NEUTROPHIL-ACTIVATING PROTEIN 1/INTERLEUKIN 8 STIMULATES THE BINDING ACTIVITY OF THE LEUKOCYTE ADHESION RECEPTOR CD11b/CD18 ON HUMAN NEUTROPHILS

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Neutrophil-activating protein 1/interleukin 8 (NAP-1/IL-8)[1] was first identified as a 72 amino acid peptide secreted by monocytes in response to bacterial LPS, with the properties of activating and attracting polymorphonuclear leukocytes (PMN) in vitro (1). Subsequently several different types of cells, including macrophages and endothelial cells, were found to synthesize NAP-1/IL-8 upon stimulation with TNF or IL-1. Sequence data indicate that NAP-1/IL-8 is homologous to a group of peptides including platelet basic protein, platelet factor 4, and macrophage inflammatory protein 2. In vivo accumulation of PMN at sites of injection of NAP-1/IL-8 suggests that it may participate in the recruitment of PMN into inflamed tissue, and NAP-1/IL-8 has been identified in skin lesions of patients with psoriasis (1).

Several lines of evidence indicate that the CD11/CD18 complex of leukocyte adhesion receptors may participate in the adhesion of PMN to the vascular endothelium which is necessary for extravasation. mAbs specific for CD18 are effective in blocking the adherence of stimulated PMN to endothelium in vivo (2) and in vitro (3, 4), and patients genetically deficient in the CD11/CD18 complex exhibit poor recruitment of PMN to Rebuck skin windows (5). Here we describe the response of CD11/CD18 on PMN to NAP-1/IL-8, with particular emphasis on the numerically dominant member, CD11b/CD18. This receptor not only recognizes ligands on endothelium but also binds to complement protein C3bi (6), fibrinogen (7), and LPS (8).

Materials and Methods

Reagents and Buffers. Recombinant human NAP-1/IL-8 (9) was generously supplied by the Sandoz Research Institute, Vienna, Austria. Human fibrinogen depleted of plasminogen was

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[1] *Abbreviations used in this paper:* AI, attachment index; EC, endothelial cells; fNLLP, formyl-norleucyl-leucyl-phenylalanine; HSA, human serum albumin; NAP-1/IL-8, neutrophil-activating protein 1/interleukin 8; PDB, phorbol dibutyrate; PI, phagocytic index; PMN, polymorphonuclear leukocytes.

a generous gift of Dr. Liliana Ossowski (The Rockefeller University, New York, NY). Fibronectin was obtained from the New York Blood Center (New York, NY). fMLP, formyl-norleucyl-leucyl-phenylalanine (fNLLP), PMA, phorbol dibutyrate (PDB), and aprotinin were purchased from Sigma Chemical Co., St. Louis, MO. Pyrogen-free human serum albumin (HSA) was supplied by Armour Pharmaceutical, Kankakee, IL. Buffers used were: PBS (137 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 8 mM phosphate, pH 7.4); PD (PBS without $CaCl_2$ or $MgCl_2$ but with 0.02% NaN_3); and HAP (pyrogen-free PBS [Sigma], 0.05% glucose, 0.5 mg/ml HSA and 0.3 U/ml aprotinin).

Antibodies. mAb provided by colleagues were: OKM10, directed against CD11b (6), from Dr. Gideon Goldstein (Ortho Pharmaceuticals, Rariton, NJ); LeuM5, directed against CD11c (10), from Dr. Louis Lanier (Becton Dickinson & Co., Mountain View, CA); 543, directed against CR1 (11), from Dr. Robert Schreiber (Washington University, St. Louis, MO); and T2G1, directed against fibrinogen (12), from Dr. Bohdan Kudryk (New York Blood Center, New York, NY). 3G8, directed against Fc_γ receptor III (Fc_γRIII, CD16) (13); W6/32, directed against HLA-A,B,C (14); and IB4, directed against CD18 (6), were as described.

Cell Preparation. Human PMN were isolated from freshly drawn, normal blood on Ficoll-Hypaque gradients (15) and suspended at a final concentration of 2×10^6 cells/ml in HAP buffer.

Cytoplasts were prepared from cytochalasin B-treated PMN exactly as described (16), washed five times to remove the cytochalasin, and suspended to a concentration of 4×10^6/ml in HAP buffer. Preparations of cytoplasts were previously characterized by enzyme assays and electron microscopy to be free of granular constituents (4).

