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Characterization of C1 inhibitor binding to neutrophils

N.-S. CHANG,* R. J. BOACKLE† & R. W. LEU‡ *University of Massachusetts Medical Center, Department of Biochemistry, Worcester, Massachusetts, †Division of Oral Biology, Department of Stomatology, Medical University of South Carolina, Charleston, South Carolina and ‡The Samuel Roberts Noble Foundation Inc., Ardmore, Oklahoma, U.S.A.

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SUMMARY

In a previous study we have isolated neutrophil membrane proteins that non-covalently bind to native C1-INH (105,000 MW) and a non-functional, degraded C1-INH (88,000 MW; C1-INH-88). To further characterize the binding nature, we have designed a novel kinetic C1 titration assay which enables not only a quantification of the removal of fluid-phase C1-INH by neutrophils, but also a concomitant measure of residual C1-INH function. Native C1-INH, when adsorbed to EDTA-pretreated neutrophils, lost its function in the inhibition of fluid-phase C1. The non-functional C1-INH-88, which is probably devoid of a reactive centre, was found to block the binding of native C1-INH to neutrophils. Pretreatment of neutrophils with serine esterase inhibitors did not abrogate binding capacity of the cells for C1-INH, whereas the binding affinity for C1-INH was lost when the cells were pretreated with trypsin. An array of human peripheral blood leucocytes and several lymphoid cell lines has surface binding sites for C1-INH, but not on human erythrocytes and U937 cells. Binding was further confirmed using (i) C1-INH-microsphere beads to neutrophils, in which the binding was blocked when pretreating neutrophils with excess C1-INH or with trypsin, and (ii) radiolabelled C1-INH to neutrophils, which was competitively blocked by unlabelled non-functional C1-INH-88. Desialylation of C1-INH significantly reduced its binding affinity for neutrophils, indicating that the membrane receptor sites on neutrophils could be specific for the binding of sialic acid residues on C1-INH. Overall, our studies indicate that neutrophils or other leucocytes possess specific surface binding sites for the sialic acid-containing portion of C1-INH.

INTRODUCTION

Human plasma C1 inhibitor (C1-INH) is a heavily glycosylated single-chain polypeptide with a molecular weight (MW), of 105,000.[1-4] C1-INH regulates activation of the classical pathway of complement by inhibiting the proteolytic activity of activated C1 ($C\bar{1}$).[4-5]C1-INH also regulates several plasma esterolytic enzymes, including activated Hageman factor, kallikrein, plasma thromboplastin antecedent and plasmin.[6-8] C1-INH is a member of the serpin family of protease inhibitors. However, the amino acid sequence of the glycosylated region in C1-INH, which is located within the amino terminal 120 residues, is not homologous with other serine protease inhibitors.[9] The purpose of heavy glycosylation in C1-INH is unknown and, most interestingly, the glycosylation is not related to the C1-INH regulatory function on esterolytic enzymes.[4]

Modulation of immune cell functions by C1-INH or by its fragments is not well defined. However, there are reports indicating that C1-INH is capable of modulating leucocyte chemotaxis in responding to activated complement fragments.[10-12] Patrick *et al.*[10] showed that C1-INH enhanced neutrophil chemotaxis in response to zymogen-activated plasma, C5a and N-formyl-met-leu-phe (FMLP). The chemotactic-enhancing activity of C1-INH may be associated with its anti-protease activity, since C1-INH complexed with C1s lost its effect on chemotaxis. However, there is no direct evidence that neutrophil surface-associated proteases are related to C1-INH-mediated chemotactic enhancement.[10] Funk *et al.* demonstrated that infusion of human C1-INH into the circulation of rabbits caused an immediate drop in the white blood cell count, and simultaneously a marked decrease in white blood cell adhesion to the endothelium.[13] Osther and co-workers demonstrated that C1-INH is associated with the surfaces of various cancer cell types and leukaemia cells, and suggested that the cell-associated C1-INH provides a protecting mechanism for cancer cells fending off complement attack.[14-16] Moreover, Randazzo *et al.*[17] isolated functional C1-INH from the cell membrane of a monocytic cell line, U937. Interestingly, patients suffering from cancer may have significant higher serum C1-INH levels than healthy subjects or patients suffering from non-malignant diseases.[18]

