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Neutrophil Inhibitory Factor Abrogates Neutrophil Adhesion by Blockade of CD11a and CD11b β_2 Integrins

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ABSTRACT

We studied the basis of inhibition of polymorphonuclear leukocyte (PMN) adhesion induced by neutrophil inhibitory factor (NIF), a 41-kDa CD11/CD18 β_2 integrin-binding protein isolated from the canine hookworm (*Ancylostoma caninum*). NIF blocked PMN adhesion in a concentration-dependent manner with complete blockade occurring at ~10 nM NIF. Because CD11a and CD11b β_2 integrins are functionally active on stimulated PMNs, and yet NIF is postulated to inhibit only CD11b integrin by binding to its I domain, we evaluated the contributions of CD11a and CD11b β_2 integrins in the mechanism of inhibition of PMN adhesion to endothelial cells. We observed an additive inhibitory effect (>90% inhibition) of PMN adhesion to endothelial cells when NIF was used in combination with anti-CD11b monoclonal antibodies, which alone at saturating concentrations reduced PMN adhesion by only 50%. NIF also prevented aggregation of phorbol ester-stimulated JY lymphoblastoid cells that expressed only the functionally active CD11a, suggesting that NIF also can inhibit CD11a-dependent response. We transduced the NIF cDNA into human dermal microvessel endothelial cells in which NIF synthesis and release prevented PMN adhesion to the transduced human dermal microvessel endothelial cells. These data indicated that the potent antiadhesive effect of NIF may be the result of inhibition of CD11a and CD11b β_2 integrins on PMNs. Moreover, the strategy of NIF release from transduced endothelial cells suggests the feasibility of blocking the CD11a- and CD11b β_2 integrin-dependent PMN adhesion and PMN migration responses specifically at sites of endothelial cell activation.

Neutrophil inhibitory factor (NIF), a 4-kDa antiadhesive glycoprotein has been isolated and cloned from the canine hookworm *Ancylostoma caninum* (Moyle et al., 1994). NIF at nanomolar concentrations inhibits polymorphonuclear leukocyte (PMN) adhesion to the endothelium (Moyle et al., 1994; Barnard et al., 1995) and PMN adhesion-dependent generation of H_2O_2 (Moyle et al., 1994). In an ex vivo guinea pig lung model, we showed that NIF infusion also prevented PMN-mediated lung vascular injury (Barnard et al., 1995). In addition, NIF release in a transgene mouse model prevented lipopolysaccharide-induced PMN infiltration into lungs, and the resultant lung vascular injury (Zhou et al., 1998). Other studies in a rat model of focal cerebral ischemia showed that NIF blocked PMN infiltration into ischemic brain tissues (Jiang et al., 1995). These effects of NIF were ascribed to inhibition of PMN adhesion to the microvascular endothelium (Moyle et al., 1994; Barnard et al., 1995).

Because CD18 integrins are required for firm and stable PMN adhesion to the endothelium (Smith et al., 1989; Springer 1990; Butcher, 1991; Malik and Lo, 1996), proteins such as NIF that interfere with CD18 function may have applications in inhibiting PMN adhesion to the vascular endothelium and transendothelial PMN migration. Two members of the CD18 integrins on PMNs, CD11a and CD11b, mediate the firm PMN adhesion by binding to different sites on intracellular adhesion molecule-1 (ICAM-1) (Diamond and Springer, 1993). Studies showed that NIF inhibited CD11b function by binding to the I domain, contained in a stretch of ~200-amino acid sequence critical for ICAM-1 binding (Muchowski et al., 1994; Rieu et al., 1994). Because anti-CD11b monoclonal antibodies (mAbs) at saturation concentrations only inhibited PMN adhesion by ~50% (Lo et al., 1989), NIF binding to CD11b cannot fully explain the effects of NIF in abrogating PMN adhesion to endothelial cells in both in vitro and in vivo studies (Moyle et al., 1994; Barnard et al., 1995). In the present study, we show that inhibition of PMN induced by NIF requires the functional inactivation of both

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ABBREVIATIONS: NIF, neutrophil inhibitory factor; PMN, polymorphonuclear leukocyte; ICAM-1, intercellular adhesion molecule-1; mAb, monoclonal antibody; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HMEC, human dermal microvascular endothelial cells; HUVEC, human umbilical vein endothelial cell; HBSS, Hanks' balanced salt solution; PMA, phorbol-12-myristate-13-acetate; TNF-α, tumor necrosis factor alpha; FACS, fluorescence-activated cell-sorting analysis; HLA, human leukocyte antigen; CM, conditioned medium.

CD11a and CD11b β_2 integrins. In addition, we show by the gene transfer approach that stable transfection of endothelial cells with NIF cDNA resulted in NIF release, which in turn prevented PMN adhesion by inhibiting the functions of CD11a and CD11b β_2 integrins on PMNs.

