A Novel Member of the Integrin Receptor Family Mediates Arg-Gly-Asp–stimulated Neutrophil Phagocytosis

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Abstract. Human neutrophils (PMN) express a heterodimeric receptor that has ligand binding specificity for the Arg-Gly-Asp (RGD) sequence within many adhesive proteins. A monoclonal antibody, B6H12, which binds to this receptor, inhibits both RGD-mediated ligand binding and stimulation of IgG-mediated phagocytosis by fibronectin, fibrinogen, vitronectin, von Willebrand's factor, and collagen type IV. By several criteria this receptor is neither a known very late antigen, a known cytoadhesin (gp IIb/IIIa–vitronectin receptor), nor a member of the LFA-1, Mac-1, p150,95 group of integrin receptors. Ligand binding via this receptor is rapidly inactivated by products of the myeloperoxidase-hydrogen peroxide-halide system of PMN. We conclude that this receptor, for which we propose the name leukocyte response integrin, is a signal-transducing molecule on PMN which may have a significant early role in modulation of PMN function at inflammatory sites.

THE integrin superfamily of adhesive receptors are transmembrane heterodimeric molecules which function in cell–matrix and cell–cell adhesion (Hynes, 1987). They are thought to function in adhesion processes by serving as transmembrane links between the extracellular environment and the cytoskeleton (Ruoslahti and Pierschbacher, 1987; Buck, 1987). As such they are intimately involved in many complex cell processes including thrombosis, hemostasis, cell maturation, embryogenesis, lymphocyte killing, and phagocytosis. The integrins can be roughly classified into three groups: (*a*) the very late antigens (VLAs)[1] first described on T lymphocytes, including the fibronectin (Fn) receptor from human placenta and osteosarcoma cells (Hemler et al., 1987); (*b*) cytoadhesins, including the platelet gp IIb/IIIa and the vitronectin receptor (VnR) (Ginsberg et al., 1988); and (*c*) LFA-1, Mac-1, p150,95, leukocyte-specific adhesion receptors, including the complement receptor for C3bi (CR3) (Anderson and Springer, 1987). The heterodimers in each group are comprised of distinct alpha chains noncovalently linked to a common beta chain. Because of this, monoclonal antibodies to the beta chain of each group can be used to classify new receptors as members of one or another group within the integrins. As an example, the Fn receptor was shown to be a member of the VLA group because it bound A-1A5, a monoclonal antibody which recognizes all VLA beta chains.

In addition to significant structural and sequence homology, the integrins also exhibit ligand-binding similarities. Several of these receptors were first discovered because of their binding to extracellular matrix proteins via an Arg-Gly-Asp (RGD) sequence in the matrix ligands. Our laboratory has recently characterized the RGD-binding proteins of human neutrophils (PMN) and monocytes by affinity chromatography of cell lysates on RGD-Sepharose (Brown and Goodwin, 1988). We showed that both phagocytes express a heterodimeric receptor distinct from the LFA-1, Mac-1, p150,95 family which exhibits immunological cross-reactivity with gp IIb/IIIa on platelets. Phagocytes undergo a number of important functional changes during recruitment to an inflammatory or infected site. These include changes in the receptors expressed at the plasma membrane, activation of new metabolic pathways, increases in oxygen consumption and the production of reactive oxygen metabolites, and augmentation of phagocytosis. Extracellular matrix proteins have been shown to mediate some of these physiologic changes, especially enhancement of ingestion of opsonized particles by monocytes and macrophages. This enhancement is dependent on recognition of the RGD sequence within these matrix proteins (Brown and Goodwin, 1988; Wright and Meyer, 1985). We hypothesized that the molecule identified by affinity chromatography on RGD-Sepharose might be the phagocyte receptor involved in extracellular matrix–stimulated ingestion.

Here we report the definitive identification of the phago-

1. *Abbreviations used in this paper*: CBD, cell-binding domain; cc, cytochrome c; E, sheep erythrocytes; Fg, fibrinogen; Fn, fibronectin; LAD, leukocyte adhesion deficiency; MPO, myeloperoxidase; PI, phagocytic index; PMN, human neutrophils; PRBP, placental RGD-binding proteins; VLA, very late antigens; Vn, vitronectin; VnR, vitronectin receptor; vWF, von Willebrand's factor.

cytosis-enhancing receptor of PMN. We have prepared polyclonal and monoclonal antibodies which inhibit both RGD-dependent ligand binding and extracellular matrix–stimulated ingestion by human PMN. The monoclonal antibody B6H12, which inhibits RGD-mediated phagocytosis enhancement, recognizes an antigen on PMN that is distinct from any other previously described integrin and has a broad specificity for RGD-containing proteins. Interestingly, ligand binding by this receptor is rapidly lost upon activation of the myeloperoxidase (MPO)–H_2O_2–halide system in PMN, perhaps suggesting that this receptor is important early in the inflammatory response of PMN, before significant respiratory burst or degranulation occur.

Materials and Methods

Special Reagents

The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO): chicken egg albumin (ovalbumin), catalase (bovine liver; 52,000 U/mg), cytochrome c (cc) (type XIV; pigeon heart), and fibrinogen (Fg). A 10-fold concentrated stock of Hank's balanced salt solution (HBSS) was purchased from Gibco Laboratories (Grand Island, NY). Human vitronectin (Vn) was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Human fibronectin and the chymotryptic cell-binding domain (CBD) of 105–110 kD were purified as described (Bohnsack et al., 1986). Collagen type IV and laminin were kind gifts of Dr. Hynda Kleinmann, National Institute of Dental Research, Bethesda, MD; von Willebrand's Factor (vWF) was the kind gift of Dr. Sam Santoro, Washington University, St. Louis, MO. The synthetic peptides GRGDSC and GDGDSC were prepared by the Peptide Chemistry Facility, Washington University School of Medicine, and KYAVTGRGDS was the gift of Dr. Steven Adams, Monsanto Corp., St. Louis, MO. GRGDSC, GDGDSC, or C as a control was linked to cc via bromacetyl succinamide (Bernatowicz and Matsueda, 1986). The molar ratio of incorporation of peptides or cysteine to cc was ~8:1, as determined by titration of free sulfhydryls with 5,5-dithio-*bis*-(2-nitrobenzoic acid).