In previous studies, we have isolated two neutrophil membrane C1-INH binding proteins (C1-INHbp) with molecular

Correspondence: Dr N.-S. Chang, Guthrie Research Institute, Guthrie Square, Sayre, PA 18840, U.S.A.

weights of 60,000 and 90,000, when separated under reducing SDS–PAGE.[19] However, C1-INHbp migrates as doublets of 58,000, 64,000 and of 77,000, 84,000 MW under non-reducing SDS–PAGE. The C1-INHbp was isolated using CNBr-activated Sepharose 6MB columns conjugated with functional C1-INH or a partially degraded, non-functional C1-INH (88,000 MW; C1-INH-88). Both native C1-INH and degraded C1-INH-88 had a binding affinity of 1.1×10^8 M^{-1} to the 60,000 MW C1-INHbp. This binding protein had no detectable protease activity and the mobility of C1-INH in agarose was retarded in the presence of the C1-INHbp.[19]. Whether these proteins are responsible for C1-INH membrane-anchoring on cancer cell surfaces is unknown. In this report, we further examined the binding characteristics of C1-INH to neutrophils. By using complement-mediated haemolysis assay,[19,20] we report here that preincubation of C1-INH to neutrophils or other leucocytes results in removal of fluid-phase C1-INH functional activity.

MATERIALS AND METHODS

Isolation of C1 inhibitor

Isolation of functional (native) and degraded C1-INH from human citrated outdated plasmas involved chromatographic steps of Affi-gel blue (Bio-Rad, Richmond, CA), hydroxyapatite (Bio-Rad), heparin–agarose (Sigma, St Louis, MO) and DEAE–Sephacel columns (Sigma).[19] Chromatography of plasmas on the Affi-gel blue column yielded three populations of C1-INH of 83,000, 105,000 and 88,000 MW, respectively. Subsequent purification using hydroxyapatite and heparin agarose columns also allowed separation of these three C1-INH species. Purified C1-INH was finally obtained using DEAE–Sephacel. The native C1-INH (105,000 MW) was functionally active in restricting C1 haemolytic activity,[19,20] whereas the degraded forms (83,000 and 88,000 MW) were nonfunctional, and are probably devoid of reactive centres.

Human neutrophils, monocytes, lymphocytes and other cell lines

Isolation of human peripheral blood neutrophils was performed using a modification of the method of Boyum.[21] Thirty millilitres of fresh whole blood were mixed with 20 ml of 0·01 M EDTA-VBS buffer (0·01 M EDTA in 5 mM barbital or veronal buffer; pH 7·4)[19] and with 10 ml of 6% T-500 Dextran (Pharmacia, Uppsala, Sweden). After standing for 30 min at 4°, the leucocyte-rich supernatant fluid was recovered, layered on a Ficoll–Hypaque gradient (Pharmacia) and centrifuged at 500 ***g*** for 20 min. Neutrophils were recovered from the pellet and treated with hypotonic phosphate-buffered saline (PBS; 1:10 diluted with H_2O) to eliminate contaminating erythrocytes. The isolated neutrophils were > 99% pure as verified by checking the nucleus morphology using 10% acetic acid treating the cells. Peripheral lymphocytes and monocytes were isolated from fresh blood by directly centrifuging the blood on the Ficoll–Hypaque gradient. Mononuclear cells were recovered from the upper layer of the gradient. Monocytes were separated from lymphocytes either using a G-10 Sephadex column or by adhesion to a plastic Petri dish at 37° for 3 hr. Additional cell lines were used including Raji cells, RPMI-1788 human IgM-λ chain secreting cells, T-lymphoblastoid cells (Molt-3) and a human monocytic cell line (U937).