Materials and Methods

Vector Construction. An 850-bp *Eco*RI fragment containing the entire NIF cDNA was subcloned into the retroviral plasmid pLNCX (provided by Dr. A. Dusty Miller, Fred Hutchinson Cancer Research Center, Seattle, WA). This 6.2-kilobase plasmid contains a neomycin resistant gene and the immediate early promoter of the human cytomegalovirus (CMV) that drives the NIF cDNA. The full-length NIF cDNA, neomycin resistant gene, and CMV promoter are situated between two long terminal repeats required for DNA integration into the host cell genome. Orientation of NIF cDNA relative to the CMV promoter in pLNCX was verified by restriction enzyme analysis (Sambrook et al., 1989). The ligated DNA (pLNC-NIF) was transformed into *Escherichia coli* DH5α competent cells (Life Technologies, Gaithersburg, MD).

Cell Culture. PA317 cells (American Type Culture Collection, Rockville, MD) were used to produce amphotropic viral particles (Miller and Buttimore, 1986; Miller and Rosman, 1989). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose and 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), and were grown to confluency and passaged every 2 to 3 days.

Human dermal microvascular endothelial cells (HMEC), supplied by Dr. Edwin W. Ades (Emory University, Atlanta, GA) (Ades et al., 1992), were cultured in endothelial basal medium MCDB 131 (Life Technologies) with 10 μg/ml human epidermal growth factor (Collaborative Medical Products, Bedford, MA), 1 μg/ml hydrocortisone (Sigma Chemical Co., St. Louis, MO), and 10% FBS (Hy-Clone Laboratoires Inc., Logan, UT). HMEC were grown to confluency and passaged every 2 to 3 days. We also used human umbilical vein endothelial cells (HUVECs) for some studies. These cells were harvested and cultured as described previously (Jaffe et al., 1973).

Generation of Retroviral Particles Containing NIF cDNA. Both pLNCX and pLNC-NIF constructs were introduced into packaging PA317 cells with the standard lipofectamine transfection method (Life Technologies). Briefly, the cells were plated at a density of 5×10^5 cells/60-mm dish on day 1. On day 2, the cells were incubated in serum-free and antibiotic-free PA317 medium containing 6 μl/ml lipofectamine and 1 μg/ml pLNC-NIF or the control pLNCX plasmid DNA. On day 3, the cells were washed two times with Hanks' Balanced Salt Solution (HBSS), and covered with serum-containing culture medium. Cells were selected for expression of neomycin phosphotransferase by incubation in the presence of G418 (500 μg/ml) (Life Technologies) starting on day 4 and continuing to day 13. The cell medium was changed every 2 to 4 days during the selection and cells were passaged 1:2 when confluent. PA317 cells surviving G418 selection (i.e., the "producer cells") were then grown to confluency in absence of G418, and the conditioned medium containing the replication-incompetent retroviral particles was used to transduce the endothelial cells as described below.

Transduction of Endothelial Cells with NIF cDNA. Cells were plated at 5×10^5 cells/60-mm dish on day 1. On day 2, fresh conditioned medium from confluent producer PA317 cells was collected and filtered through a 0.45-μm nylon filter, mixed with endothelial cell medium at a 1:1 ratio, and added to the HBSS-washed cells, then the incubation was continued. These steps were repeated on days 3 and 4. On day 5, the endothelial cells were washed with HBSS and fed with endothelial cell medium. The endothelial cells were selected for expression of neomycin phosphotransferase with 500 μg/ml G418 from day 7 to at least day 16. The medium was changed every 3 to 4 days during selection. The NIF-transduced endothelial cells had identical growth characteristics and cell morphology as the nontransduced cells (data not shown).

Northern Blot Analysis. Total RNA was isolated from confluent endothelial cells with TriReagent (Molecular Research Center, Inc., Cincinnati, OH). Twenty micrograms of RNA/sample was size-fractionated by electrophoresis through 1% agarose-2.2 M formaldehyde gel and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were hybridized with a ^{32}P (800 μCi/mmol; Amersham Corp., Arlington Heights, IL) labeled 850-bp *Eco*RI NIF cDNA fragment generated by random priming as described in Rahman et al. (1999). After stringent washes, autoradiography was performed by exposing membranes to X-ray film at −70°C for 1 to 5 days. NIF mRNA was present in NIF-transduced HMEC up to 20 passages (data not shown).

Protein Analysis. Endothelial cells were plated on 60-mm dishes and grown to 80% confluency. The growth medium was removed, and the cell monolayers were washed two times with PBS. Methionine-free DMEM with 4.5 g/l glucose (Life Technologies) was added and the cells were incubated for 2 to 3 h at 37°C in 5% CO_2. The medium was then replaced with fresh methionine-free DMEM containing 150 μCi [^{35}S]methionine (Translabel; ICN Radiochemicals, Irvine, CA), and allowed to incubate for 24 h. NIF was immunoprecipitated by adding 5 μl (1:200 dilution) of rabbit polyclonal antibody raised against the recombinant NIF (Corvas International, San Diego, CA) to the aliquots of conditioned medium from the plates, and incubated overnight at 4°C with gentle rocking. The immunocomplex was pelleted with protein G-Sepharose 4FF (Pharmacia Inc., Piscataway, NJ), rinsed three times with wash buffer (0.05% Tween 20, 120 mM NaC1, 2 mM $CaCl_2$, 20 mM Tris-HC1, pH 8), solubilized by heating in sample buffer, and subjected to electrophoresis through SDS-polyacrylamide gels (Laemmli, 1970). The gels were soaked in 1% glycerol and dried, and the ^{35}S radiolabelled bands were visualized by autoradiography at −70°C for 1 to 3 days.

