Effects of Human Complement Component 1 Inactivator on Neutrophil Chemotaxis and Chemotactic Deactivation

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This study was undertaken to ascertain the relationship between complement-derived chemotactic factors and complement component 1 inactivator (C1INA) enhancement of neutrophil chemotaxis. Studies were also designed to determine whether the C1s reactive or binding site on C1INA was functional in altering chemotactic responsiveness of neutrophilic leukocytes. Chemotaxis was assessed by determining cell migration in micropore filters. C1INA was found to enhance the chemotactic response to zymosan-activated plasma, C5a, and *N*-formyl-L-methionyl-L-phenylalanine and to bring the response of chemotactically deactivated cells to normal. In contrast, C1INA inhibited the chemotactic response to trypsin and EAC4^{oxy}2-activated C3. Complexes of C1INA and C1s failed to mediate the usual C1INA-enhanced response. Artificially produced C5-deficient plasma, when treated with zymosan, failed to support chemotaxis or to produce chemotactic deactivation. C1INA was without effect when this activated plasma was used as a source of chemotactic factors. We conclude from these data that C1INA enhancement of neutrophil chemotaxis to activated plasma is associated with C5-derived chemotactic fragments. The effects of C1INA are apparently related to the C1s reactive or binding site(s) on the C1INA molecule. We suggest that C1INA may play a homeostatic role in neutrophil chemotaxis.

Human complement component 1 inactivator (C1INA) represents one of at least eight proteinase inhibitors found in human plasma. This glycoprotein with α -globulin mobility is present in trace quantities (150 to 350 μ g/ml) and possesses specificity for active proteinases of the complement, blood clotting, fibrinolytic, and kallikrein systems (12).

In addition to possessing reactivity for several soluble plasma serine proteinases, C1INA has been reported to alter leukocyte function. Smith et al. (26) demonstrated that human C1INA caused a severalfold enhancement of polymorphonuclear leukocyte (PMN) chemotaxis when using human plasma activated with lipopolysaccharide (LPS), zymosan, and antigen-antibody complexes. The enhancing influence of C1INA occurred at physiological concentrations, and C1INA had to be present simultaneously with leukocytes in chemotaxis assays. These earlier experiments (26) led to the suggestion that C1INA was enhancing the cell's response to C5-associated chemotactic factors. Goetzl (6) reported that a brief exposure of neutrophils to C1INA caused a slight enhancement of chemo-

taxis when trypsin-activated C5 was used as the chemotactic agent. Remold and Rosenberg (23) described an effect of C1INA on the response of guinea pig macrophages after exposure to migration inhibitory factor.

In the present report, we have assessed the effect of physiological amounts of C1INA on the chemotactic response to activated C3 and C5 and to *N*-formyl-L-methionyl-L-phenylalanine (fMet-Phe). We have also studied the effect of C1INA on chemotactic deactivation induced with zymosan-activated plasma (ZAP) and a chemotactic fragment(s) of C5. We report here that C1INA enhances PMN chemotaxis to fMet-Phe and C5 fragment(s) but inhibits the response to trypsinized C3. With the aid of C5-deficient plasma (C5DP), we present evidence that the enhancing influence of C1INA with activated serum is associated with C5. We also demonstrate that C1INA opposes chemotactic deactivation. Finally, we present evidence to suggest that the C1s reactive site or binding site(s) on C1INA participates in its augmenting influence on chemotaxis.

MATERIALS AND METHODS

Chemical reagents. All chemicals used were reagent or analytical grade and were obtained from the following sources: *N*-acetyl-L-tyrosine ethyl ester from

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Aldrich Chemical Co., Milwaukee, Wis.; bovine serum albumin, trypsin, epsilon amino caproic acid, phenylmethylsulfonyl fluoride, and polybrene from Sigma Chemical Co., St. Louis, Mo.; soybean trypsin inhibitor from Calbiochem, La Jolla, Calif.; sodium dodecyl sulfate from Pierce Chemical Co., Rockford, Ill.; *Salmonella typhimurium* LPS from Difco Laboratories, Detroit, Mich.; Hanks balanced salt solution (HBSS) with phenol red from GIBCO Laboratories, Grand Island, N.Y.; sodium diatrizoate (Hypaque) from Winthrop Laboratories, New York, N.Y.; Sephadex, concanavalin A-Sepharose 4B, and protein A-Sepharose 4B from Pharmacia Fine Chemicals, Inc., Piscataway, N.J.; diethylaminoethyl cellulose from Schleicher & Schuell, Inc., Keene, N.H.; Hypatite C from Clarkson Chemical Co., Inc., Williamsport, Pa.; Dowex AG2-X10 from Bio-Rad Laboratories, Richmond, Calif.; fMet-Phe from Andrlis Chemical Co., Bethesda, Md.; and zymosan from Nutritional Biochemicals Corp., Cleveland, Ohio.

