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Research Article

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Collagen-induced Release of Interleukin 1 from Human Blood Mononuclear Cells

Potentiation by Fibronectin Binding to the $\alpha_5\beta_1$ Integrin

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Abstract

PBMC express cell surface receptors for extracellular matrix components known as integrins. We have recently shown that ligand binding to one PBMC integrin, the collagen receptor $\alpha_2\beta_1$, stimulates the secretion of interleukin 1 (IL-1). We have now investigated the role of fibronectin (Fn), an adherence protein that has binding sites for both PBMC and collagen, in the generation of the IL-1 response to collagen. In contrast to collagen, Fn did not stimulate IL-1 release but Fn-depleted serum decreased the release of IL-1 induced by collagen. A polyclonal antiserum directed against Fn also decreased the collagen-induced IL-1 secretion. The IL-1 response to collagen from cells incubated in Fn-depleted serum was restored by the addition of either purified Fn or the 120-kD cell-binding fragment of Fn, which contains the cell-binding site but not the collagen-binding domain. Smaller Arg-Gly-Asp (RGD) peptides failed to enhance the PBMC response to collagen but inhibited in a concentration-dependent fashion the potentiating effect Fn. As expected, a MAb against the $\alpha_2\beta_1$ collagen receptor decreased collagen-induced IL-1 release. However collagen-induced IL-1 release was also inhibited by a MAb against the $\alpha_5\beta_1$ Fn receptor. The effect of the two MAbs was not additive, suggesting that the occupancy of both receptors by ligands is required in order for collagen to induce an maximal response from PBMC. The mechanism by which Fn exerts its effect remains unknown. However, flow-cytometric analysis revealed that Fn does not alter expression of the $\alpha_2\beta_1$ receptor on PBMC. These data demonstrate a potentiating effect of Fn on the collagen-induced secretion of IL-1 from human PBMC and suggest that this effect is mediated via the integrin $\alpha_5\beta_1$. These findings indicate a complex interactive role for specific integrin receptors in the regulation of the mononuclear cell immune response. (*J. Clin. Invest.* 1992. 89:61–67.) Key words: integrin • collagen • fibronectin • mononuclear cells • interleukin 1

Introduction

Interleukin 1 (IL-1) is a family of several low molecular weight proteins produced by mononuclear and other mammalian cells, in response to infection, injury, and immune stimuli (1, 2). IL-1 secretion in areas of inflammation or tissue repair is the result of a multistep interaction between mononuclear cells and the surrounding tissues. These events include chemotactic recruitment of PBMC by degradation products of extracellular matrix proteins such as collagen, fibronectin (Fn),[1] and elastin (3–5), and adherence to the extracellular matrix, an event that precedes and is necessary for the synthesis and the release of cytokines (6, 7). Among the constituents of the extracellular matrix that interact with mononuclear cells, collagen appears to have the most profound effects. Adherence to collagen has been shown to alter the phenotypic characteristic of monocytes (8), enhance complement receptor- and FC receptor-mediated phagocytosis (9), and stimulate the production of IL-1 (10, 11). These events are likely to be the result of specific receptor-mediated interactions between mononuclear cells and collagen. PBMC express several types of cell-surface receptors that mediate their binding to other cells, immune products, and extracellular matrix (12, 13). Among them are the integrins, an evolutionarily conserved family of heterodimeric transmembrane glycoproteins that bind to constituents of the extracellular matrix (14). These receptors are composed of an α and a β chain and are classified in subfamilies depending on their β subunit. The β_1 subfamily, also known as very late antigens (VLAs), includes receptors for collagen, laminin, and Fn (15). Recently, we have collected evidence that an integrin mediates the stimulatory effect of collagen on PBMC IL-1 secretion by demonstrating that a monoclonal antibody to the $\alpha_2\beta_1$ receptor blocks the IL-1 response to collagen (11). Since PBMC express other β_1 integrins that recognize ubiquitous adherence proteins, additional extracellular matrix-receptor interactions modulating the secretion of IL-1 from human mononuclear cells are likely. In this study, we have investigated the role of fibronectin in the generation of the IL-1 response to collagen. We report that Fn does not directly stimulate the secretion of IL-1, but increases the PBMC response to collagen. This effect appears to be due to the functional engagement of the $\alpha_5\beta_1$ integrin receptor and does not depend on the binding of Fn to collagen.

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Methods

Unless otherwise specified, reagents and media were from the Sigma Chemical Co. (St. Louis, MO).

