CD66 Nonspecific Cross-reacting Antigens Are Involved in Neutrophil Adherence to Cytokine-activated Endothelial Cells

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Abstract. Neutrophil adherence to cytokine-activated endothelial cell (EC) monolayers depends on the expression of the endothelial leukocyte adhesion molecule-1 (ELAM-1). The ligand for ELAM-1 is the sialylated Lewis-x antigen (SLex) structure. The selectin LAM-1 (or LECAM-1) has been described as one of the SLex-presenting glycoproteins involved in neutrophil binding to ELAM-1. Other presenter molecules have not yet been described.

Our data demonstrate that the carcinoembryonic antigen (CEA)-like surface molecules on neutrophils—known as the nonspecific cross-reacting antigens (NCAs)—are involved in neutrophil adherence to monolayers of IL-1-β-activated EC. The NCAs are recognized by CD66 (NCA-160 and NCA-90) and CD67 (NCA-95). Because NCA-95 and NCA-90 have previously been found to be phosphatidylinositol (PI)-linked, paroxysmal nocturnal hemoglobinuria (PNH) neutrophils (which lack PI-linked surface proteins) were tested as well. PNH neutrophils showed a diminished binding to activated EC. CD66 (on PNH cells still recognizing the transmembrane NCA-160 form) still inhibited the adherence of PNH cells to IL-1-β-activated EC, but to a limited extent.

Soluble CEA(-related) antigens inhibited normal neutrophil adherence as well, whereas neutrophil transmigration was unaffected. Sialidase-treatment as well as CD66 preclearing abolished the inhibitory capacity of the CEA(-related) antigens. The binding of soluble CEA antigens to IL-1-β-pretreated EC was blocked by anti-ELAM-1. These soluble antigens, as well as the neutrophil NCA-160 and NCA-90, both recognized by CD66 antibodies, presented the SLex determinant.

Together, these findings indicate that the CD66 antigens (i.e., NCA-160/NCA-90) function as presenter molecules of the SLex oligosaccharide structures on neutrophils that bind to ELAM-1 on EC.

Migration of neutrophils from the bloodstream to inflammatory sites requires the expression of the CD18 integrin subfamily (LFA-1, CR3, and p150,95) (Harlan, 1985; Anderson et al., 1985; Kuijpers and Roos, 1989; Springer, 1990). In the neutrophil adherence to the vascular lining, endothelial ICAM-1 may function as a cellular ligand for LFA-1 and CR3 (Rothlein et al., 1986; Diamond et al., 1990). The accessory role of the human selectin family (endothelial leukocyte adhesion molecule 1 [ELAM-1], GMP-140, and LAM-1) (Bevilacqua et al., 1989; Johnston et al., 1989; Tedder et al., 1989) is well documented.

Unless activated, the endothelial cells do not express the selectin members. Upon activation of endothelial cells (EC) with thrombin or histamine, GMP-140 is momentarily upregulated by fusion of intracellular Weibel-Palade bodies with the plasma membrane (Bonfanti et al., 1989; Hattori et al., 1989). In contrast, ELAM-1 expression depends on de novo synthesis, and is induced by the inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF). Its expression is transient with a peak at 4–6 h (Bevilacqua et al., 1989; Luscinskas et al., 1989). Recent evidence suggests a role for LAM-1 on neutrophils in the recruitment to inflammatory lesions (Watson et al., 1991; Smith et al., 1991).

The ligands recognized by ELAM-1 and GMP-140 are similar but not necessarily identical (Zhou et al., 1991; Berg et al., 1991). Sialidase treatment of neutrophils abolishes binding to either of the two selectins. Moreover, antibodies directed to the sialylated Lewis-x antigen (SLex) (Lowe et al., 1990; Goelz et al., 1990; Phillips et al., 1990; Walz et al., 1990) block both interactions (Polley et al., 1991). Unrelated to these sialylated ligands, a group of sulfated galactosyl ceramides (sulfatides) have been discovered as func-

1. Abbreviations used in this paper: CEA, carcinoembryonic antigen; EC, endothelial cells; ELAM-1, endothelial leukocyte adhesion molecule 1; FMLP, formyl-methionyl-leucyl-phenylalanine; HSA, human serum albumin; IL-1, interleukin-1; Le, Lewis-x antigen; NCA, nonspecific cross-reacting antigen; PI, phosphatidylinositol; PNH, paroxysmal nocturnal hemoglobinuria; r, recombinant; SLex, sialylated Lewis-x antigen.
tional ligands for GMP-140 (but not ELAM-1) (Aruffo et al., 1991).