Preparation of C1INA. Human C1INA was prepared and evaluated as described by Pensky and Lepow (21) and with modifications of Reboul et al. (22). These modifications include the incorporation of 2 mM phenylmethylsulfonyl fluoride instead of diisopropylfluorophosphate, 20 mM ethylenediaminetetraacetic acid (EDTA), 10 mM epsilon amino caproic acid, and 1.2 mg of polybrene per ml in the polyethylene glycol precipitation. An additional purification step was added to this protocol that included gel filtration in Sephadex G-200 with 10% glycerol in the elution buffer.

C1INA was also further purified from C1INA generously donated by the American National Red Cross, Bethesda, Md. Highly purified C1INA was also given to us by Jack Pensky (Department of Medicine, Case Western Reserve School of Medicine, Cleveland, Ohio). The specific activity of the C1INA prepared in our laboratory ranged from 50 to 180 *N*-acetyl-L-tyrosine ethyl ester esterolytic units per optical density at 280 nm (16). Immunoelectrophoretic analysis revealed one precipitin arc with α_2 mobility against anti-whole human serum. Polyacrylamide gel isoelectric focusing (pH 3 to 10) demonstrated a single broad band with an apparent pI of 4.0. Sodium dodecyl sulfate-gel electrophoresis demonstrated a major and minor band pattern consistent with that reported by Harpel and Cooper (10). The protein content (18) of individual preparations is noted in Results. Unless otherwise noted, C1INA was used at approximate physiological concentrations of 7 esterolytic units per ml (1).

Chemotactic factors and complement components. Human C1s, C2, C3, and C5 were prepared according to published procedures (9, 19, 20, 29; I. H. Lepow, E. W. Todd, R. D. Smink, Jr., and J. Pensky, *Fed. Proc.* 24:446, 1965). C3 (100 μ g/ml) was treated with 1% trypsin (wt/wt) for 90 s at 30°C, pH 7.4, and the reaction was terminated by addition of 3% soybean trypsin inhibitor. Chemotactic activity was also generated from C3 with EAC4^{oxy2}. A cell button of 2×10^9 EAC4^{oxy2} was incubated with 1 mg (in 1.0 ml) of C3 for 60 min at 37°C. The cells were removed by centrifugation, and the supernatants were tested for chemotactic activity. A low-molecular-weight chemotactic factor was isolated from human serum according

to Gallin and Rosenthal (5). We designate this preparation "C5 fragment(s)" since it does not represent a homogeneous entity, and we express the content in reaction mixtures as micrograms per milliliter on a basis of protein content (Lepow et al., *Fed. Proc.* 24:446, 1965). fMet-Phe was dissolved in HBSS and used in chemotaxis assays at 10^{-6} M. ZAP was prepared by incubating zymosan (10 mg/ml) with fresh human plasma for 30 min at 37°C, diluting 10-fold with HBSS, and removing the zymosan particles by centrifugation and subsequent filtration through filters (pore size, 0.2 μ m; Millipore Corp., Bedford, Mass.). C1s was isolated from the eoglobulin fraction of human serum followed by sequential ion-exchange chromatography on diethylaminoethyl and triethylaminoethyl cellulose. The specific activity of highly purified C1s was 214 U/optical density unit at 280 nm.

Preparation of C5DP. Goat antiserum to human C5 was prepared as follows. The precipitin band was excised from 12 replicate agar immunoelectrophoresis gels which were developed with purified C5 and anti-C5 in the presence of 0.01 M EDTA. The precipitin bands were allowed to stand for several days at 4°C in several changes of physiological saline containing 0.02% sodium azide. The washed, opaque immune precipitates were emulsified in Freund complete adjuvant and injected subcutaneously into multiple sites in one goat. Two weeks later the injection procedure was repeated; 6 weeks after the initial injection, the antiserum was harvested, heat inactivated, made 0.02% with sodium azide, and frozen at -80°C.