PMN Isolation and Labeling. PMNs were isolated from whole human blood donated by healthy volunteers with a single-step separation over a sodium metrizoate/Dextran solution (Polymorphprep, Nycomed Pharma AS, Oslo, Norway) followed by hypotonic lysis of erythrocytes. The viability of the isolated PMNs was >95%. PMNs were labeled with the fluorescent dye calcein/AM as described in Marks et al. (1991).

PMN Adhesion Assay. Endothelial cells were seeded at 5×10^4 cells/well in 96-well plates and grown to confluence over 3 days. PMN adhesion to fibrinogen or endothelial cells was determined as described in Barnard et al. (1995). Phorbol-12-myristate-13-acetate (PMA) (Sigma Chemical Co.) was added to the wells at final concentrations of 10 or 20 nM to activate PMNs, and the cells were incubated for 10 min at 37°C in 5% CO_2. In a control group, the NIF polyclonal Ab (final concentration of 150 nM) was added to endothelial cells 30 min before the adhesion assay. For assays evaluating tumor necrosis factor-alpha (TNF-α)-induced increase in endothelial adhesivity, TNF-α was added to endothelial cell monolayers at a final concentration of 1000 U/ml and incubated for 3 h at 37°C in 5% CO_2 to activate endothelial cells. The anti-NIF polyclonal (150 nM) or anti-ICAM 1 mAb RR1/1 [10 μg/ml final concentration (supplied by Dr. R. Rothlein, Boehringer-Ingelheim, Ridgefield, CT)] was added to endothelial cell monolayers 30 min before addition of the labeled PMNs.

Flow Cytometry. We used fluorescence-activated cell-sorting analysis (FACS) to determine the expression of cell adhesion molecules on endothelial cells and PMNs with specific mAbs. Endothelial cells or PMNs were harvested and incubated with antiadhesion molecules mAbs (10 μg/ml) at 4°C for 20 min. Cells were washed followed by incubation with fluorescein isothiocyanate-labeled $F(ab)'_2$ fragments of goat anti-mouse antibody at 10 μg/ml for 20 min at 4°C. Cells were washed before FACS analysis.

To monitor the expression of adhesion molecules on JY lymphoblastoid cells, we used FACS to detect CD11a (mAb TS2/4), CD11b (mAb CBRM1/20), ICAM-1 (mAb RR1/1), human leukocyte antigen (HLA) (mAb W6/32) on either unstimulated JY cells or JY cells stimulated with PMA (200 ng/ml, 18 h). These antiadhesion receptor

mAbs were obtained from 5th Workshop and Conference on Human Leukocyte Differentiation Antigens, 1993, Boston, MA.

JY Lymphoblastoid Cell Aggregation Assay. We adopted an established JY cell aggregation assay to detect NIF's ability to block JY cell aggregation (Rothlein et al., 1985). Briefly, 2×10^5 cells in 100 μl RPMI 1640 medium with 10% FBS were added to a flat-bottomed 96-microtest plate. PMA (200 ng/ml, 18 h) was then added, viewed under an inverted microscope, and JY cell aggregation was scored. Scores ranged from 0 to 5+, where 0 indicated that no cells were in clusters; 1+ indicated <10% of the cells were in aggregates; 2+ indicated that 10 to 50% of the cells were in aggregates; 3+ indicated that 50 to 100% of the cells were in small, loose, aggregated clusters; 4+ indicated that up to 100% of the cells were aggregated in large clusters; and 5+ indicated that 100% of the cells were in large, compact aggregates.

Statistical Analysis. Data are presented as means ± S.E. Comparisions between experimental groups were made by ANOVA and the Wilcoxon test with significance value set at $P < .05$.

Results

NIF Inhibits CD11a- and CD11b-Dependent PMN Adhesion. We determined the ability of NIF to inhibit CD11b function with the in vitro fibrinogen PMN binding assay, which is dependent solely on CD11b integrin (Diamond and Springer, 1993). NIF prevented binding of PMA-activated PMNs to fibrinogen in a dose-dependent manner with complete blockade of PMN binding occurring at ~10 nM NIF (Fig. 1a). Anti-CD11b mAb (OKM10) also abrogated PMN binding to fibrinogen as expected (Fig. 1a, inset). We also determined the effects of NIF in preventing CD11a- and CD11b-dependent PMN adhesion to endothelial cells. As shown in Fig. 1b, NIF prevented PMA-induced PMN adhesion to endothelial cells in a dose-dependent manner (with full inhibition occurring at ~10 nM); the inhibition induced by NIF was the same as that with an anti-CD 18 mAb (IB4) (Fig. 1b, inset).