Mononuclear cell cultures. PBMC cultures were prepared from blood from healthy volunteers as described (16, 17). Briefly, freshly drawn blood was fractionated on Ficoll/Hypaque, and the PBMC were

1. *Abbreviations used in this paper:* Fn, fibronectin.

removed from the interface and washed twice with RPMI 1640 medium. The cells were resuspended in complete medium (RPMI 1640 medium supplemented with 5% [vol/vol] heat-inactivated FBS [Sterile Systems, Logan, UT; endotoxin, 0.038 ng/ml]) at a concentration of 1×10^6 cells/ml, and 1-ml aliquots incubated in 16-mm wells of 24-well tissue culture plates for 48 h at 37°C in a humidified atmosphere of 5% CO_2/95% air. For some experiments 1-ml aliquots were allowed to adhere for 2 h at 37°C. After incubation, the nonadherent cells (enriched lymphocytes) were removed from the wells, resuspended in 1-ml medium and incubated for 48 h. The adherent population (enriched monocytes) was washed twice with RPMI 1640 to remove any remaining nonadherent cells. The adherent cells were then incubated in 1 ml of complete medium for 48 h. The adherent population was subsequently stained for the monocyte-macrophage–specific enzyme α naphthyl acetate esterase and was found to be > 95% monocytes. Monocytes composed 20.2±4.4% of the entire original mononuclear population isolated by Ficoll density gradient centrifugation. The media were then collected, passed through 0.22 μm filters and stored at −20°C until assayed for IL-1. In all experiments, conditioned media were assayed for endotoxin by the chromogenic Limulus amebocyte lysate assay (Whittaker M. A. Bioproducts, Walkersville, MD). Endotoxin was not detected at the level of sensitivity of the assay (≥ 10 pg/ml).

IL-1 assay. The PBMC-conditioned media were assayed for IL-1 activity (IL-1α and IL-1β) by assessing the increment in mitogen-induced proliferation of the helper T cell line D10.G4.1 (D10. cells) as previously described (16, 17). The IL-1 standard used in the assays was ultrapure IL-1 (Genzyme Corp., Cambridge, MA), except for the IL-1 used in the neutralization assay, which was recombinant IL-1α or IL-1β (Genzyme). D10. cell proliferation was measured by the colorimetric method with 3-(4,5-dimethylthiazol-2-Y1)-2,5-diphenyl tetrazolium bromide (MTT), as described (18), and was converted to U/ml of IL-1 activity by performing a log-logit transformation of the serial dilution curves and determining the dilution of the test sample that yielded a value corresponding to 50% of the standard IL-1 maximum activity. The interassay and the intrassay variabilities were 29 and 10%, respectively. Recovery of added recombinant IL-1 β was ≥ 90. The minimum amount of LPS capable of stimulating IL-1 secretion was 10–100 pg/ml.

Since PBMC may secrete agents in addition to IL-1 that are comitogenic in the T cell assay (19–21), we verified our findings as indicative of the presence of IL-1 by demonstrating inhibition of the PBMC-conditioned media effect in the presence of monoclonal anti-IL-1α and anti-IL-1β antibodies (kindly provided by John Kenney, Syntex Corp., Palo Alto, CA). These neutralization experiments were performed by incubating serial dilutions of the PBMC culture medium for 2 h with anti-IL-1α (1:20 dilution), anti IL-1β (1:200 dilution), or control serum at 37°C before assay of the D10. cells.

Preparation of test materials. Intact type I collagen (a generous gift of Dr. Howard Welgus, Washington University, St. Louis, MO) was extracted from rat tails according to the method of Piez et al. (22). This material was used for all the experiments requiring the coating of a surface with collagen. Heat-denatured type I collagen was prepared by incubating native collagen in a water bath at 90°C for 10 min. This material was used as source of soluble collagen to be added to cells cultured on a plastic surface. Fn and Fn fragments (the 65/75-kD carboxy-terminal fragment containing the variable splicing CS-1 site that binds to the integrin $\alpha_4\beta_1$ and the RGD-containing 120-kD cell adhesive fragment) were isolated from bovine plasma as previously described (23), and were judged greater that 95% pure by SDS-PAGE and protein staining (Phast System; Pharmacia, Inc., Piscataway, NJ). Fn-depleted serum was obtained by affinity chromatography of FCS on a gelatin–Sepharose column. The flow through fraction contained no detectable Fn upon examination by SDS-PAGE and protein staining. GRGDSP and GRGESP peptides were purchased from Telios Pharmaceuticals (San Diego, CA).