In immunoelectrophoresis and Ouchterlony analyses, the antiserum was found to possess strong anti-C5, anti-human serum albumin (HSA), and anti-immunoglobulin G (IgG) activities. When immunodiffusion patterns were allowed to develop for more than 2 days with normal human serum (NHS), an additional faint band was seen that possessed slow beta mobility and a slow diffusion rate. Anti-HSA activity was removed via precipitation with stoichiometric amounts of HSA. Anti-IgG activity was removed by absorption with IgG-Sepharose 6B. This preparation did not demonstrate reactivity with serum genetically deficient in C5, kindly given to us by John Leddy, The University of Rochester Medical Center, Rochester, N.Y.

After the protein content was adjusted to 5%, the IgG fraction of the anti-C5 was isolated by caprylic acid precipitation according to the method of Steinbach and Audran (28). Insolubilization of anti-C5 activity was effected by treatment with protein A-Sepharose. Protein A-anti C5 yielded approximately 20 mg of protein per ml of packed resin.

Fifteen milliliters of normal human plasma was treated by stirring for 45 min at room temperature with 5 ml of packed resin in the presence of 0.01 M EDTA. A second treatment for 90 min at 4°C and 30 min at room temperature rendered the plasma deficient in C5 as ascertained by immunoelectrophoresis (25) and rocket electrophoresis (32) with commercial anti-C5. In addition, when C5DP was used as an immunogen in a single sheep, no anti-C5 activity was detectable after multiple administrations over a 3-month period. The unidentified antigen precipitated by our antiserum (>48 h of developing time) described above was still present in the absorbed C5DP.

The C5DP was dialyzed against HBSS and frozen

at -80°C until ready for use. A sham controlled, Sepharose-protein A-absorbed plasma was treated in an identical fashion with regard to incubations, dialysis, and freezing. This plasma subsequently served as a positive stimulus control when activated with zymosan.

The C5DP was assessed for single complement component activity and total hemolytic activity and compared with the C5DP sham control as well as with fresh NHS (Table 1). Relative complement component concentrations were also assessed via rocket electrophoresis (Table 2). The only complement component that was totally exhausted by the absorption procedure was C5. With the exception of C1q and properdin, all remaining components were relatively unaltered. C1q and properdin concentrations were compromised approximately 50%. Quantitative 50% hemolytic complement assays carried out in tubes showed C5DP, C5DP sham, and NHS to contain 0.0, 34.4, and 45.7 U/ml, respectively, which agreed well with the microtiter procedure.

Preparation of leukocytes. Blood leukocytes containing at least 95% PMN were isolated according to English and Andersen (2). Consistently less than 2% of the leukocytes were blood monocytes or lymphocytes. No platelets were observed in these preparations, and the erythrocyte-to-PMN ratio was approximately 2:1. PMN viability was greater than 98% as determined by eosin exclusion.

Chemotaxis. PMN chemotactic responsiveness was determined by a modified Boyden technique using blind-well chambers (Neuroprobe, Inc., Bethesda, Md.) and single or double micropore filters (pore size, $3\ \mu\text{m}$). The chambers contained 4×10^4 PMN/ mm^2 of exposed filter surface. The chemotactic response was evaluated in three ways. (i) The PMN accumulating on the lower surface of the filter after 3 h of incubation at 37°C were counted. In double-filter experiments, counts of cells that had entered the second filter were included. Ten $40\times$ microscopic fields were counted on each filter of duplicate or triplicate determinations. The double-filter technique was used in experiments where the interpretation of results would be seriously affected by cells falling off the lower surface of the top filter. (ii) The distribution of PMN throughout the filters was assessed as described by Zigmond and Hirsch (33). (iii) Leading fronts (33) were estimated by measuring the depth of migration into the filter of the first two PMN in each field after 90-min incubation times.

Presentation of data. The data are expressed as the mean $\pm$ standard error of the mean; n represents

the number of separate experiments. The paired Student t test was used to assess significance.