Flow Cytometry. Cells were exposed to mAb (5 μg/ml in PD, 0.1% HSA) for 15 min at 0°C, washed, and incubated with fluoresceinated affinity-purified goat anti-mouse IgG $F(ab')_2$ (7 μg/ml in PD, 0.1% HSA) (Tago Inc., Burlingame, CA) for 45 min at 0°C. Fluorescence was measured on a Becton Dickinson FACScan cell analyzer. The mAb T2G1 (antifibrinogen) was used as a negative control in all experiments, and the mean fluorescence channel derived from this antibody, which was the same as that obtained by omitting the primary antibody, was subtracted from those of experimental samples.

Preparation of Opsonized Erythrocytes. Sheep erythrocytes (E) were coated with IgG or IgM as previously described (17). Erythrocytes coated with C3bi (EC3bi) were prepared by incubating EIgM in C5-deficient human serum (Sigma Chemical Co.) as described (18). Erythrocytes coated with the biosynthetic precursor of LPS, lipid IVa, were prepared as described (8).

Assays for Attachment and Phagocytosis of Ligand-coated Erythrocytes. Assays for rosetting and phagocytosis by adherent PMN or cytoplasts were essentially as previously described (17). Monolayers of adherent cytoplasts were formed by plating the cytoplasts and centrifuging the plates for 5 min at 150 *g*. The attachment index (AI) is the number of erythrocytes bound per 100 PMN. The phagocytic index (PI) represents the number of erythrocytes ingested by 100 PMN, counted after hypotonic lysis of uningested erythrocytes. Data are shown for representative experiments, since variation between preparations of PMN from different individuals precludes averaging of results.

Adhesion of PMN to Fibrinogen-coated Surfaces. Adhesion of PMN to fibrinogen-coated substrates was performed as described (7). The number of adherent PMN within a ×40 field were counted using a phase contrast microscope. All samples were run and counted in triplicate and averaged.

Isolation and Culture of Human Endothelial Cells. Human umbilical vein endothelial cells (EC) were isolated from fresh umbilical cords and cultured as previously described (4). Primary EC cultures were subsequently seeded onto Terasaki plates that had been precoated with human fibronectin, and EC were cultured for 2-3 d before use in adhesion assays. EC monolayers were confluent by this time with typical cobblestone morphology, intact junctions (4), and expression of factor VIII-related antigen (19).

Adhesion of PMN to EC. EC monolayers were fixed with 0.25% glutaraldehyde in PBS at 4°C for 5 min, incubated with 50 mM glycine, 0.15 M NaCl for 5 min, washed with PBS four to five times, and stored at 4°C. Adhesion of PDB-stimulated PMN to fixed EC monolayers was CD18-dependent, since the adhesion was blocked by pretreating the PMN with mAb IB4 (anti-CD18) (data not shown). The details of the adhesion assay have been published elsewhere (4).

Results and Discussion

NAP1/IL-8 Causes Increased Expression of CD11b/CD18. The expression of CD11b/CD18 on the surface of PMN as measured by flow cytometry increased in a concentration-dependent manner in response to NAP-1/IL-8 (Fig. 1, *stars*). The effect was apparent after exposure to 10^{-9} M NAP-1/IL-8, which appears to correspond to the threshold concentration, and reached a maximum at 5×10^{-8} M. The average increase in expression of CD11b/CD18 observed over several experiments was 2.9-fold, with a range of 2.1–4.9-fold. The expression of CD11b/CD18 in response to 5×10^{-8} M NAP-1/IL-8 was rapid, reaching a plateau by 15 min and remaining at that level for at least 90 min (Fig. 2, *open circles*). These results are consistent with the observation that NAP-1/IL-8 induces the rapid secretion of specific granules from PMN (20), and specific granules serve as intracellular storage compartments for CD11b/CD18 (21). Maximum upregulation of CD11b/CD18 was observed at concentrations of NAP-1/IL-8 that induce PMN chemotaxis, a rise in cytosolic free calcium, granule exocytosis (22, 23), and transient actin polymerization (Detmers, P., unpublished observations).