All test materials contained < 10 pg/ml endotoxin in the chromogenic Limulus amebocyte lysate assay (data not shown). In order to further rule out a significant LPS contamination, all the experiments were conducted with and without polymixin B (500 μg/ml), an antibiotic that at low concentrations blocks level of LPS ≤ 1 ng/ml (24). As previously reported (11) polymixin B inhibited the LPS but not the collagen-induced PBMC response. Moreover, treatment with this antibiotic failed to affect the results of any of the experiments described below (data not shown).

Flow cytometry. In these experiments, PBMC were cultured on a Fn-coated surface or with soluble Fn (1–100 μg/ml for both sets of experiments) for 48 h. The nonadherent mononuclear cells were then removed from the PBMC cultures and resuspended in tissue culture medium. The adherent mononuclear cells were incubated with EDTA for 20 min at 37°C, resuspended by a rubber policeman, washed twice with PBS and pooled with the nonadherent cells. The suspension of adherent and nonadherent cells was then washed with PBS containing 1% BSA. For some experiments nonadherent monocytes were removed and discarded 2 h after plating, before the addition of soluble Fn. These cells were cultured for an additional 46 h in the presence of Fn and then resuspended with a rubber policeman as described above. Subsequently, 1×10^6 cells were incubated with the monoclonal antibodies P1E6 or P1D6, which recognize the α_2 or α_5 integrins, respectively, at a concentration of 10 μg/ml or at saturating concentrations as recommended by the manufacturer in PBS with 1% BSA for 20 min at 4°C. Cells were washed once with PBS containing 1% BSA. Cells were then incubated with a secondary goat anti-mouse F(ab')2 fragment coupled to fluorescein at 10 μg/ml (Tago Inc., Burlingame, CA) for 30 min at 4°C, washed twice in PBS with 1% BSA and resuspended in PBS with 1% BSA. Fluorescein-labeled monocytes and lymphocytes were gated and analyzed with a FACScan® flow cytometer (Becton Dickinson and Co., Mountain View, CA).

Antibodies. The P1H5 monoclonal antibody directed against the $\alpha_2\beta_1$ integrin cell surface collagen receptor was generously provided by Dr. William G. Carter (Fred Hutchinson Cancer Research Center, Seattle, WA). Monoclonal antibody 10E5 directed against the platelet membrane glycoprotein IIb-IIIa complex was kindly provided by Dr. Barry S. Coller (SUNY, Stonybrook, NY). The P1D6 monoclonal antibody against the $\alpha_5\beta_1$ receptor and the monoclonal antibody P1E6 against the $\alpha_2\beta_1$ were purchased from Telios Pharmaceuticals (San Diego, CA). The monoclonal antibody D9b against the Fn collagen–binding site was generously provided by David L. Hasty (University of Tennessee, Memphis, TN). The control monoclonal antibody M-37 (that binds to the 20-kD carboxy-terminal portion of the 60-kD Fn collagen–binding domain and does not block collagen binding to Fn) and the polyclonal antibody against Fn were kindly provided by Dr. John A. McDonald (Washington University, St. Louis, MO). The characterization and specificity of these antibodies have been previously documented (25–29). All antibodies were used in IL-1 release assays at a concentration of 10 μg/ml.

Statistical methods. Group mean values were compared by two-tailed Student's t test or analysis of variance, as appropriate. Subsequent mean comparison tests were performed by Tukey's honestly significant difference test.

Results

Fn potentiates the collagen-induced secretion of IL-1 from PBMC. As shown in Table I, unstimulated PBMC incubated in uncoated plastic wells for 48 h released small, but measurable, amounts of IL-1 (2.1±0.8 U/ml). As previously reported (11) incubation of PBMC in wells coated with rat tail type I collagen or in the presence of soluble heat-denatured collagen resulted in significant increases in IL-1 secretion, as judged by the D10. cell-proliferation assay.