RESULTS

C1INA effect on chemotaxis associated with activation of C3. In previous experiments (26), addition of C1INA to the cell suspension during the chemotaxis assay resulted in a significant drop in the chemotactic response to trypsin-activated human C3. In those studies, the test cells were mixed blood leukocytes and were suspended in 10% plasma. In this report, we used purified blood neutrophils suspended in HBSS containing 0.5 to 2.0% bovine serum albumin, and the C1INA was prepared by a different procedure than previously used (11). When C1INA was added to the cell compartment of the chemotaxis chamber, the chemotactic response to trypsin-activated human C3 was $13.0 \pm 9.0\%$ of the counts from chambers without C1INA ($P < 0.001$). The response of the same cell suspensions to LPS-activated serum was enhanced ($378 \pm 56\%$, $P < 0.05$) by addition of the same C1INA preparation to the cell compartment at the same concentration.

That the inhibited response to activated C3 was not due to the trypsin method of activation is indicated by experiments with EAC $^{oxy}2$ -activated C3. Addition of C1INA to the cell compartment resulted in responses $34.6 \pm 23.8\%$ of control ($n = 3$).

Analysis of the effect of C1INA on trypsin-activated C3 chemotaxis at an earlier incubation time than used for the above experiments dem-

TABLE 2. Rocket electrophoretic analysis of C5DP compared with sham-treated plasma and NHS

Determination	Analysis ^a					
	C1q	C3	C4	P ^b	C1INA	C5
C5DP vs C5DP sham ^c	52	98	97	55	93	Undetected
C5DP vs NHS ^d	32	80	94	60	95	Undetected

^a Relative percentile concentrations determined via rocket immunoelectrophoresis.

^b Properdin.

^c C5DP sham control assumed to be 100%.

^d NHS assumed to be 100%.

TABLE 1. Hemolytic analysis of C5-deficient human plasma, C5-deficient sham control, and fresh NHS

Plasma or serum	Hemolytic analysis ^a									
	C1	C2	C3	C4	C5	C6	C7	C8	C9	CH ₅₀
C5DP	1.5	3.2	3.2	16.0	— ^{b*}	32.0	12.0	12.0	4.0	0*
C5DP sham	4.0	9.6	6.4	32.0	64.0	48.0	24.0	24.0	8.0	64*
NHS	12.0	6.4	19.2	32.0	128.0	48.0	64.0	32.0	8.0	96*

^a Values represent dilution ($\times 1,000$) effecting 50% lysis in microtiter plate assay except C5 values (*) in C5DP and all 50% hemolytic complement (CH₅₀) values where the dilution is equivalent to that shown. Assayed by Cordis Corp., Miami, Fla.

^b None detected.

onstrated that cells in the presence of C1INA moved shorter distances into the filter and fewer total cells migrated into the filters. The leading-front analysis with C1INA resulted in a response of 70 μ m in 90 min compared with 130 μ m at the same interval without C1INA ($n = 2$). Estimates of total cells in the filters after 90 min of incubation were made by counting cells at 10- μ m intervals throughout the filter. The results showed that with C1INA there were $69.2 \pm 9.8\%$ of the number seen in assays without C1INA ($n = 6$, $P < 0.001$).

These experiments thus confirm and extend the previous work and indicate that neither plasma nor leukocytes, other than neutrophils, contributed to the observed inhibition or enhancement by C1INA. Furthermore, C1INA prepared by a different method gave the same effects at the same concentrations used previously.

C1INA effect on chemotaxis associated with C5. We previously conjectured that the augmenting effect of C1INA on neutrophil chemotaxis using activated plasma was dependent on a C5-associated product (26). We tested this supposition by using an artificially produced C5DP. The results of two separate experiments are shown in Table 3. Addition of zymosan to C5DP failed to increase chemotactic activity over background levels. Addition of C1INA to the cell compartment, when using zymosan-activated C5DP, did not alter cell migration into the filters. Enhancement of the chemotactic response to sham control ZAP occurred as usual.

When the C5 fragment(s) preparation was used as the chemotactic factor, addition of C1INA to the cell compartment resulted in a significant enhancement (3.26-fold, $P < 0.001$, $n = 6$) of response (Fig. 1). Figure 2 shows similar results in terms of the cell distribution throughout the micropore filter. The results clearly show that C1INA caused more cells to respond to C5 fragments as reflected in higher cell counts at each 10- μ m increment tested. The leading-front analysis of chemotaxis did not discern the C1INA-associated enhancement ($88.2 \pm 2.5 \mu$ m

with C1INA, $88.4 \pm 2.8 \mu$ m without C1INA; $n = 5$).