Whereas anti-CD11a and anti-CD11b mAbs each inhibited PMN adhesion by ~50% in response to PMA, the combination of these two mAbs resulted in complete inhibition (Fig. 2). This indicated the participation of both CD11a and CD11b in mediating PMN adhesion. The anti-CD18 mAb also abrogated PMN adhesion (Fig. 2), confirming the CD18-dependence of the response. To demonstrate that NIF exerted its inhibitory effect by interfering with both CD11a- and CD11b-dependent PMN adhesion, we evaluated the effects of varying concentrations of NIF in combination with CD11a or CD11b mAb. We found that NIF was equally efficacious in inhibiting function of both CD11b and CD11a because NIF had the same maximal effect on PMN adhesion in the presence of either antibody. In the presence of a saturating amount of anti-CD11b mAb, the inhibitory effect was achieved at 10^{-8} M NIF ($P < .05$). In contrast, in the presence of anti-CD11a mAb, 10^{-9} M NIF was required to inhibit PMN adhesion to the same degree (Table 1). Thus, these data indicate that NIF is about one order of magnitude more potent in inhibiting CD11b than CD11a.

NIF Prevents Aggregation of JY Lymphoblastoid Cells Expressing CD11a. To address whether NIF binding to CD11a accounts for its potent antiadhesive effect, we used JY lymphoblastoid cells, which aggregate in a CD11a/CD18-dependent manner following PMA stimulation (Rothlein et al., 1985). We first showed by FACS analysis the cell surface expression of CD11a on JY cells. Unstimulated JY cells expressed CD11a (Fig. 3), CD11c (data not shown), CD18 (data not shown), ICAM-1 (Fig. 3) as well as HLA (data not shown); however, CD11b was not detectable (Fig. 3). Upon PMA stimulation (200 ng/ml, 18 h), the expression of CD11a, CD11b, ICAM-1, and HLA remained the same. In two of three experiments, we observed a slight decrease in CD11c as well as CD18 antigens (data not shown). As shown in Table 2, mAbs directed against CD11a, CD18, and ICAM-1 inhibited JY cell aggregation, whereas mAbs directed against CD11b and HLA had no effect; thus, JY cell aggregation was dependent on binding of CD11a to ICAM-1. NIF was shown to inhibit JY cell aggregation following PMA stimulation (Table 2).

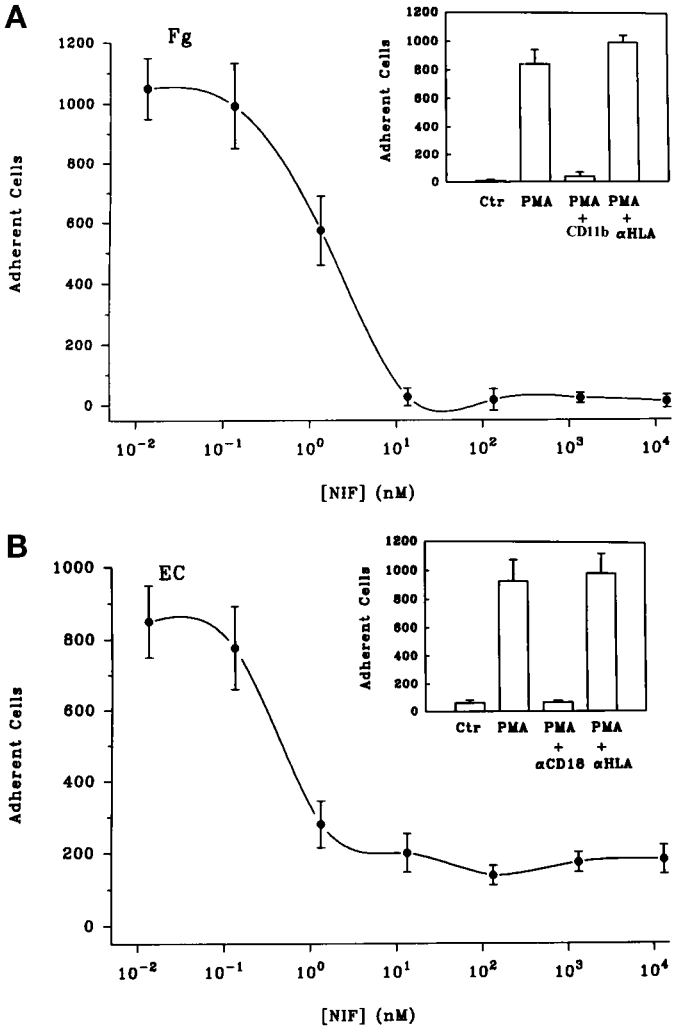

Fig. 1. a, NIF prevents PMN binding to fibrinogen (Fg) immobilized onto glass slides with human serum albumin (1 mg/ml). PMNs were layered onto the fibrinogen-coated surface and immediately activated by PMA treatment (10 nM, 10 min) in the absence and presence of indicated concentrations of recombinant NIF. PMN adhered to fibrinogen in a CD11b-dependent manner as indicated by inhibition with anti-CD11b mAb (OKM10, 10 μg/ml) (inset). NIF produced a dose-dependent inhibition of PMN binding to fibrinogen. Data represent means ± S.E. of three separate experiments. b, NIF inhibits PMN adhesion to endothelial cells (EC). PMNs were layered onto confluent HUVEC monolayer and recombinant NIF at different concentrations was added. PMNs were activated by PMA treatment (10 nM, 10 min) and adhesion was allowed to proceed at 37°C. PMNs adhered to HUVEC in a CD18-dependent manner as indicated by inhibition with anti-CD18 mAb (IB4, 10 μg/ml) (inset). NIF inhibited PMN adhesion to HUVEC in a dose-dependent manner. Data represent means ± S.E. of three separate experiments.