Purification of RGD-binding Proteins from Human Placenta

Membranes were isolated from fresh human placenta, processed, and solubilized exactly as described (Calderon et al., 1988). Protein was incubated with wheat germ agglutinin, and adherent proteins were eluted with 0.5 M *N*-acetylglucosamine (GlcNAc) in a 0.5 M NaCl, 0.05 M PO_4, pH 7.4, buffer containing PMSF, iodoacetamide, and 50 mM octyl glucoside. The eluate was dialyzed to remove the GlcNAc and applied to an -IFN-Sepharose column. Unbound protein was washed through with octylglucoside-containing buffer and applied to a column bearing KYAVTGRGDS. Bound proteins were eluted by EDTA (Brown and Goodwin, 1988). SDS-PAGE analysis showed only two Coomassie-stained bands which had relative molecular masses on both unreduced and reduced gels consistent with the VnR. Since this protein pool contained more than one protein (see below) we have called it the placental RGD-binding proteins (PRBP).

Monoclonal and Polyclonal Antibodies

mAb A1A5 tissue culture supernatant was the gift of Dr. Martin Hemler, Dana Farber Cancer Center, Harvard Medical School, Boston, MA. Goat polyclonal anti-Fn receptor (VLA-5) was the gift of Dr. Rudolph Juliano, University of North Carolina, Chapel Hill, NC. Polyclonal antibody to PRBP was produced in rabbits. The IgG was isolated from serum by caprylic acid precipitation and DEAE chromatography (Steinbuch and Audran, 1969). Fab fragments were prepared by papain digestion. Monoclonal antibodies were produced by immunization of BALB/c mice with purified PRBP. Spleen cells were fused with the nonsecreting myeloma cell line P3X63AG8.6.5.3. Hybridoma culture supernatants were screened for antibodies that reacted with purified PRBP by ELISA. Reactive antibodies were screened further for binding to PMN by indirect immunofluorescent staining and fluorescence flow cytometry. Antibodies 7G2, B3F12, 6H12, and 3F12 were reactive with purified PRBP by ELISA but unreactive with PMN. These mAbs also immunoprecipitated VnR from detergent-solubilized placental membrane preparations. The identity of the immunoprecipitated material as authentic VnR was confirmed by limited NH_2-terminal sequencing of the alpha chain. mAb 7G2 reacted with the VnR beta chain by Western blot; none of the others reacted on Western blots, suggesting that they recognized conformational epitopes on VnR. mAb B6H12 was reactive both with purified PRBP by ELISA and with PMN by fluorescence flow cytometry. Antibodies were produced in the form of tissue culture supernatants or ascites in pristane-primed mice for further characterization. IgG was purified from ascites by caprylic acid precipitation and DEAE chromatography (Steinbuch and Audran, 1969).

Isolation of PMN

PMN were isolated from heparinized blood from normal volunteers and from a single patient with leukocyte adhesion deficiency (LAD) by the method of Boyum (1968) with modifications (Gresham et al., 1987). In some experiments the erythrocyte lysis step was omitted to prevent possible damage to the PMN during the hypotonic lysis procedure.

Immunoprecipitation and SDS-PAGE Analysis

For cell surface labeling, 30–100 × 10^6 PMN were iodinated by chloroglycoluril (Markwell and Fox, 1978) in the presence of 25 μM paranitrophenylparaguanidinobenzoate and 0.5% NaN_3 for 30 min at 0°C. Cells were solubilized in Hepes buffer containing 200 mM octylglucoside, 20 mM iodoacetamine, 2 μM pepstatin, 2 μM leupeptin, 25 μM paranitrophenylparaguanidinobenzoate, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.4. Total placental membrane proteins were labeled with ^{125}I using chloroglycoluril after detergent solubilization. Protein-bound and free iodide were separated on Sephadex G-25 columns. Aliquots of labeled proteins were incubated with monoclonal or polyclonal antibodies for 2 h at 4°C and then for an additional hour with either anti-mouse Ig Sepharose (Cooper Biomedical, Inc., Malvern, PA) or Protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ), respectively. Immunoprecipitates were analyzed on 6% SDS–polyacrylamide gels, and autoradiography was performed as described (Maizel, 1971).

Opsonization of Sheep Erythrocytes

Sheep erythrocytes (E) were purchased from Whittaker M.A. Bioproducts (Walkersville, MD). EIgG were prepared as described (Gresham et al., 1987) using a 1:500 dilution of rabbit IgG anti-E (Diamedix, Miami, FL).

Phagocytosis Assay

PMN phagocytosis was assessed by a fluid-phase assay as described (Gresham et al., 1987, 1988). PMN were suspended in HBSS containing 4.2 mM $NaHCO_3$, 10 mM Hepes, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 1% ovalbumin, pH 7.4 ($HBSS^{++}$-1% ovalbumin). The reaction mixtures contained 1.0 × 10^5 PMN, the indicated antibody concentrations, the indicated stimulatory ligands, 5,000 U/ml catalase, and 15 μl of EIgG (5.0 × 10^8/ml) in a final volume of 115 μl. The assay tubes were incubated at 37°C in 5% CO_2 for 30 min. The noningested E were lysed with 0.83% ammonium chloride. Phagocytosis was assessed by light microscopy and quantitated as a phagocytic index (PI), the number of EIgG ingested/100 PMN.

cc-RGDS Binding Assay

cc-RGDS was radioiodinated by chloroglycoluril (Markwell and Fox, 1978) for 15 min at 0°C. PMN (5.0 × 10^5) in $HBSS^{++}$-1% ovalbumin were incubated with 10 μg of radiolabeled cc-RGDS in the presence of 5,000 U/ml of catalase and 293 μg of unlabeled cc-C in a final volume of 250 μl. The reaction mixtures were incubated in 1.5 ml eppendorf tubes for 30 min at 37°C. The mixtures were overlayered on Versilube (General Electric Co., Wilmington, MA) and centrifuged at 12,000 *g* to assess pellet-associated radioactivity. Specific binding was determined by subtracting the radioactivity bound in the presence of 293 μg of unlabeled cc-RGDS from the total radioactivity bound in the absence of cold cc-RGDS. Unlabeled cc-C was included in the reaction to control for any binding due to cc and not specifically due to the RGDS peptide. For inhibition experiments, inhibitors were included during the entire incubation at the indicated concentrations. Antibodies were incubated with the PMN at the indicated concentrations for 15 min at room temperature before the addition of the other reactants.

Fluorescence Flow Cytometry

PMN (10^6) were stained with excess murine mAbs and FITC $F(ab')_2$ anti-

mouse Ig (Tago Inc., Burlingame, CA) at 0°C. After washing with PBS the cells were resuspended in 0.3 ml 0.5% paraformaldehyde in PBS before analysis on a FACS IV (Becton Dickinson & Co., Sunnyvale, CA).

