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A POSSIBLE ROLE FOR COLD INSOLUBLE GLOBULIN IN CHEMOTACTIC FACTOR MEDIATED POLYMORPHONUCLEAR LEUKOCYTE ADHERENCE TO PLASTIC

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We examined the potential role of fibronectin in chemotactic factor stimulation of neutrophil adherence to plastic. Monoclonal antibody to human fibronectin significantly reduced chemotactic peptide stimulation of adherence but did not reduce adherence stimulated by phorbol myristate acetate or aggregation stimulated by either agent. Stimulation of neutrophils by chemotactic peptide was also associated with loss of cell surface fibronectin detected by immunofluorescence or binding of radiolabeled collagen. These data suggest that chemotactic peptides stimulate neutrophils to release Fn and that Fn mediates the attachment of neutrophils to plastic surfaces.

INTRODUCTION

Fibronectin (Fn) or cold insoluble globulin is an adhesive glycoprotein which has been demonstrated to circulate in human plasma (1-3) and also has been associated with the plasma membrane of in vitro cultured human fibroblasts (4, 5), epithelial cells (6) and endothelial cells (7). Recently, Fn has been demonstrated in inflammatory and secretory cells including human platelets and leukocytes (8-10). Several observations have suggested a potential role for Fn in inflammation. First, human platelets were shown to release Fn into supernatant fluids during the course of thrombin-induced aggregation (11). Secondly, Fn increased the motility and phagocytic capacity of human polymorphonuclear leukocytes (PMN) (12). More recently, PMN have been shown to synthesize and deposit Fn at sites of attachment to gelatin-coated plastic (10). Our previous observations demonstrated that

Abbreviations: Fn, fibronectin; FMLP, n-formyl-methionyl-leucyl-phenylalanine; HBSS, Hank's balanced salt solution; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocyte.

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chemotactic factors enhance PMN adhesiveness to surfaces (13, 14). We report here that antibody to human Fn impairs chemotactic factor stimulation of PMN adhesiveness to plastic.

MATERIALS AND METHODS

Adherence and Aggregation Assays

Purified PMN (>98% pure) were prepared as previously described (14) from heparinized venous blood obtained from normal volunteers. One million PMN suspended in Hank's balanced salt solution (HBSS, KC Biological, Lenexa, KA) were added to 16 mm plastic tissue culture wells (Flow Laboratories, Hamden, CT), and adherent cells after 60 min incubation with chemotactic factors at 37°C were quantitated by Coulter Counter (Coulter Electronics, Hialeah, FL). In experiments with the mouse monoclonal antibody to human Fn (Pel Freeze Biological, Rogers, AZ), 10^6 PMN were preincubated with antibody for 10 min at 37°C before incubation with chemotactic factors. Aggregation of a stirred suspension of PMN (10^7/ml) was quantitated using a platelet aggregometer-recorder system (Chronolog Corporation, Havertown, PA) as previously described (15). To obtain optimal aggregation curves, stirred neutrophils were preincubated with cytochalasin B 5 μg/ml (Sigma Chemical Co., St. Louis, MO) for 2 min before addition of the aggregating stimulus.

Immunofluorescence

One million PMN were incubated with or without the synthetic chemotactic agent n-formyl methionyl-leucyl-phenylalanine (FMLP, Sigma Chemical Company, St. Louis, MO) 10 μM for 60 minutes at 37°C on a rotary shaker. At the end of this period the cells were washed in cold HBSS and incubated at 4°C with 1:50 dilution of rabbit antifibronectin antiserum or preimmune rabbit serum prepared as previously described (16). At the end of one hour the cells were washed and incubated for 60 minutes at 4°C with fluoresceinated goat anti-rabbit IgG (Alpha Gamma Laboratories, Sierra Madre, CA), then washed and placed on a clean microscope slide and examined for fluorescence with a Zeiss epiilluminated fluorescence microscope.

