EFFECTS OF HUMAN α1 INHIBITOR ON THE CHEMOTACTIC RESPONSIVENESS OF HUMAN NEUTROPHILIC LEUKOCYTES

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ABSTRACT

EFFECTS OF HUMAN $C\bar{1}$ INHIBITOR ON THE CHEMOTACTIC RESPONSIVENESS OF HUMAN NEUTROPHILIC LEUKOCYTES

By

David Y. Liu

$C\bar{1}$ inhibitor has been shown to enhance and inhibit neutrophil chemotactic responsiveness under appropriate conditions by a direct effect on cells. To further define the nature of this effect, the influence of $C\bar{1}$ inhibitor on complement and noncomplement mediated chemotaxis was investigated.

The presence of highly purified human $C\bar{1}$ inhibitor with either mixed blood leukocytes suspended in 10% plasma or isolated polymorphonuclear leukocytes suspended in 0.5% bovine serum albumin resulted in significant inhibition of the chemotactic response to human C3-derived chemotactic factors. This C3-related chemotactic activity was generated from highly purified human C3 with either trypsin or the cellular intermediate, $EAC4^{oxy}2$. A comparison of the migrating cell distribution in micropore filters in the absence and presence of $C\bar{1}$ inhibitor was performed. Mixed blood leukocytes responding to trypsin-activated C3 at 45, 90, and 135 minutes were quantitated in 10 micron increments. At each time interval $C\bar{1}$ inhibitor was associated with a greater number of cells near the top of the filter and a shorter migration distance into the filter.

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The presence of $C\bar{1}$ inhibitor with either mixed blood leukocytes in 10% plasma or isolated granulocytes in 0.5% albumin resulted in significant inhibition of the chemotactic response to C5-derived chemotactic factors. C5-related chemotactic activity was generated from highly purified human C5 with trypsin or isolated from lipopolysaccharide-activated guinea pig serum as the 15,000 molecular weight fraction from gel filtration.

The effect of $C\bar{1}$ inhibitor on the neutrophil chemotactic response to N-formylmethionylphenylalanine was examined. $C\bar{1}$ inhibitor only when present with the neutrophils significantly enhanced their chemotactic response to 10^{-6}M N-formylmethionyl-phenylalanine. An analysis of the filter distribution of migrating neutrophils revealed a significantly higher number of cells in a zone nearest the upper surface of the filter at 90 and 135 minutes in the presence of $C\bar{1}$ inhibitor and suggested an increase in the number of cells throughout the filter in the presence of $C\bar{1}$ inhibitor.

Another cytotaxin mediated phenomenon, chemotactic deactivation, was examined for its possible susceptibility to the action of $C\bar{1}$ inhibitor. A 10% zymosan-activated plasma filtrate was used as both the deactivating agent and the cytotaxin. Neutrophils, which were deactivated by incubating with zymosan-activated plasma filtrate for 30 minutes and then extensively washed, were unresponsive to subsequent chemotactic stimulation. The presence of $C\bar{1}$ inhibitor during the incubation of neutrophils with zymosan-activated plasma filtrate resulted in partial prevention of deactivation, whereas complete reversal of deactivation was obtained by adding $C\bar{1}$ inhibitor to previously deactivated and washed neutrophils.

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The results of this study lend support to the conclusion that $C\bar{1}$ inhibitor can directly affect the chemotactic response of human neutrophils. Well-defined conditions for the participation of $C\bar{1}$ inhibitor in either enhancement or inhibition are now clearly established so that the basic mechanism of $C\bar{1}$ inhibitor function can be further elucidated. Moreover, the ability of $C\bar{1}$ inhibitor to reverse chemotactic deactivation, which has hitherto been considered irreversible, may be relevant to the understanding of the chemotactic response of human neutrophils.

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By

David Y. Liu

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Dedicated to Jenny

for her endearing patience and understanding

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INTRODUCTION

Approximately 10% of the total protein content in plasma consists of a group of proteins characterized by their ability to inhibit proteolytic enzymes. These proteinase inhibitors have a potential pathophysiologic significance. Inherited deficiencies of several inhibitors are associated with disease: α_1-antitrypsin deficiency predisposes to emphysema and hepatic cirrhosis, antithrombin III deficiency is characteristic of thrombo-embolic disease, and $\overline{C1}$ inhibitor deficiency is associated with hereditary angioneurotic edema. The proteolytic enzyme inhibitors regulate and modulate four interrelated protease systems of the blood: coagulation, fibrinolysis, kallikrein and complement. Several of these inhibitors have been found to have a profound influence upon cell motility. This new dimension of their regulatory function is currently under intense investigation.

It is my intention to describe studies pertinent to the understanding of the regulation of human neutrophil chemotaxis by $\overline{C1}$ inhibitor. This is done in two major sections. In the first, research concerning the structure and function of $\overline{C1}$ inhibitor is extensively reviewed. This involves a discussion of its control functions and the resulting biological activities manifested in inflammatory and hemostatic reactions. It is hoped that such a

discussion will provide a biochemical basis for understanding the participation of ClINH in the chemotactic response of neutrophils.

The second part examines the studies pertaining to the evolution of leukocyte chemotaxis as an extremely complex phenomenon. Attention is focused on efforts to characterize the various chemotactic stimuli. This will be supplemented by information on regulators of the chemotactic response whether the target of their action is the cell or the stimulus. Finally, this review will emphasize the significance of chemotaxis as an investigative tool which has been used to extend our comprehension of such fundamental biological phenomena as cell motility.

The work described in this dissertation was formulated in an attempt to identify and characterize the nature of the effect of $\overline{Cl}$ inhibitor on neutrophil motility.

LITERATURE REVIEW

Biochemical Nature of $C\bar{1}$ Inhibitor

Structure

The existence of a serum inhibitor of the first component of complement, $C\bar{1}$, was first demonstrated by Ratnoff and Lepow (1957). $C\bar{1}$ inhibitor (ClINH) is an α_2-glycoprotein that is antigenically identical to α_2-neuraminoglycoprotein (Pensky and Schwick, 1969). Purified ClINH preparations demonstrated two bands on SDS-acrylamide gel electrophoresis with apparent molecular weights of 105,000 and 96,000 daltons (Harpel and Cooper, 1975). Both components are chemically, immunologically, and functional identical to ClINH. Each of these components appeared to consist of a single polypeptide chain since their apparent molecular weight was not altered by disulfide bond cleavage. Haupt and associates (1970) report a molecular weight for ClINH of 104,000 daltons as determined by sedimentation equilibrium ultracentrifugation with a 35% carbohydrate content. ClINH has an $s_{20,w}$ of 3.67 and an isoelectric point between 2.7-2.8. Analysis of ClINH isolated from the plasma of two patients with hereditary angioneurotic edema showed an apparent molecular weight of 109,000 daltons, an inability to form a complex with $C\bar{1}s$ or plasmin, and a slightly different amino acid content (Harpel, Hugli and Cooper, 1975).

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Various procedures have been reported for the purification of ClINH. They all utilize standard biochemical techniques such as salt fractionation, ion exchange chromatography, gel filtration, adsorption chromatography, and preparative electrophoresis (Haupt et al., 1970; Schreiber, Kaplan and Austen, 1973a; Harpel and Cooper, 1975; Nagaki, Hashimoto and Inai, 1976; Pensky and Lepow, in press). High purity, moderate yields, long preparation times, and low specific activities are all common characteristics of these procedures. Three assays most commonly used for the detection and quantification of ClINH functional activity include the inhibition of the esterolytic activity of $C\bar{1}$ using the synthetic amino acid esters N-acetyl-L-tyrosine ethyl ester (Levy and Lepow, 1959) and N-α-acetyl-L-lysine methyl ester (Harpel, 1970); and the inhibition of the activity of $C\bar{1}$ in immune hemolysis (Gigli, Ruddy and Austen, 1968).

Physiologic Function

$C\bar{1}$ inhibitor controls the first step in the activation of and inhibits enzymes in all four proteolytic systems of the blood: coagulation, fibrinolysis, kinin formation, and complement. These enzymes include $C\bar{1}$ and its subcomponents $C\bar{1}s$ and $C\bar{1}r$ (Levy and Lepow, 1959; Lepow and Leon, 1962; Gigli, Ruddy and Austen, 1968; Ratnoff et al., 1969; Nagaki, Iida and Inai, 1974), kallikrein (Ratnoff et al., 1969), PF/Dil (Kagen and Becker, 1963), activated Hageman factor and plasma thromboplastin antecedent (Forbes, Pensky and Ratnoff, 1970), activated Hageman factor fragments (Schreiber,

Kaplan and Austen, 1973b), and plasmin (Ratnoff et al., 1969; Schreiber, Kaplan and Austen, 1973a).