Results

Catalase Is Required to Demonstrate Both cc-RGDS Binding and Stimulation of PMN Fc Receptor-mediated Phagocytosis

Our previous data indicated that while monocytes express both an integrin which binds to RGD-Sepharose and a structurally distinct integrin which binds preferentially to Sepharose bearing the Fn CBD, this is not the case for PMN. Instead, PMN express a single integrin-like receptor which binds preferentially to RGD-Sepharose columns (Brown and Goodwin, 1988). Because this presented a simpler system for determination of the characterizations of the RGD-binding receptor involved in phagocytosis enhancement, we chose to investigate extracellular matrix stimulation of PMN phagocytosis. Fn has been reported to stimulate ingestion of C3b-opsonized E by fMLF- or C5a-stimulated PMN (Pommier et al., 1984*a*), but not to stimulate the ingestion of EIgG by PMN (Wright et al., 1983*a*). We wanted to avoid the added complication of chemotactic peptide stimulation of PMN, as the expression of other adhesive receptors (i.e., CR3) which bind ligand via an RGD sequence (Wright et al., 1987) is markedly enhanced by these stimuli. We therefore decided to investigate further the ability of extracellular matrix proteins to stimulate EIgG ingestion. Because many adhesive receptors are present on PMN, and matrix proteins such as Fn may interact with cell surfaces via several domains, we developed a nonphysiologic ligand which would interact with PMN only by an RGD sequence. We linked either the hexapeptide GRGDSC, GDGDSC, or the single amino acid C as controls to pigeon heart cc via a bromacetyl succinamide linkage. The amino acid sequence for this cytochrome has been determined and does not contain an RGD sequence. These ligands are referred to as cc-RGDS, cc-DGDS, or cc-C and were used to assess RGDS-stimulated PMN Fc receptor-mediated phagocytosis. As shown in Fig. 1 *A*, in the presence of catalase, cc-RGDS stimulated PMN ingestion of EIgG in a dose-dependent manner with an optimal concentration of 40 μg/ml. As with other stimuli that affect PMN Fc receptor-mediated ingestion, the dose-response curve is biphasic (Gresham et al., 1987). This clearly distinguishes the dose-response of RGDS stimulation on PMN from that on monocytes (Pommier et al., 1983). In preliminary experiments we discovered that the inclusion of catalase was necessary to consistently observe cc-RGDS-stimulated ingestion (Fig. 1 *A*). Ingestion performed in the presence of catalase incubated at 100°C for 10 min was significantly reduced (Fig. 1 *A*). cc-C or cc-DGDS up to concentrations of 320 μg/ml had no effect on EIgG ingestion even in the presence of catalase (data not shown). The effect of catalase on cc-RGDS-stimulated ingestion was dose-dependent (Fig. 1 *B*). The inclusion of catalase also slightly enhanced nonstimulated ingestion of EIgG (Fig. 1 *B*). This effect of catalase and inhibitors of the MPO-H_2O_2-halide system on PMN IgG Fc- and complement-mediated ingestion has been reported (Stendahl et al., 1984; Gaither et al., 1987). In addition, the inclusion of either 10 mM NaN_3 (an inhibitor of MPO) or 10 mM methionine (a competitor for the damaging oxidant) also revealed consistent stimulation of ingestion by cc-RGDS. These data indicated that the MPO-H_2O_2-halide system was generating an oxidant which damaged either some step in the pathway for RGD-mediated enhancement of phagocytosis or the receptor responsible for cc-RGDS-stimulated ingestion. To examine the latter possibility, we examined the effect of catalase on the binding of radiolabeled cc-RGDS to PMN under the conditions of the phagocytosis assay. In the presence of catalase, a ninefold increase in the amount of cc-RGDS specifically bound was observed over the amount bound in the absence of catalase (Table I). These data indicated that at least one effect of catalase was on the binding of the cc-RGDS ligand. To examine whether catalase also protected some step in the process of RGD-stimulated ingestion, we assessed the effect of catalase on EIgG ingestion by PMN adherent to solid-phase Vn. In this assay the catalase was present only during the adherence of the PMN and was washed away before the addition of the EIgG. Vn contains an RGDS sequence and enhances monocyte ingestion of EC3b (Brown and Goodwin, 1988). As shown in Fig. 2, even though catalase was present only during the adherence of the PMN to either the control or the Vn-coated surface, it significantly enhanced Vn-stimulated ingestion of EIgG. These data and those in Table I indicated that the effect of catalase is on the interaction of the ligand with the PMN surface and not a general one on the phagocytic process. All subsequent assays were performed in the presence of catalase.

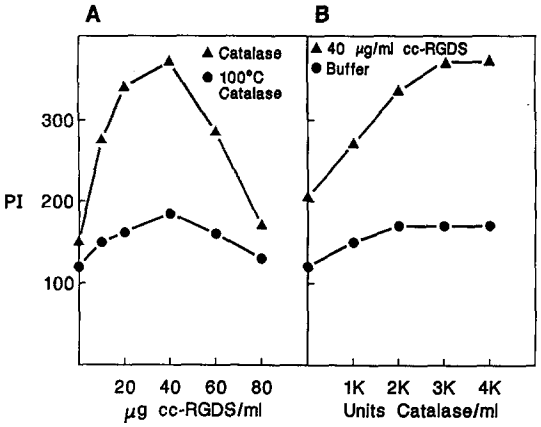

Figure 1. Effect of catalase on cc-RGDS-stimulated ingestion of EIgG. (*A*) PMN and EIgG were incubated with the final concentrations of cc-RGDS (20, 40, 60, or 80 μg/ml) in the presence of either 5,000 U/ml of native catalase (▲) or catalase heated at 100°C for 10 min (●). After 30 min at 37°C, phagocytosis was assessed as a PI, the number of EIgG ingested/100 PMN. (*B*) PMN and EIgG were incubated with increasing concentrations of catalase in the presence (▲) or absence (●) of 40 μg/ml of cc-RGDS. After 30 min at 37°C, phagocytosis was assessed as a PI.

Table I. Effect of Catalase on cc-RGDS Binding

Catalase	RGDS-specific cpm bound/10^6 PMN
+	30,267 ± 1,930 SEM, n = 10
−	3,394 ± 931 SEM, n = 5

10^6 PMN were incubated with 40 μg of ^{125}I-cc-RGDS in the presence (+) or absence (−) of 5,000 U of catalase/ml for 30 min at 37°C. The PMN were centrifuged through oil and the pellet-associated counts assessed. Specific binding was calculated as described in Materials and Methods.