The expression of CD11c/CD18 (p150,95) and CR1 increased on average 2.0-fold in response to 5×10^{-8} M NAP-1/IL-8 (range 1.7–2.7-fold and 1.5–2.3-fold, respectively) (data not shown). Upregulation of CR1 was very rapid and reached a maximum by 15 min (data not shown), consistent with the rapid release of a separate vesicular compartment (Morel, F., and B. Dewald unpublished observations). No change was observed for HLA-A,B,C (range 1.0–1.1), while $Fc\gamma RIII$ exhibited a very slight increase in expression, or in some cases, no change at all (average 1.1-fold, range 1.0–1.6-fold) (data not shown).

NAP1/IL-8 Enhances the Binding Activities of CD11b/CD18: Attachment of EC3bi. Freshly isolated PMN bind few EC3bi, but increased binding of EC3bi was observed at concentrations of NAP-1/IL-8 as low as 10^{-9} M, and 5×10^{-8} M NAP-1/IL-8 caused the AI to increase on average 10.9-fold (range 1.3–37.0-fold) (Fig. 1, *closed circles*).

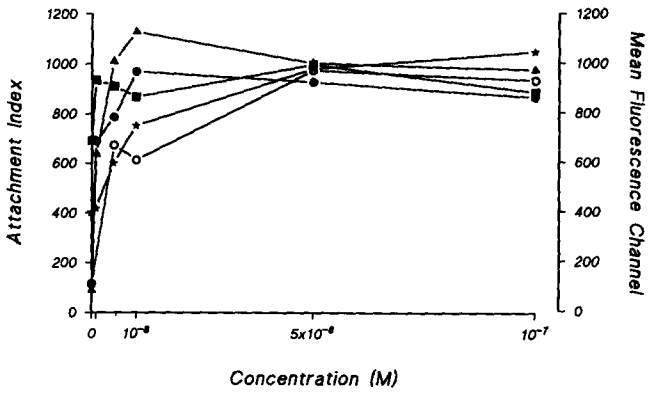

FIGURE 1. Expression of cell surface CD11b/CD18 and binding of EC3bi and EIVa to PMN treated with NAP-1/IL-8. Expression of CD11b/CD18 on the surface of PMN incubated 60 min with NAP-1/IL-8 was measured using mAb OKM10 and flow cytometry as described in Materials and Methods (mean fluorescence channel, *stars*). Similar results were obtained in eight separate experiments. Binding of EC3bi (AI) to PMN was measured by adding EC3bi for the last 30 min of a 60-min incubation at 37°C with NAP-1/IL-8 (*filled circles*). Binding of EIgG under the same conditions served as a control (*filled squares*). Similar results were obtained in seven experiments. Binding of EC3bi to PMN stimulated with fMLP for the last 10 min of a 60-min incubation at 37°C was measured by adding the EC3bi for the last 30 min of the 60-min incubation (*open circles*). Similar results were obtained in nine experiments. Binding of EIVa to PMN incubated at 37°C for 30 min with NAP-1/IL-8 was also measured (*filled triangles*). Similar results were obtained in five separate experiments.

Under the same conditions binding of EIgG increased slightly in response to NAP-1/IL-8 (average of 1.5-fold, range 1.0–1.9) (Figure 1, *squares*).

Binding of EC3bi also increased an average of 5.3-fold in response to 5×10^{-8} M fMLP (range 1.3–20.7-fold) (Fig. 1, *open circles*). We previously reported that fMLP caused enhanced phagocytosis of EIgG and increased expression of CD11b/CD18 without a rise the binding activity of CD11b/CD18 (24). These observations were made repeatedly with a single batch of fMLP that may have been chemically modified in such a way as to give a result that does not represent a normal response to formyl peptides. Our present observations of strong enhancement of the binding activity of CD11b/CD18 by fMLP were obtained with a different batch of fMLP and have been confirmed by similar results with a different formyl peptide, fNLLP (data not shown).

Binding of EC3bi in response to NAP-1/IL-8 increased rapidly and appeared to reach a plateau by 15 min (Fig. 2, *squares*). In some experiments we observed a biphasic response with a further increase in binding of EC3bi between 30 and 60 min.