Loss of $\overline{C1s}$ or $\overline{C1r}$ activity is associated with the formation of a 1:1 molar complex between the inhibitor and the enzyme without peptide bond cleavage (Nagaki, Iida and Inai, 1974; Harpel and Cooper, 1975). Plasmin and ClINH form a 1:1 molar complex with concomitant loss of plasmin activity and degradation of ClINH (Harpel, 1970; Harpel and Cooper, 1975; Nagaki, Hashimoto and Inai, 1976). The reaction between kallikrein and ClINH is suggested to follow second order kinetics leading to an inactive 1:1 molar complex (Gigli et al., 1970; Harpel, Mosesson, and Cooper, 1975). These complexes are all resistant to denaturation by SDS or urea (Harpel, Mosesson and Cooper, 1975). Acidic mucopolysaccharides enhance the ability of ClINH to block the esterolytic and hemolytic activities of $\overline{C1s}$ (Nagaki and Inai, 1976; Rent et al., 1976). At this time there is little known concerning the chemical mechanisms of ClINH binding to its target protease. The necessity of peptide bond cleavage for complex formation to be established between proteolytic enzymes and their naturally occurring inhibitors continues to be a controversial question. Nevertheless, ClINH has a broad specificity for serine esterases. As more information is gathered it would not be surprising if ClINH was found to express a broad specificity similar to α_2-macroglobulin for active serine-proteinases and endopeptidases.

Phylogeny and Ontogeny

Adolphs (1973) reported the presence of a specific inhibitor of guinea pig $C\bar{1}$ in normal murine serum. Further up the phylogenetic tree Donaldson and Pensky (1970) found identical ClINH antigens only in some anthropoid primates.

Synthesis of ClINH by hepatic tissue obtained from human embryos aged only 29 days has been observed by Gitlin and Biasucci (1969). Immunofluorescent techniques revealed that ClINH is in 5-10% of normal hepatic parenchymal cells (Johnson et al., 1971).

Pathology

The discovery of ClINH acquired a clinical significance when an inherited absence of the function of ClINH was diagnosed in patients with hereditary angioneurotic edema (Landermann et al., 1962; Donaldson and Evans, 1963). This disease is characterized by intermittent localized swelling of the skin and the mucous membranes and frequent deaths from laryngeal edema.

ClINH deficiency is an autosomal dominant trait. The usual form of the disease is characterized by extremely low levels of ClINH. In one variant form an immunochemically normal but functionally deficient inhibitor was described (Rosen et al., 1965). This protein exhibits genetically determined variations in electrophoretic mobility, binding capacity to $C\bar{1}s$, and ability to inhibit $C\bar{1}s$ esterolytic activity.

However, it has been suggested that many of these cases may be artifacts induced by a spontaneous conversion of plasma prekallikrein into kallikrein by the Hageman factor which occurs exclusively in

the cold (Van Royen et al., 1976). Low levels of functional ClINH are due to the binding of ClINH by kallikrein. Laurell and Martensson (1971) described an inactive ClINH which was found in molecular association with albumin. This functional and antigenic deficiency is due to impaired synthesis. There is normal catabolism of ClINH and immunofluorescent analysis revealed no inhibitor antigen on liver sections from patients with this form of edema (Johnson et al., 1971).

In patients with ClINH deficiency, intradermal injection of $C\overline{1}s$ produces a wheal followed by the development of local angioedema. Patient plasma will spontaneously generate complement-dependent kinin activity. This may reflect episodic activation of Hageman factor followed by conversion of plasminogen to plasmin. The plasmin would then activate $C\overline{1}$ with subsequent depletion of $C\overline{4}$ and $C\overline{2}$. These enzymatic activities would normally have been regulated by ClINH.

The pathology associated with ClINH is suggested by the discovery of ClINH on the membrane of human carcinoma and blast leukemic cells using immunofluorescence (Osther and Linnemann, 1973a,b). It was hypothesized that ClINH is a blocking factor coating neoplastic cells resulting in inhibition of the cytotoxic activity of complement.

Chemotaxis

This section of the literature review presents those aspects of research concerned with the study of the in vitro migration of neutrophilic polymorphonuclear leukocytes. Information concerning

the history of chemotaxis and the present methodological techniques utilized in chemotaxis is provided. The structural and functional aspects of the cytotaxins and regulators of chemotaxis are emphasized along with the cellular biochemistry of the chemotactic response. Furthermore, the role of chemotaxis in inflammatory reactions is scrutinized.

Historical Perspective

Theodor Leber (1891) performed the first definitive study of leukocyte chemotaxis. Using the capillary tube technique he tested substances for their ability to attract leukocytes into the capillary. By observing the cells continuously, Leber demonstrated the active migration of cells into the test site. He postulated that the force bringing leukocytes into an inflammatory lesion was chemotaxis and a foreign substance was essential to initiate cell migration.

Metchnikoff (1893) combined these findings with others to formulate a phagocytic theory. Metchnikoff realized that both types of phagocytes, macrophages and polymorphonuclear leukocytes are probably chemotactically responsive. From an observation that the two different types of phagocytes constituted different proportions in different lesions with each type phagocytizing different species of bacteria, Metchnikoff implied a cell specificity in the migrational response. His speculations concerning the mechanisms of cell motility and the influence of host substances on chemotaxis remain relevant to this day.

The work of Von Sicherer (1899) was significant for its attempt to make the distinction between chemotactic migration and migration resulting from vascular permeability changes. This involved performing chemotaxis in vitro rather than introducing capillary tubes into body cavities as had traditionally been done. Experiments of Comandon (1917) introduced an in vitro technique for measuring chemotaxis by directly observing cell movement between a slide and coverslip with time-lapse cinematography. McCutcheon and colleagues (reviewed by McCutcheon, 1946) studied the random and chemotactic migrations of leukocytes suspended in plasma while using the slide and coverslip method. Their investigations were notable for the degree of quantitation in the experiments performed. The skin window technique developed by Rebuck and Crowley (1955) allowed the in vivo examination of the migration of inflammatory cells. In response to the stimulus, either the test substance or trauma, cells migrate from the dermis to the coverslip. This method is not quantitative unless the coverslip is replaced by a chamber (Perillie and Finch, 1964); however, this method finds application in conjunction with in vitro chemotaxis assays for clinical studies of abnormal leukocyte function.

Menkin (1938) isolated a chemotactic factor from inflammatory exudates. This substance was active in vitro or in vivo, ninhydrin-positive, dialyzable, heat-stable, and water-soluble. Menkin named his factor, leucotaxine. He also reported that a similar substance could be obtained by the action of trypsin on serum proteins. Meier and Schär (1958) reported the chemotactic activity of gram-negative bacterial lipopolysaccharides with no analogous activity from

gram-positive bacteria. Meier and Schär (1955) also observed the in vitro chemotactic activity of chicken plasma protein-anti-chicken plasma protein precipitate. The observation that heat-inactivated antisera reduced the chemotactic activity was not associated with complement.

Nature

Chemotaxis is defined as the accumulation of cells at a particular locus resulting from directional migration in a chemical gradient of a soluble diffusible substance (Wilkinson, 1974b). In order to understand directional migration it is a necessity to understand the mechanisms of random migration. Robineaux (1964) reported the anterior extension of a hyaloplasmic membrane in a moving cell before the initiation of locomotion into which cellular contents flowed. The migrating cell had an adhesive elongated tail which remained adherent to the surface until broken away by the force of forward movement.