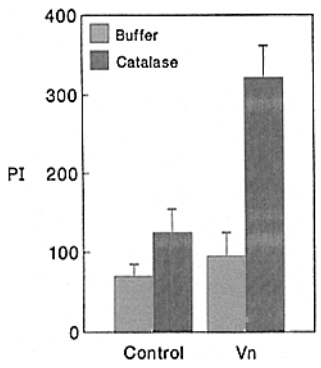

Figure 2. Effect of catalase on ingestion of EIgG by PMN adherent to control or V-coated surfaces. Plastic Lab-Tek chambers were coated with either carbonate buffer, pH 9.6 (*Control*), or 10 μg/ml of Vn in carbonate buffer (*Vn*) for 2 h at 37°C. After washing the chambers, PMN (2.5 × 10^4/well) were adhered in the presence or absence of 5,000 U/ml catalase for 45 min at 37°C. Catalase was removed by extensive washing. The EIgG were added and phagocytosis assessed after 30 min at 37°C.

mAb B6H12 and Polyclonal Fab Anti-PRBP Inhibit Both cc-RGDS Binding and RGD-stimulated Phagocytosis

To facilitate isolation of the extracellular matrix receptor involved in phagocytosis enhancement, we prepared a polyclonal (rabbit) and several monoclonal antibodies to human PRBP isolated from placenta. The polyclonal and a single monoclonal antibody, B6H12, bound to PMN as assessed by fluorescence flow cytometry analysis. We tested the effects of these antibodies on cc-RGDS–stimulated ingestion. As shown in Fig. 3, *A* and *B*, both antibodies inhibited cc-RGDS–stimulated ingestion in a dose-dependent manner with complete inhibition at ~30 nM (B6H12) and 80 nM (Fab anti-PRBP). This dose of either antibody had no effect on EIgG ingestion by buffer-treated PMN (Fig. 3, *A* and *B*); however, at concentrations of ≥16 μg/ml, treatment of PMN with either antibody significantly stimulated ingestion in the absence of cc-RGDS. The PI for PMN incubated with 100 nM B6H12 was 334, and for PMN incubated with 320 nM Fab anti-PRBP was 309. These values were very similar to those obtained with cc-RGDS stimulation in the same experiment (PI = 330 and 332, respectively). Three other monoclonal antibodies made to the PRBP (B3F12, 6H12, and 3F12) did not bind to PMN and did not have any effect on either cc-RGDS–stimulated or nonstimulated ingestion of EIgG. All these antibodies immunoprecipitated VnR from placenta (determined by NH_2-terminal sequencing of the immunoprecipitated alpha chain) and stained endothelial cells (data not shown). We also tested an antibody to the VnR beta chain, mAb 7G2 (Brown and Goodwin, 1988), which binds to platelets and can immunoprecipitate VnR from placenta and gp IIb/IIIa from platelets. This mAb also did not bind to PMN and did not affect either cc-RGDS–stimulated or nonstimulated ingestion by PMN. PMI-1, an anti IIb mAb (Ginsberg et al., 1986), also does not bind to PMN (Brown and Goodwin, 1988). Immunoprecipitation with B6H12 was unable to remove VnR from placental membrane extracts, as determined by subsequent immunoprecipitation with 3F12 or 7G2 (data not shown). In addition, neither polyclonal antibody to the Fn receptor (anti-VLA-5) (Brown and Juliano, 1986) nor mAb A-1A5 (anti-VLA beta chain) (Hemler et al., 1987) had any effect on either cc-RGDS–stimulated or nonstimulated ingestion of EIgG. The effect of mAb B6H12 and Fab anti-VnR was specific for cc-RGDS–stimulated phagocytosis because neither antibody had any effect on EIgG ingestion stimulated by phorbol ester treatment of PMN (data not shown). These data suggest that B6H12 recognizes an antigen which, while it binds to the RGD sequence, is not VnR, gp IIb/IIIa or a VLA.

To assess whether B6H12 or Fab anti-PRBP prevented phagocytosis enhancement by inhibiting ligand binding, we examined the binding of radiolabeled cc-RGDS to antibody-treated PMN. As shown in Table II, mAb B6H12 and Fab anti-PRBP reduced cc-RGDS binding by 93.8 and 95.7%, respectively. In contrast, mAb B3F12 reduced cc-RGDS binding only by 7.4%. These data indicated that the polyclonal and the monoclonal antibody B6H12 inhibit cc-RGDS–stimulated phagocytosis because they inhibit binding of the cc-RGDS ligand to the cell surface.

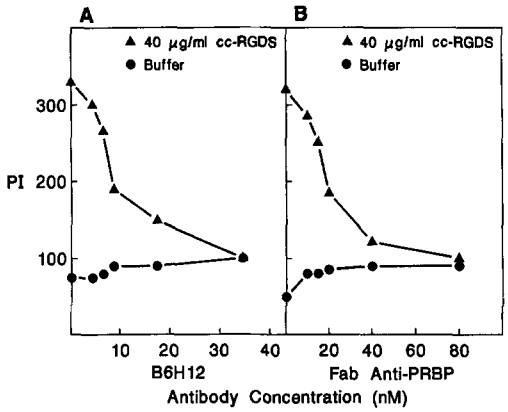

Figure 3. Effect of mAb B6H12 and polyclonal Fab anti-VnR on cc-RGDS–stimulated ingestion of EIgG. PMN were incubated with the indicated concentrations of either mAb B6H12 (*A*) or Fab polyclonal anti-VnR (*B*) for 15 min at room temperature. Without washing, the EIgG were added and the mixture incubated with (▲) or without (●) 40 μg/ml of cc-RGDS. After 30 min at 37°C, phagocytosis was assessed as a PI.

Table II. Inhibition of cc-RGDS–specific Binding to PMN

Inhibitors	cc-RGDS specific cpm/10^6 PMN*	Inhibition‡
		%
cc-C (1.16 mg/ml)	30,173 ± 1,748 SEM, *n* = 11	0
GRGDSC (500 μg/ml)	6,144 ± 2,905 SEM, *n* = 3	79.6
Peptide 32 (500 μg/ml)	39,442 ± 7,800 SEM, *n* = 2	0
Fn (500 μg/ml)	7,106 ± 3,568 SEM, *n* = 3	76.5
Fab anti-VnR (4 μg/ml)	1,312 ± 867 SEM, *n* = 5	95.7
B6H12 (4 μg/ml)	1,877 ± 1,811 SEM, *n* = 3	93.8
B3F12 (4 μg/ml)	27,948 ± 5,701 SEM, *n* = 3	7.4

* 10^6 PMN were incubated with 40 μg of ^{125}I-cc-RGDS with the indicated concentrations of unlabeled inhibitors in the presence of 5,000 U catalase/ml for 30 min at 37°C. The PMN were centrifuged through oil and the pellet-associated counts assessed. Specific binding was calculated as described in Materials and Methods.