Conversely, incubation of PBMC in Fn-coated wells or with soluble Fn (0.1–100 μg/ml for both set of experiments), caused no increase in IL-1 secretion. In order to determine

Table I. *Effect of Health-Denatured Rat Tail Type I Collagen and Fibronectin on IL-1 Activity from Monocytes, Lymphocytes, and Total Mononuclear Cells (PBMC)*

Stimulus	IL-1 activity		
	Monocytes	Lymphocytes	PBMC
		U/ml	
None	2.3±0.9	0.9±0.4	2.1±0.8
Collagen (*100 μg/ml*)	3098.5±424.4*	33.6±6.3	2987.6±391.5*
Fibronectin (*100 μg/ml*)	2.0±0.7	1.5±0.9	1.8±0.3

Cells were incubated with soluble heat-denatured rat tail type 1 collagen or Fn. These substances were added to the culture wells after the plating of the cells. The magnitude of the IL-1 response to collagen was dose dependent within the range tested (0.1–100 μg/ml). Fn had no effect on IL-1 release at any of the concentrations tested (0.1–100 μg/ml). Similar results were obtained by culturing PBMC on either type 1 collagen or Fn-coated wells. * $P < 0.0001$.

whether collagen induces IL-1 release from monocytes, lymphocytes, or both, heat–denatured type I collagen was added to adherent monocytes or lymphocytes depleted of monocytes. As shown in Table I, monocytes, but not lymphocytes, released increased amounts of IL-1 when incubated with collagen. When soluble Fn was added to these cultures neither monocytes nor lymphocytes responded with an increased secretion of IL-1. When PBMC were cultured on collagen-coated wells in either serumfree conditions or with a Fn-depleted serum, the release of IL-1 was significantly lower (Fig. 1) than from PBMC cultured in 5% serum. The addition of exogenous Fn (50 μg/ml) to Fn-depleted serum restored the ability to potentiate the IL-1 response to collagen. The collagen-induced IL-1 secretion was also significantly decreased by a polyclonal antibody against Fn. These data suggest that although Fn does not stimulate IL-1 release, it has a potentiating effect that enhances the IL-1 response to collagen. To determine whether the potentiating effect of Fn occurs in monocytes, lymphocytes, or both, additional experiments were carried out by adding heat–denatured type I collagen to adherent monocytes or lymphocytes depleted of monocytes cultured in plastic with Fn-depleted serum. As shown in Table II adherent monocytes responded to collagen with a lower secretion of IL-1 than monocytes cultured with 5% serum. This response was similar to the one observed culturing PBMC in the same experimental conditions. Addition of 50 μg/ml of exogenous Fn to the adherent monocytes cultures restored a full IL-1 response to collagen. Isolated lymphocytes cultured in Fn-depleted serum failed to respond to collagen. The addition of exogeneous Fn to collagen-stimulated lyphocytes also had no effect.

The collagen-induced IL-1 secretion was almost completely abolished (Table III) by an anti–IL-1β antibody and, in part, by an anti–IL-1α antibody indicating that IL-1β was responsible for the majority of the D10. cell–proliferation activity.

The $\alpha_5\beta_1$ integrin receptor mediates the potentiating effect of Fn on IL-1 release. To determine whether specific integrin receptors mediate the IL-1 response to collagen and Fn, neutralization experiments were carried out with antiintegrin monoclonal antibodies (Fig. 2). As previously reported, the P1H5 antibody directed against the $\alpha_2\beta_1$ integrin, a cell surface collagen receptor, decreased the IL-1 release elicited by culturing PBMC on collagen-coated wells. The antibody P1D6, which recognizes the $\alpha_5\beta_1$ Fn receptor, also decreased significantly the IL-1 response to collagen. The effect of the two antibodies was not additive because when PBMC were cultured on collagen-coated wells with both the P1H5 and the P1D6 antibody, inhibition of IL-1 release was similar to that observed when each of the two antibodies was added alone to the PBMC cultures. The control antibody 10E5, had no effect on the collagen induced IL-1 release.

The potentiating effect of Fn is not the result of Fn binding to collagen. Since Fn possesses a collagen-binding site in addition to the Arg-Gly-Asp–(RGD) containing cell-binding site recognized by the $\alpha_5\beta_1$ integrin, the possibility exists that Fn may facilitate the interaction of collagen with the $\alpha_2\beta_1$ receptor by anchoring collagen to the PBMC surface so as to properly present this molecule to its integrin receptor. This hypothesis was tested by investigating whether the 120-kD Fn fragment, a polypeptide that possesses the $\alpha_5\beta_1$ receptor–binding site but not the collagen-binding site, mimics the effect of native Fn on collagen-induced IL-1 secretion. These experiments were carried out using Fn-depleted serum. The 120-kD fragment, either added to the culture medium or coated on the culture wells, did not alter the baseline secretion of IL-1. Moreover, PBMC cultured in collagen-coated wells with 120-kD Fn fragment added released amounts of IL-1 (Fig. 3) that were similar to those produced by PBMC cultured in the presence of native Fn. The control 65/75-kD Fn fragment failed to support the IL-1 response to collagen. These data suggest that the potentiating effect of Fn is the result of the functional engagement of the $\alpha_5\beta_1$ receptor and not of the facilitation of PBMC interaction with collagen. That the binding of Fn to collagen is not required for inducing the IL-1 response, was further demonstrated (Table IV) by the failure of the antibody D9b, which inhibits Fn binding to collagen (27), to block the potentiating effect of Fn. As expected, pretreatment of PBMC with the control antibody M-37 also failed to block the permissive effect of the 120-kD Fn fragment on the collagen-induced IL-1 secretion.