The studies of Ramsey (1972a,b) present detailed observations of human neutrophil movement using phase contrast cinematography. Ramsey termed the flattened extension of a cell a lamellipodium. The random formation of lamellipodia and the adherence of one of these into which cytoplasm flowed are characteristics of migration. The direction of migration is determined by which lamellipodia received the cell contents. The remaining lamellipodia formed the adherent tail and retraction fibers. Ramsey did not consider these events to be characteristic of amoeboid movement. It was shown that chemotaxis did not involve changes in rate of migration but was

attributable to directional movement only. Ramsey concluded that cell movement in the presence and absence of cytotaxin is similar except that in the former case cell movements are directional. However, Keller and Sorkin (1966) found that, depending on the test conditions, the chemotactic substance enhanced random migration and induced directional migration. By photographic tracing of cell movement they demonstrated that cells present in a uniform concentration of cytotaxin exhibited a greater degree of migration than in the absence of cytotaxin. In the presence of a gradient of attractant neutrophils showed strong directional migration.

The question of how a chemotactic substance can cause the directional movement of a cell is difficult to answer. A hypothesis that offers some explanation is that cells possess multiple surface recognition sites which detect the stimuli. Several investigators have provided some evidence for this. Using a micromanipulator, Ramsey (1972a) showed that individual cells could respond repeatedly to moving the stimulus to another location. In fact, chemotactically active cells were observed to move towards the center of a gradient by a convoluted path rather than by a direct route. This supports the results of Cornely (1966) which showed the capability of leukocytes to reverse the direction of migration in response to reversing the gradient of chemotactic serum. This indicated that cells are continuously responsive to a chemotactic gradient. Zigmond (1974), based on time lapse observations, postulated a spatial rather than a temporal mechanism of detecting a gradient for the cell. It is not understood how this spatial mechanism provides the cell with information to selectively form pseudopods. It was also gugested

by Keller and Sorkin (1967b) that cells recognize a chemotactic gradient through cell surface receptors. Keller and Bessis (1975) confirmed these studies with an analysis of the behavior of heat-polarized leukocytes. In the absence of chemotactic stimulation, heat-induced pseudopod formation occurs randomly in all directions. In the presence of stimulation the proximal metapod and the distal pseudopod migrated independently towards the chemotactic target. This indicated that different sections of the cell are able to recognize and respond to chemotactic stimulation independently of each other.

Measurement

In 1962, Boyden introduced a revolutionary techniuqe for the investigation of chemotaxis of macrophages and polymorphonuclear leukocytes. In this system and in all following modifications the principle remains the same. A micropore filter separates the cell containing upper compartment from the lower compartment containing the chemotactic substance. The filter membrane was of such pore size that leukocytes could not pass through it except by active movement towards a chemotactic stimulus. At a given time the filters are removed and stained. The measurement of migration can take one of two forms: (1) count the number of cells which have migrated to a certain depth of the filter, usually the lower surface (Boyden, 1962); or (2) measure the distance traveled by the leading front of cells (Weksler and Hill, 1969; Zigmond and Hirsch, 1973).

Another refinement of these techniques is to study the distribution of cells in the filter (Zigmond and Hirsch, 1973). This is

accomplished by counting cells in a series of planes at 10 micron intervals of the filter. The distribution profile of random migration resembles one side of a normal frequency distribution curve and significant deviations from this distribution are indications of directional migration (Zigmond and Hirsch, 1973; Wilkinson, 1974).

Since investigators have noticed that a variable proportion of cells on the bottom side of the filter becomes detached, several modifications have been introduced. Keller et al. (1972) proposed the use of two filters, the lower being impermeable to the detached cells. Others (Frei, Baisero and Ochsner, 1974) suggested a direct count of cells that have fallen into the lower compartment. In addition, a radioassay employing chromium-51 labeled cells was described as a means to increase accuracy and efficiency (Goetzl and Austen, 1972a; Gallin, Clark and Kimball, 1973). A complete departure from the Boyden system was reported by Nelson, Quie and Simmons (1975). Their system utilized agarose as a medium for migration with separate wells for the cells and chemotactic substance.

Chemotactic Factors

Complement-Derived

There is much evidence now that the activation of complement by a variety of substances causes the liberation of chemotactic factors. Boyden (1962) was the first to speculate upon the importance of complement as a possible source of chemotactic activity in serum activated by immune reactions. Similar results were reported by Keller and Sorkin (1965). It was later shown that normal rabbit

serum but not plasma possesses chemotactic activity for rabbit neutrophils and incubation with immune complexes increases the chemotactic activity in plasma and serum (Borel and Sorkin, 1969). When another inflammatory agent, endotoxin, was incubated with fresh mammalian sera there was evidence for the generation of rabbit neutrophil chemotactic activity by activation of the complement system (Snyderman, Gewurz and Mergenhagen, 1968).

Trimolecular Complex $\overline{C567}$. The initial reports of Ward, Cochrane and Muller-Eberhard (1965, 1966) on the characterization of complement components involved in neutrophil chemotaxis in vitro identified C5, C6, and C7 of rabbit serum as the components of a high molecular weight complex exhibiting chemotactic activity. It was their belief that C7 was required for generation of the chemotactic factor associated with human complement. Later studies demonstrated the potential importance of other complement factors in chemotaxis. Stecher and Sorkin (1969) generated chemotactic activity in C6-deficient rabbit serum, as did Snyderman, Phillips and Mergenhagen (1970). Snyderman, Gewurz and Mergenhagen (1968) could not generate activity from C5-deficient mouse serum using endotoxin and could only generate a low molecular weight factor in normal serum. No complement-associated chemotactic activity was found in the high molecular weight fraction of normal activated serum. These results suggest that C5 was essential for the generation of chemotactic activity in whole serum. However, a more recent report by Lachmann, Kay and Thompson (1970) confirmed the chemotactic activity of purified human $\overline{C567}$. It was found that the chemotactic activity generated by

mixing $\overline{C56}$ with C7 is preempted if the $\overline{C56}$ is first available to bind hydrophobic surfaces. This suggests that $\overline{C567}$ may exhibit a transient action under physiological conditions. Ward and Zvaifler (1971) reported the presence of $\overline{C567}$ in the synovial fluids of human rheumatoid arthritis patients which was active for rabbit neutrophils. Human $\overline{C567}$ purified by Arroyave and Muller-Eberhard (1973) was utilized for chemotactic activity by Berenberg and Ward (1973).

C5-Derived Factors. Largely through the work of Snyderman and associates (Snyderman et al., 1969; Snyderman, Phillips and Mergenhagen, 1970), the low molecular weight neutrophil chemotactic factor in activated guinea pig sera was demonstrated to be a peptide derived from C5. This chemotactic activity for rabbit neutrophils was found in sera activated either by endotoxin or immune complexes. Evidence for this fragment being derived from C5 was as follows: molecular weight of 15,000 on gel filtration coincidental with a cleavage product of exogenously added ^{125}I C5, and inhibition of activity by anti-C5 and not anti-C3. Similar results were obtained for rabbit serum. Other investigators have reported different results (Clark, Frank and Kimball, 1973). Normal and C4-deficient guinea pig sera activated by immune complexes or endotoxin when fractionated revealed both a 17,000 and a 10,000 molecular weight factor that were antigenically related to C5. Both fractions also caused bluing when injected intradermally into Evan's blue treated guinea pigs, suggesting anaphylatoxin activity.

Human serum behaves almost identically to guinea pig serum when activated by inflammatory agents. Snyderman and Mergenhagen (1972)

activated human serum with endotoxin or aggregated human gamma globulins. A 15,000 molecular weight cytotaxin was isolated and characterized as heat stable and antigenically related to C5. Similar chemotactic fragments which have the characteristics of C5a are generated by activation of the classical and alternate pathways of complement (Gallin, Clark and Frank, 1975).

Chemotactic activity can thus be generated from C5 by activation of whole serum. C5-derived factors have also been shown to exist by experiments utilizing purified reagents. C5a was produced by treatment of guinea pig C5 with $EAC\overline{1423}$ (Shin et al., 1968). This fragment possessed chemotactic activity for rabbit polymorphonuclear leukocytes as well as anaphylactic activity for guinea pig ileum. The action of $EAC\overline{1423}$ on human C5 resulted in the appearance of a chemotactic factor (12,500 daltons) for rabbit neutrophils with no muscle-contracting activity (Ward and Newman, 1969). Trypsin cleavage of human C5 resulted in the appearance of two chemotactic peptides (8,500 and 12,500 daltons), neither having contractile activity. Ward et al. (1973) studied two bacterial proteinases produced by *Serratia marcescens* and group A, β-hemolytic *Streptococcus*. Each proteinase was able to cleave several chemotactic factors from purified human C5 and only the streptococcal proteinase produced a C5-related factor in human serum.