NIF Release by Transduced Endothelial Cells Prevents PMN Adhesion. We transduced the NIF cDNA into endothelial cells and determined NIF mRNA expression by Northern analysis (Fig. 4a). The synthesis and release of NIF protein by the transduced HMEC were demonstrated by metabolically labeling the cells with [^{35}S]methionine. As shown in Fig. 4b, a 41-kDa protein band following immunoprecipitation was evident on the autoradiographs from SDS-polyacrylamide gel electrophoresis. The amount of NIF protein released from the transduced endothelial cells was 110 ± 5 ng NIF/10^6 endothelial cells/24 h (n = 3). NIF was undetectable in the vector-transduced or control-nontransduced HMEC. Conditioned medium from NIF-transduced cells prevented PMN adhesion by ~85% (Fig. 5). The inhibitory activity of the conditioned medium was fully neutralized by the anti-NIF antibody, indicating the authenticity of NIF as the sole inhibitor (Fig. 5). In other experiments, PMNs adhered minimally (~5%) to both vector-transduced or NIF-transduced endothelial cells under the basal condition (Fig. 6). However, in coculture experiments, challenge with PMA (10 and 20 nM) increased PMN adhesion to vector-transduced endothelial cells, whereas PMA did not significantly increase PMN adhesion to NIF-transduced endothelial cells (Fig. 6).

FACS analysis showed significant increase in ICAM-1 expression, but not E-selectin expression in the NIF- and vector-transduced HMEC following TNF-α treatment (data not shown). This finding is consistent with evidence that HMEC do not express E-selectin (Chen et al., 1997). The anti-ICAM-1 mAb prevented the increased PMN adhesion to vector-transduced HMECs activated with TNF-α (Fig. 7), indi-

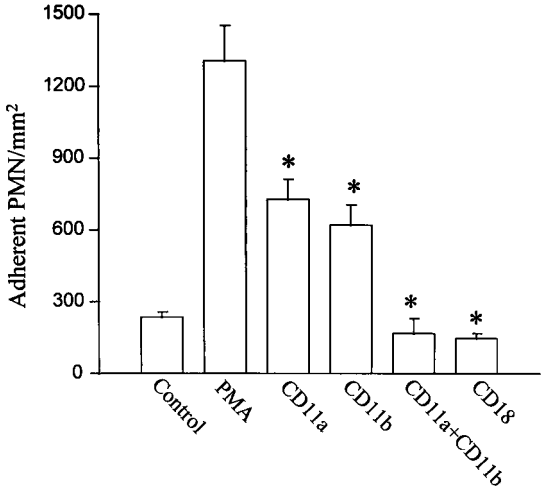

Fig. 2. NIF blocks PMN adhesion to endothelial cells equivalent to anti-CD18 mAb. PMA-treated PMNs were incubated with either anti-CD11a mAb (TS1/22, 10 μg/ml), anti-CD11b mAb (OKM10, 10 μg/ml), or anti-CD18 mAb (IB4, 10 μg/ml). Recombinant NIF (1 μM) was added to the medium containing PMN treated with either anti-CD11a mAb or anti-CD11b mAb to determine the additive effects of NIF with these mAbs. Data represent means ± S.E. of three separate experiments. *, difference from the PMA-treated control (P < .05).

TABLE 1

Concentration-dependent effects of NIF on PMN adhesion to endothelial cells in combination with anti-CD11a or CD11b mAb

PMN adhesion assay was carried out as described in *Materials and Methods*. PMN were first incubated with either anti-CD11a mAb (TS1/22) or anti-CD11b/CD18 (OKM10) mAbs at a saturating concentration (20 μg/ml). NIF at varying doses from 10^{-7} to 10^{-11} M was added to mAb-treated PMN prior to the PMN adhesion assay. PMN were layered onto HUVEC monolayers and immediately activated with phorbol dibutyrate (PDB) (100 ng/ml). Values represent number of adherent PMN ± S.E. from four separate experiments.

	PMN Adhesion
Basal	81 ± 9
PDB	1758 ± 326
PDB + αCD11a	1100 ± 274
PDB + αCD11a + NIF	
10^{-7} M	110 ± 5[a]
10^{-8} M	100 ± 17[a]
10^{-9} M	131 ± 25[a,b]
10^{-10} M	874 ± 217[a]
10^{-11} M	987 ± 180[a]
PDB + αCD11b	1205 ± 230
PDB + αCD11b + NIF	
10^{-7} M	90 ± 7[a]
10^{-8} M	112 ± 15[a]
10^{-9} M	743 ± 176[a]
10^{-10} M	975 ± 200[a]
10^{-11} M	850 ± 186[a]

[a] Different from PDB-stimulated PMN positive control (p < .05).
[b] Different from corresponding value with αCD11b at 10^{-9} M NIF (p < .05).