Chemotaxis associated with fMet-Phe. The use of fMet-Phe allowed us to test the effects of C1INA on a chemotactic agent distinct

FIG. 1. Effect of human C1INA on neutrophil chemotaxis with LPS-activated plasma, trypsinized C3, C5 fragment(s), and fMet-Phe. All results were normalized to percentage of positive control (100%), represented by the horizontal line. Hatched bars depict the chemotactic responses when C1INA was present with PMN in the cell compartment. Open bar, 8 U of C1s-complexed C1INA per ml present with PMN during chemotaxis; 3-h incubation. Means $\pm$ standard error, $n = 7$.

FIG. 2. Effect of human C1INA on PMN distribution in micropore filters. Incubation time, 90 min. Symbols: (—) C5 fragment(s) (40 μ g/ml); (---) C5 fragment(s) + C1INA. C1INA concentration, 8 U/ml. Brackets represent ± 1 standard error of the mean.

TABLE 3. Lack of C1INA enhancement with C5DP

Group	Cell compartment	Stimulus compartment	% of control* $\pm$ SEM
1	C5DP sham	C5DP sham	56.3 ± 5.9
2	C5DP sham	C5DP sham + Z	100
3	C5DP sham + C1INA	C5DP sham + Z	163.5 ± 2.3
4	C5DP	C5DP	18.3 ± 1.9
5	C5DP	C5DP + Z	23.5 ± 2.0
6	C5DP + C1INA	C5DP + Z	23.5 ± 1.4

* Control, group 2 (zymosan [Z]-activated C5DP sham). Double-filter technique was used in all experiments. $n = 4$; group 2 and 3, $P < 0.001$. SEM, Standard error of the mean.

from serum factors. The results indicate (Fig. 1) that C1INA in the cell suspension can enhance chemotactic responsiveness without addition of serum or plasma. The observed enhancement was $186 \pm 15\%$ of control ($P < 0.01$, $n = 6$). C1INA obtained from Jack Pensky produced similar results (186% of response without C1INA) (data not shown). Concentrations of C1INA less than 4.0 U/ml produced no effect. C1INA-mediated enhancement of responses to activated serum also did not occur when C1INA was less than 4.0 U/ml (26).

Analysis of the effects of C1INA on fMet-Phe chemotaxis by the leading-front technique failed to show any difference from controls (cells responding to fMet-Phe in the absence of C1INA). However, estimates of the total cells in the filter showed that chambers containing C1INA had 123% ($n = 3$) the number of cells in controls without C1INA. This effect was the same as with the C1INA-induced enhancement of the chemotactic response to activated serum, but less pronounced.

Preincubation of PMN in C1INA for 30 min at 37°C did not change the response of the cells to fMet-Phe if the C1INA was removed before the chemotaxis assay (percentage of control chemotactic response to fMet-Phe after removal of C1INA, 93.7 ± 9.6 ; without removal of C1INA, 290 ± 36.5 ; $n = 3$, $P < 0.01$). This indicated that preincubation with C1INA had no effect.

C1INA effect on chemotactic deactivation. ZAP and C5 fragment(s) produced two striking effects on neutrophil migration in chemotaxis chambers. If placed in the lower compartment, both chemotactic agents induce a significant increase in cell migration. If neutrophils are preincubated in a high concentration of these agents before placement in the chamber, they show a significant decrease in the chemotactic response (chemotactic deactivation). Since previous experiments (26) have shown that C1INA significantly enhances the first effect, we were

prompted to ascertain whether C1INA could modify chemotactic deactivation as well.

Optimal conditions for deactivation were determined and found to be both dose and time dependent. Chemotactic deactivation was near maximum with a 10% ZAP concentration and a 30-min cell incubation period and with 40 μ g of the C5 fragment(s) preparation per ml and a 15-min incubation period. ZAP-treated PMN showed a chemotactic response that was 13% of the response of cells preincubated in 10% autologous plasma for 30 min. The response to C5 fragment(s) was 27% of control (i.e., HBSS preincubation). C5DP activated with zymosan was much less effective in producing deactivation (Table 4) when compared with normal human serum and plasma and with sham controlled serum.