C5-derived chemotactic factors can also result by the cleavage of C5 with enzymes endogenous to cells. Lysosomal fractions from rabbit neutrophils, peritoneal and alveolar macrophages show strong chemotactic activity for rabbit neutrophils in the presence of fresh normal serum (Borel, Keller and Sorkin, 1969). Lysates of lysosomal

granules from rabbit neutrophils contain a neutral enzyme which cleaves human C5 into chemotactic fragments (Ward and Hill, 1970). The enzyme cleaves C5, but not C3, into chemotactic fragments of variable molecular weights, depending upon the conditions of interaction. This has been confirmed for human peripheral leukocytes (Taubman, Goldschmidt and Lepow, 1970). Venge and Olsson (1975) demonstrated that chymotrypsin-like cationic proteins of human granulocytes can generate a chemotactic activity for human neutrophils from C5. These enzymes are confined to the azurophil (primary) cytoplasmic granules of the cell. A C5-cleaving enzyme in lysosomes is released from neutrophils during phagocytosis (Ward and Zvaifler, 1973). The lysosomal granules of human platelets contain a protein fraction that acts upon C5 to liberate chemotactic activity for human neutrophils (Weksler and Coupal, 1973) in a manner very similar to that described for rabbit neutrophils by Ward and Hill (1970). The expression of chemotactic activity for rabbit neutrophils is observed if acidic proteinases from beef lung and either peritoneal or pulmonary rabbit macrophages are incubated with guinea pig C5 (Snyderman, Shin and Dannenberg, 1972). Rabbit kidney cells infected with herpes simplex virus release a factor which directly cleaves guinea pig C5 in serum or in isolation to produce a neutrophil chemotactic factor (Brier et al., 1970). Similarly, fluids from chicken embryos infected with either Newcastle disease virus or mumps virus are associated with the elaboration of a chemotactic factor for rabbit neutrophils from human C5 (Ward, Cohen and Flanagan, 1972). The release of these cytotaxin generators may be secondary to virus-induced cytotoxicity and could be lysosomal enzymes.

The use of purified enzymes and complement components has provided meaningful information on the mechanisms of the generation of complement-derived chemotactic factors in vitro. Nevertheless, this does not necessarily provide information for predicting events in vivo. The association of C5 with disease states has been well documented. In the case of tissue injury mediated by immune complexes, C5-derived products have been isolated. In experimental immunologic vasculitis where deposits of immune complexes and complement proteins develop in blood vessel walls, there exists a correlation between the presence of chemotactic fragments of C5 and the accumulation of neutrophils (Hill and Ward, 1972). Cleavage products of C5 have been isolated within the first few hours after the fixation of complement by immune complexes. Tissue extracts from rats depleted of complement show no cytotaxins despite the presence of immune complexes and neutrophils fail to accumulate in the vessel walls. Two chemotactic fragments antigenically related to C5 were found in the synovial fluids of humans with rheumatoid arthritis (Ward and Zvaifler, 1971). In these fluids there was an extraordinary accumulation of neutrophils. Snyderman, Phillips and Mergenhagen (1971) demonstrated the presence of chemotactic C5 fragments in vivo after the injection of endotoxin into peritoneal cavities of guinea pigs or mice and before the influx of leukocytes. C5-deficient mice exhibited none of these characteristics after injection of endotoxin. These data suggest an important function for C5 in the early accumulation of granulocytes in inflammatory exudates.

The physical or chemical nature of these C5-derived chemotactic factors is not well known. The relation of these cytotaxins to anaphylatoxin is not clear. It was recently reported that pure human C5a anaphylatoxin isolated from serum was inactive at 10^{-6}M for inducing human neutrophil chemotaxis (Fernandez, Henson and Hugli, 1975). However, Shin et al. (1968) observed both biological activities associated with the same molecular fragment generated from purified guinea pig C5. Furthermore, it appears that treatment of C5 with trypsin (Ward and Newman, 1969) or lysosomal enzymes (Ward and Hill, 1970), and the activation of guinea pig (Clark, Frank and Kimball, 1973) and human serum (Snyderman and Mergenhagen, 1972) with endotoxin or immune complexes all result in a heterogeneous population of chemotactically active fragments derived from C5. Nilsson, Mandle and McConnell-Mapes (1975) alluded to this possibility based on their structural investigations of C5 subunit structure and modification by trypsin and $\overline{C423}$.

C3-Derived Factors. The paper by Bokisch, Muller-Eberhard and Cochrane (1969) contributed much to an understanding of the formation and characteristics of the biologically active C3 fragments. Treatment of human C3 with trypsin, C3 convertase ($\overline{C42}$), and the C3 inactivator complex (CoVF$\overline{B}$) gave preparations that were chemotactic for rabbit neutrophils. Smith et al. (1975) confirmed these results for human neutrophil activity generated by treatment of human C3 with trypsin. Opferkuch, Snyderman and Borsos (1973) reported an increase in chemotactic activity for rabbit neutrophils present in the supernatant of mixtures of EA$\overline{C42}$ and rat serum containing EDTA as the

number of $\overline{42}$ sites increased. When streptokinase and human plasminogen are added to human and rabbit serum, and purified human C3, a chemotactic fragment of C3 is generated (Ward, 1967). It is chemotactic for rabbit neutrophils in vitro, causes accumulation of rat neutrophils in vivo, and increases rat vascular permeability. Ward et al. (1973) documented the ability of proteinases derived from *Serratia marcescens* and groupa A, β-hemolytic *Streptococcus* to generate chemotactic fragments from human C3. By antigenic analysis of activated serum, it was discovered that only treatment with *Serratia* proteinase resulted in a C3-related factor, chemotactic for rabbit neutrophils. Allantoic fluid from chicken embryos infected with Newcastle disease virus or mumps virus contains human C3 cleaving activity for the production of rabbit neutrophil cytotaxin (Ward, Cohen and Flanagan, 1972). The studies of Hill and Ward (1969) indicate the presence of a serine esterase in rat heart tissue which can cleave C3, isolated or in serum, into chemotactic fragments. Other tissue generators of C3-derived cytotaxins were found to reside in the azurophil cytoplasmic granules of human leukocytes (Venge and Olsson, 1975).

The in vivo significance of C3-derived chemotactic factors has been indirectly established in two situations. In experimental myocardial infarcts in rats, C3 cleavage products appear in soluble extracts of infarcted tissue (Hill and Ward, 1971). If the rats are depleted of serum C3, there is no chemotactic activity in the myocardium and no intense neutrophil accumulation. The etiological agent may be the heart tissue protease previously described by Hill and Ward (1969). Another example is a class of human patients with

inflammatory nonrheumatoid arthritis featuring numerous granulocytes in the joint fluids. A majority of these patients have C3-derived chemotactic factors in their synovial fluids (Ward and Zvaifler, 1971). In addition, many of these fluids contain a C3-cleaving enzyme, capable of producing chemotactic activity from C3. These studies suggest a nonimmunologic function for C3 participation in the acute inflammatory response to nonspecific tissue injury.

The chemical nature of C3a anaphylatoxin has been studied in depth. However, knowledge of the relationship of structure to chemotactic function is still inadequate. It was recently reported that pure human C3a anaphylatoxin isolated from yeast cell activated serum is chemotactically ineffective over a range of 10^{-5} to 3×10^{-7} M (Fernandez, Henson and Hugli, 1975). This was supported by Shin et al. (1969), who presented evidence that guinea pig C3a anaphylatoxin isolated from lipopolysaccharide-activated serum is not chemotactic for rabbit neutrophils. Extended treatment of C3a with trypsin yields a fragment retaining chemotactic and losing anaphylactic activity (Bokisch, Muller-Eberhard and Cochrane, 1969).

C3$\overline{B}$ and CoVF$\overline{B}$. Ruddy, Austen and Goetzl (1975) consistently found chemotactic activity for human neutrophils in mixtures of highly purified CoVF, B, and $\overline{D}$. In fact, the chemotactic activity of CoVF$\overline{B}$ correlated with its hemolytic activity. Furthermore, mixtures of C3b, B, and $\overline{D}$ could be demonstrated to have chemotactic activity. This is evidence for the association of the appearance of chemotactic activity with the formation of properdin pathway convertases into necessary complexes.