Functional C1-INH assay

Neutrophils or other leucocytes were washed thoroughly with 0·01 M EDTA-VBS buffer in order to remove any possible cell-bound C1 molecules[22] and then resuspended in an iso-osmotic $DGVB^{2+}$ buffer (dextrose-gelatin-metal-veronal buffer),[19,20] before incorporating into the C1-INH functional testing. The functional assay for C1-INH restriction of fluid-phase C1 haemolytic activity has been described previously.[19,20,23] Briefly, various concentrations of C1-INH were incubated with rate limiting levels of functionally purified human C1 (2 units; Cordis Laboratories, Miami, FL) at 30° for 10 min. Then, to the mixtures, $EAC4^{gp}$ cells (sheep erythrocytes coated with antibodies and excess guinea-pig C4, 5×10^7 cells/ml) and excess guinea-pig C2 were added, consecutively, with subsequent 10 min incubation at 30°. Eventually, excess guinea-pig C3–C9 reagents were added and continuous measurement of haemolysis was monitored at 700 nm as a function of decreasing turbidity at 37° using a 4-cuvette automatic kinetic spectrophotometer (Gilford, Oberlin, OH). The times to half-lysis ($t_{\frac{1}{2}}$) of erythrocytes were measured and the percentages of C1 functional inhibition by C1-INH compared to buffer controls were calculated as described.[23] Briefly, $t_{\frac{1}{2}}$ (min) values were obtained by using various concentrations of C1 in lysing sheep erythrocytes. A linearly regressed line or equation was derived from the inverse of $t_{\frac{1}{2}}$ ($1/t_{\frac{1}{2}}$) versus the inverse concentrations (or dilutions) of C1. When pretreated with C1-INH, the residual quantity of functional C1 was determined by using the measured $t_{\frac{1}{2}}$ and the standard regressed line (or equation). Percentage of C1 inhibition by C1-INH was calculated by substracting the residual C1 concentration from the original C1 concentration divided by the original C1 concentration.

By employing this assay protocol, four tests were simultaneously run in the kinetic spectrophotometer (i) C1-INH preincubated with (or preadsorbed by) neutrophils at 30° for 10 min, followed by step-wise addition of C1 and the rest of the reagents for testing erythrocyte haemolysis; (ii) without prior C1-INH and neutrophil preincubation, C1-INH was mixed simultaneously with neutrophils and C1 and incubated at 30° for 10 min, and the rest of the test performed step-wise; (iii) C1 alone was incubated with $EAC4^{gp}$ for 10 min at 30° in the presence of neutrophils, following by step-wise addition of the rest of the reagents (base-line control); (iv) C1-INH was incubated with C1 at 30° for 10 min, followed by adding neutrophils, $EAC4^{gp}$ and others (positive C1 inhibition control). The turbidity of neutrophils (or other cell types used) did not contribute to more than 6% of the absorbance (turbidity) at 700 nm.

In time–course studies, native C1-INH was incubated with neutrophils at 30° for various time durations before testing the functional activity of C1-INH. Excess amounts of either non-functional C1-INH-88 or heat-denatured C1-INH (60° for 30 min) were mixed with native C1-INH and co-incubated with neutrophils for 10 min at 30°, prior to step-wise addition of C1 and other complement reagents for haemolytic testing. Controls consisted of either C1-INH-88 or native C1-INH alone in incubation with neutrophils.

Neutrophil trypsinization

Neutrophils ($1{\cdot}75 \times 10^6$/ml) were treated with trypsin (62·5 μg/ml; Sigma) for various time durations at 37°, followed by the

addition of soybean trypsin inhibitor (200 μg/ml; Sigma) and washing the cells three times with $DGVB^{2+}$ buffer. The treated neutrophils were then incubated with C1-INH at 30° for 10 min before addition to the C1-INH assay. In these experiments untreated cells served as controls.

Neuraminidase treatment of C1-INH

To remove the sialic acid residues of C1-INH, native C1-INH (24 μg/ml) was treated with agarose bead-conjugated neuraminidase (Sigma) (45·6 unit/ml) for 1·0 and 2·0 hr at room temperature and the neuraminidase beads were then removed by centrifugation (500 ***g***, 10 min). Enzyme-treated C1-INH samples were then dialysed against VBS to remove free sialic acid prior to testing C1-INH functional activity. Removal of sialic acid from C1-INH was demonstrated using rocket immunoelectrophoresis, according to Seta *et al.*[24]

Neutrophils with enzyme inhibitor treatment

Neutrophils ($1{\cdot}25 \times 10^6$ cells/ml) were treated with enzyme inhibitors, including soybean trypsin inhibitor (2000 unit; Sigma) or phenylmethylsulphonyl fluoride (PMSF, 10 mM; Calbiochem, La Jolla, CA), for 30 and 60 min at 37°, and then washed three times with $DGVB^{2+}$ prior to incorporation in the C1-INH functional assay system.