‡ Calculated from the mean cpm. Percent inhibition = 100 × (1 − [mean cpm in the presence of inhibitor]/[mean cpm in the presence of cc-C]).

mAb B6H12 Recognizes a Cell Surface Heterodimer Distinct from Previously Described Members of the Integrin Receptor Family

To ascertain the structure of the receptor recognized by the Fab anti-VnR and mAb B6H12 and compare it to the structure of the receptor isolated by RGD affinity chromatography (Brown and Goodwin, 1988), we surface labeled PMN and immunoprecipitated with the polyclonal anti-PRBP and several of the monoclonal antibodies produced against purified PRBP from placenta. Both the polyclonal antibody and mAb B6H12 immunoprecipitate a heterodimer of 130–140 kD and 110 kD upon reduction (Fig. 4, lanes *2* and *4*). Neither mAb 7G2 (anti-gp IIb/IIIa) nor mAb B3F12 (anti-VnR) immunoprecipitated any detectable proteins from PMN. However, both mAb 7G2 and mAb B3F12 but not mAb B6H12 immunoprecipitated a heterodimer from iodinated placental membrane proteins of 130 and 100 kD upon reduction (data not shown). To further distinguish the B6H12 antigen from the gp IIb/IIIa antigen, surface-labeled platelets were immunoprecipitated with mAb 7G2 and the precipitate was compared under nonreducing conditions to the mAb B6H12 immunoprecipitate from PMN (Fig. 5, lanes *2* and *3*). As is apparent, these two antibodies immunoprecipitate heterodimers with completely distinct alpha and beta chain relative molecular masses. The PMN heterodimer cannot be immunoprecipitated with an antibody that recognizes the beta chain of the gp IIb/IIIa–VnR family (mab 7G2) and is therefore distinct from these cytoadhesins.

We next investigated the possibility that the B6H12 antigen was a member of the LFA-1, Mac-1, p150,95 family of cell adhesion receptors. As shown in Fig. 6, mAb B6H12 binds normally to PMN from a patient with LAD (Anderson and Springer, 1987) as assessed by FACS analysis. This patient has been found previously (Kishimoto et al., 1987) to lack expression of all members of this family, and in our own studies PMN from this patient failed to express any beta chain antigen as assessed by FACS analysis using antibody to the beta chain, mAb IB4 (Wright et al., 1983*b*) (data not shown). In addition, binding of radiolabeled cc-RGDS was assessed on both normal and LAD PMN; normal PMN bound 11,978 RGDS-specific cpm/10^6 PMN while the LAD PMN bound 12,616 RGDS-specific cpm/10^6 PMN. Moreover, our previous work indicated that treatment of PMN lysates with mAb IB4 did not immunoprecipitate the PMN receptor which bound to RGD-Sepharose (Brown and Goodwin, 1988). These data indicated that the B6H12 antigen is not a member of the LFA-1, Mac-1, p150,95 family of cell adhesion receptors. Finally, we immunoprecipitated antigens recognized by polyclonal anti-VLA-5 from surface-labeled PMN. Two bands at ~160 and 130 kD were seen on autoradiograms of gels run on reduced samples. These bands

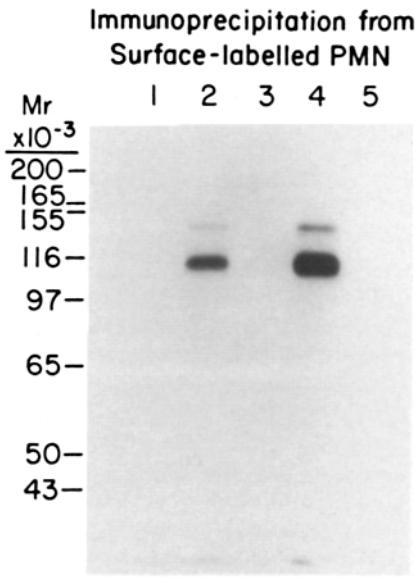

Figure 4. Immunoprecipitation from surface-labeled PMN with mAb B6H12 or polyclonal anti-VnR. Surface-iodinated PMN lysates were immunoprecipitated with the following antibodies: (lane *1*) preimmune rabbit serum; (lane *2*) rabbit polyclonal anti-VnR; (lane *3*) mAb 7G2 (anti-VnR beta chain); (lane *4*) mAb B6H12; and (lane *5*) mAb B3F12 (anti-VnR). The immunoprecipitates were analyzed under reducing conditions (50 mM DTT) by SDS-PAGE.

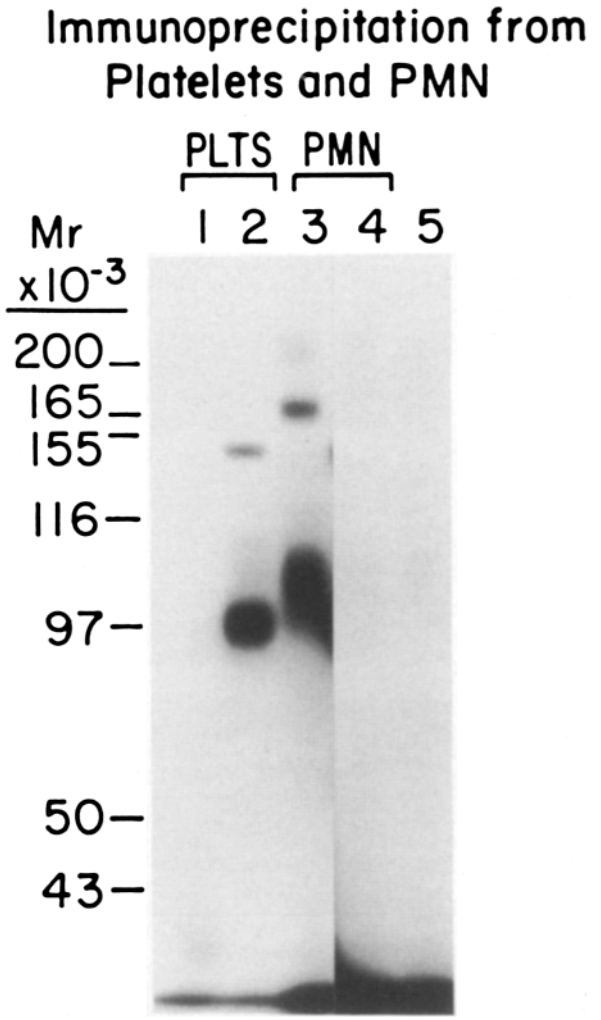

Figure 5. Immunoprecipitation from surface-labeled platelets and PMN with mAb B6H12 and mAb 7G2. Surface-iodinated platelets (lanes *1* and *2*) and PMN (lanes *3–5*) were immunoprecipitated with the following antibodies: (lanes *1* and *3*) mAb B6H12; (lanes *2* and *4*) mAb 7G2; and (lane *5*) mAb B3F12. The precipitates were analyzed under nonreducing conditions by SDS-PAGE.