Non-Complement Factors

Chemotactic factors not associated with the complement system but still found in the plasma have been identified and characterized within the last several years. A serum-derived chemotactic mediator system, the anaphylatoxin-related binary peptide leucotactic system, has been well described by Wissler (1972a,b) and Wissler, Stecher and Sorkin (1972a,b). These experiments present evidence for a two peptide system, consisting of classical anaphylatoxin (CAT) and a co-cytotaxin (CCT). Neither of these peptides is chemotactic by itself, but a mixture of the two causes chemotactic migration of cells. Contact activation of normal guinea pig, rat or hog serum with hydrophilic, insoluble substances of high molecular weight, e.g., dextran, yeast or immune complexes, leads to the formation of these peptides. CAT had anaphylatoxin activity in purified form. For chemotaxis, a gradient of CAT but not of CCT was essential. Furthermore, CCT could be replaced by various nucleotides such as ATP, cyclic AMP or GTP. The magnitude of the chemotactic activity was found to be strongly dependent on the absolute concentrations and the molar ratio of the two peptide components.

Chemotactic factors can be liberated during the activation of plasma enzyme systems other than complement. Borel and Sorkin (1969) reported that serum clotted on glass became chemotactically active. The chemotactic activity of kallikrein was investigated (Kaplan, Kay and Austen, 1972; Weiss, Gallin and Kaplan, 1974). Biological activity of kallikrein coincident with chemotactic activity was separated from glass-clotted serum. This activity for human neutrophils was also generated when prekallikrein was converted

to kallikrein by Hageman factor fragments. In support of this were the experiments showing that reconstitution of Fletcher factor-deficient serum with prekallikrein completely corrected the chemotactic deficiency associated with kaolin-activated Fletcher factor-deficient serum. The chemotactic defect was also corrected by addition of intact activated Hageman factor, suggesting that the absolute contribution of kallikrein to the chemotactic activity of kaolin-activated normal serum is small. The coagulation and fibrinolytic pathways of plasma participate in the generation of chemotactic activity. Kay, Pepper and Ewart (1973) first observed that clot supernatants, prepared by thrombin action on human fibrinogen, were chemotactic for human leukocytes. Subsequently, fibrino-peptide B was identified as a chemotactic agent in the supernatant. The enzymatic lysis of fibrin (Stecher and Sorkin, 1972) and collagen (Chang and Houck, 1970) can lead to the generation of chemotactic factors in vitro.

Investigations into the discovery and characterization of chemotactic factors present in inflammatory tissue have proceeded in the last few years. Hayashi and associates (Yoshida, Yoshinaga and Hayashi, 1968) isolated a chemotactic factor from rabbit Arthus and burned skin lesions. Termed leucoegresin, this cytotaxin was characterized as being antigenically and physicochemically closely identical to rabbit IgG (Yoshinaga et al., 1971; Yamamoto, Yoshinaga and Hagashi, 1971). Leucoegresin was produced locally in the skin lesion site and in vitro from rabbit and hyman IgG by the action of inflammatory neutral SH-dependent proteases (Hayashi et al., 1969; Yoshinaga et al., 1971). The chemotactic activity was also

produced by papain action on traditionally papain resistant subclasses of IgG (Yoshinaga et al., 1972). These results suggest that IgG is subject to structural changes due to enzymatic action leading to the formation of neutrophil leucoegresin.

Proteins have been studied for the effect of tertiary structural changes on their chemotactic activity. Human serum albumin, which is not chemotactic in its native form, becomes chemotactic for human neutrophils after denaturation by acidification, alkalation, or reduction and alkylation, or modification of the albumin structure by conjugation with synthetic non-polar side groups (Wilkinson and McKay, 1971, 1972). The major conformational changes which accompany the transition of hemoglobin to globin lead to the acquisition of chemotactic activity (Wilkinson, 1973).

In view of the potent chemotactic activity of cyclic AMP for slime molds, its potential effect on neutrophil migration has been investigated by several laboratories. Cyclic AMP was reported to be chemotactic for neutrophils by Leahy, McLean and Bonner (1970). This was confirmed by Gamow and Barnes (1974), who also presented evidence for positive and negative chemotaxis towards cyclic GMP. Kaley and Weiner (1971) failed to find activity for cyclic AMP, dibutyryl cyclic AMP or cyclic GMP. Tse, Phelps and Urban (1972) actually found cyclic AMP to be inhibitory for random and chemotactic migration. The evidence is confusing at best.

Kaley and Weiner (1971) and Higgs, McCall and Youlten (1975) also reported prostaglandin E_1 to be chemotactic for rabbit neutrophils. Turner, Campbell and Lynn (1975) did not find this to be true, but did discover that mild aerobic oxidation of arachidonic

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(catabolic precursor of prostaglandin E_1) and eicosapentaenoic acids yielded strong chemotactic activity for human granulocytes. A component of the chemotactic oxidized arachidonic acid was identified as 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid which could also be generated by the action of a lipoxygenase on arachidonic acid (Turner, Tainer and Lynn, 1975). Lipoxygenase is an aggregation-activated platelet enzyme.

Cell-associated chemotactic factors are diverse with regard to their characteristics and origin. Eosinophil chemotactic factor of anaphylaxis from the guinea pig lung attracted guinea pig neutrophils only when neutrophils comprised 90% of the total cell population (Kay, Shin and Austen, 1973). Dialyzable transfer factor from human leukocytes was discovered to contain a potent chemotactic agent in vitro for human granulocytes and in vivo for monkey granulocytes (Gallin and Kirkpatrick, 1974). The phagocytosing neutrophil releases chemotactic factors for other neutrophils. The appearance of chemotactic activity following human and rabbit neutrophil phagocytosis of urate or calcium pyrophosphate crystals has been reported (Tse and Phelps, 1970). Spilberg, Mandell and Wochner (1974) described this chemotactic material as lysosome-derived and synthesized de novo. Chemotactic activity was recovered in the supernate of leukocytes rotated in a globulin-coated tube (Zigmond and Hirsch, 1973). Similar activity was obtained by repeatedly freezing and thawing horse leukocytes confirming the data of Cornerly (1966), who subjected the neutrophil to sucrose lysis and the lysosome granule to freezing and thawing. Chick embryos infected with either Newcastle disease virus or mumps virus and

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monkey kidney cells infeqted with mumps virus elaborate chemotactic activity for rabbit neutrophils. Guinea pig lymphocytes, when stimulated in vitro by specific antigen, produce a chemotactic stimulus for guinea pig neutrophils (Ward, Remold and David, 1970). This may have relevance to the experiments of Ramseier (1969), who has shown that mixed leukocyte cultures produce a factor that causes neutrophil accumulation when injected into the skin.

"Immunechemotaxis" is a concept recently proposed from observations that immune reactions occurring on the surface of the responding neutrophil elicit the chemotactic response of that cell (Jensen and Esquenazi, 1975; Williams et al., 1976).

There is still very little known about chemotactic factors produced by bacteria. Keller and Sorkin (1967a) found culture filtrates from *Staphylococcus albus* and *Escherichia coli* to be chemotactic for neutrophils. Ward, Lepow and Newman (1968) characterized their bacterial factors as low molecular weight substances, the elaboration of which was related to log phase growth. An electrophoretically isolated lipid moiety of *E. coli* chemotactic factor was found to be chemotactic for human neutrophils (Tainer, Turner and Lynn, 1975). The chromatographic and chemical properties of the *E. coli* lipid cytotaxins were similar to those of a previously reported cytotaxin synthesized from arachidonic acid. The isolated peptide components were not chemotactic. Schiffman et al. (1975) found chemotactic activity in the lipid material from *E. coli* chemotactic factor in addition to low molecular weight, anionic, heat stable peptides possessing blocked N-terminal groups. Free carboxyl groups are required for their activity. Possibly related to these

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findings are those of Schiffman, Corcoran and Wahl (1975) documenting the chemotactic activity of synthetic N-formylmethionyl peptides.

Cellular Biochemistry of Chemotaxis

The chemotactic response is a complex sequence of events involving the recognition of chemotactic factors which is then translated into directional migration. An overall coherent scenario has yet to be established. In analyzing the mechanisms of the chemotactic response, investigators have attempted to alter the response through biochemical and pharmacological manipulations. This approach yields results which must be interpreted with caution. Many of these manipulations utilize agents which act at different levels, some of which may be irrelevant to directional migration.