The selectin member LAM-1 was reported to be involved in the neutrophil adherence to ELAM-1 (Kishimoto et al., 1991; Picker et al., 1991). Although LAM-1 on neutrophils bears SLeα, its role as the predominant presenter of critical oligosaccharide determinants must be limited to the initial phase of adherence: LAMA-negative neutrophils still bind oligosaccharide presenters must exist on the neutrophil surface.

For several reasons carcinoembryonic antigen (CEA)-related molecules are of potential importance in the ELAM-1 interactions: (a) colon carcinoma cell lines expressing CEA bind to cytokine-activated EC (Dejana et al., 1988; Benjamin et al., 1990); (b) The CEA(-like) antigens possess adhesivesis properties (Oikawa et al., 1989; Benchimol et al., 1989); (c) neutrophils express several CEA(-like) nonspecific cross-reacting antigens (NCAs) (Buchegger et al., 1984; Audette et al., 1987; Kuroki et al., 1990) with >80% homology between CEA and NCAs (Thompson et al., 1987); and (d) CEA(-like) antigens are highly glycosylated molecules with multiple sialyl and fucosyl residues (Kobata et al., 1989; Thomas et al., 1990). The CEA family is a group of proteins that belongs to the immunoglobulin supergene family (Williams and Barclay, 1988).

In our hands, three different CEA-like surface antigens can be distinguished on neutrophils by immunoprecipitation and Western blotting techniques. The CD66 and CD67 mAb directed to granulocyte-specific antigens (Majdic, 1989; Stockinger, 1989; Van der Schoot et al., 1989a,b) have recently been identified to bind to the antigens NCA-160, NCA-95, and NCA-90 (Van der Schoot et al., 1989a, 1990; Watt et al., 1991). NCA-160 (binding CD66) is a transmembrane phosphoprotein (Van der Schoot et al., 1989a), NCA-95 (binding CD67) and NCA-90 (binding CD66) are strictly phosphatidylinositol (PI)-linked surface proteins (Van der Schoot et al., 1989b; Kolbinger et al., 1989; Hefta et al., 1990; Berling et al., 1990).

In this paper, we describe the role of the membrane-intercalated NCA proteins in neutrophil adherence to and transmigration across cytokine-treated EC. The functional importance of these proteins is corroborated by the ability of soluble CEA to specifically inhibit these neutrophil interactions.

**Materials and Methods**

**Reagents**

Formyl-methionyl-leucyl-phenylalanine (FMLP), PMA, protein A-Sepharose, cytochrome c-activated Sepharose, and dimethyl pimelate were purchased from Sigma Chem. Co. (St. Louis, MO). FMLP and PMA were dissolved in DMSO at 1,000× the final concentration for cell stimulation, and were stored at -20°C. The rabbit anti-human CEA serum was obtained from Dakopatts (Glostrup, Denmark). Purified CEA product prepared from PCA extracts of liver metastasis of human colonic adenocarcinoma was purchased from Zymed Labs. Inc. (South San Francisco, CA). The following purified murine mAbs (IgG1) were used: Neo-618, 26/3/13, 4/3/17, CD66 CLB-gran10 (I4H4), 49.30, CD67 CLB-B13.9, MUS, 80H3, and N1 (Table I) (Berling et al., 1990; Tetteroo et al., 1986). Neo-618 and 49.30 were purchased from Sanbio (Uden, The Netherlands) and 80H3 from DiaNova (Hamburg, Germany). (None of the anti-CEA/NCA mAbs used in the present study induced any neutrophil activation measured as NADPH oxidase activity, neutrophil aggregation, change in intracellular Ca²⁺ or cAMP level [not shown]). In additional experiments, the CD15 mAb CLB-B4.3 (IgM) (Tetteroo et al., 1984) or CSLEX-1 (IgM) (Fukushima et al., 1984) to SLeα were used as indicated. CSLEX-1 was provided by Dr. Paul Terasaki (University of California Medical School, Los Angeles, CA). In control experiments CD18 mAb CLB-LFA1/1 (Majdic et al., 1984) or an ELAM-1 mAb (Van der Schoot et al., 1989) Fab(β) fragments were used as described before (Kuijpers et al., 1991). The basal incubation medium for cell suspensions consisted of a 1:1 mixture of RPMI-1640 and Medium 199 (Gibco Laboratories, Paisley, UK), supplemented with 0.5% (wt/vol) human serum albumin (HSA).