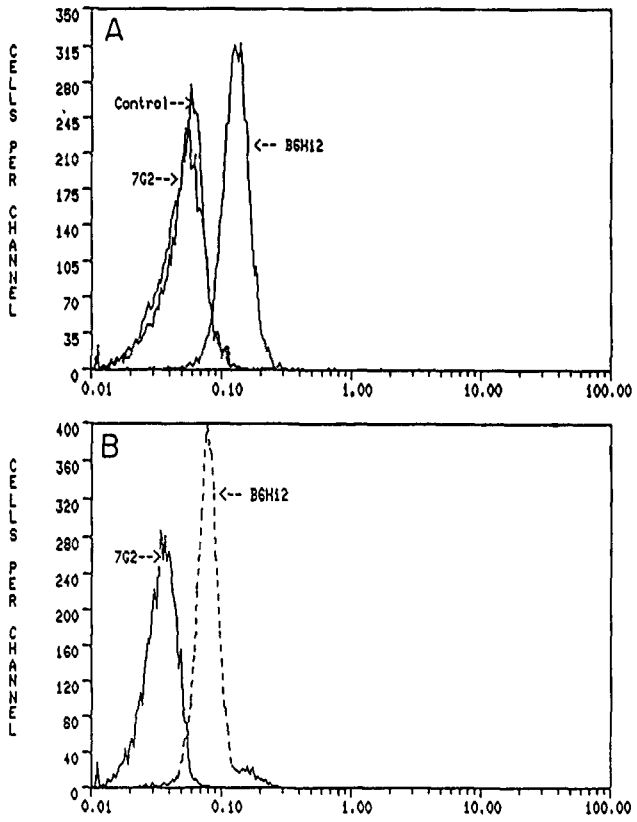

Figure 6. mAb B6H12 profile of normal and LAD PMN as analyzed by FACS. Normal PMN (*A*) and LAD PMN (*B*) were analyzed by indirect immunofluorescence for mAb B6H12. Both normal and LAD PMN express the B6H12 antigen. LAD PMN are unable to express the Mac-1, LFA-1, p150,95 because of an abnormality in beta chain synthesis.

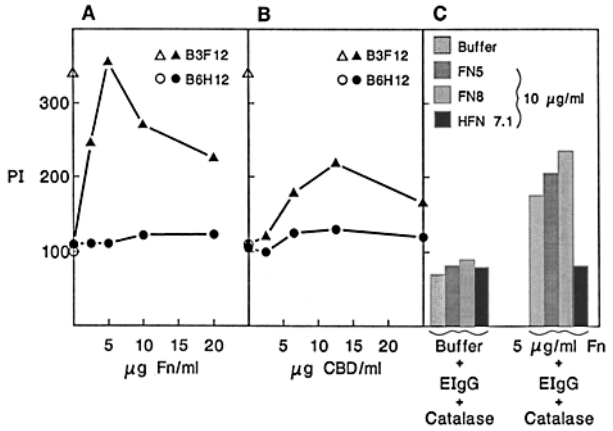

Figure 7. Fn and its CBD stimulate ingestion of EIgG in a manner dependent on B6H12 antigen. (*A* and *B*) PMN were incubated with 5,000 U/ml catalase and a 1:120 dilution of tissue culture supernatant containing either mAb B6H12 (●) or mAb B3F12 (▲) for 15 min at room temperature. Without washing, the EIgG were added and the mixture was incubated with either increasing concentrations of Fn (*A*), increasing concentrations of its purified CBD (*B*), or 40 μg/ml cc-RGDS (*open symbols*). After 30 min at 37°C, phagocytosis was assessed. (*C*) Fn (5 μg/ml) or buffer were incubated with either buffer, or mAb Fn5, Fn8, or HFn7.1 at 10 μg/ml for 30 min at room temperature. PMN, 5,000 U/ml catalase, and EIgG were added and phagocytosis assessed after 30 min at 37°C.

did not align with the immunoprecipitates from anti-PRBP (data not shown), but are consistent with the reported molecular masses of either VLA-2 or VLA-4 (Hemler et al., 1987). Together with the data that neither A1A5 nor the polyclonal anti-VLA-5 inhibit cc-RGDS-stimulated phagocytosis, these data suggest that the cell surface receptor recognized by B6H12 is not a VLA. In concert, these data demonstrate that the B6H12 antigen represents a previously unrecognized RGD-binding receptor.

Many RGDS-containing Proteins Stimulate PMN Fc Receptor-Mediated Ingestion via a B6H12-dependent Mechanism

Because cc-RGDS, but not cc-DGDS nor cc-C, was able to augment PMN phagocytosis of EIgG significantly, we concluded that the RGD(S) sequence was responsible for phagocytosis enhancement. This conclusion was substantiated by the fact that unlinked GRGDSC peptide inhibited specific cc-RGDS binding by 79.6% while an irrelevant peptide had no effect (Table II). This inhibition was not limited to free RGDS peptide but could be demonstrated also by a protein containing an RGDS sequence, Fn, which inhibited specific cc-RGDS binding by 76.5%. These data indicated that the mAb B6H12 recognized a receptor which might recognize the RGD sequence of many proteins.