The nature of chemotactic recognition is unlikely to be as specific as the interaction of antibody with antigen. One recognition pattern is that nonpolar groups are necessary for chemotactic recognition. This has largely evolved from the studies of Wilkinson (1973, 1974) and Wilkinson and McKay (1972, 1972). Cells theoretically detect a soluble protein which has many exposed non-polar side groups and many mutually repulseive groups for the prevention of polymerization and insolubilization. Native proteins upon denaturation undergo conformational changes with non-polar groups forced to the exterior. Cells detect these changes without a requirement for stereospecific binding by a receptor. Interactions may take place through hydrophobic bonding. Indeed, it was found that surface activity and chemotactic activity of proteins are related

(Wilkinson, 1974a). Other mechanisms may involve interactions at the membrane level of a different type than the one proposed by Wilkinson. Ionic interactions between cell surface and chemotactic molecules may possibly be influencing chemotaxis. Gallin, Durocher and Kaplan (1975) demonstrated a high degree of association between changes in cell surface charge and chemotactic activity. Human chemotactic factors reduced the negative surface charge on human granulocytes.

An important aspect of chemotactic recognition is that of cell-specific chemotaxis. A mechanism for this was suggested by Wissler, Stecher and Sorkin (1972). Their hypothesis proposes that cell-specificity is governed by the proportions of the two peptides CAT and CCT in the chemotactic mixture. Both rabbit neutrophils and guinea pig eosinophils responded to certain proportions of CAT and CCT, while at other proportions only one of the cell types migrated. These effects need to be confirmed in a homologous system since CAT and CCT were generated from pig serum. Further evidence of this type of regulation is provided by the work of Kay, Shin and Austen (1973) which demonstrated a marked synergism between ECF-A and C5a in their ability to stimulate eosinophil chemotaxis. Electrophoretically isolated farctions of *E. coli* chemotactic factor yielded free protein components with no chemotactic activity, a fee lipid moiety that is chemotactic for both rabbit alveolar macrophages and human neutrophils, and lipoprotein complexes that only stimulate macrophage migration (Tainer, Turner and Lynn, 1975). In this case, proteins act as modifiers of target cell specificity.

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Anaerobic glycolysis is probably the major energy source for neutrophil chemotaxis. Chemotaxis is diminished by inhibitors of anaerobic glycolysis such as iodoacetate (Carruthers, 1966) and inhibited to a lesser degree by uncouplers of oxidative phosphorylation (Ward, 1966). This suggests that oxidative metabolism is used but is not essential for the chemotactic response. This was confirmed by Goetzl and Austen (1974a). They found the ability of a chemotactic factor to stimulate glucose metabolism of human neutrophils through aerobic glycolysis or the hexose monophosphate shunt is associated with a maximal chemotactic response, but alone this stimulation is not sufficient for chemotaxis. Ouabain inhibits chemotaxis but is reversed by raising external K^+ concentration (Ward and Becker, 1970a).

Agents, such as prostaglandins, theophylline and epinephrine, which increase intracellular cyclic AMP of rabbit neutrophils, decrease spontaneous motility and chemotactic responsiveness (Rivkin, Rosenblatt and Becker, 1975). Cholera toxin increases cyclic AMP levels and inhibits chemotaxis. Chemotactic stimuli have no significant effect on intracellular cyclic AMP levels. The experiments of Estensen et al. (1973) suggested that chemotaxis is enhanced by agents which increase cellular cyclic GMP concentration such as phorbol myristate acetate, acetylcholine, imidazole, and 8-bromo cyclic GMP. Until more is known about the chemotactic response, the exact relationship between cyclic nucleotides and neutrophil migration remains nebulous.

Some biochemical and ultrastructural information is known concerning the relationship of cellular structures and chemotaxis.

Neutrophils exposed to C5a show a transient increase in the number of microtubules (Goldstein et al., 1973), and colchicine, which prevents tubulin polymerization, inhibits chemotactic migration (Caner, 1965). Bryant et al. (1966) reported optimal leukocyte migration in the presence of divalent cations. C5-derived chemotactic factor has been reported to cause a redistribution of granulocyte intracellular calcium out of the cytoplasm and into a granule fraction in association with microtubule polymerization (Gallin and Rosenthal, 1974). The mechanism of chemotactic factor alteration of intracellular calcium is unknown.

Cytochalasin B is a fungal metabolite which disrupts contractile microfilament systems. It has a complex effect on human and rabbit neutrophils, stimulating or inhibiting it, depending on the drug concentration (Becker et al., 1972). However, the role of microfilaments in migration is confused by the findings of Zigmond and Hirsch (1972) that cytochalasin B suppressed glycolysis by inhibiting sugar transport into leukocytes. Cytochalasin B also causes a decrease in the volume of rabbit neutrophils, an effect similar to that observed with chemotactic factors (Hsu and Becker, 1975).

Drugs which inhibit RNA and protein synthesis such as puromycin and actinomycin D were reported by Carruthers (1967) to have a slight inhibitory effect on neutrophil random migration, but a complete inhibitory effect on chemotactic migration.

The complement-derived chemotactic factors as well as *E. coli* chemotactic factor induce the release of lysosomal enzymes from rabbit neutrophils without a similar effect on cytoplasmic enzymes (Becker et al., 1974a,b). No statistical correlation was found

between the abilities of the chemotactic factors to stimulate migration and to induce release of lysosomal enzymes.

The chemotactic response of rabbit neutrophils to complement-derived and *E. coli* chemotactic factors involves the activation of a serine esterase (Ward and Becker, 1969; Ward and Becker, 1970b; Becker, 1972). Activation of proesterase 1 was shown to be associated with chemotactic activity and a correlation between the degree of activation and level of chemotactic activity was established (Becker, 1972). The nature and mechanism of esterase 1 activity during the chemotactic response remains to be determined. There is no information in regard to the manner by which chemotactic factors activate proesterase 1 and there is no evidence concerning the cell location of proesterase 1.

A phenomenon related to esterase 1 activity is deactivation (Ward and Becker, 1968; Becker, 1972). Rabbit neutrophils incubated with $\overline{C567}$ or C5a are cross-deactivated to $\overline{C567}$, Cta, C3a, or *E. coli* chemotactic factor (Becker, 1972). Deactivation was also observed with human neutrophils using kallikrein and C5a (Goetzl and Austen, 1974b), human eosinophils using histamine (Clark, Gallin and Kaplan, 1975) and ECF-A and C5a (Wasserman et al., 1975). Kallikrein and C5a cross-deactivated to each other (Goetzl and Austen, 1974b). $\overline{CoVFB}$ and $\overline{C3B}$ cross-deactivated to kallikrein (Ruddy, Austen and Goetzl, 1975), ECF-A and C5a cross-deactivated to each other (Wasserman et al., 1975), and histamine cross-deactivated to C5a (Clark, Gallin and Kaplan, 1975). Deactivation of human neutrophils by kallikrein did not result in a reduction of hexose monophosphate shunt stimulation after the introduction of kallikrein (Goetzl and

Austen, 1974a). Deactivation is prevented by the same phosphonates that inhibit chemotaxis and the inhibition profiles are the same for the two processes (Ward and Becker, 1968). This indicated that esterase 1 is involved in both chemotaxis and deactivation. Recently, Becker (1975) has presented preliminary evidence that activation of esterase 1 is also involved in spontaneous motility. Deactivated neutrophils at the present time are not well characterized with respect to functions.

The possibility that activation of esterase 1 is important for spontaneous motility may suggest a role for serine esterases in a phenomenon essential to motility. It has been suggested that cell surface proteases mi ht function in cell attachment (Whur, Payne and Koppel, 1974). Grinnell (1975) provided some recent evidence for this possibility. He found that cell attachment to a substratum in serum-containing medium was inhibited when protease inhibitors were added to the medium. Furthermore, proteolytic activation of serum-coated substrata resulted in an enhanced rate of cell attachment.