Binding of fluorescent C1-INH microspheres to neutrophils

Fluorescent microspheres (Covalent Technology Corp., Redwood City, CA) were coated with native C1-INH (3 μg protein/100 μl beads) according to the manufacturer's instruction. Neutrophils ($3{\cdot}0 \times 10^6$ cells/ml) were plated on coverslips for 1 hr at 37° and washed with $DGVB^{2+}$ buffer twice prior to incubating with aliquots of C1-INH microspheres at 4° for 1 hr. Following washing the coverslips twice with cold $DGVB^{2+}$, the binding of C1-INH microsphere beads to neutrophils was examined under a fluorescent microscope. Binding was counted as positive if more than three beads bound to each individual neutrophil.[25] Approximately 50 neutrophils were counted each time and the procedure was repeated three times from other independent experiments. Binding experiments were also performed using microspheres coated with albumin and desialylated C1-INH. Other studies included neutrophils pretreated with trypsin (62·5 μg/ml) or precoated with native C1-INH (3 μg/per coverslip) before testing the binding of C1-INH microspheres.

Binding of [^{125}I] C1-INH to neutrophils

Labelling of native C1-INH or non-functional C1-INH-88 with ^{125}I was performed using the Iodo-Bead method, described previously.[19] The labelled C1-INH had a specificity activity of $2{\cdot}5 \times 10^6$ c.p.m./μg. Binding of non-functional [^{125}I]C1-INH-88 to neutrophils (2×10^6 cells) at 4° for 90 min was performed according to Celada *et al.*[26] When indicated, more than 50-fold excess quantities of unlabelled native C1-INH were added to competitively inhibit the binding of [^{125}I]C1-INH-88 to neutrophils. Alternatively, the binding of radiolabelled native C1-INH to neutrophils was performed and excess quantities of unlabelled C1-INH-88 were used to block the binding of [^{125}I]C1-INH to neutrophils.

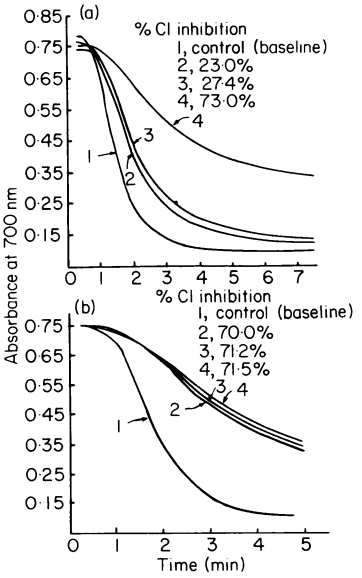

Figure 1. Typical curves of functional C1-INH binding assays. Haemolysis was mediated by rate-limiting levels of C1 with an excess of other complement components, which was measured a function of decreasing turbidity (absorbance at 700 nm) versus time (min) at 37°. Percentage of C1 inhibition by C1-INH was calculated according to the time of half-lysis of erythrocytes.[19,20] (a) C1-INH, when directly interacted with fluid-phase C1 for 10 min at 30°, could maximally inhibit the rate of haemolysis (Line no. 4) compared to the control using C1 alone (Line no. 1). Pre-adsorption of C1-INH with Raji cells (8×10^4 cells) for 10 min at 30° resulted in a functional loss of C1-INH in inhibiting fluid-phase C1 (Line no. 2). A functional loss of C1-INH was also observed when directly mixing C1-INH with both C1 and Raji cells at 30° for 10 min (Line no. 3). (b) Identical conditions were tested by preadsorbing C1-INH with human erythrocytes (1×10^6 cells), however, there was no C1-INH functional depletion observed.