Collagen Binding Assay

Human collagen was prepared by pepsin digestion of human cadaver dermal collagen; pepsin was inactivated by 1 N NaOH and the collagen precipitated by repeated exposure to 10% NaCl in glacial acetic acid. The collagen was resolubilized in 0.5 M acetic acid, dialyzed against phosphate buffered saline pH 7.4 and lyophyllized. For the binding assay, denatured collagen (41°C for 60 min) was acetylated with ^{3}H-acetic anhydride according to the method of Levine et al. (17) and dialyzed repeatedly against phosphate buffered saline pH 7.4. The specific activity of the ^{3}H-collagen was 32,000 cpm/μg, and the radioactivity was 90% precipitable by 10% trichloroacetic acid. In the binding assay, 5 x 10^{6} PMN in HBSS were preincubated in buffer or with chemotactic factors for 60 min at 37°C on a rotator. Following this 200,000 cpm of ^{3}H-collagen (6.8 μg) was added to duplicate tubes for 10 min at 37°; the cells were diluted with cold HBSS (4°C), immediately centrifuged at 1000 g and washed with cold buffer. PMN associated radioactivity was determined by scintillation spectroscopy. Nonspecific binding (in the presence of 100 fold excess cold collagen) was less than 20%. ^{3}H-collagen binding was maximal at 10 min incubation and was reversed in the presence of excess unlabeled collagen or Fn.

Reagents

Fn was isolated from fresh human citrated plasma by affinity chromatography on gelatin-Sepharose (18). Phorbol myristate acetate (PMA, Consolidated Midland Corp., Brester, NY) was purchased.

RESULTS

The chemotactic factor FMLP 10 μM increased adherence to plastic from baseline of 112 x 10^{3} cells to 205 x 10^{3} cells per well (Figure 1). Monoclonal IgM antibody to human fibronectin (1/50 dilution) reduced FMLP-stimulated adherence by 83% without significantly affecting baseline levels of adherence. Control supernates from the parent myeloma cell line did not affect basal or stimulated adherence. Phorbol myristate acetate also stimulated PMN adherence to plastic from baseline 105 x 10^{3} cells/well to 240 x

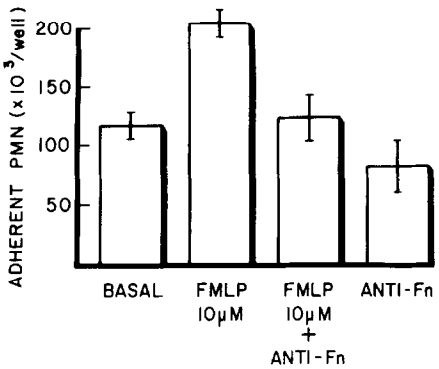


Figure 1. Inhibition of FMLP-stimulated adhesive to plastic by mouse monoclonal anti-Fn. Results are expressed as Mean ± SEM of 5 experiments. Anti-Fn (1/50 dilution) reduced FMLP stimulation ($p<0.005$, paired t-statistic) but not baseline adherence.

10^3 cells/well at a concentration of 10 ng/ml. In contrast to our findings with FMLP, adherence induced by the surface active agent PMA was not reduced significantly by the same concentrations of anti-Fn (data not shown). Exogenous Fn 1 μg/ml and 10 μg/ml had no apparent effect on baseline PMN adherence to plastic; however, antibody inhibition of FMLP-stimulated adherence was reversed by preincubation of monoclonal anti-Fn (1/50 dilution) with Fn 10 μg/ml without affecting baseline adherence (Table 1).

Next, we assessed cell-cell adherence by aggregometry. Exogenous Fn at 10 μg/ml did not induce aggregation nor did it affect aggregation induced by FMLP (10 μM) or PMA (10 ng/ml). Moreover anti-Fn had no significant

Table 1. Effect of Fn on PMN adherence to plastic*

Treatment	Adherent Cells/Well x 10^3
None	108 ± 10
FMLP 10 μM	204 ± 11
FMLP + Anti-Fn	124 ± 24**
FMLP + Fn (10 μg/ml) + Anti-Fn	188 ± 25†
Fn + Anti-Fn	90 ± 19