**Granulocyte Isolation**

Granulocytes from healthy controls or patients suffering from paroxysmal nocturnal hemoglobinuria (PNH) were purified from 30-50 ml of whole blood anticoagulated with 0.4% (wt/vol) trisodium citrate (pH 7.4), essentially as described (Roos and de Boer, 1986). After density gradient centrifugation over isotonic Percoll (1.076 g/ml), the interface containing the mononuclear cells was removed, and erythrocytes were lysed by treatment for 10 min with ice-cold isotonic NH₄Cl solution (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.4). Granulocytes were resuspended in incubation medium. Purity of the granulocytes was >98% (>95% neutrophils).

**Chromium Labeling of Neutrophils**

Freshly purified neutrophils were radioiodinated with 125I by direct labeling to Galin et al. (1973). Briefly, neutrophils (10⁷ cells/ml) were incubated with 1 μCi ¹²⁵I (¹²⁵I) (100 μCi/g; New England Nuclear, Boston, MA) in incubation medium (containing 0.1% HSA instead of 0.5% HSA) at 37°C for 1 h under gently shaking conditions and were subsequently washed. Viable activity after labeling was >95%.

**Endothelial Cell Culture**

EC were cultured following standard procedures essentially as described before (Kuijpers et al., 1991, 1992b). In short, EC were isolated from human umbilical cord veins according to Jaffe et al. (1973) with minor modifications (Willems et al., 1982). Cells were first cultured in plastic flasks (80 cm²) precoated with fibronectin. The culture medium consisted of an equal mixture of RPMI-1640 and Medium 199, supplemented with 20% (vol/vol) heat-inactivated human serum, penicillin (100 U/ml; Gibco Laboratories), streptomycin (100 μg/ml; Gibco Laboratories), fungizone (2.5 μg/ml; Gibco Laboratories), and glutamine (2 mM). After one or two passages with trypsin/EDTA (Gibco Laboratories), EC were subcultured to confluent monolayers on tissue culture-treated polycarbonate membranes (80-μm pore size; 24-mm diameter) of Transwell cell culture chamber inserts (Costar Data Packaging, Cambridge, MA).

**Transfected Cell Lines**

HeLa cells stably transfected with CEA (HeLa-CEA), NCA-95 (HeLa-CNGM6), NCA-90 (HeLa-NCA), or—as a control—the plasmid only (HeLaNeo) have been described before by Berling et al. (1990). The murine pre-B cells (pMRB101) and the ELAM-1-transfected pre-B cells (pMRB107) were kindly provided by M. Robinson (Cell Tech Ltd., Slough, Berkshire, UK) and have been successfully used before (Leeuwenberg et al., 1992).

**Adherence and Migration Assay**

The confluent monolayers were washed twice with basal incubation medium prewarmed to 37°C. Prewarmed incubation medium with or without an optimal amount of the chemoattractant FMLP (10 nM) was added to the lower chambers (Kuijpers et al., 1992b). CD15-labeled neutrophils (10⁷ cells/ml) preincubated with purified mAb at 20 μg/ml for 10 min before addition of the neutrophils to the upper chamber and remained present during the assay.

The chamber plates were then incubated at 37°C in a 5% CO₂ incubator for 30 min (or other times as indicated). After 30 min, neutrophil fractions were collected, and diluted with a fixed amount of incubation medium that had been preincubated with purified mAb at 20 μg/ml for 10 min before addition of the neutrophils to the upper chamber and remained present during the assay. Radioactivity was determined in three cell fractions: "luminal" (i.e., the content of the upper compartment with a fixed volume of washing buffer), "abluminal" (i.e., the content of the lower compartment plus a fixed volume of buffer used to rinse..."
the basal side of the membrane), and "adhering cell" fraction (i.e., the complete membrane, carefully cut out of its container). Recovery was >92%. Adhesion was measured as radioactivity found in the "membrane fraction," and migration was measured as radioactivity found in the abluminal fraction (lower compartment). The results were expressed as percentage of radioactivity added to the chambers.