RESULTS

C1-INH functional assay

Preliminary tests were performed to examine whether leucocytes adsorbed C1, thereby resulting in C1-INH binding to cell-associated C1. When Raji cells were tested for C1 binding, C1 molecules were found to be only less than 10% adsorbed by Raji cells ($1{-}9 \times 10^4$ cells). Similarly, neutrophils ($1{-}9 \times 10^5$ cells) removed less than 5·5% of the total rate-limiting amount of functional C1 molecules. The amount of C1 adsorption by leucocytes was substracted from each experiment versus control. Figure 1a shows the kinetics of the haemolysis assay. Line 1 represents a curve of sheep erythrocyte haemolysis mediated by a rate-limiting level of functional C1 molecules in the presence of Raji cells. Next, we tested whether Raji cells could absorb C1-INH activity. As compared to the base-line control (Line no. 1), C1-INH exerted its maximal inhibitory effect on C1 (73% C1 inhibition) when directly incubated with C1 in the fluid phase (Line no. 4). However, C1-INH pre-adsorbed with Raji cells for 10 min at 30° prior to the interaction with C1 resulted in a significant decrease in C1-INH functional activity (23·0% C1 inhibition, i.e. 68·5% C1-INH functional loss; Line no. 2). Simultaneous mixing of C1-INH, Raji cells and C1 (Line no. 3) at 30° for 10 min also resulted in a functional decrease of C1-INH activity (27·4% C1 inhibition, i.e. 62·5% C1-INH func-

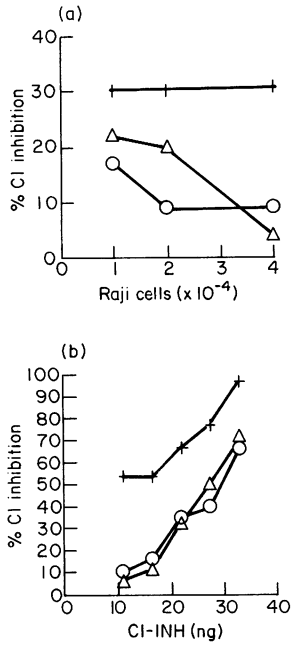

Figure 2. Raji cells and C1-INH interactions. (a) C1-INH (10 ng) was pre-adsorbed by various numbers of Raji cells ($1–5 \times 10^5$ cells/ml) by incubating at 30° for 10 min, before step-wise addition of C1, $EAC4^{gp}$ and C3–C9 according to the standard assay procedures (△, pre-adsorbed). C1-INH was also co-incubated with a mixture of Raji cells and C1 at 30° for 10 min, followed by functional testing (○, co-incubation). Total functional C1-INH activity (or unadsorbed) was measured by directly incubating C1-INH with C1 at 30° for 10 min (+, total activity). (b) Similar studies were performed by pre-adsorbing various concentrations of C1-INH with a fixed number of Raji cells (9×10^4) at 30° for 10 min (△), or coincubation of C1-INH with a mixture of Raji cells and C1 (○). Total C1-INH functional activity was also measured (+).

tional loss). Since Raji cells adsorbed non-significant amounts of C1, the C1-INH functional depletion was, therefore, due to direct binding of C1-INH to Raji cells. No significant difference was noted when C1-INH was preincubated with Raji cells (Line no. 2) or added to the assay simultaneously with Raji cells and C1 (Line no. 3). As a negative control, identical experiments were performed using human erythrocytes. As shown in Fig. 1b, no functional depletion of C1-INH was observed with human erythrocytes, demonstrating that human erythrocytes do not have C1-INH binding activity.

The removal of C1-INH activity was a function of cell number such that increasing the number of Raji cells added to the assay resulted in reduction C1-INH activity (Fig. 2a). Again, functional depletion of C1-INH was observed when C1-INH was mixed simultaneously with the cells and C1, with no apparently significant difference to the results when pre-adsorbing C1-INH by the cells (Fig. 2a). Similar studies performed using various quantities of C1-INH with a fixed number of the cells also resulted in decreases of C1-INH function in inhibiting C1 functional activity (Fig. 2b). Consistently, simultaneous coincubation of C1-INH, C1 and Raji cells resulted in C1-INH functional depletion, with no significant differences to those results obtained when pre-adsorbing C1-INH by Raji cells (Fig. 2b). Similar results were obtained using neutrophils in the C1-INH binding tests (data not shown).

