RESEARCH COMMUNICATION

Stimulation of neutrophil adhesion by antibodies recognizing CD15 (Le^x) and CD15-expressing carcinoembryonic antigen-related glycoprotein NCA-160

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The carbohydrate antigen, CD15 (Le^x), and its sialylated derivative have recently been shown to be involved in the binding of neutrophils to the endothelial lectins, E-selectin and P-selectin. Neutrophil NCA-160, a carcinoembryonic antigen (CEA)-related glycoprotein, is the major carrier of CD15, which is also expressed on the common β_2 chain of leucocyte integrins. Rabbit IgG antibodies directed against CEA, which cross-react with neutrophil NCAs, increase the adhesion of neutrophils to plastic. This effect is also observed with F(ab')₂ and Fab antibody fragments and a monoclonal antibody (mAb) recognizing the same antigen. Anti-CD15 mAbs inhibit adhesion at higher concentrations, but augment adhesion at lower concentrations. Anti-CEA and anti-CD15 antibodies cause the homotypic adhesion of neutrophils demonstrable by light microscopy and flow cytometry. Anti-(integrin β_2 chain) mAbs inhibit both adhesion to plastic and homotypic adhesion. These results suggest that binding of ligand to NCA-160 is able to trigger neutrophil adhesion events which have been shown to be integrin mediated. Anti-CD15 mAbs do not, however, induce a respiratory burst from neutrophils.

INTRODUCTION

Neutrophils are the primary cells of the acute inflammatory response and major effector cells of humoral immunity. In order to reach inflammatory or infected sites, the neutrophils must emigrate from the bloodstream under the influence of chemotactic stimuli. The first stages of this emigration involve the low-level adhesion of the cells to endothelium in the process known as 'rolling adhesion' [1]. Subsequent to this rolling adhesion, changes in both the endothelium and the leucocyte result in a much stronger adhesion which causes arrest of the cell and allows migration through the vessel wall (reviewed in [2] and [3]). This stronger adhesion is mediated by glycoproteins of the integrin family, particularly CR3 and CD11b/CD18 (reviewed in [4]). The initial rolling adhesion is not integrin dependent [1], but is mediated by endothelial lectins which recognize carbohydrates on the neutrophil surface [5].

Two specific lectins mediating neutrophil adhesion have been identified on activated endothelium, i.e. E-selectin (ELAM-1) and P-selectin (GMP140), while a third, related molecule, L-selectin, is found on the leucocytes themselves [6–10]. It is clear, therefore, that carbohydrate groups on the phagocytic cell surface play a vital role in effector function of the cells. The carbohydrates Lewis^x (Le^x, CD15, 3-fucosyl-*N*-acetyl-lactosamine) and its sialylated derivative (sLe^x) have been shown to be involved in the interaction of neutrophils with E-selectin and P-selectin on endothelial cells [11,12] and P-selectin on platelets [13]. These carbohydrates may also mediate the L-selectin-dependent leucocyte-leucocyte interaction [14].

The fine specificity of the Le^x derivatives recognized by the selectins has been studied extensively. Sialic acid appears to be more critical for the interaction between E-selectin and neutrophils [15,16], with P-selectin apparently having a broader speci-

ficity for carbohydrate groups than E-selectin [17]. The closely related sialylated Lewis^x structure has also been implicated in E-selectin binding, although the relative affinity of the different Lewis groups is unclear [18,19].

Le^x and sLe^x are expressed on glycolipids and on several glycoproteins on the neutrophil surface [20–22]. The exact nature of (s)Le^x-containing ligands recognized by the endothelial selectins has not been determined. It has also been shown that the receptor for P-selectin is glycoprotein rather than glycolipid [23]. Although these carbohydrates are expressed on a number of proteins recognized as adhesion molecules, the relative importance of the different glycoproteins and their role in neutrophil adhesion has not been established. Binding of the selectins does appear to be a prerequisite to the enhanced integrin-mediated adhesion needed for the arrest of the cell [24–26]. The selectin receptor must be capable of transducing a signal which facilitates activation of the leucocyte integrins, although the mechanism of signal transduction has not been elucidated.

NCA-160 is one of several heavily glycosylated neutrophil-membrane glycoproteins recognized by antibodies against carcinoembryonic antigen (CEA), to which it is closely related [27]. Carbohydrate accounts for 59% of the mass of the mature NCA-160 molecule [27]. It is the major neutrophil glycoprotein expressing Le^x, CD15 [22]. The function of this molecule is not known. However, a number of molecules in the CEA family have been shown to have cell-adhesion activity [28–30]. In this paper we demonstrate the marked stimulation of neutrophil adhesion by anti-CD15 antibodies and antibodies recognizing NCA-160.