The effect of C1INA on ZAP-induced chemotactic deactivation was assessed by addition of C1INA to the cell compartment during the

TABLE 4. *Decreased chemotactic deactivation associated with C5DP*

Group	Preincubation ^a	Chemotactic stimulus	Count $\pm$ SEM ^b	P^c
1	NHS	NHS	136 $\pm$ 30	
2	NHS	ZA-NHS	393 $\pm$ 39	
3	NHS	ZA-sham	616 $\pm$ 92	
4	NHS	ZA-C5DP	122 $\pm$ 25	
5	NHS	ZA-NHPC	365 $\pm$ 63	
6	ZA-NHS	ZA-NHS	107 $\pm$ 28	< 0.001
7	ZA-sham	ZA-NHS	96 $\pm$ 20	< 0.005
8	ZA-C5DP	ZA-NHS	295 $\pm$ 29	NS
9	ZA-NHPC	ZA-NHS	136 $\pm$ 28	< 0.001

^a PMN preincubated for 30 min at 37°C in 10% NHS or zymosan-activated (ZA) serum source as indicated, washed twice in HBSS, resuspended to 2×10^6 PMN/ml in 10% NHS, and placed in the cell compartment. NHPC, Fresh normal human plasma from donor source for C5DP.

^b PMN count from double-filter technique; 2.5-h incubation; $n = 4$. SEM, Standard error of the mean.

^c As compared with group 2. NS, Not significant.

TABLE 5. *Effects of C1INA on chemotactic deactivation*

Group	Preincubation ^a	Cell compartment	Stimulus compartment	% of control $\pm$ SEM ^b	n^c
1	10% P1, HBSS	10% P1, HBSS	10% P1, HBSS	0	18
2	10% P1, HBSS	10% P1, HBSS	10% ZAP, HBSS	100	18
3	10% ZAP, HBSS	10% P1, HBSS	10% ZAP, HBSS	13.3 $\pm$ 6.0	18
4	10% ZAP, HBSS, 7 U of C1INA/ml	10% P1, HBSS	10% ZAP, HBSS	56.6 $\pm$ 1.0	5
5	10% ZAP, HBSS	10% P1, HBSS, 7 U of C1INA/ml	10% ZAP, HBSS	122.7 $\pm$ 9.2	5

^a Cells were preincubated 30 min at 37°C with the substances listed and then washed three times in HBSS. P1, Fresh human plasma.

^b SEM, Standard error of the mean.

^c Number of experiments done in triplicate. Groups 2 and 3, $P < 0.001$; 3 and 4, $P < 0.01$; 3 and 5, $P < 0.001$; 2 and 5, $P > 0.4$. Mean cell counts $\pm$ SEM for group 1 = 95 ± 17 cells/high-power field and for 2 = 414 ± 68 cells/high-power field.

chemotaxis assay or by addition to the cell suspension during the preincubation (deactivation) period (Table 5). When C1INA was added to the PMN-ZAP mixture during the preincubation step and then removed along with the ZAP by washing the cells before the chemotaxis assay, there was significantly higher chemotactic activity when compared with cells preincubated in ZAP alone. In separate experiments we determined that no further increase in the chemotactic response resulted from increasing the concentration of C1INA from 7 to 22 U/ml during ZAP preincubation. Furthermore, if PMN pretreated with ZAP were tested for chemotactic responsiveness in the presence of 7 U of C1INA per ml, their response was no different from that of cells preincubated in normal plasma (100% control).

In additional experiments (Table 6), PMN were sequentially preincubated in 10% ZAP for 30 min, washed, and then incubated for 30 min in C1INA. If C1INA was washed from the cells before the chemotaxis assay, no alteration in the deactivated state was detected (compare groups 3 and 4). If C1INA was retained with the cells during the chemotaxis assay, the response was the same as for cells preincubated in normal plasma (compare groups 2 and 5). This experiment shows that sequential treatment of cells with ZAP and C1INA did not alter the deactivated state (group 4). C1INA had to be present simultaneously with the chemotactic stimulus in order to alter the deactivation (group 5).

The effect of C1INA on C5 fragment(s)-induced chemotactic deactivation was also evaluated. The chemotactic response of C5 fragment(s)-deactivated cells was significantly increased by addition of C1INA to the cell suspension in chemotaxis chambers (Table 7). The deactivated neutrophils responded to at least normal levels in the presence of C1INA.