The report by Lentnek, Schreiber and MacGregor (1976) demonstrated that human granulocyte adherence is augmented in inflammatory states and that this augmentation is mediated by inflammatory plasma factors. The importance of granulocyte adhesiveness to the vascular endothelium for the initiation of leukocyte migration from venules has been shown in hamsters by Atherton and Born (1972). Zigmond and Hirsch (1972) have suggested that a possible effect of cytochalasin B on leukocyte motility is to cause an increase in adhesiveness of the cells to their substrate (Carter, 1967). In

addition, the relationship between cell adherence and motility may also involve surface charge. In the amoebae there is an inverse correlation between cell adhesiveness and negativity of amoebae surface charge (Ambrose and Forrester, 1968). Bone marrow granulocyte precursor cells were described as having high negative surface charge, poor distensibility, low adherence, and poor pseudopod formation (Lichtman and Weed, 1972). The negative surface charge of human granulocytes was decreased when cells were incubated with chemotactic factors (Gallin, Durocher and Kaplan, 1975). Their findings support the concept that an appropriate alteration in cell adhesiveness may be critical for the chemotactic response of human neutrophils.

Modulators

Agents which are known to modulate neutrophil chemotaxis exert their activity at any of three levels: (1) formation of chemotactic factor, (2) interaction with preformed chemotactic factors, and (3) interference with cellular events involved in the chemotactic response.

A recent study (Walker et al., 1975) demonstrated that a preparation from normal human plasma reduced human leukocyte chemotaxis only when it was added to serum before activation with zymosan. This low molecular weight (<500) anti-inflammatory agent did not affect cell function directly and it was concluded that the fraction selectively inhibits the release or generation of serum chemotactic factors in vitro.

Substances which interact with chemotactic factors are found in the serum and as cellular constituents. Normal human serum was found to contain an inactivator of chemotactic factors for rabbit neutrophils (Berenberg and Ward, 1973). It directly and irreversibly inactivates the chemotactic fragments of C3 and C5, $\overline{C567}$, and *E. coli* chemotactic factor. Further purification of this inactivator revealed two inactivators, a 7S β-globulin specific for C3-derived cytotaxin and a 4S α-globulin specific for the C5-derived cytotaxin (Till and Ward, 1975). Both inactivators were effective against kallikrein and *E. coli* chemotactic factor. Furthermore, the β-globulin inactivator possessed the ability to inactivate C3a and C5a anaphylatoxins, while the α-globulin inactivator could only inhibit C3a anaphylatoxin (Ward, Data and Till, 1974). It is still confusing as to whether or not chemotactic factor inactivator is different from anaphylatoxin inactivator (Bokisch and Muller-Eberhard, 1970) and the $\overline{C567}$-INH of reactive lysis (McLeod, Baker and Gewurz, 1975). Clinically, elevated serum levels of the chemotactic factor inactivator were found in patients with Hodgkin's disease, indicating a possible cause for deficient skin inflammatory reactions in these patients (Ward and Berenberg, 1974). Sera deficient in α_1-antitrypsin are also deficient in the chemotactic factor inactivator (Ward and Talano, 1973). A similar acting inactivator has been observed in tumor cells and their ascitic fluid, normal rat tissues and serum (Brozna and Ward, 1975). Other serum inhibitors of chemotactic factors have been associated with chronic, hypocomplementemic glomerulonephritis (Gewurz et al., 1967), cirrhosis (DeMeo and Andersen, 1972), skin test anergy (Van Epps,

Palmer and Williams, Jr., 1974) and reticulum cell sarcoma (Ruutu et al., 1975).

Cell-derived products exhibit inhibitor activities towards chemotactic factors. Incubation of neutrohil granule lysates or post-phagocytic media with C5-related cytotaxins resulted in progressive inactivation of chemotactic activity (Wright and Gallin, 1975). The chymotrypsin-like cationic proteins of human granulocytes abolish chemotactic activity upon incubation with C5 chemotactic fragments (Venge and Olsson, 1975).

The serum protease inhibitors, ClINH and α_2-macroglobulin reduced the chemotactic activity of kallikrein and plasminogen activator simultaneously with the suppression of kinin-generating and plasminogen-activating activities, respectively (Goetzl and Austen, 1974). This is presumably accomplished by interaction with an intact serine esterase active site.

These two inhibitors in addition to α_1-antitrypsin directly affect the chemotactic response of human neutrohils. ClINH reversibly enhances and inhibits the neutrophil chemotactic response to activated plasma and trypsin-activated C3, respectively, without affecting spontaneous motility (Smith et al., 1975). Goetzl (1975) confirmed some of these results and found that α_1-antitrypsin, α_2-macroglobulin, and TLCK caused irreversible enhancement of random migration and inhibition of chemotaxis. In addition, ClINH slightly enhanced the chemotactic response to trypsin-activated C5. Clinical studies of serum inhibitors of neutrophil chemotactic response acting directly on the cell have been reported (Ward and Schlegel, 1969;

Smith et al., 1972; Soriano et al., 1973). These patients are characterized by a severe susceptibility to infections.

The regulation of the chemotactic response can proceed through factors normally present in human leukocytes. Incubation of mononuclear or polymorphonuclear cells in acidic medium, with endotoxin or with starch particles, releases a neutrophil-immobilizing factor (Goetzl and Austen, 1972). This factor irreversibly suppresses chemotactic and random migration without affecting other cell functions (Goetzl et al., 1973). Products of human activated lymphocytes when fractionated revealed the existence of a leukocyte inhibitory factor which specifically inhibits human neutrophil migration (Rocklin, 1974).

Clinical Disorders

The descriptions of syndromes in which isolated deficiencies of chemotactic function are accompanied by clinical manifestations add support to the concept of an essential role for chemotactic migration in the inflammatory response. Examples of primary defects of chemotaxis in which the patient's serum contains inhibitors of chemotaxis were described in the previous section. Other types of chemotactic malfunctions are those in which there is an intrinsic defect of neutrophil movement or the patient's serum is inadequate as a source of chemotactic stimuli.

Intrinsic disorders of neutrophil movement and other cell functions are reported to be associated with chronic granulomatous disease (Steerman et al., 1971), acquired granulomatous disease (Singh et al., 1972), granulocytasthenia (Higgins, Swanson and

Yamazaki, 1970) and pyoderma gangrenosum (Miller and Dooley, 1973). Abnormalities of only neutrophil movement are the Chediak-Higashi syndrome (Clark and Kimball, 1971), lazy leukocyte syndrome (Miller, Oski and Harris, 1971), diabetes mellitus (Mowat and Baum, 1971) and familial chemotactic defect (Miller et al., 1973).

The second group of patients can be distinguished by the inability of their serum to provide adequate chemotactic stimulus for their own neutrophils or for control neutrophils. Gewurz et al. (1967) reported observations of failures to generate cytotaxins in C2 deficient serum. Alper et al. (1970) described a patient with Klinefelter's syndrome, C3 deficiency and an inability to generate chemotactic activity until C3 was added to the serum. Miller and Nilsson (1970) found a deficiency of serum chemotactic activity in a child with C5 functional deficiency and normal serum levels of C5. Weiss, Gallin and Kaplan (1974) discovered an abnormality of chemotactic activity in serum from Fletcher factor deficiency cases.

Chemotaxis in Inflammation

It is the responsibility of investigators to demonstrate that the in vitro observation of leukocyte directional migration using techniques such as the Boyden chamber is a significant aspect of the inflammatory response in vivo. However, in vivo manipulations invariably begin a chain reaction of events that may influence cell movement through mechanisms other than those essential for directional migration. This makes interpretation of a direct cause-effect relationship between experimental manipulation and chemotactic migration very difficult. Nevertheless, there is strong presumptive evidence of an in vivo correlate of chemotaxis.

Buckley (1963) produced tiny heat injuries in the rabbit ear without damaging the capillaries. Granulocytes could be observed migrating towards the site of injury. Actively motile granulocytes moving at random were seen near the center of injury and peripheral cells were moving directionally towards the center. Hurley (1963) studied leukocyte emigration from rat skin using electron microscopy and intravenous injection of visible markers. Hurley demonstrated that leukocytes but not the injected particles or plasma protein passed through the vascular endothelium. Histamine treatment caused the reverse situation to occur. Therefore, Hurley could dissociate the two phenomena of leukocyte emigration and vascular permeability.