MATERIALS AND METHODS

Materials

Biochemicals were purchased from Sigma Chemical Co. Ltd,

Abbreviations used: CEA, carcinoembryonic antigen; NCA, non-specific cross-reacting antigen; mAb, monoclonal antibody; Le^x, Lewis^x; sLe^x, sialylated Le^x; MC2, mouse anti-CD15 mAb; PBS, phosphate-buffered saline.

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Fig. 1. Phase-contrast microscopy showing neutrophils incubated for 30 min at 37 °C in the presence of (a) PBS or antibodies recognizing (b) CD43, (c) CD15, (d) CEA, (e) CD18 or (f) CD11b

Table 1. Antibody-induced aggregation of neutrophils assayed by flow cytometry

Purified human neutrophils, either fixed with 0.5% para-formaldehyde or untreated, were incubated at 37 °C for 30 min with various antibodies. Aggregation was assayed by cytofluorimetry using forward light scatter (approximate particle size) as the parameter.

Antibody	Antigen	Mean forward scatter (arbitrary units)	
		Viable cells	Fixed cells
Control (PBS)	—	24.8	24.0
DF-T1	CD43	25.0	24.9
MC2	CD15	129.3	24.9
Anti-CEA Ig	NCA	113.6	25.2
H52	CD18	25.7	26.3
MN41	CD11b	25.0	24.9

Poole, U.K., unless otherwise stated. Mouse anti-CD15 mAb, MC2, was produced in this laboratory [31]. Rabbit polyclonal anti-CEA immunoglobulin, absorbed against blood-group antigens A and B and human plasma, was purchased from Dako Ltd, High Wycombe, Bucks, U.K. This antibody recognizes a number of glycoproteins expressed on the neutrophil surface [22]. Rat monoclonal YTH71.3 (anti-CD66) was a gift from H. Waldmann (Department of Pathology, Cambridge University, Cambridge, U.K.); H52 (anti-CD18) from Dr. T. August (John Hopkins University, Baltimore, MD, U.S.A.); MN41 (anti-CD11b) from the Fourth International Leucocyte Typing Work-

shop; DFT1 (anti-CD43) from Dr. D. Flavell (Southampton University, Hants., U.K.) and MY-43 (anti-Fc γ receptor) from Dr. L. Shen (Dartmouth Medical School, NH, U.S.A.). Non-immune rabbit IgG was purified from normal serum by ion-exchange chromatography [32].

Isolation of neutrophils

Neutrophils were isolated from heparinized blood from healthy volunteers by discontinuous density-gradient centrifugation as described previously [33]. Briefly, whole blood was layered on to an equal volume of Lymphoprep (specific gravity 1.077 g/ml) (Nycomed, Oslo, Norway), above an equal volume of dense Ficoll/hypaque (specific gravity 1.119 g/ml) (Ficoll 400 was from Pharmacia LKB, Milton Keynes, Bucks., U.K., and sodium hypaque was from Sterling Research Laboratories, Guildford, Surrey, U.K.). After centrifugation at 400 *g* for 30 min, the neutrophils were harvested from the Lymphoprep-Ficoll/hypaque interface, pelleted, washed and resuspended in appropriate buffer. For adhesion assays the buffer was 0.15 M-NaCl/0.5 mM-MgCl₂; for aggregation assays it was phosphate-buffered saline (PBS)/0.1 mM-CaCl₂/0.1 mM-MgCl₂/0.1 mM-MnSO₄. The average yield was 1.0 × 10⁶ neutrophils/ml of blood, and the average purity was > 95% neutrophils.

Purification of IgM from mouse ascitic fluid

Mouse ascitic fluid containing anti-CD15 IgM was filtered through sterile 0.2 μ m-pore-size filters (Flow Laboratories, Rickmansworth, Herts., U.K.) and 200 μ l samples of filtered ascites were then applied to an f.p.l.c. Superose 6 gel-filtration column (Pharmacia, Milton Keynes, Bucks., U.K.), which had been equilibrated with PBS. The column was run at a flow rate of 0.5 ml/min. IgM eluted in the first protein peak, free from contaminating proteins.

Preparation of immunoglobulin fragments

Fab' fragments of rabbit IgG were prepared by papain digestion [32]. Fc fragments of antibody were removed by incubation with protein A-Sepharose (Pharmacia), equilibrated with 0.1 M-sodium phosphate, pH 7.0. F(ab')₂ fragments of rabbit IgG were prepared by pepsin digestion [34]. Fragments of the Fc region were removed by gel filtration on Sephacryl S-300 (Pharmacia), equilibrated in PBS. Both Fab and F(ab')₂ fragments appeared to be homogeneous by the criterion of SDS/PAGE.