EC monolayers were pretreated with optimal concentrations of human recombinant IL-1-β (rIL-1-β) (10 U/ml) or TNF-α (50 ng/ml). The cytokine was left for 4 h with the cells, followed by extensive washing of the monolayer with prewarmed incubation medium before use. Under these conditions, no chemoattractant was added to the lower chamber. The "spontaneous" transmigration is driven by EC-derived platelet-activating factor and IL-8, as described elsewhere (Kuijpers et al., 1992a). rIL-1-β was a gift of Dr. P. T. Lomedico (Hoffmann-La Roche, Nutley, NJ). Human recombinant TNF-α (rTNF-α) was a gift of Dr. A. Creasy (Cetus, Oakland, CA).

Immunoprecipitation and Western Blotting
Lysis of 125I-labeled neutrophils was performed in Hepes medium containing 1% NP-40 (vol/vol), protease inhibitors PMSF (1 mM), Na-p-tosyl-l-lysine-chloromethyl ketone (1 mM), leupeptin (20 μg/ml), soybean trypsin inhibitor (20 μg/ml) (Sigma Chem. Co.), and EDTA (5 mM). After an overnight preclearing step with protein A-Sepharose, immunoprecipitation was performed with the rabbit anti-CEA antiseraum, or the murine mAb CLB-gran10 chemically cross-linked to protein A-Sepharose by dimethyl pimelic acid, or CLB-B13.9 (Tettoreto et al., 1986) coupled to goat anti-mouse Ig on cyogen bromide-activated Sepharose. Sham precipitates were performed with CLB-CD2 mAb coupled to protein A-Sepharose. Samples were run with reduced 5-15% gradient SDS-PAGE together with prestained markers from Gibco-BRL Life Technologies (Breda, The Netherlands). For autoradiography of the 125I-labeled immunoprecipitates, X-AR5 film (Eastman Kodak Co., Rochester, NY) was used in combination with intensifier screen (Cronen Lighting Plus, Du Pont Co., Newtow, CT). In experiments with nonradiolabeled neutrophils, cell lysates or immunoprecipitates were (subsequent to 5-15% reduced SDS-PAGE) electrophoretically blotted to nitrocellulose sheets at 4°C according to standard procedures. After the initial blocking of nitrocellulose with BSA (1% [wt/vol] in 10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 8.0), the sheets were incubated with primary antibody at ~10 μg/ml for 1 h. After extensive washing with the Tris/Tween buffer, the alkaline phosphatase-conjugated anti-mouse Ig antibody (Promega Corp., Madison, WI) was used in the final incubation step for the subsequent enzymatic detection of electrophorized proteins recognized by the primary antibody.

Immunoprecipitation of 125I-radiolabeled or immunoblotting of nonlabeled purified CEA was performed in the same way as described for neutrophil lysates. After immunoprecipitation of the CEA product with CSLEX-1 or CLB-B4.3 (coupled to cyogen bromide-activated Sepharose), the precipitates were washed and resuspended in a 0.5-M glycine buffer, pH 11, for 1 h. After removal of the Sepharose beads, the soluble fraction was neutralized to pH 7.0 and reprecipitated with CLB-gran10 (IH4).

Results

Inhibition of Neutrophil Adhesion to IL-treated EC
The enhanced neutrophil adherence to cytokine-treated EC can be attributed to a large extent to the expression of ELAM-1, but migration completely depends on functional CD18 (Smith et al., 1988; Luscinskas et al., 1989; Hakker et al., 1991). A series of anti-NCA mAbs—directed to common, shared, or specific epitopes (Table I)—was tested for its effect on these processes. Inhibition was found with some mAbs, of which 49.30 most potently interfered with the neutrophil adherence to EC pretreated with rIL-1-β (Fig. 1) or TNF-α (not shown). Under these conditions, neutrophil transmigration was not significantly affected with any of the mAbs tested. Inhibition of adherence by 49.30 (directed to NCA-160 and NCA-90) was maximal when EC were shortly pretreated with cytokines (rIL-1-β or TNF-α for 4–8 h) to induce an optimal expression of ELAM-1. mAb 49.30 could not be evaluated when the EC monolayers had been pretreated for 24 h with rIL-1-β or rTNF-α, because neutrophil adherence and transmigration were almost absent at that time. Neutrophil adherence and migration across monolayers of resting EC along a gradient of FMLP were unaffected by 49.30 (not shown).