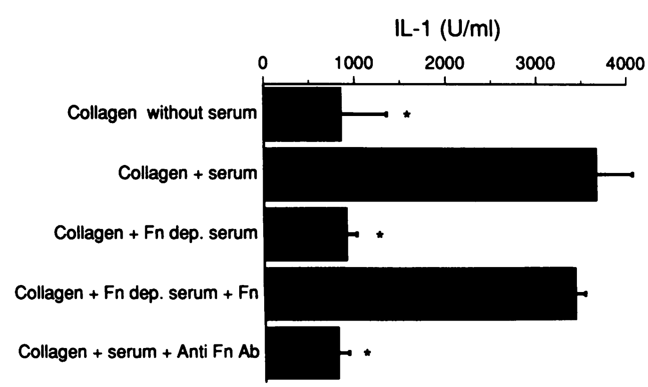

Figure 1. Effect of Fn deprivation or Fn blocking on the collagen-induced IL-1 secretion. PBMC were incubated in culture wells coated with rat tail type I collagen for 48 h in serum free or with either 5% heat-inactivated FCS or 5% Fn-depleted serum. Fn or a polyclonal anti-Fn antibody were also added to the culture media as described in the text. The culture media was then assayed for IL-1. *$P < 0.001$ compared to PBMC incubated with Fn-containing serum.

Table II. Effect of Fn Deprivation on the Collagen-induced IL-1 Secretion from Peripheral Blood Monocytes, Lymphocytes, and Total Mononuclear Cells (PBMC)

Stimulus	IL-1 activity		
	Monocytes	Lymphocytes	PBMC
		U/ml	
Collagen + serum	3648.4±529.5	41.5±8.0	3743.1±578.7
Collagen + Fn-depl. serum	1159.7±134.5*	29.8±5.6	1264.5±201.0*
Collagen + Fn-depl. serum + Fn	3247.6±486.4	27.9±4.3	3104.5±419.1

Cells were incubated in plastic with soluble heat-denatured rat tail type 1 collagen (100 μg/ml) and either 5% serum or 5% Fn-deprived serum. These substances were added to the culture wells after the plating of the cells. Both monocytes and PBMC secreted a lower amount of IL-1 (* $P < 0.01$) when cultured with Fn-depleted serum than with Fn-containing serum. Addition of exogenous Fn (50 μg/ml) restored a full IL-1 response to collagen. Fn deprivation had no effect on the lymphocyte response to collagen.

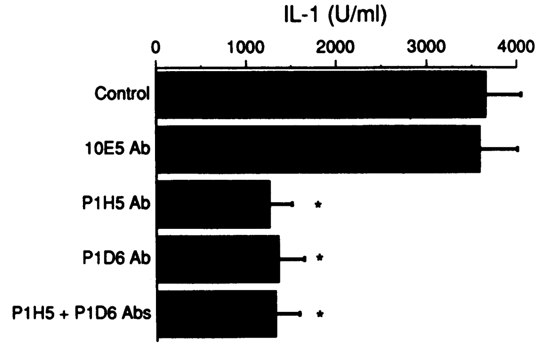

Figure 2. Effect of the monoclonal antibody P1H5 directed against the $\alpha_2\beta_1$ integrin collagen receptor and the monoclonal antibody P1D6 that recognizes the $\alpha_5\beta_1$ Fn receptor on the collagen-induced IL-1 secretion. PBMC were preincubated in the presence of the indicated antibody (10 μg antibody/ml final concentration) for 2 h and then cultured on collagen-coated wells for an additional 46 h. A control antibody 10E5 directed against the platelet membrane IIb-IIIa complex had no effect. Similar results were obtained when PBMC were cultured on plastic and stimulated with soluble heat-denatured collagen. The spontaneous release of IL-1 from unstimulated PBMC was not affected by any of the antibodies used in this study (data not shown). *$P < 0.001$ compared to controls.