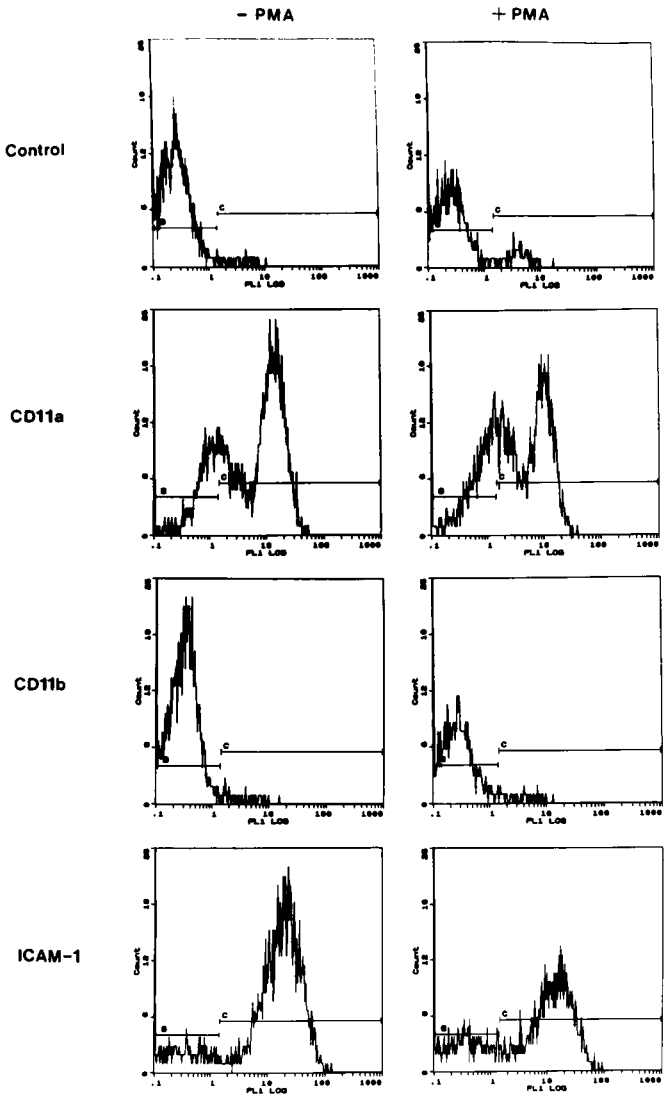

Fig. 3. FACS analysis of JY lymphoblastoid cells. JY lymphoblastoid cells were treated with PMA (200 ng/ml, 18 h) and FACS analysis was performed to determine the surface expression of CD11a, CD11b, or ICAM-1. The mAbs used were anti-CD11a (TS2/4), anti-CD11b (CBRM1/20), anti-ICAM-1 (RR1/1), and anti-HLA (W6/32) as control. Control unstimulated JY lymphoblastoid cells were used for comparison. The JY cells did not express CD11b and PMA stimulation also did not alter CD11b expression. Data are representative of three separate experiments.

cating that the adhesion response was ICAM-1-dependent in these cells. The inhibition of PMNs induced by anti-ICAM-1 mAb was similar to that observed in NIF-transduced cells (Fig. 7).

TABLE 2

NIF and anti-CD11a mAb inhibit PMA-induced aggregation of JY lymphoblastoid cells

JY cells were stimulated with PMA (200 ng/ml) for 18 h and the degree of aggregation was scored as described in *Materials and Methods.* MAbs (10 μg/ml) or NIF (1 μM) were added. Aggregation scores of JY cells from five separate fields within 1-μl well were obtained. Values represent an average of aggregation scores from triplicate microtiter wells.

Treatment	Aggregation Scores		
	Expt. 1	Expt. 2	Expt. 3
Basal	0	1+	0
PMA	3+	3+	4+
PMA + anti-CD11a	1+	0	1+
PMA + anti-CD11b	4+	3+	4+
PMA + anti-CD18	1+	1+	0
PMA + anti-ICAM-1	0	0	1+
PMA + anti-HLA	3+	3+	3+
PMA + NIF	1+	1+	1+