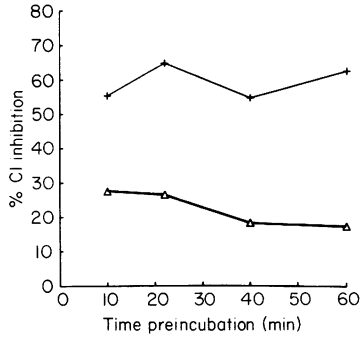

Figure 3. Time–course assay. C1-INH was preadsorbed with neutrophils at 30° for several time durations before testing its residual functional activity against C1 (△). Total activity of C1-INH was measured using time-incubated C1-INH samples at 30° (+).

Table 1. Competitive binding*

	C1-INH-88 (ng)	% functional C1-INH adsorbed by neutrophils
1	0	50 ± 13·2 ($n=3$)
2	500	18·0
3	750	10·1
4	1000	0·0

* Neutrophils ($3·3 \times 10^5$) were incubated with a mixture of functional C1-INH (25 ng) and various quantities of degraded C1-INH-88 for 10 min at 30°, before testing inhibition of C1 haemolytic function by C1-INH. Note that degraded C1-INH-88 blocked the binding of functional C1-INH to neutrophils.

By incubating C1-INH with neutrophils, or C1-INH alone at 30° for various times before testing C1-INH function, we observed that the depletion of C1-INH functional activity had achieved a maximal extent during the initial 10-min incubation, with little increases in functional depletion following prolonged interaction with neutrophils (Fig. 3). These results indicate that the initial rate of C1-INH binding to neutrophils was relatively rapid, followed by gradual approach to an equilibrium of binding. No significant functional decreases were observed by incubating C1-INH aone at 30° up to 80 min.

Furthermore, when excess amounts of non-functional degraded C1-INH-88 were co-incubated with the mixture of functional C1-INH and neutrophils, the functional depletion of C1-INH by neutrophils was reduced (Table 1). Similar results were obtained when using heat-inactivated C1-INH in competing with functional C1-INH binding to neutrophils. In addition to neutrophils and Raji cells, the following cells were capable of binding C1-INH: human peripheral blood lymphocytes, monocytes, RPMI-1788, and Molt 3. Autologous lymphocytes and monocytes were also found to be positive for the C1-INH binding. Cells not found to bind C1-INH were human erythrocytes and U937 cells.

Table 2. PMSF pretreated neutrophils and C1-INH interactions*

		% functional C1-INH adsorbed by neutrophils (mean ± SD; $n=3$)
1	Untreated cells (control)	55·7 ± 11·0
2	30 min PMSF pretreated cells	40·6 ± 5·7†
3	60 min PMSF pretreated cells	57·5 ± 6·9‡

* Neutrophils were pretreated with PMSF for 30 or 60 min prior to incubating with C1-INH for 10 min at 30°, followed by performing C1 functional inhibition tests.

† $P > 0·5$ (t-test versus control).

‡ $P > 0·5$ (t-test versus control).

Table 3. Trypsinization of neutrophils*

Time (min) of neutrophil trypsinization	% functional C1-INH adsorbed by neutrophils
Control (untreated cells)	91·0
10	84·7
20	41·1
40	49·0
60	36·8
80	5·0

* Neutrophils were pretreated with trypsin for various time durations prior to testing the binding of functional C1-INH. The longer neutrophils were treated with trypsin, the less C1-INH was adsorbed by neutrophils.

Protease sensitivity of C1-INH binding

As shown in Table 2, pretreatment of neutrophils with PMSF did not abrogate the capacity of neutrophils to absorb C1-INH. Similar results were obtained when pretreating neutrophils with soybean trypsin inhibitor (data not shown). However, pretreatment of neutrophils with trypsin abrogated C1-INH binding to neutrophils (Table 3). The longer neutrophils were treated with trypsin, the less C1-INH was absorbed by neutrophils.

Role of sialic acid in C1-INH

Following neuraminidase-treatment of C1-INH for 1 and 2 hr, there was 59·6% and 100% recovery of C1-INH function, respectively, compared to the control experiments using untreated C1-INH for the binding. Thus, treatment of native C1-INH with neuraminidase resulted in a significant reduction of C1-INH binding to neutrophils. The removal of sialic acid on C1-INH did not reduce its inhibitory activity for C1, which confirms a previous report by Minta.[27]

Binding of C1-INH microspheres to neutrophils

In agreement with our results using the C1-INH functional assays, microsphere beads coated with native C1-INH bound to neutrophils with more than 10 beads binding per cell (Table 4). However, there was only 45·9 ± 11·7% of positive binding of total neutrophils. Desialylated C1-INH microspheres had significantly reduced binding affinity to neutrophils. Neutrophils pretreated with trypsin or presaturated with native C1-INH also had a reduced binding capability for C1-INH microsphere beads.