RGD peptides decrease the potentiating effect of Fn. The effects of small peptides known to bind to the $\alpha_5\beta_1$ receptor was investigated by performing additional experiments in Fn-depleted serum. In these studies we found (Table V) that both the GRGDSP peptide and the control GRGESP peptide failed to enhance the IL-1 response to collagen. However, the GRGDSP (but not the GRGESP peptides) competed with intact Fn. At 1 mg/ml, the GRGDSP peptide blocked the effect of Fn on collagen-induced IL-1 secretion.

Fn binding to the $\alpha_5\beta_1$ receptor does not increase the expression of the $\alpha_2\beta_1$ integrin. The accumulated data indicate that the functional engagement of the $\alpha_5\beta_1$ receptor potentiates the effect of collagen binding to the $\alpha_2\beta_1$ receptor on IL-1 secretion. One possible explanation for this phenomenon is that ligand binding to the $\alpha_5\beta_1$ receptor increases the expression of the $\alpha_2\beta_1$ receptor. To test this hypothesis a flow cytometric analysis of both monocytes and lymphocytes was performed of PBMC cultured with Fn for 48 h. The results showed that culture with soluble Fn or on a Fn substrate does not increase the expression of the $\alpha_2\beta_1$ receptor on either monocytes or lymphocytes above control levels. Moreover, no changes in the expression of $\alpha_2\beta_1$ were observed when adherent monocytes were cultured with soluble Fn for 48 h and then subjected to the flow cytometric analysis (data not shown).

Table III. Neutralization of PBMC IL-1 Activity (U/ml) with Monoclonal Anti-human IL-1α and IL-1β Antibodies

Stimulant	Antibody		
	None	Anti-IL-1α	Anti-IL-1β
		U/ml	
Recombinant IL-1α	322±33	0	266±41
Recombinant IL-1β	469±47	422±51	9±1
Collagen (*100 μg/ml*)			
1:1	3456±475	2956±385	263±58
1:4	951±108	604±77	89±12
1:16	148±22	106±21	13±2

Conditioned media derived from PBMC cultured with collagen (100 μg/ml) were serially diluted and assayed for their ability to induce D.10 cell proliferation in the absence or the presence of neutralizing antibodies to IL-1α or IL-1β. Conditioned medium (100 μl) was incubated with a 1:20 dilution of anti–IL-1α antibody or a 1:200 dilution of anti–IL-1β for 2 h and then assayed for D.10 cell proliferation (final volume 200 μl).

Discussion

The proliferation, differentiation, and metabolic activity of cells is regulated by their interaction with extracellular matrices. Circulating mononuclear cells are no exception because they are armed with cell surface integrin receptors able to recognize collagen and other extracellular matrix components (11, 14). PBMC binding to collagen, an event that triggers the synthesis and release of IL-1 (11), is mediated, at least in part, via the integrin $\alpha_2\beta_1$. PBMC also express integrin receptors for Fn, a protein that binds to collagen and is a substrate for cell adherence and migration (30, 31). In this study, we investigated the effects of Fn on the collagen-induced IL-1 secretion. We observed that Fn, in contrast to collagen, did not stimulate IL-1 release. However, Fn binding did potentiate the stimulatory effect of collagen on IL-1 release.

The stimulatory effect of collagen on IL-1 release and the potentiation of these phenomena by Fn were observed in cultures of PBMC and adherent monocytes but not in cultures of nonadherent cells. Moreover, the effects of both collagen and Fn were similar in the PBMC and the adherent monocyte cultures. Thus, although a contribution of other blood cellular elements cannot be entirely excluded, the accumulated data point to the monocytes as the source of the adherence proteins-induced IL-1 activity.