When mAb 49.30 was used in combination with CD18 or anti-ELAM-1, additive inhibition was only observed with CD18 (Fig. 2). The inhibition of neutrophil adherence by anti-ELAM-1 mAb was high, because binding of neutrophils to ELAM-1 results in activation of the adhesive CD18 proteins (Lo et al., 1991). Consequently, the anti-ELAM-1 mAb inhibits neutrophil adherence more efficiently than CD18 (Kuijpers et al., 1991). To study the ELAM-1-dependent binding only, the neutrophils were ATP depleted by sodium iodoacetate before use (Kuijpers et al., 1989, 1991). As shown in Fig. 3, mAb 49.30 significantly inhibited the neutrophil adherence also under these conditions. Migration of ATP-depleted neutrophils was absent, either across cytokine-treated EC (Fig. 3) or along a gradient of FMLP (not shown).

<table>
<thead>
<tr>
<th>Table I. Reactivity of Anti-NCA mAbs with Either HeLa Cells Transfected With CEA(-related) Antigens or with Neutrophils*</th>
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<tr>
<td></td>
</tr>
<tr>
<td>Neo-618</td>
</tr>
<tr>
<td>26/3/13</td>
</tr>
<tr>
<td>4/3/17</td>
</tr>
<tr>
<td>CLB-gran10</td>
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<tr>
<td>49.30</td>
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<tr>
<td>CLB-B13.9</td>
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<tr>
<td>80H3</td>
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<tr>
<td>MUS</td>
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<td>N1</td>
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* The HeLa transfectants have been previously described (Berling et al., 1990). Control transfected HeLa cells have been designated HeLa-Neo. Surface staining of HeLa cells and purified neutrophils was measured by cytometry on a FACScan (Becton and Dickinson Immunocytometry Sys., Mountain View, CA), essentially as described before (Berling et al., 1990; Kuijpers et al., 1991). Mean fluorescence intensity ± SEM of three to seven experiments. The procedures for immunoblotting of neutrophil lysates are described in Materials and Methods.

† Nonblotting mAb; antigen has been characterized by immunoprecipitation (Materials and Methods).
Adhesion of Neutrophils from PNH Patients to IL-treated EC

The NCA-95 and NCA-90 antigens are membrane attached through a lipid PI anchor. Neutrophils of PNH patients are deficient in the expression of these proteins, including the NCA-90 and NCA-95 antigens (Grunert et al., 1990; Van der Schoot, C. E., T. W. Kuipers, G. Nagel, F. Grunert, M. Daams, and A. E. G. Kr. von dem Borne, manuscript submitted for publication). Moreover, the amino acid sequence deduced from the cDNA CGM6 (Berling et al., 1990) and NCA (Thompson et al., 1987; Neumaier et al., 1988; Oikawa et al., 1987)—representing NCA-95 and NCA-90, respectively—had already suggested PI-anchored surface molecules. Two mAbs (MUS and NI), which selectively recognized NCA-90 mAb without binding to NCA-160 or NCA-95 (Table I), were noted to have a significant inhibiting effect on neutrophil adherence (Fig. 1). Therefore, PNH neutrophils which completely missed the expression of several PI-linked surface antigens tested but expressed transmembrane molecules at a normal level (Table II), were studied to further discriminate between the role of NCA-160 versus NCA-90 in adherence and in the inhibition found with 49.30.

These PNH neutrophils bound significantly less to rIL-1-β-pretreated EC than did normal control neutrophils. Moreover, NCA-90-deficient neutrophils were still sensitive to inhibition of 49.30 (Fig. 4), whereas MUS and NI were without effect (not shown). Together, the results obtained with mAb 4/3/17, NI, and MUS (Table I, Fig. 1) and the subsequent observations made with PNH neutrophils are in keeping with a role for NCA-160 as well as NCA-90 in neutrophil adherence to cytokine-treated EC.

Adhesion of LAM-1-negative Neutrophils to IL-treated EC

Whereas the expression of NCA-160, NCA-95, and NCA-90...
is upregulated during neutrophil activation (Tetteroo et al., 1986; Van der Schoot, C. E., T. W. Kuijpers, G. Nagel, F. Gruner, M. Daams, and A. E. G. Kr. von dem Borne, unpublished observations), LAM-1 is known to be rapidly shed from the plasma membrane (Jutila et al., 1990; Kishimoto et al., 1991; Kuijpers et al., 1992b). Under normal conditions, all neutrophils added to the upper compartment express LAM-1, as we have described before (Kuijpers et al., 1992a,b). After pretreatment of neutrophils for 30 min at 37°C with 10 nM FMLP, the neutrophils have become LAM-1-negative, nonaggregated cells (Kuijpers et al., 1991). However, the adherence of these neutrophils to rIL-1-β-activated EC was neither downmodulated, nor inhibited to a significantly larger extent by mAb 49.30 (Table III).