Table 4. Binding of C1-INH-microsphere beads to neutrophils*

	Microspheres coated with	Neutrophils % binding ($n=4$)
1	Albumin	0·50 ± 0·07
2	C1-INH	45·9 ± 11·7
3	Desialylated C1-INH	11·1 ± 7·8
4	C1-INH: binding to trypsin-treated neutrophils	6·6 ± 4·2
5	C1-INH: binding to C1-INH-pretreated neutrophils	6·1 ± 4·3

* See Materials and Methods for experimental procedures.

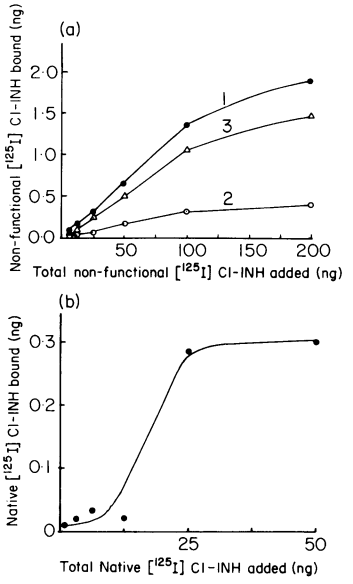

Figure 4. Binding of native [^{125}I]C1-1NH and non-functional [^{125}]C1-INH-88 to neutrophils. (a) Various quantities of [^{125}I] C1-INH-88 were incubated with neutrophils at 4° for 90 min in the presence or absence of more than 50-fold excess of unlabelled, native C1-INH. (b) similar experiments were performed using native [^{125}I]C1-INH binding to neutrophils in the presence or absence of more than 50-fold excess of unlabelled C1-INH-88. The saturation binding curve (a, Line 3) was obtained by subtracting the non-specific binding of [^{125}I]C1-INH-88 (a, Line 2), from the total binding of [^{125}I]C1-INH-88 to neutrophils (a, Line 1). The non-specific binding of [^{125}I]C1-INH-88 was determined from the amount of [^{125}I]C1-INH-88 binding to neutrophils in the presence of excess native C1-INH. An identical approach was used to study the binding of native [^{125}I] C1-INH to neutrophils (b). Only the saturation binding curve is shown.

Binding of [^{125}I] C1-INH to neutrophils

Figure 4a illustrates the binding of non-functional [^{125}I] C1-INH-88 to neutrophils, in which the binding was blocked when

using 50-fold excess unlabelled native C1-INH. A saturation binding curve was obtained from the difference between the total binding of [^{125}I]C1-INH-88 to neutrophils and the non-specific binding of [^{125}I] C1-INH-88 to neutrophils (Fig. 4a). Similar approaches were employed and a saturation binding curve was also obtained when using less quantities of native [^{125}I] C1-INH binding to proportionally reduced numbers of neutrophils (Fig 4b).

DISCUSSION

As a serine protease inhibitor, C1-INH plays a critical role in homeostatically controlling proteolytic enzymes.[4] However, the functional purpose of heavy glycosylation in C1-INH still remains unknown. In our previous observations,[19] as well as in these studies, we provide evidence that C1-INH binding to neutrophils and other leucocytes is independent of inhibitor and target enzyme interactions. The glycosylated portion of C1-INH appears to be responsible for binding to leucocytes, since removal of sialic acid in this region caused a significant reduction of C1-INH binding to leucocytes.