Increased Expression of CD11b/CD18 is Not Necessary for Enhanced Binding of EC3bi. We used PMN cytoplasts to determine if ligand binding was enhanced by NAP-1/IL-8 in the absence of newly expressed receptors. Flow cytometry with mAbs OKM10 (anti CD11b) and LeuM5 (anti CD11c) confirmed that neither of these two receptors increased on the cytoplast surface in response to NAP-1/IL-8 (data not shown). However, NAP-1/IL-8 increased the binding of EC3bi to cytoplasts about threefold without changing the attachment of EIgG (Table I). Thus, increased expression of CD11b/CD18 is not required to observe enhanced ligand-binding activity in response to NAP-1/IL-8, and the cytokine must therefore cause a qualitative change in CD11b/CD18 on the cell surface. One possible mechanism for increased receptor avidity in the absence of increased receptor expression is receptor aggregation. CD11b/CD18 moves into small aggregates on PMN in response to stimuli that enhance binding of EC3bi

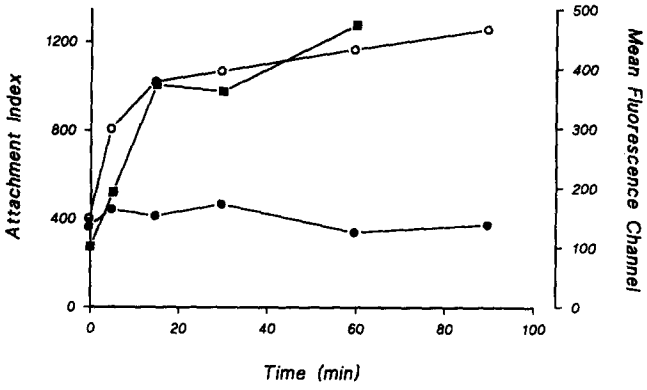

FIGURE 2. Expression of cell surface CD11b/CD18 and attachment of EC3bi to PMN treated with NAP-1/IL-8: time course. For measuring expression of CD11b/CD18, PMN were incubated at 37°C for a total of 90 min, with either HAP (*filled circles*) or 5×10^{-8} M NAP-1/IL-8 (*open circles*) added for the indicated times. The cells were then cooled on ice, and cell surface expression of CD11b/CD18 was measured using mAb OKM10 and flow cytometry as described in Materials and Methods (mean fluorescence channel). Similar results were obtained in three additional experiments. For measuring attachment of EC3bi (AI), PMN were incubated for a total of 60 min at 37°C with 10^{-8} M NAP-1/IL-8 added for the indicated times (*filled squares*). The 0 min sample received HAP buffer instead of NAP-1/IL-8. These data were confirmed by seven additional experiments.

TABLE I
NAP-1/IL-8 Stimulated Binding of EC3bi by PMN Cytoplasts

Exp.	Concentration of NAP-1/IL-8	Attachment index*	
		EC3bi	EIgG
1	None	67	114
	10^{-8} M	157	129
	5×10^{-8} M	187	106
2	None	35	364
	10^{-8} M	112	315
	5×10^{-8} M	107	328

* Cytoplasts were incubated for a total of 60 min at 37°C with HAP buffer or NAP-1/IL-8 with EC3bi added for the final 30 min of the incubation. AI is described in Materials and Methods.

(24), and the time course of aggregation corresponds precisely with the time course of increased binding of C3bi (24) or EC (4).

NAP-1/IL-8 Does Not Promote Phagocytosis of EC3bi. In six separate experiments, 1 h of incubation at 37°C with concentrations of NAP-1/IL-8 ranging from 10^{-9} to 10^{-7} M failed to induce phagocytosis of EC3bi, although ingestion of EIgG was the same as in controls or slightly enhanced (average increase of 1.3-fold in the PI at 5×10^{-8} M NAP-1/IL-8 [range 1.0–2.0]) (data not shown). NAP-1/IL-8 was therefore capable of stimulating ligand binding by CD11b/CD18 without promoting the signals required to accomplish phagocytosis.

NAP-1/IL-8 Enhances the Binding of LPS to PMN. CD11b/CD18 recognizes LPS through a binding site that is distinct from that which binds C3bi (25). 5×10^{-8} M NAP-1/IL-8 increased the binding of erythrocytes coated with the biosynthetic precursor of LPS, lipid IVa (EIVa), by an average of 16.1-fold (range 4–50-fold), and a large enhancement of EIVa binding was observed at concentrations as low as 10^{-9} M (Fig. 1, *triangles*). NAP-1/IL-8 thus stimulated two different ligand-binding activities of CD11b/CD18. This represents the first description of regulation of the LPS-binding activity of CD11/CD18, and this enhanced ability of PMN to recognize LPS could improve antimicrobial defense.