Inhibition of Adherence by Soluble CEA(-related) Antigen

A soluble CEA product was purchased and tested to substantiate our findings with anti-NCA mAbs with respect to neutrophil adherence. Neutrophil adherence to cytokine-treated

Table III. The Role of LAM-1 in the Neutrophil Adherence to Cytokine-activated EC Monolayers

<table>
<thead>
<tr>
<th>Additions</th>
<th>LAM-1-positive neutrophils</th>
<th>LAM-1-negative neutrophils</th>
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<tbody>
<tr>
<td>Control mAb</td>
<td>37 ± 2.2</td>
<td>38 ± 2.8</td>
</tr>
<tr>
<td>CD18 (CLB-LFA1/1)</td>
<td>19 ± 3.0</td>
<td>17 ± 4.9</td>
</tr>
<tr>
<td>αELAM-1 (ENA-2)</td>
<td>7 ± 0.6</td>
<td>8 ± 1.1</td>
</tr>
<tr>
<td>CD66 (49.30)</td>
<td>16 ± 2.0</td>
<td>14 ± 3.2</td>
</tr>
</tbody>
</table>

Mean ± SEM of four to eight experiments. Adherence is expressed as the percentage of 3Cr-labeled neutrophils that adhered to the EC on the filter (see Materials and Methods). The control mAb used is an isotype-matched anti-TNP IgGl mAb.

Figure 4. Comparison of adherence to rIL-1-β-pretreated EC between normal control neutrophils and PNH neutrophils deficient in PI-linked antigens such as NCA95/90. Mean ± SEM of three experiments. Bars marked by an asterisk display a significant effect (p < 0.05) as compared to the respective neutrophil adherence to IL-1-pretreated EC.

Figure 5. (A) The effect of soluble CEA(-related) antigens on neutrophil adherence to and migration across monolayers of EC. Neutrophils either migrated across resting EC toward FMLP (10 nM) in the lower compartment, or across monolayers of rIL-1-β-pretreated EC without exogenously added chemoattractants. Black bars, adherence; open bars, migration. Mean ± SEM of four to eight experiments. Bars marked by an asterisk display a significant effect (p < 0.05). (B) The effect of preclearing with CD66 CLB-gran 10 or control mAb CD9 on the inhibitory capacity of the soluble CEA(-related) antigens on neutrophil adherence to and migration across rIL-1-β-pretreated EC. Black bars, adherence; open bars, migration. Mean ± SEM of three experiments. Bars marked by an asterisk display a significant effect (p < 0.05). (C) The effect of sialidase (Vibrio cholerae) on the inhibitory capacity of CEA(-related) antigens on the neutrophils adherence to and migration across rIL-1-β-pretreated EC. Black bars, adherence; open bars, migration. Mean ± SEM of three experiments. Bars marked by an asterisk display a significant effect (p < 0.05).
Figure 6. Autoradiogram of the $^{125}$I-labeled CEA product (lane 1) and immunoprecipitates with CD66 CLB-gran10 (lane 2), and CD67 CLB-B13.9 (lane 3a) developed after 2-2.5 h, or (lane 3b) after 48 h. Lane 4 is a sham precipitate after 48 h. The bands below 44 kD in lane 3b probably represent trace contaminants in the CEA product that have nonspecifically bound to the goat anti-mouse/cyanoogen bromide-activated Sepharose, as used for CD67 immunoprecipitation (cf. Materials and Methods).

When the CEA product was precleared by CLB-gran10 before testing, no inhibition of neutrophil adherence to rIL-1-β-pretreated EC was observed anymore, whereas the CEA product precleared with CD9 mAb still inhibited neutrophil adherence to these ELAM-1-expressing EC (Fig. 5 B). Moreover, direct interaction of the CEA product with ELAM-1 was indicated by time course considerations and substantiated by the significant inhibition by anti–ELAM-1 mAb (Fig. 7 A), as well as the direct interaction of ELAM-1–transfected murine pre–B cells to the CEA product coated on polystyrene (Fig. 7 B). Anti–ELAM-1 as well as 49.30 mAbs blocked the binding for >85% (not shown).