C1s effect of C1INA-augmented chemotaxis. Stoichiometric amounts of purified C1INA and C1s were mixed together and incubated at 37°C for 15 min. The mixture was then applied to a previously calibrated Sephadex G-100 column and eluted with HBSS containing

10% glycerol. Complexed C1INA was located via rocket electrophoresis with anti-C1INA, and the fractions were pooled and concentrated by ultrafiltration. An 8 U/ml equivalent of complexed C1INA (determined by rocket electrophoresis) was used in chemotaxis assays (Table 8). Untreated C1s was also chromatographed through the same column and subsequently used in chemotaxis assays for comparison. The chemotactic response of C1INA-C1s complexes or C1s alone was no different from 100% control responses.

DISCUSSION

The data in this report provide compelling evidence that C1INA-mediated enhancement of neutrophil chemotaxis to activated plasma is

TABLE 7. *Effect of C1INA on C5 fragment(s)-induced deactivation*

Group	Cell compartment	Stimulus compartment	Response ^a
1	PMN, 2% BSA ^b	C5 fragment(s)	100
2	Deactivated PMN, 2% BSA	C5 fragment(s)	27.2 ± 7.6
3	Deactivated PMN, 2% BSA, C1INA	C5 fragment(s)	140 ± 26

^a Counts were normalized by setting the chemotactic response of untreated PMN to 100%. Groups 1 and 2, $P < 0.001$; 2 and 3, $P < 0.001$; double-filter technique; $n = 5$.

^b BSA, Bovine serum albumin.

TABLE 8. *Effect of C1INA-C1s complexes on chemotaxis*

Group	Cell compartment	Stimulus compartment	% Response ^a
1	2% BSA	HBSS	0.3
2	2% BSA, C1INA-C1s	HBSS	0.6
3	2% BSA	C5a	100
4	2% BSA, C1INA-C1s	C5a	103.4 ± 2.5
5	2% BSA, C1s ^b	HBSS	0.6
6	2% BSA, C1s	C5a	97.0 ± 3.5

^a Groups 3 and 4, $P > 0.25$; $n = 4$.

^b C1s concentration, 80 U/ml or 8.0 C1INA equivalents.

TABLE 6. *Requirement for ZAP in C1INA reversal of chemotactic deactivation*

Group	First preincubation ^a	Second preincubation	Cell compartment	Stimulus compartment	Counts ± SEM ^b
1	10% P1, HBSS	10% P1, HBSS	10% P1, HBSS	10% P1, HBSS	148 ± 27
2	10% P1, HBSS	10% P1, HBSS	10% P1, HBSS	10% ZAP, HBSS	305 ± 26
3	10% ZAP, HBSS	10% P1, HBSS	10% P1, HBSS	10% ZAP, HBSS	108 ± 26
4	10% ZAP, HBSS	10% P1, C1INA, HBSS	10% P1, HBSS	10% ZAP, HBSS	106 ± 21
5	10% ZAP, HBSS	10% P1, HBSS	10% P1, C1INA, HBSS	10% ZAP, HBSS	331 ± 83

^a Cells were preincubated for 30 min at 37°C with the substances listed, washed three times in HBSS, and then preincubated for an additional 30 min in the substances listed under column "Second preincubation." After the second preincubation, cells were washed three times in HBSS and tested for chemotactic response. $n = 6$. P1, Fresh human plasma.

^b Groups 1 and 2, $P < 0.005$; 2 and 3, $P < 0.001$; 3 and 4, $P > 0.4$. SEM, Standard error of the mean.

associated with the fifth component of complement. We have arrived at this conclusion because C1INA greatly enhanced the chemotactic response to the isolated chemotactic fragment(s) of C5 and was without effect when using activated plasma deficient in C5. These results confirm our earlier speculation (26) that enhanced chemotaxis to activated plasma due to C1INA was associated with a C5 product(s).

The chemotaxis-enhancing activity of C1INA may be related to its antiproteinase activity. We entertain this possibility because isolated complexes of C1INA and C1s from gel filtration columns were completely ineffective in demonstrating enhancement of chemotaxis.