The potentiating effect of Fn on collagen-induced IL-1 release is linked to its ability to bind and functionally engage the

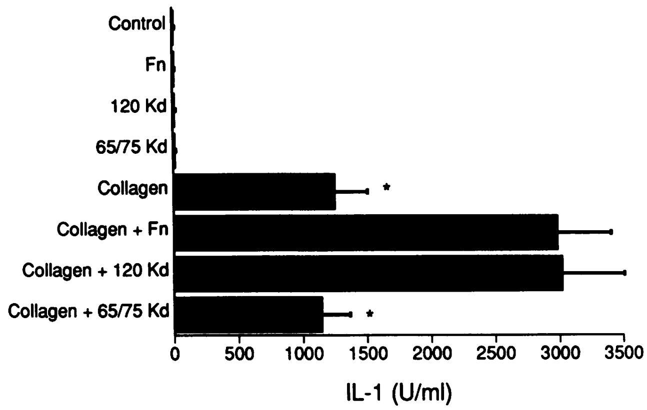

Figure 3. Effect of 120-kD Fn fragment on the collagen-induced IL-1 release. PBMC were incubated in culture wells coated with rat tail type I collagen for 48 h with 5% Fn-depleted serum and either native Fn or the 120-kD Fn fragments added to culture medium. The culture media was then assayed for IL-1. A control 65/75-kD Fn fragment had no effect on the IL-1 response to collagen. *$P < 0.001$ compared to PBMC incubated with collagen and Fn or collagen and 120-kD Fn fragment.

$\alpha_5\beta_1$ receptor because antibodies directed to this Fn receptor blocked the secretion of IL-1, while the 120-kD Fn fragment, which contains the RGD adhesive sequence, mimicked the effects of the native molecule. The 120-kD fragment does not contain the collagen-binding domain suggesting that the effect of Fn is not due to its ability to bind collagen and cells simultaneously and, therefore, to facilitate the interaction between PBMC and collagen molecules. Fn is produced by many mammalian cells (31). Thus, it could be argued that endogenously produced Fn could compete with the 120-kD fragment and be the actual enhancer of the IL-1 response. However, this is unlikely as a lower IL-1 response to collagen was observed in all the experiments carried out without exogenous sources of Fn and pretreatment with antibodies against the Fn collagen-binding site did not alter the ability of PBMC to respond to the 120-kD fragment. Interestingly, the carboxy-terminal 65/75 kD chymotryptic fragment of Fn, which contains the CS-1 variable splicing site responsible for binding to the integrin $\alpha_4\beta_1$ (30), did not affect IL-1 release. This further supports our results suggesting that the effects of Fn on IL-1 release are mediated exclusively via the integrin $\alpha_5\beta_1$.

The inability of RGD peptides to evoke the response observed with Fn or its 120-kD fragment may suggest that the RGD sequence is not the functional domain that accounts for the potentiating effect of Fn. Other determinants of a more extended binding site may be involved. Alternatively, the ability of this domain to activate the $\alpha_5\beta_1$ receptor may depend on its conformational status. Several studies showing a dichotomy between the effects of Fn and RGD peptides in different systems have been published, and include the activation of complement receptors in monocytes and the induction of metalloproteinase gene expression in fibroblasts (32, 33).

The observations described in this report are consistent with the hypothesis that the induction of a maximal IL-1 release requires the interaction of PBMC with collagen and Fn via two distinct integrin receptors, $\alpha_2\beta_1$ and $\alpha_5\beta_1$, respectively. The potentiation caused by ligand binding to the $\alpha_5\beta_1$ receptor appears to result from an enhancement of the $\alpha_2\beta_1$-mediated effects of collagen on IL-1 release. Although PBMC express several collagen receptors (14), the lack of additive effects of the anti-$\alpha_2\beta_1$ and the anti-$\alpha_5\beta_1$ antibodies suggests that the binding of ligand to the $\alpha_5\beta_1$ receptor does not regulate the effect of collagen on IL-1 release mediated by receptors other than the $\alpha_2\beta_1$ integrin.

An increased integrin-mediated adhesiveness as a result of a nonintegrin receptor engagement has been described for the β_1 (34) and β_2 integrins (LFA-1) in T cells (35), the β_2 and β_3 receptors in polymorphonucleated cells (36), and the gpIIb/IIIa receptor in platelets (37). In addition, other studies have linked fibronectin and the $\alpha_5\beta_1$ integrin to signal transduction (33, 38). However potentiation of a cytokine release as a result of an interaction between two β_1 integrins has not been previously reported.