NAP-1/IL-8 Enhances Binding of PMN to Fibrinogen. CD11b/CD18 recognizes a portion of the γ-chain of fibrinogen containing the amino acid sequence Lys-Glu-Ala-Gly-Asp-Val (KQAGDV) (7). NAP-1/IL-8 at a concentration of 5×10^{-8} M increased the binding of PMN to fibrinogen immobilized on plastic substrates by an average of 4.3-fold (range 1.6–9.4-fold), while it had no effect on the binding of PMN to control, HSA-coated substrates (Table II). NAP-1/IL-8 may thus accelerate the interaction of PMN with fibrin deposits at sites of tissue injury.

NAP-1/IL-8 Stimulates Binding of PMN to Endothelium. At a concentration of 5×10^{-8} M NAP-1/IL-8, PMN adherence to unstimulated EC increased on average 1.6-fold (Table II). In contrast, PDB caused an average 3.4-fold increase in PMN adherence, indicating that NAP-1/IL-8 was a relatively mild stimulus of PMN-EC interaction. These results confirm those obtained by others (26). Since EC may be stimulated to synthesize NAP-1/IL-8 (1), local secretion of NAP-1/IL-8 may promote sufficient adhesion of PMN to EC in inflamed tissue to enable transmigration to occur.

TABLE II

NAP-1/IL-8 Enhanced Adhesion of PMN to Fibrinogen-coated Substrates and EC Monolayers

Conditions	Adhesion to fibrinogen* (PMN adherent per field)		Adhesion to EC‡ (PMN adherent per mm^2)			
	HSA	Fibrinogen	Exp. 1	Exp. 2	Exp. 3	Exp. 4
No stimulus	25	30	235	168	232	596
NAP-1/IL-8						
1×10^{-9} M	–	–	290	234	258	635
1×10^{-8} M	44	83	345	258	423	668
5×10^{-8} M	35	137	325	300	447	880
1×10^{-7} M	35	116	320	252	341	905
PMA, 33 ng/ml	32	230	–	–	–	–
PDB, 300 ng/ml	–	–	680	798	856	1,440

* PMN were added with NAP-1/IL-8 or HAP buffer to Terasaki plates coated with fibrinogen or pyrogen-free HSA for 60 min at 37°C. PMA-treated PMN were incubated for 15 min at 37°C. Nonadherent cells were removed by washing, and the adherent cells were fixed in glutaraldehyde before counting.

‡ Fluorescent PMN challenged with NAP-1/IL-8 or PDB were added to EC monolayers and coincubated for 15 min at 37°C. Unattached PMN were removed by washing, and adherent PMN were quantitated with an inverted microscope equipped for epifluorescence.

Summary

The cytokine NAP-1/IL-8 is produced by a variety of different cells in response to inflammatory stimuli and elicits several biological responses from PMN. Experiments presented here demonstrate that PMN exposed to NAP-1/IL-8 expressed increased amounts of CD11b/CD18, as well as CD11c/CD18 and CR1, on their cell surface, while expression of $Fc\gamma RIII$ and HLA-A,B,C remained essentially unchanged. Increased CD11b/CD18 and CD11c/CD18 appears to correspond with the release of specific granules by NAP-1/IL-8. NAP-1/IL-8 was also a potent stimulator of several of the binding activities of CD11b/CD18. Ligation of EC3bi by CD11b/CD18 was rapidly enhanced by NAP-1/IL-8, but phagocytosis of the ligated particles was not induced by the agonist. In addition, enhanced binding of EC3bi was observed in the absence of an increase in receptor expression as shown with PMN cytoplasts. NAP-1/IL-8 promoted additional adhesive interactions between CD11b/CD18 and the biosynthetic precursor of LPS, lipid IVa, fibrinogen, and endothelial cells, suggesting that NAP-1/IL-8 may promote leukocyte adhesion in vivo that could lead to recruitment of PMN to sites of tissue inflammation.

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