The effect of C1INA on the motility of human neutrophils depends on the chemotactic stimulant. When cells are migrating toward activated plasma, C5 fragment(s), or fMet-Phe, the addition of C1INA to the cell suspension significantly increases the number of migrating cells. When activated C3 is in the lower compartment, the addition of C1INA significantly inhibits the movement of cells into the filter. Other plasma factors or leukocytes are not involved since C1INA exerts its effects in serum-free systems (e.g., cells migrating toward fMet-Phe) and in mixed leukocytes (26) and isolated neutrophils, but seems to influence the cells only at the time they are responding to a chemotactic stimulus. Preincubation of cells in C1INA was without effect if C1INA was removed before cells were placed in the chemotaxis chamber.

The principal chemotactic activity in activated plasma appears to be a fragment of C5. Patient's sera deficient in C5 exhibit very low chemotactic activity (16), and we find that artificial depletion of C5 essentially removes chemotactic activity. The chemotactic activity of C5a and C5a desarginine in serum has been established (13). There is also considerable evidence that C3a lacks chemotactic activity (4, 13). We have not ruled out the possibility that our C3 preparations contained trace amounts of C5 sufficient to cause a chemotactic response (4) when exposed to trypsin. However, our results with the trypsin- and EAC4^{oxy}2-treated C3 preparations demonstrate a qualitatively different effect of C1INA than was found with chemotaxis toward C5 fragment(s) isolated from activated plasma. Whether or not this effect is linked to a C3-derived chemotactic factor remains to be assessed.

The deactivating effects of preincubation in ZAP may be the result of one or several different factors present in activated plasma. Preincubation of PMN with kallikrein (15), plasminogen activator (14), C3B (25), or the products of the activation of complement (e.g., a chemotactic

fragment of C5 [7]) causes significantly less than the expected cellular response to a concentration gradient of the same chemotactic substance in a Boyden-type chamber. Though the active factor(s) responsible for ZAP-induced deactivation is unknown, the cells can be returned to apparently normal responsiveness by addition of C1INA to the cell suspension in the chemotaxis assay. Furthermore, C1INA significantly reduces deactivation when present in the preincubation fluids. C1INA is, therefore, not causing its effects solely by altering the physical conditions necessary for *in vitro* chemotaxis (e.g., changing the gradient of cytotoxin or modifying the filter surface). Rather, C1INA seems to be capable of altering the cell's response to the deactivating agent. Our results indicate a role for C5 in these events since zymosan-activated C5DP produced much less deactivation than controls and C1INA reversed the deactivation caused by C5 fragment(s).

Recent evidence indicates that chemotactic deactivation is associated with hyperadhesiveness and reduced motility (3, 27). However, neutrophils deactivated with C5 fragment(s) appear to respond normally to rechallenge with the same chemotactic factor (8, 27), and their motility is normal if substratum adhesiveness is reduced (T. L. York, unpublished data). It is interesting to speculate that C1INA may in some way influence the chemotactic factor-induced changes in neutrophil adhesiveness and thereby enhance the migration of deactivated cells. In recent experiments, we have found that an inhibitor of trypsin-like enzymes, *N*- α -*p*-tosyl-L-lysine chloromethyl ketone, will block the enhancement of adhesiveness caused by a chemotactic stimulus.

There is support in the literature that synthetic serine esterase inhibitors suppress chemotactic deactivation. Phosphonate and aromatic amino acid esters have been reported by Ward and Becker (30) to partially prevent deactivation of rabbit neutrophils by chemotactic factors derived from activated rabbit serum. Thus, synthetic esterase inhibitors influence chemotactic deactivation in a fashion similar to that reported here with a naturally occurring serine esterase inhibitor. Since C1INA and phosphonate inhibitors are both known to irreversibly alter the active site of serine esterases, we hypothesized an interaction of C1INA with the activatable esterase associated with rabbit neutrophil chemotaxis. However, experiments designed to directly test this hypothesis failed to show that C1INA altered the action of or the activation of esterase I on rabbit neutrophils (R. A. Patrick and E. L. Becker, unpublished observations).

The C1s reactive site or the C1s binding site on C1INA is apparently involved in its augmentation of neutrophil chemotaxis. It is clear from this report that C1INA directly influences the chemotactic responsiveness of human neutrophils. Since C1INA effects now appear to be associated with the major plasma-containing chemotactic factor (C5 fragment[s]), it is possible that a homeostatic role in neutrophil chemotaxis is exerted by this α -globulin inhibitor.

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