We examined the effect of Fn on PMN Fc receptor-mediated phagocytosis, and, as shown in Fig. 7 *A*, Fn stimulated ingestion in a dose-dependent manner. As with cc-RGDS stimulation (Fig. 1 *A*), the dose-response curve was biphasic. Fn-mediated augmentation of ingestion was completely abrogated by treatment of the PMN with mAb B6H12 but not mAb B3F12. However, nonstimulated or baseline levels of EIgG ingestion were never affected by mAb B6H12 treatment. The CBD of Fn which contains the RGDS sequence also stimulated EIgG ingestion, though much less efficiently than the intact Fn molecule (Fig. 7 *B*). This is in contrast to monocyte ingestion which is not augmented by the purified CBD fragment (Bohnsack et al., 1986; Brown and Goodwin, 1988). mAb B6H12 and not mAb B3F12 completely abrogated CBD-stimulated ingestion by PMN (Fig. 7 *B*). To further localize the region of the Fn molecule which was involved in phagocytosis enhancement, an optimal dose of Fn was incubated with various monoclonal antibodies which recognize distinct domains of Fn before stimulation of PMN. As shown in Fig. 7 *C*, neither Fn8, which recognizes the NH_2 terminus of Fn, nor Fn5, which recognizes a site in the CBD NH_2 terminal to the RGDS sequence (Bohnsack et al., 1986), had any effect on Fn-stimulated ingestion of EIgG. However, HFn7.1, which recognizes a site close to the RGDS sequence in the CBD, completely abrogated Fn-stimulated phagocytosis. A similar effect of HFn7.1 has been observed for Fn-stimulated monocyte phagocytosis (Bohnsack et al., 1986). These data indicated that the CBD of the Fn molecule which contains the RGDS sequence was responsible for augmentation of PMN Fc receptor-mediated ingestion by intact Fn and that mAb B6H12 recognized the receptor which mediated this enhancement.

We investigated other extracellular matrix proteins which

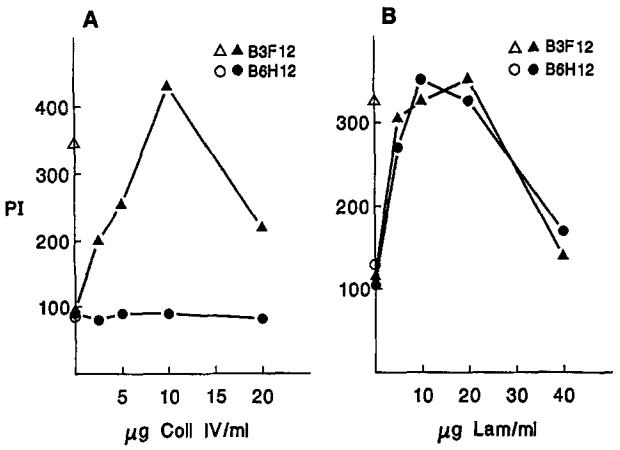

Figure 8. Effect of mAb B6H12 on collagen type IV- and laminin-stimulated ingestion of EIgG. (*A* and *B*) PMN and 5,000 U/ml of catalase were incubated with a 1:20 dilution of tissue culture supernatant containing either mAb B3F12 (▲) or mAb B6H12 (●) for 15 min at room temperature. Without washing, the EIgG were added and the mixture was incubated with either increasing concentrations of collagen type IV (*A*), increasing concentrations of laminin (*B*), or 40 μg/ml of cc-RGDS (*open symbols*). After 30 min at 37°C, phagocytosis was assessed.

have been reported to contain RGD sequences for their ability to augment ingestion via the B6H12 antigen. As shown in Fig. 8, *A* and *B*, both collagen type IV and laminin enhanced PMN Fc receptor-mediated ingestion; however, mAb B6H12 inhibited only collagen-stimulated ingestion and not laminin-stimulated ingestion. Therefore, the B6H12 antigen was not able to recognize an RGD sequence in all extracellular matrix proteins. This is not surprising for laminin, because it has been shown to interact with nonintegrin receptors via domains completely distinct from RGD sequence (Graf et al., 1987).

Because the B6H12 receptor appeared to recognize several proteins with RGD sequences, it appeared to function like

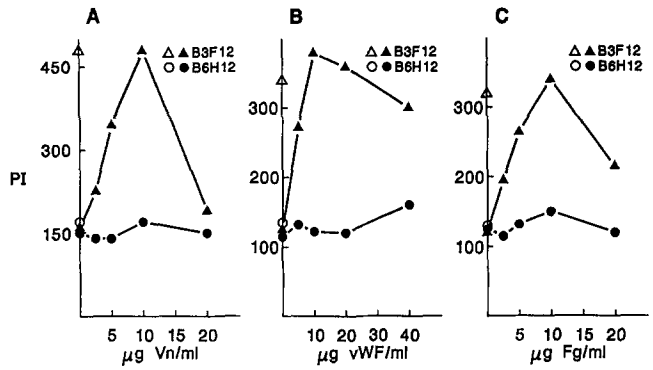

Figure 9. Effect of mAb B6H12 on Vn-, vWF-, and Fg-stimulated ingestion of EIgG. (*A–C*) PMN and 5,000 U/ml catalase were incubated with a 1:20 dilution of tissue culture supernatant containing mAb B3F12 (▲) or mAb B6H12 (●) for 15 min at room temperature. Without washing, the EIgG were added and the mixture was incubated with either increasing concentrations of Vn (*A*), increasing concentrations of vWF (*B*), increasing concentrations of Fg (*C*), or 40 μg/ml of cc-RGDS (*open symbols*). After 30 min at 37°C, phagocytosis was assessed.

the gp IIb/IIIa receptor of platelets which binds Vn, vWF, and Fg, as well as Fn. We also investigated these proteins for augmentation of ingestion via the B6H12 antigen. As shown in Fig. 9, all three of these ligands stimulated PMN ingestion of EIgG in a dose-dependent manner. Moreover, the stimulated ingestion by all of these ligands was prevented by treatment of the PMN with mAb B6H12 but not mAb B3F12. These data indicated that the B6H12 receptor recognized RGD sequences in many proteins and mediated phagocytosis stimulation by many, but not all, adhesive proteins.

Discussion

Data from our laboratory indicate that receptor-mediated phagocytosis by PMN is a recruited function at inflammatory sites (Gresham et al., 1988). In this regard, various integrins expressed on PMN may exert a significant regulatory influence on PMN phagocytic function. Therefore, assessment of the structure, ligand-binding specificity, and mechanisms for regulation of these receptors is a primary goal of our laboratory. To facilitate isolation of the RGD-binding receptors involved in regulation of phagocytic function, we have developed an assay for the effects of various adhesive proteins on PMN phagocytosis. This assay allowed us to investigate the effect of polyclonal and monoclonal antibodies which we and others produced against various integrin receptors. Two factors led to the successful development of this assay: (*a*) the use of a nonphysiologic ligand, cc-RGDS, allowed us to examine ligand binding as well as RGDS-stimulated ingestion without interference from other domains in adhesive proteins which may be involved in binding but which do not augment phagocytosis (Pommier et al., 1984*b*; Bohnsack et al., 1986); and (*b*) the presence of catalase in the reaction mixture allowed us to observe RGDS-stimulated EIgG ingestion by PMN consistently.