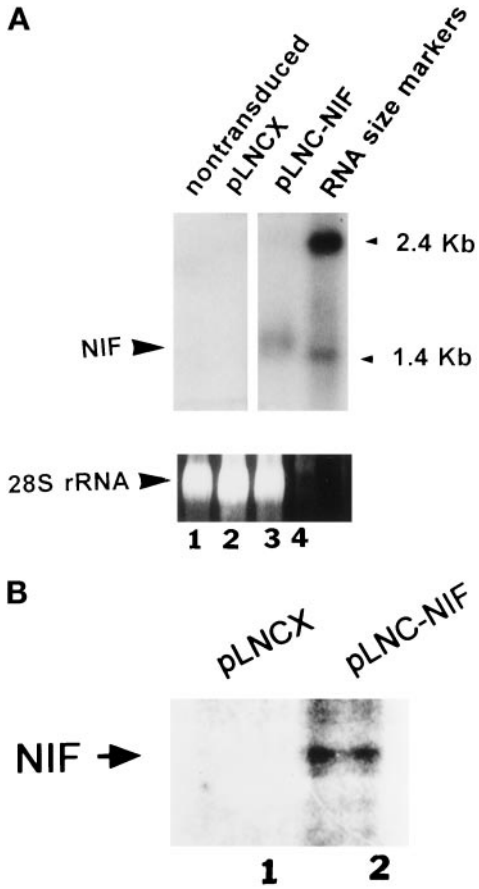

Fig. 4. a, Northern blot analysis of pLNCX- and pLNC-NIF-transduced HMEC. NIF mRNA (1.4 kilobases) was visualized by autoradiography after hybridization with ^{32}P labeled NIF cDNA probe. NIF mRNA expression was detected in pLNC-NIF-transduced HMEC but not in the control pLNCX- or nontransduced cells. 28S rRNA expression was performed to demonstrate equal loading of RNA samples in each lane. The results are representative of three separate experiments. b, Protein analysis of conditioned medium obtained from pLNCX- or pLNC-NIF-transduced HMEC. Cells were incubated with [^{35}S]methionine and the secreted NIF protein was immunoprecipitated from conditioned media with an anti-NIF polyclonal antibody. Immunoprecipitated protein was size-fractionated through SDS-denaturing gel by electrophoresis and bands were visualized by autoradiography. NIF band (41 kDa) was present in media from pLNC-NIF HMEC (lane 2) and absent in media from control pLNCX cells (lane 1). Results are representative of three separate experiments.

Discussion

NIF is a 41-kDa glycoprotein released by the canine hookworm (*A. caninum*) as a part of its defense response against phagocytic cells (Moyle et al., 1994). NIF was shown to bind to the Mac-1 subunit (CD11b) of the CD11b/CD18 integrin (Zhang and Plow, 1996, 1997). This is suggested to be a primary factor contributing to the action of NIF in preventing

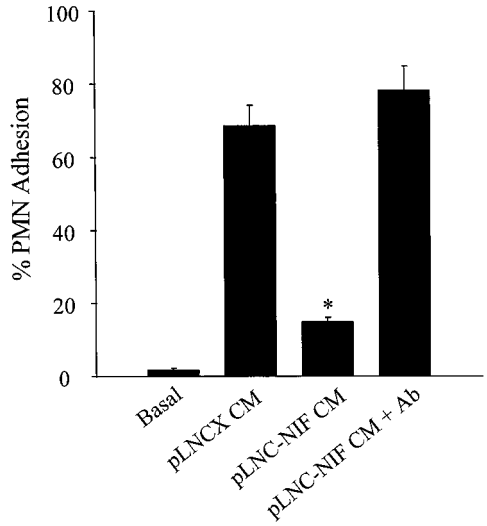

Fig. 5. Effects of conditioned medium (CM) from NIF-transduced HMEC on PMN adhesion to endothelial cells. CM obtained from either pLNCX- or pLNC-NIF-transduced HMEC was added to monolayers of control endothelial cells. PMNs were layered onto endothelial monolayers and activated with PMA treatment (20 nM, 10 min) and the PMN adhesion was determined as described in *Materials and Methods*. In some experiments, conditioned medium from NIF-transduced HMEC was treated for 30 min with NIF antibody before the PMN adhesion assay. Data represent means ± S.E. and are representative of four separate experiments with eight replicates each. *, difference from PMA-treated control groups ($P < .05$).

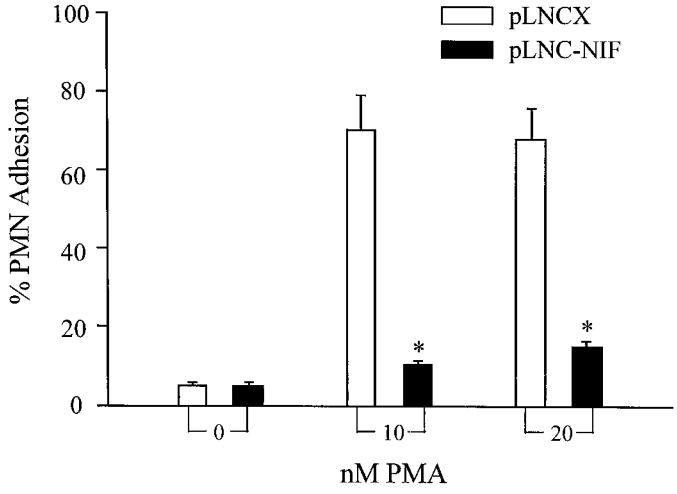

Fig. 6. Effects of PMA on PMN adhesion to NIF-transduced HMEC. PMNs were layered onto to PLNCX- or pLNC-NIF-transduced HMEC and then activated with PMA (10 or 20 nM, 10 min). Adhesion assay was carried out as described in *Materials and Methods*. Data represent means ± S.E. and are representative of three separate experiments with six replicates each. *, difference from the PMA-treated vector control ($P <$ .05).