*Results are mean ± SEM of 3-6 experiments
**Differs from FMLP alone, $p<0.005$, paired t-statistic
†Fn and Anti-Fn (1/50 dilution) were incubated 15 min at 37°, then added to 10^6 PMN for additional 10 min at 37°C before exposure to chemotactic factor. Differs from Fn + anti-Fn, $p=0.026$, paired t-statistic

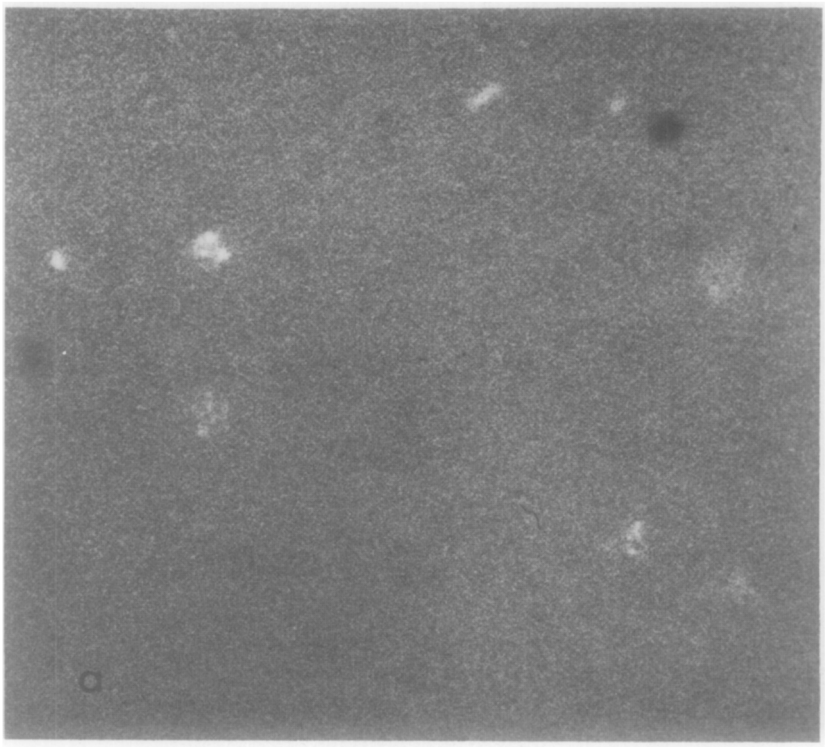
effects on FMLP- or PMA-induced aggregation over a range of concentrations from 1/10 to 1/100 dilution.

These data suggested that expression of Fn at the cell surface or release into the incubation media was necessary for FMLP-stimulated adherence to plastic. Accordingly, we looked for alterations in cell surface Fn using an indirect fluorescent antibody technique. Unstimulated PMN demonstrated marked rim fluorescence as shown in Figure 2A. Fibronectin appeared as fluorescence at the rim of every PMN examined. In some cells peripheral aggregates of fluorescent-staining Fn were seen. In contrast, the majority of cells incubated with FMLP 10 μM for 60 min and washed free of FMLP showed a marked loss of immunofluorescence (Figure 2B).

In the next series of experiments, we utilized the observations that Fn binds to collagen (19). Using ^{3}H labelled collagen as a quantitative probe for surface associated Fn, unstimulated PMN bound a maximum of 329 ± 90 pM collagen per 10^{6} PMN at 10 min at 37°C (Table 2). In contrast, PMN prestimulated with FMLP 10 μM for 60 min then incubated with ^{3}H-collagen bound 60% less collagen under the same conditions (Table 2). Diminshed binding was not due to direct interference with binding by FMLP, since FMLP added simultaneously with ^{3}H-collagen did not inhibit binding significantly compared to the unstimulated control.