In the preliminary tests, we have excluded the possibility that the binding of C1-INH to leucocytes was due to adsorption of C1-INH to cell-bound C1. We determined that leucocytes bound non-significant amounts (< 10%) of C1 in the assay system. Most of the added C1 molecules became bound to sheep erythrocytes (EAC4) that eventually resulted in red cell lysis by late complement components. By taking advantage of such properties, the assay enabled us to assess the binding of C1-INH to leucocytes and simultaneously measure the residual fluid-phase C1-INH functional activity. Evidence that C1-INH binds to specific sites on leucocytes, but not membrane-associated esterases, are: (i) kinetic patterns were completely shifted when using certain cell types, such as erythrocytes or U937 cells, that did not bind C1-INH (Fig. 1b); (ii) desialylated C1-INH was still functional in the C1 assay but could not bind to neutrophils; (iii) PMSF- or soybean trypsin inhibitor-treated neutrophils still retained their binding affinity for C1-INH (Table 2); (iv) non-functional C1-INH-88 was able to block the binding of native C1-INH to neutrophils (Table 1); (v) binding of C1-INH to neutrophils was further substantiated by using conventional methods employing radioiodinated ligands (Fig. 4) and C1-INH-microspheres (Table 4); (vi) isolated neutrophil membrane C1-INH binding proteins did not covalently interact with native C1-INH, as previously described.[19]

The carbohydrate composition of C1-INH reveals high contents of sialic acid, galactose and N-acetyl galactosamine.[4,28] Minto reported that desialylation of C1-INH results in rapid clearance by hepatic asialoglycoprotein receptors *in vivo*.[27] However, it is unclear whether C1-INH mediated modulation of leucocyte chemotactic movement is due to binding of C1-INH to sialic acid-specific receptors on leucocyte surfaces. Gesel *et al.*[29,30] have reported that macrophages express specific binding sites for sialic acid-containing glycoconjugates, in which the expression of binding sites can be up-regulated by treating macrophages with interferons, tumour necrosis factor and dexamethasone. However, the receptor sites have not been isolated and characterized yet. Whether this receptor binds C1-INH is also unknown. In this study, it is demonstrated that removal of sialic acid residues of C1-INH did not reduce its function, which is consistent with a previous report.[27] Notably, the neuraminidase-treated C1-INH significantly reduced its binding affinity for neutrophil surface binding sites, which was observed using both the haemolytic assay and the C1-INH microsphere bead binding assay (Table 4). These results suggests that cell-surface receptors are probably specific for sialic acid binding, or the sialic acid residues may have enhanced the binding of C1-INH polypeptide backbone to neutrophil surface receptors by charge interactions. We have microsequenced the NH-terminal amino acid sequence of the 60,000 MW C1-INHbp (data not shown) and searched for sequence homology in the GenBank. We found that this sequence (16 residues determined) does not match any sequence of known protein, and, most importantly, this binding protein is not identical to the receptor for galactose and N-acetylgalactosamine.[31,32]

The most intriguing phenomenon in our assay is that simultaneous incubation of C1-INH with the mixture of neutrophils (or other leucocytes) and C1 resulted in a functional decrease of C1-INH activity with no significant difference to those results observed when C1-INH was preincubated with neutrophils prior to interaction with C1. These results indicate that under low ionic strength conditions C1-INH probably has a greater affinity for neutrophils than that of fluid-phase activated C1. Such immediate binding interactions could be important in mediating a rapid drop of white blood cell number in the circulation when the blood C1-INH level is raised.[13]

Although C1-INH and serum C1-INH levels have been shown to associate with various cancer cells[14-16] and malignant diseases,[18] such association may be a defence mechanism for cancer cells to prevent complement attack. Randazzo *et al.*[17] have isolated functionally active C1-INH on U937 cell surfaces. However, the mechanism for C1-INH membrane anchoring on U937 cells is not clear. In our studies, U937 cells were not shown to express surface binding sites for C1-INH. A possible explanation is that secreted C1-INH from U937 cells may occupy the membrane binding sites. Another possibility is that U937 cells probably do not have membrane C1-INH binding sites, simply because the cells require further differentiation, such as stimulation with interferons or other cytokines, in order to express the C1-INH binding proteins. In some cases of acquired C1-INH deficiency, which are associated with B-cell lympho-proliferative diseases, C1-INH is assumed to be depleted as a consequence of complement activation caused by anti-idiotypic antibodies directed against monoclonal immunoglobulin expressed on B-cell surfaces.[4] However, whether C1-INH is also consumed by adsorbing to B cells and becoming internalized remains to be investigated.

In summary, our studies have shown that the glycosylated portion of C1-INH is responsible for binding to leucocytes, which is different from the region-flanking reactive centre that is essential for functional inhibition of serine proteases by C1-INH.

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