PMN adhesion to endothelial cells. The specific NIF binding domain may be the I domain because the deletion of the D248 PLGY region of the I domain abolished the interaction with NIF but did not affect the binding to C3bi (Zhang and Plow, 1996). This finding suggests that the binding sites for NIF and C3bi to CD11b are overlapping but not identical. Analysis of these sites with a recombinant glutathione *S*-transferase fusion protein containing the I domain also showed that NIF binds to this domain in a manner inhibited by the anti-I domain antibody (Muchowski et al., 1994; Rieu et al., 1994). In the present study, we showed that NIF blocked the interaction of PMNs with fibrinogen (a CD11b/CD18 ligand), which occurs as the result of NIF binding to CD11b I domain. Although previous studies have implicated the importance of NIF interactions with the CD11b I domain, NIF also may bind to other sites (Rieu et al., 1994; Zhang and Plow, 1996). Moreover, because the I domain exists in CD11a (Rieu et al., 1994), it is plausible that NIF also can impair the function of CD11a integrin. Binding to both CD11a and CD11b may explain NIF's potent inhibition of PMN adhesion to endothelial cells, equivalent to that of anti-CD18 antibodies but exceeding that of anti-CD11b antibodies (Graf et al., 1996).

We showed that NIF prevented the aggregation of JY lymphocytes that solely expressed CD11a. We also demonstrated that NIF in the presence of anti-CD11b mAb had an additive inhibitory effect in preventing PMN adhesion. In the presence of anti-CD11b mAb, it required 10^{-8} M NIF to inhibit PMA-induced PMN binding to endothelial cells, whereas in the presence of anti-CD11a mAb, it required 10^{-9} M NIF to inhibit PMN adhesion. Thus, the binding of NIF to CD11a may be one order of magnitude less efficient than binding to CD11b. Because NIF can potentially inactivate both integrins, these findings help to explain the potent action of NIF in preventing adhesion of activated PMNs to vascular endothelial cells (Muchowski et al., 1994; Rieu et al., 1994) and intravascular PMN sequestration and airspace PMN migration in vivo (Barnard et al., 1995; Zhou et al., 1998).

The above-mentioned studies indicate that NIF release from *A. caninum* and its binding to both CD11a and CD11b β_2 integrins on phagocytes provide a formidable mechanism for evading the immunological response of phagocytic cells. Because a similar strategy can be used by cells releasing NIF to evade PMNs, we stably transfected endothelial cells with NIF cDNA by the retroviral-mediated transduction method, and determined whether the released NIF modified PMN adhesion to endothelial cells. NIF cDNA integration and expression in endothelial cells were evident by the high level of NIF mRNA expression, which persisted up to 20 passages. The NIF-transduced endothelial cells released NIF protein into the cell culture medium. Moreover, the conditioned medium from the NIF-transduced cells inhibited the adhesion of PMNs to endothelial cells activated by 20 nM PMA (used to produce the maximal PMN adhesion response). The results also showed that PMN adhesion to the NIF-releasing endothelial cells activated by TNF-α or PMA was inhibited (>90%). The anti-NIF antibody prevented these effects, indicating that the released NIF was responsible for the protective effect.

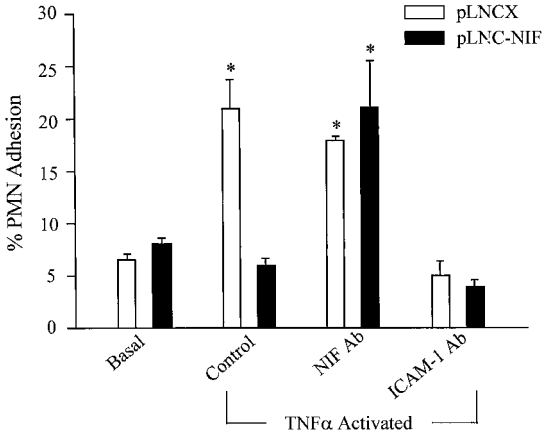

Fig. 7. Effect of TNF-α on PMN adhesion to NIF-transduced HMEC. pLNCX- and pLNC-NIF-transduced HMEC were treated for 3 h with TNF-α (1000 U/ml). Anti-NIF polyclonal or anti-ICAM-1 mAb (RR 1/1) antibodies were added 30 min before the PMN adhesion assay. Data represent means ± S.E. and are representative of three separate experiments with eight replicates each. *, difference from the untreated control (basal) group ($P < .05$).

ICAM-1 binding to members of CD18 integrin family mediates the stable PMN adhesion to endothelial cells. We observed that there was no increase in E-selectin expression in NIF-transduced endothelial cells consistent with the absence of E-selectin expression in HMEC (Chen et al., 1997) used to transduce NIF cDNA. Thus, ICAM-1 expression (rather than E-selectin) was responsible for PMN adhesion to the TNF-α-activated endothelial cells releasing NIF. This finding is concordant with the observation that the inhibition of PMN adhesion to endothelial cells with anti-ICAM-1 mAb (RR1/1) was similar to the response with anti-CD18 mAb. The finding that mAb RR1/1 blocks domain I of ICAM-1 (Berendt et al., 1992), the site recognized by CD11a (Staunton et al., 1990), coupled with the present observation that RR1/1 prevented PMN adhesion, further supports the contention that the inhibitory action of NIF involves binding of NIF to CD11a I domain.

In summary, the present data suggests that NIF inhibits the function of both CD11a and CD11b integrins on PMNs and that may explain the potent antiadhesive function of NIF. Moreover, the strategy of endothelial cell gene transfer to release NIF suggests the feasibility of using this approach to prevent CD11a- and CD11b-dependent PMN adhesion and migration specifically at sites of expression of endothelial adhesion molecules.

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