similarities to $\alpha_5\beta_1$. Like $\alpha_5\beta_1$, Mac-1 recognizes and leads to ingestion of infectious agents and other opsonized material. Mac-1 binds its C3bi ligand efficiently only after cell activation (65). However, it is unclear that increased β_2 integrin avidity after activation represents a change in affinity; other mechanisms such as receptor clustering (24) may account for increased binding of cells to ligand. To date, changes in affinity of the β_2 integrins have not been documented. It is interesting that $\alpha_v\beta_3$ ligation has opposite effects on $\alpha_5\beta_1$ and $\alpha_M\beta_2$ function in leukocytes (62), perhaps suggesting differences in the mechanism of activation of these receptors.

$\alpha_v\beta_3$ may affect the function of other integrins as well. In this light, it is important to reconsider the mechanisms by which antibodies to or ligation of $\alpha_v\beta_3$ regulate cell functions in vivo and in vitro. While data showing a role for $\alpha_v\beta_3$ in phenomena as diverse as increase in $[Ca^{++}]_i$ in endothelial cells on adhesion to fibronectin (57) and in melanoma metastasis (27, 49) have been interpreted as resulting directly from adhesion of $\alpha_v\beta_3$ to a ligand, an alternative explanation is that anti-$\alpha_v\beta_3$ or even $\alpha_v\beta_3$ ligand could affect the function of $\alpha_5\beta_1$ or other integrins.

Finally, what is the physiologic role for conversion of mac-

rophage $\alpha_5\beta_1$ from a high affinity phagocytic receptor to a low affinity adhesion receptor by $\alpha_v\beta_3$ ligation? Expression of $\alpha_5\beta_1$ in monocytes has been shown to precede expression of $\alpha_v\beta_3$ during differentiation into macrophages. Perhaps expression of $\alpha_v\beta_3$ during differentiation permits receptor specialization: $\alpha_5\beta_1$ assumes primarily an adhesive function in the tissue macrophage, while $\alpha_5\beta_1$ becomes the primary receptor for recognition and phagocytosis of fibronectin-opsonized particles. A role for $\alpha_v\beta_3$ in the recognition of fibronectin-opsonized material, rather than adhesion, could explain the intralumenal localization of $\alpha_v\beta_3$ on endothelial cells (19).

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