The sequence of events that link ligand binding of these receptors and IL-1 release remain to be elucidated. At least two mechanisms can be envisioned. Fn binding to $\alpha_5\beta_1$ may affect the function of $\alpha_2\beta_1$ by regulating its expression or its affinity to collagen. By flow cytometric analysis, we were unable to detect quantitative changes in $\alpha_2\beta_1$ receptor expression in either monocytes or lymphocytes incubated with Fn. An effect of Fn binding on the affinity of the $\alpha_2\beta_1$ integrin for collagen cannot be discounted since ligand binding may depend on changes in its conformation, a mechanism described for the activation of the platelet integrin gpIIb/IIIa (37). Alternatively, ligand binding to the $\alpha_5\beta_1$ receptor could potentiate the stimulatory effects of collagen on IL-1 release by facilitating or enhancing an intracellular signal directly involved in the synthesis and release of IL-1. Our knowledge of the signal transduction pathways activated by ligand binding to integrins is limited. Receptor clustering (33) and phosphorylation (39), pH changes (40), and transmembrane calcium fluxes (41) have all been implicated.

Fn and collagen are present in most extracellular matrices (42). However, Fn expression is markedly increased in reactive tissues at sites of inflammation and tissue repair (42, 43). Che-

Table IV. Effect of the Antibody D9b Against the Fn Collagen-binding Domain and the Control Antibody M-37m on the Collagen-induced IL-1 Secretion

Antibody	Collagen	Collagen + Fn	Collagen + 120 kD
		U/ml	
—	645±78*	2318±156	2022±240
Ab D9b	697±94*	2295±187	2312±321
Ab M-37	721±105*	2398±195	2146±297

All the experiments were conducted in Fn-depleted serum to eliminate exogenous sources of Fn. Rat tail type I collagen (100 μg/ml) was coated on the culture wells before the addition of the cells and the other test substances. Fn (50 μg/ml), the 120-kD fragment (50 μg/ml), and the antibodies were added to the culture medium after the PBMC were allowed to adhere on the collagen substrate of 2 h. Both the antibody D9b, which blocks the collagen binding domain on the Fn molecule, and the antibody M-37, which binds to the 20-kD carboxy-terminal portion of the 60-kD Fn collagen-binding domain and does not block collagen binding to Fn, did not decrease the ability of native Fn or the 120-kD fragment to potentiate the collagen-induced IL-1 secretion demonstrating that this effect does not require the binding of Fn to collagen. * $P < 0.0001$ compared to either collagen + Fn or collagen + 120-kD fragment.

Table V. Effect of RGD-containing Peptides on the Collagen-induced IL-1 Release

Peptide	Collagen (100 μg/ml)	Collagen (100 μg/ml) + Fn (50 μg/ml)
	U/ml	
—	1462±185	2479±315[a]
GRGDSP (*1 mg/ml*)	1528±208	1314±191[c]
GRGESP (*1 mg/ml*)	1531±133	2132±243[b]

All experiments were carried out in Fn-depleted serum to eliminate endogenous sources of Fn. The GRGDSP or the control GRGESP peptides were coated on the culture wells before the addition of the PBMC. Heat-denatured collagen and native Fn were added to the culture medium after the PBMC and were allowed to adhere on the RDG peptides for 2 h. [a], $P < 0.01$ Compared to collagen alone; [b], $P < 0.05$ compared to collagen + GRGESP; [c], $P < 0.01$ compared to either collagen + Fn or collagen + Fn + GRGESP.

motactic Fn fragments are released in areas of tissue remodeling (44), providing a signal for homing monocytes and perhaps modulating their IL-1 response. This may be of particular importance in pathologic states, such as the adult respiratory distress syndrome, characterized by increased Fn expression and remodeling of the lung parenchyma (30). Local release of IL-1 appears also to be relevant in chronic disorders such as osteoporosis. During bone remodeling, fragments of collagen and other bone matrix constituents are released into the local microenvironment (45). These degradation products serve as potent stimulators of monocytic release of IL-1 (11), a potent stimulator of bone resorption (46) that has been linked to postmenopausal bone loss (47). During this process, Fn may provide additional signals both for the recruitment of monocytes and subsequent release of IL-1, an event that, in turn, is important for maintaining or amplifying the bone resorption process. Both systemic calciotrophic hormones such as $1,25(OH)_2D_3$, and local factors such as TGFβ, regulate the synthesis of matrix components such as collagen and Fn as well as expression of matrix receptors (48, 49) providing further complexity to the mechanism regulating IL-1 secretion and, perhaps, a means for fine tuning the monocyte immune response.

The present study further elucidates the role of specific integrin receptors in the regulation of the PBMC immune response and describes a regulatory interaction between two β_1 integrins. Moreover, the ability of extracellular matrix proteins to modulate IL-1 release via the integrin receptors provides an experimental model to investigate the signaling processes activated by ligand binding to integrins and to examine the biological effects of extracellular matrices on immune cells.

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