DISCUSSION

Our data demonstrate that a mouse monoclonal antibody to human Fn inhibited chemotactic factor stimulated PMN adhesiveness to plastic but not PMN aggregation. Further, upon stimulation, PMN lose fluorescent-staining surface Fn and bind less ^{3}H-collagen than unstimulated cells. We were unable to detect alterations in aggregation by preincubation of PMN with various dilutions of mouse hybridoma anti-Fn. This contrasts with observation in the literature which suggest that Fn has lectin-like activity agglutinating sheep erythrocytes (20) and increasing the aggregation of chick embryo and hamster kidney cells (21). Furthermore, Fn has been observed at the cell surface during the thrombin stimulation of plate-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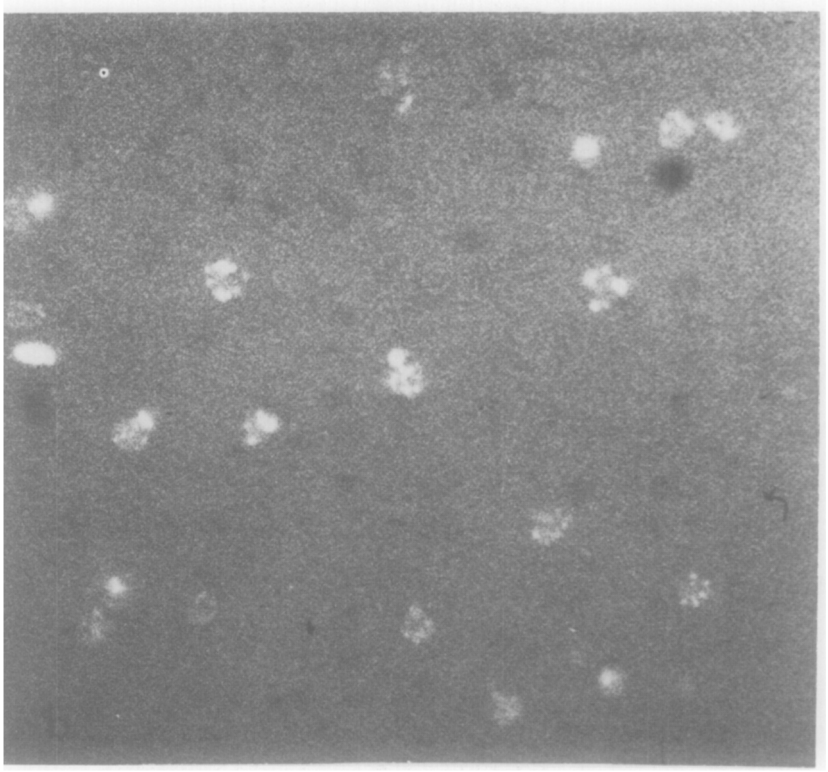

Table 2. PMN binding of ^{3}H collagen*

Conditions	Collagen binding pM/10^6 PMN
Unstimulated PMN	330 ± 90
FMLP-stimulated PMN	130 ± 20**
FMLP-post added	260 ± 60†

*Results are Mean ± SEM of 6 experiments. Binding assessed after 10 min incubation with ^{3}H collagen at 37°C

**Differs from unstimulated PMN, $p<0.05$ student's t-test

†FMLP 10 μM added simultaneously with ^{3}H collagen

let secretion and aggregation (8). The conditions we employed to induce optimal aggregation by FMLP require the addition of cytochalasin B to render PMN secretory (15). Cytochalasin can induce release Fn from the surface of fibroblasts (22); thus, the experimental conditions we used may have limited detection of altered aggregation mediated by changes in cell surface Fn. On the other hand, PMA induced aggregation, which did not require cytochalasin, also was unaffected by anti-Fn.

The data presented here, although consistent with loss of surface Fn activity detectable by fluoresceinated antibody or radiolabeled collagen probes, do not distinguish clearly among release of Fn from the cell surface, hydrolysis of surface Fn by proteases or accelerated turnover and internalization. However, our data do support the observations by Weissman, et al. (10) that PMN deposit Fn at sites of attachment to substrates. Our observations are consistent with the hypothesis that Fn is released by PMN following chemotactic factor stimulation and that Fn mediates the attachment of stimulated PMN to plastic surfaces.

Figure 2. Immunofluorescent staining Fn in resting (a) and stimulated (b) neutrophils. These photographs are representative of 3 separate experiments.

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