Figure 7. (A) Binding of the $^{125}$I-labeled CEA product to monolayers of rIL-1-β-pretreated EC. Binding to EC pretreated for 4 h with rIL-1β (black bars) was significantly blocked by prior incubation of the monolayers with anti–ELAM-1 mAb (white bars; $p < 0.05$). Mean ± SEM of three experiments. (B) Direct interaction of ELAM-1-transfected cells with the CEA product coated on polystyrene. Murine pre–B cells transfected with human recombinant ELAM-1 (pMRBI07; black bars) and control murine pre–B cells (pMRBI01; white bars) were used at 10⁶ cells/well for adherence to the CEA product or to HSA as a control protein. After four washes with basal incubation medium, adhering cells were microscopically scored. Represented is the mean of 10 high-power fields (120 x) per well of three experiments in triplicate.

Oligosaccharides SLε and Leε Present on CEA(-related) Antigens

Sialidase-treated neutrophils have previously been found to
lack ELAM-1 binding. Subsequently, the crucial oligosaccharide for ELAM-1 binding was found to be SLe\(^a\) (Lowe et al., 1990; Goelz et al., 1990; Phillips et al., 1990; Walz et al., 1990; Polley et al., 1991). CEA-related antigens are well-documented glycoproteins of which oligosaccharide chains constitute 30–50\% of the relative molecular weight (Kobata et al., 1989; Thompson et al., 1991). CEA contains mainly type II polylactosamine ([Galβ1-4GlcNAc\(_{β}\)] side chains (in contrast to the type I Galβ1-3GlcNAc isomers) (Chandrasekaran et al., 1983; Yamashita et al., 1987)—just like most glycoproteins on granulocytes (Spooncer et al., 1984; Fukuda et al., 1984, 1986). These chains bear fucose and sialic acid residues at positions potentially critical for Le\(^a\) and its sialylated form in >50\% of its oligosaccharide structure (Chandrasekaran et al., 1983).

Both the NCA-160 and NCA-90 were immunoblotted by the anti-SLe\(^a\) mAb CSLEX-1. NCA-160 was also positively stained in Western blot with anti-Le\(^a\) mAb CLB-B4.3 (Fig. 8), as was shown before by Stocks et al. (1990). In some experiments, NCA-95 was immunoblotted with anti-Le\(^a\) as well (not shown). The soluble CEA product was found to be positive for SLe\(^a\) and Le\(^a\) determinants in an ELISA (not shown). Immunoprecipitation with anti-SLe\(^a\) mAb CSLEX-1 or anti-Le\(^a\) mAb CLB-B4.3 demonstrated that both the 180- and 150-kD bands were SLe\(^a\) positive. The CEA band of 180 kD was not fully α-2,3 sialylated at the Le\(^a\) determinants, as indicated by the immunoprecipitation of the high molecular weight band also with anti-Le\(^a\) (Fig. 9 A). As already indicated by the difference in mobility of the high molecular weight bands of CEA in Fig. 9 A, the level of sialylation directly influenced electrophoretic mobility: sialidase treatment of the CEA product induced an apparent increase in the 180 and 150 kD (SLe\(^a\)-positive) bands of ∼10 kD (Fig. 9 B). Both the SLe\(^a\)- and Le\(^a\)-positive 180-kD band were recognized by the monospecific anti-CEA mAb 26/3/13 (not shown).

When the CEA product was sialidase treated before use, there was no inhibition of neutrophil adherence to rIL-1β-pretreated EC to be seen anymore. This was not caused by trace amounts of sialidase, because sialidase-treated HSA did not show any effect (Fig. 5 C).