Adhesion of neutrophils to plastic microtitre-plate wells

Adhesion of neutrophils was assayed using a modification of the method described elsewhere [35]. Neutrophils were isolated as described above and resuspended at a density of 2.5 × 10⁶ neutrophils/ml. Cell suspension (100 μ l) was placed in the wells of a 96-well e.l.s.a. plate (Flow Laboratories) to which was immediately added 10 μ l of antibody or buffer. The plates were incubated at 37 °C for 30 min, and non-adherent cells were removed following addition of 200 μ l of buffer, which was then tipped out of the plate. After a second washing with 200 μ l of buffer, which was also tipped out, cells were covered with 100 μ l of buffer. The original cell suspension was used to give measurements of 100% activity. Aliquots (100 μ l) of 1 mg of *p*-nitrophenyl phosphate/ml in 0.1 M-sodium carbonate, pH 10.2, were added to each well and the plates incubated for 30 min at 37 °C. Finally, 100 μ l of 2 M-NaOH was added to each well and the absorbance at 405 nm was read on a Dynatech MR1000 e.l.s.a.

plate reader. All tests were done in triplicate; percentage adhesion was calculated by the following equation:

$$\frac{A_{405} \text{ in experimental well}}{A_{405} \text{ of total cell suspension}} \times 100 = \text{Adherent cells (\%)}$$

Aggregation of neutrophils

Neutrophils were resuspended at 10^6 cells/ml; 0.5 ml of this solution was placed in wells of a 24-well tissue-culture plate (Flow Laboratories) containing 5 μ l of antibody (antibody was ascitic fluid or commercial preparations as supplied), or control buffer. The plate was incubated at 37 °C for 30 min with shaking, and the amount of aggregation was assayed using light microscopy and flow cytometry (FACScan, Becton-Dickinson, Oxford, U.K.). For flow cytometry, 10000 events were analysed and the mean of the parameter forward scatter (giving an approximate measure of particle size) was taken as a measure of aggregation.

Neutrophil chemiluminescence

A Dynatech Microlite ML1000 luminometer was used. Chemiluminescence microtitre plates (Dynatech, Billingham, Sussex, U.K.) were coated with F(ab')₂ sheep anti-(mouse IgM) antibody (Caltag Laboratories) by incubating 100 μ l of this antibody in PBS in microtitre-plate wells overnight at 4 °C. The coated wells were then incubated with mAbs MC2 or MY43, followed by washing. Neutrophil chemiluminescence elicited by the bound antibodies was measured using luminol as enhancer, essentially as described [36].

RESULTS

The effects of incubation of neutrophils at 37 °C for 30 min in the presence of different antibodies are shown in Fig. 1. It is clear that anti-CD15 mAb, MC2, or the anti-(CEA) immunoglobulin cause neutrophils to adhere to one another forming large homotypic aggregates. Erythrocytes contaminating the neutrophil preparations did not adhere to these aggregates. This stimulation of aggregation was not observed with normal rabbit IgG or with any of the other antibodies tested (anti-CD11b, anti-CD18, anti-CD43, anti-CD45), or in the absence of added antibody. Table 1 shows the results of flow-cytometric analysis of the cell aggregates, forward light scatter being the parameter used as a measure of particle size.

Fixation of the cells with 0.5% paraformaldehyde did not reduce binding of any of the antibodies to the cells when measured by indirect immunofluorescence (results not shown), but it did eliminate the aggregation response caused by anti-CD15 or anti-CEA antibodies. These results demonstrate that antibodies which recognize epitopes on neutrophil NCAs elicit a response which causes the cells to aggregate, and that this response is dependent on viability of the cell.

Another characteristic of activated neutrophils is their increased adhesion to plastic [37]. Fig. 2 shows the effect which mAbs MC2 (anti-CD15), H52 (anti-CD18) or anti-(CEA) IgG had on the adhesion to plastic of freshly prepared neutrophils. H52 caused a clear dose-dependent inhibition of adhesion, with the highest concentrations of antibody (50-fold dilution of mouse ascitic fluid) reducing adhesion to 23 % of the control level. This is consistent with previous reports which stated that the adhesion of neutrophils to plastic is integrin dependent [37]. MC2 inhibited adhesion at the higher concentrations tested such that a 10-fold dilution of ascitic fluid reduced adhesion to the same level as the

higher concentrations of anti-CD18 antibody. However, at lower concentrations of MC2 adhesion was augmented. Incubation with anti-CEA immunoglobulins increased the level of neutrophil adhesion to plastic at all concentrations tested. The results in Fig. 2 show a single experiment where assays were carried out in triplicate. Similar results were obtained on at least eight occasions using different preparations of antibodies.