The protective effect of catalase indicated that an oxidant being generated during the incubation was preventing cc-RGDS augmentation of Fc receptor-mediated ingestion. This fact probably explains the previously reported failure to detect Fn enhancement of PMN ingestion of EIgG (Wright et al., 1983*a*). Like catalase, NaN_3 and methionine could protect cc-RGDS stimulation, indicating that it is likely that the MPO-H_2O_2–halide system of PMN was generating an oxidant (possibly HOCl) which prevented phagocytosis enhancement. This system has been shown to partially inhibit both IgG Fc- and complement-mediated ingestion by PMN (Stendahl et al., 1984; Gaither et al., 1987). As shown in Fig. 1 *B* and Fig. 2, the inclusion of catalase enhanced EIgG ingestion by buffer-treated PMN as reported (Stendahl et al., 1984). However, the most marked effect of catalase was on RGD-stimulated ingestion, which was only minimally different from baseline ingestion in the absence of catalase. The inclusion of catalase in our assay also resulted in a ninefold increase in ligand binding (Table I) and was necessary only during interaction with the ligand and not during ingestion (Fig. 2). Therefore, we concluded that in our assay the primary effect of catalase is on the interaction of the RGD ligand with the PMN and not on a step in the phagocytic process. We suggest that the receptor involved in RGDS-stimulated phagocytosis is damaged by the generated oxidants, thus inhibiting ligand binding. Thus, the generation of oxidants at inflammatory sites may actually act to inhibit the

function of this receptor. This raises the possibility that this receptor acts very early in the PMN response to the inflammatory stimulus, before significant release of MPO or activation of the respiratory burst.

Because our previous work indicated an antigenic and structural similarity of the PMN RGD-binding receptor to the gp IIb/IIIa–VnR family, we prepared polyclonal and monoclonal antibodies to purified VnR and assessed their effect on cc-RGDS–stimulated ingestion. Both polyclonal anti-VnR and a single monoclonal antibody, B6H12, bound to PMN and inhibited both cc-RGDS binding and cc-RGDS–stimulated ingestion (Fig. 3; Table II). mAb B6H12 immunoprecipitated a heterodimeric receptor similar in molecular weight to the RGD-binding proteins detected by affinity chromatography of lysates from surface-iodinated PMN (Brown and Goodwin, 1988). However, mAb B6H12 did not appear to recognize a member of the cytoadhesin family because a monoclonal antibody (mAb 7G2) that binds to the beta chain of the gp IIb/IIIa–VnR family had no effect in our assay and did not bind to PMN nor immunoprecipitate any surface-labeled proteins from PMN (Fig. 4). These data along with the failure of anti–IIb mAb PMI-1 to bind to PMN (Brown and Goodwin, 1988) indicate that, unlike a previous report (Burns et al., 1986), we cannot detect gp IIb/IIIa on PMN. Three other mAbs, which recognized placental VnR and VnR on both endothelial cells and fibroblasts (data not shown) also did not bind to nor immunoprecipitate any proteins from PMN, and had no effect on cc-RGDS–stimulated ingestion or binding (Table II). Moreover, mAb B6H12 recognizes a heterodimer on PMN which is distinct from either gp IIb/IIIa or placental VnR (Figs. 4 and 5). Since B6H12 clearly inhibited Fn-enhanced phagocytosis, the PMN integrin is not identical to the M21 membrane RGD receptor (Cherish and Spiro, 1987). These data demonstrate that this receptor is not a known cytoadhesin. Since the immunogen used to produce B6H12 consisted of all wheat germ agglutinin–binding proteins from placental membranes which also bound to RGD-Sepharose, it is likely that a small amount of B6H12 antigen was present in the placental PRBP preparation.

Several lines of evidence indicate that the receptor recognized by mAb B6H12 is also not a member of the VLA or the LFA-1, Mac-1, p150,95 groups of integrin receptors. First, PMN from a patient with LAD which do not express the beta chain antigen of the LFA-1 group, do express B6H12 antigen (Fig. 6) and bind radiolabeled cc-RGDS normally. Also monoclonal antibody to the beta chain of this group fails to remove the RGD-binding proteins from surface-labeled PMN (Brown and Goodwin, 1988). Second, mAb to the VLA beta chain (A-1A5) does not bind to granulocytes (Hemler et al., 1987). Although polyclonal anti–VLA-5 antibody did bind to granulocytes, it immunoprecipitated an antigen or antigens of different apparent relative molecular masses than that immunoprecipitated by B6H12 or anti-PRBP. In addition neither mAb A-1A5 nor polyclonal anti–VLA-5 affected cc-RGDS–stimulated ingestion. mAb B6H12 cannot recognize the VLA beta chain as it does not bind to fibroblasts (Brown, E. J., and John McDonald, unpublished observation). These data suggest that the receptor expressed on PMN recognized by mAb B6H12 is not previously described. Since it transduces the signal for increased phagocytosis, we propose the name leukocyte response integrin for this new receptor. While it may be an integrin from a new group, polyclonal anti-VnR and anti-gp IIb/IIIa both recognize it, suggesting that B6H12 antigen is immunologically related to the cytoadhesins; thus, this antigen may represent a new alpha chain in combination with the cytoadhesin beta chain. Amino acid sequence analysis of the B6H12 antigen will be required to determine its relationship to the known groups of integrins.

While this RGD-binding receptor on PMN involved in extracellular matrix–stimulated ingestion is not gp IIb/IIIa, it expresses a similar ligand binding specificity. Fn, Vn, vWF, and Fg all bound to the PMN receptor, because they enhanced PMN phagocytosis in a B6H12-inhibitable manner. Unlike gp IIb/IIIa, the PMN phagocytosis-enhancing receptor recognized by B6H12 also bound collagen type IV. Laminin, an adhesive protein which contains an RGD sequence, also enhanced PMN ingestion but did so independently of the receptor recognized by mAb B6H12. This fact is consistent with the observation that laminin interacts with nonintegrin receptors via domains distinct from the RGD sequence (Graf et al., 1987). The fact that mAb B6H12 inhibited RGD-stimulated ingestion by a number of adhesive proteins does not rule out a role for other adhesive receptors functioning in ligand binding. Fg has been reported to bind to CR3 on PMN and monocytes (Altieri et al., 1988), yet our data indicate that all Fg-stimulated ingestion is blocked by mAb B6H12. It may be, as we have hypothesized for Fn (Bohnsack et al., 1986; Brown and Goodwin, 1988), that other domains of these adhesives molecules are involved in ligand binding, but that for PMN at least, augmentation of ingestion is dependent on the RGD-sequence and is mediated by the receptor recognized by mAb B6H12.

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