**Discussion**

The function of ELAM-1 as an important adhesion molecule on inflamed endothelium in neutrophil binding in vitro (Bevilacqua et al., 1987; Luscinskas et al., 1989; Kuijpers et al., 1991) has been further substantiated by recent in vivo animal models, in which neutrophil binding and influx were blocked by anti-ELAM-1 mAb (Mulligan et al., 1991; Gundel et al., 1991). The SLe\(^a\) moieties located on glycoproteins have been shown to be essential for neutrophils to bind to ELAM-1 (Lowe et al., 1990; Goelz et al., 1990; Phillips et al., 1990; Walz et al., 1990; Polley et al., 1991). However, many surface-expressed (and soluble) glycoproteins may bear the SLe\(^a\) oligosaccharide. Therefore, SLe\(^a\) positivity alone cannot be considered as proof for involvement in ELAM-1 binding. Previously, LAM-1 on neutrophils was suggested to be
Of a series of anti-NCA mAbs (Table I), some were found to significantly inhibit neutrophil adherence to cytokine-activated monolayers of EC, whereas others did not (Fig. 1). The anti-NCA-160/NCA-90 mAb 49.30 was most effective, but only under conditions of neutrophil adherence to ELAM-1-expressing EC (Fig. 1). Adherence of PNH neutrophils (lacking NCA-95 and NCA-90 [Table II]) to cytokine-treated EC was sensitive to CD66 mAb 49.30 inhibition, but to a very limited extent (Fig. 4). Together with the data obtained with the more selective anti-NCA mAbs 4/3/17, N1, and MUS (Table I, Fig. 1), this indicates that NCA-90 and to some extent NCA-160 are involved in adherence. We did not find any inhibitory effect with the CD67 mAbs.

Glycosylation of the CEA/NCA molecules is essential. First, HeLa cells transfected with CEA (HeLa-CEA), NCA-95 (HeLa-CGM6), or NCA-90 (HeLa-NCA) did not consistently bind to ELAM-1-expressing EC. Le^a or SLex determinants were absent on the HeLa cells—transfected or not—(data not shown), which is in keeping with the fact that HeLa cells do not have any α-1,3 fucosyl transferase activity (Goelz et al., 1990). Second, the soluble CEA product lost all inhibitory capacity upon sialidase treatment (Fig. 5 C).

Although transfection studies of Oikawa et al. (1989) and Benchimol et al. (1989) have demonstrated that aggregation of CEA/NCA-transfected COS cells can occur, neutrophils (or CEA/NCA-transfected HeLa cells) continuously and stably expressing these molecules do not spontaneously form clusters. The NCA proteins on neutrophils have evolved to present molecules of the correct oligosaccharide determinants for lectin-like molecules such as ELAM-1 (Bevilacqua et al., 1989). Binding of CEA(-related) antigens to ELAM-1 was evidenced, as shown in Fig. 7, A and B.

Our data strongly indicate that, apart from LAM-1 and the NCA proteins, other SLex-bearing presenter molecules also contribute to the adherence of neutrophils to ELAM-1-expressing EC. First, LAM-1-negative neutrophils are still able to bind to cytokine-treated, ELAM-1-expressing EC (Table III). This finding corroborates a role for LAM-1 especially in the initial phase of neutrophil adherence to cytokine-treated EC (Kuijpers et al., 1991). This notion is in line with the adhesion studies at 500 s by Smith et al. (1991) and Kishimoto et al. (1991). Second, the inhibition of neutrophil adherence by CD66 mAb 49.30 was not dramatically increased upon prior LAM-1 shedding (Table III). Third, anti-ELAM-1 mAb consistently showed a stronger inhibition of neutrophil adherence to cytokine-treated EC than did 49.30 (Fig. 2).

We have found that the surface-expressed NCA molecules on neutrophils can trigger CD18 activation, hence contribut-
ing to a strengthening of neutrophil adherence to EC via CD18-dependent binding. This NCA-mediated triggering is absent in PNH neutrophils (Kuijpers, T. W., C. E. Van der Schoot, M. Hoogerwerf, and D. Roos, manuscript submitted for publication). Thus, both NCA-160 and NCA-90 are involved in neutrophil adherence but only NCA-90 seems to increase adherence via CD18 activation. We (Kuijpers, 1991) and others (Lo et al., 1991) have demonstrated the existence of such a direct ELAM-1-mediated activation of CD1/CD18 molecules.

Medoff et al. (1984) have ascribed a negative modulatory capacity to CEA in lymphocyte proliferation. We can add another possible role of circulating CEA-related antigens in plasma. Because of the presentation of certain oligosaccharide structures, CEA-like antigens may interfere with neutrophil adherence to endothelium at sites of inflammation. Possibly, a wide spectrum of serum proteins, ranging from acute phase proteins (e.g., α1-acid glycoprotein/orosomucoid [Chandrasekaran et al., 1984; Walz et al., 1990]) to oncofetal proteins (e.g., mucin-type of glycoprotein [Kannagi et al., 1986]), or soluble CEA/NCA antigens (in all kinds of cancer patients with adenocarcinomas), modulate neutrophil adherence. Further investigation is currently undertaken to elucidate whether soluble serum components can indeed have such a modulatory role in neutrophil adhesion.

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References