The dose-dependent stimulation of neutrophil adhesion by anti-CEA immunoglobulin was also observed using F(ab')₂ and monovalent Fab fragments derived from the same antibody (Fig. 3). Purified anti-CEA antibodies or antibody fragments in PBS at concentrations of 20, 10, 1 and 0.1 μ g/ml all caused an increased adherence relative to controls (non-immune rabbit IgG), as did MC2. The dose-response to the fragments suggested that the effect could not be due to the presence of undetected, undigested antibody in the preparations. Other control antibodies (DFT.1 and non-specific rabbit IgG) did not cause a change in adherence when compared with assays performed in the absence of antibody. However, YTH71.3, a mAb which recognizes CEA

Fig. 2. Effect of different concentrations of antibodies recognizing CD15 (O), CEA (□) or CD18 (Δ) on adhesion of neutrophils to plastic microtitre-plate wells

Fig. 3. Effect of purified immunoglobulins MC2 (anti-CD15), anti-CEA whole molecule, F(ab')₂ or Fab fragments on adhesion of neutrophils to plastic microtitre wells

The protein concentrations of the antibody preparations were 0.1 (□), 1.0 (▨), 10 (▩), and 20 (■) μ g/ml, the effect of non-immune rabbit IgG at 20 μ g/ml is shown as a solid bar.

Fig. 4. Stimulation of neutrophil chemiluminescence of anti-Fc α receptor antibody MY-43 (■) but not anti-CD15 antibody MC2 (○)

and NCA160 found on neutrophils [38], caused a similar response to the polyclonal anti-CEA immunoglobulins (results not shown).

DISCUSSION

We have demonstrated that antibodies which recognize epitopes on neutrophil NCA-160 cause increased adhesion to plastic and increased homotypic aggregation. Both of these phenomena are a result of the activation of the cell, and have been shown to be integrin mediated [37,39]. Since the effects of antibody fragments, even monovalent Fab' fragments, were the same as that of whole molecules, the effect cannot be due solely to cross-linking of surface receptors. It should be noted that NCA-160 is probably anchored by a transmembrane protein domain, whereas the lower-molecular-mass NCA-90 and NCA-95 are lipid-anchored. It appears that NCA-160 is able to signal to the cell, triggering processes which lead to increases in adhesion.

Increases in neutrophil adhesion were also caused by low concentrations of antibodies which recognize CD15 (Le^x). Since NCA-160 is the major carrier of CD15, it is likely that the increased adhesion is caused by activation of an adhesion mechanism working through NCA-160. At higher concentrations of anti-CD15 antibody, adhesion was inhibited. It is possible that this is due to the fact that the integrin β chain CD18 also carries the CD15 determinant [20,22,33]. Thus, although an NCA-160-dependent adhesion mechanism may be activated, the CD18-mediated adhesion is subsequently blocked, thereby masking the activation effect.

This is consistent with the observations of others [40] and our own observations (results not shown) that neutrophil adhesion stimulated by anti-CD15 antibodies was inhibited by antibodies recognizing integrin CD18. It is also consistent with the observation that the avidity of the anti-CD15 antibodies for the carbohydrate antigen on NCA-160 does appear to be greater than for the carbohydrate chains on CD18 since, although both are detected upon immunoblotting of neutrophil-membrane extracts with anti-CD15, immunoprecipitation of the same extracts with anti-CD15 antibodies invariably results in selective recovery of NCA-160 [22,33]. This higher avidity is possibly due to the presence of more repeating *N*-acetyl-lactosamine units expressing CD15 on the NCA-160.

The stimulation of neutrophil adhesion by anti-CD15 antibodies is a specific event. Anti-CD15 mAbs do not stimulate neutrophil chemiluminescence when bound to microtitre plates. However, under identical conditions, another IgM mAb, MY-43, which recognizes the neutrophil IgA Fc α receptor, is a potent

trigger of chemiluminescence (Fig. 4). These results, which have also been obtained using another CD15 antibody [41], show that the glycoproteins expressing CD15 deliver different messages to the cell from those delivered by the Fc receptors which trigger phagocytosis, respiratory burst and degranulation [36].

The mechanisms by which anti-CD15 and anti-CEA immunoglobulins cause an increase in adhesion is not clear. Our results suggest that the adhesion is integrin dependent. Several reagents have been shown to enhance integrin-mediated leucocyte adhesion [42,43], and enhanced adhesion has been shown to occur independently of an increase in surface expression [39,44,45]. Recent studies have demonstrated that a conformational modification which allows functional upregulation is required for the leucocyte integrins to mediate adhesion [46]. Antibodies recognizing a heavily glycosylated lymphocyte glycoprotein, CD44, have been shown to cause a Mg²⁺-dependent activation of the major lymphocyte integrin, LFA-1 [47].

A number of recent reports have suggested that binding of endothelial P-selectin or E-selectin to their neutrophil ligands results in activation of integrins directly or indirectly [24–26]. Our results suggest that NCA-160 might act as a receptor for the vascular selectins mediating the initial tethering of the neutrophil to the endothelium and the generation of an intracellular signal which results in integrin activation and the strong adhesion required for extravasation.

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