More recently, passive Arthus reactions in synovial tissues were introduced in rabbits depleted of neutrophils with nitrogen mustard (DeShazo, Henson and Cochrane, 1972). C6 deficient rabbits exhibited protracted inflammation and neutrophil accumulation in the synovial tissue. Arthus sites in the synovial blood vessel walls were established by intravenous injection of antigen and injection of antibody into the knee joint. Neutrophils injected into the joint space migrated from the space into the synovium towards the site of immune complex-complement reaction in the vessel walls. There was marked inhibition of migration in C6 deficient or C3-depleted rabbits. Furthermore, little or no vascular permeability existed as measured by a lack of leakage of intravenously injected ^{125}I-serum albumin out of the vessels. The review by Cochrane and Janoff (1974) summarizes the in vivo studies which indicate the

importance of complement for the accumulation of neutrophils at sites of immunological reactions.

Snyderman, Phillips and Mergenhagen (1971) investigated the time sequence of in vivo production of chemotactic factors, eventually identified as C5-derived fragments. They reported a rise and fall of neutrophil chemotactic activity in the peritoneal fluid of guinea pigs within six hours of endotoxin injection. Neutrophils appeared during the decline of chemotactic activity and increased in numbers during the first 24 hours. C5 deficient mice failed to show any of these peak patterns.

Wilkinson et al. (1973) extended the experimental procedure of Snyderman, Phillips and Mergenhagen (1971) in order to study the time course of appearance of eosinophil and macrophage-related activities. The patterns for neutrophil chemotactic activity and migration were similar to those reported previously. In addition, peaks of macrophage and eosinophil chemotactic activities appeared synchronously with the peak of chemotactic activity. Eosinophil chemotactic activity declined simultaneously with neutrophil activity but a plateau of macrophage chemotactic activity remained for several days. Macrophages and eosinophils reached the peritoneal cavity slower than neutrophils, reaching a peak several days after injection of inflammatory stimulant.

The experiments described in this section provide significant circumstantial evidence for the probability of chemotactic migration occurring in vivo. The experiments of Buckley (1963) demonstrated directional migration of leukocytes. The work of Snyderman, Phillips and Mergenhagen (1971) and Wilkinson et al. (1973) associated the

formation of chemotactic activity with the appearance of the specifically responding leukocytes which suggested the importance of a chemotactic gradient for directional migration. The data of DeShazo, Henson and Cochrane (1972) and DeShazo et al. (1972) offer an isolated in vivo correlate of complement-mediated chemotaxis. To postulate complement as a mediator of chemotactic responses in vivo would suggest that there exists an extravascular complement pool. Alper and Rosen (1967) proposed the possibility of 30% of the total vascular C3 protein existing in extravascular pools. Finally, both Hurley (1963) and DeShazo, Henson and Cochrane (1972) dissociated leukocyte migration due to chemotactic responsiveness from migration due to altered vascular permeability.

REFERENCES

REFERENCES

Adolphs, H. D. 1973. Two complement inhibitors in mouse sera. Med. Microbiol. Immunol. 158: 171-175.

Alper, C. A., Abramson, N., Johnston, R. B., Jandl, J. H., and Rosen, F. S. 1970. Increased susceptibility to infection associated with abnormalities of complement-mediated functions and of the third component of complement (C3). New Engl. J. Med. 282: 349-354.

Alper, C. A., and Rosen, F. S. 1967. Studies of the in vivo behavior of human C'3 in normal subjects and patients. J. Clin. Invest. 46: 2021-2034.

Ambrose, E. S., and Forrester, J. A. 1968. Electrical phenomena associated with cell movements. Symp. Soc. Exp. Biol. 22: 237-248.

Arroyave, C. M., and Muller-Eberhard, H. J. 1973. Interactions between human C5, C6 and C7 and their functional significance in complement-dependent cytolysis. J. Immunol. 111: 536-545.

Atherton, A., and Born, G. V. R. 1972. Quantitative investigations of the adhesiveness of circulating polymorphonuclear leukocytes to blood vessel walls. J. Physiol. (Lond) 222: 447-474.

Becker, E. L. 1972. The relationship of the chemotactic behavior of the complement-derived factors, C3a, C5a, and C567, and a bacterial chemotactic factor to their ability to activate the proesterase 1 of rabbit polymorphonuclear leukocytes. J. Exp. Med. 135: 376-387.

Becker, E. L. 1975. Enzyme activation and the mechanism of polymorphonuclear leukocyte chemotaxis. In *The Phagocytic Cell in Host Resistance*. J. A. Bellanti and D. H. Dayton, eds. Raven Press, New York.

Becker, E. L., Davis, A. T., Estensen, R. D., and Quie, P. G. 1972. Cytochalasin B. IV. Inhibition and stimulation of chemotaxis of rabbit and human polymorphonuclear leukocytes. J. Immunol. 108: 396-402.

Becker, E. L., and Showell, H. J. 1974. The ability of chemotactic factors to induce lysosomal enzyme release. II. The mechanism of release. J. Immunol. 112: 2055-2062.

Becker, E. L., Showell, H. J., Henson, P. M., and Hsu, L. S. 1974. The ability of chemotactic factors to induce lysosomal enzyme release. I. The characteristics of the release, the importance of surfaces and the relation of enzyme release to chemotactic responsiveness. J. Immunol. 112: 2047-2054.

Becker, E. L., and Ward, P. A. 1969. Esterases of the polymorphonuclear leukocyte capable of hydrolyzing acetyl DL-phenylalanine β-naphthyl ester. J. Exp. Med. 129: 569-584.

Berenberg, J. L., and Ward, P. A. 1973. Chemotactic factor inactivator in normal human serum. J. Clin. Invest. 52: 1200-1206.

Bokisch, V. A., and Muller-Eberhard, H. J. 1970. Anaphylatoxin inactivator of human plasma: Its isolation and characterization as a carboxypeptidase. J. Clin. Invest. 49: 2427-2436.

Bokisch, V. A., Muller-Eberhard, H. J., and Cochrane, C. G. 1969. Isolation of a fragment (C3a) of the third component of human complement containing anaphylatoxin and chemotactic activity and description of an anaphylatoxin inactivator of human serum. J. Exp. Med. 129: 1109-1130.

Borel, J. F., Keller, H. U., and Sorkin, E. 1969. Studies on chemotaxis. XI. Effect on neutrophils of lysosomal and other subcellular fractions from leukocytes. Int. Arch. Allergy 35: 194-205.

Borel, J. F., and Sorkin, E. 1969. Differences between plasma and serum mediated chemotaxis of leukocytes. Experientia 25: 1333-1335.

Boyden, S. 1962. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear lejcocytes. J. Exp. Med. 115: 453-466.

Brier, A. M., Snyderman, R., Mergenhagen, S. E., and Notkins, A. L. 1970. Inflammation and herpes simplex virus release of a chemotaxis-generating factor from infected cells. Science 170: 1104-1106.

Brozna, J. P., and Ward, P. A. 1975. Antileukotactic properties of tumor cells. J. Clin. Invest. 56: 616-623.

Buckley, I. K. 1963. Delayed secondary damage and leucocyte chemotaxis following focal aseptic heat injury in vivo. Experimental and Molecular Pathology 2: 402-417.

Caner, J. E. Z. 1965. Colchicine inhibition of chemotaxis. Arthritis Rheum. 8: 757-764.

Carruthers, B. M. 1966. Leukocyte motility. I. Method of study, normal variation, effect of physical alterations in environment and effect of iodoacetate. Canad. J. Physiol. Pharmacol. 44: 475-485.

Carruthers, B. M. 1967. Leukocyte motility. II. Effect of absence of glucose in medium: Effect of presence of deoxyglucose, dinitrophenyl, puromycin, actinomycin D, and trypsin on the response to chemotactic substance; effect of segregation of cells from chemotactic substance. Canad. J. Physiol. Pharmacol. 45: 269-280.

Carter, S. B. 1967. Effects of cytochalasins on mammalian cells. Nature 213: 261-264.

Chang, C., and Houck, J. C. 1970. Demonstration of the chemotactic properties of collagen. Proc. Soc. Exp. Biol. Med. 134: 22-26.

Clark, R. A., Frank, M. M., and Kimball, H. R. 1973. Generation of chemotactic factors in guinea pig serum via activation of the classical and alternate complement pathways. Clin. Immunol. Immunopathol. 1: 414-426.

Clark, R. A., Gallin, J. I., and Kaplan, A. P. 1975. The selective eosinophil chemotactic activity of histamine. J. Exp. Med. 142: 1462-1476.

Clark, R. A., and Kimball, H. R. 1971. Defective granulocyte chemotaxis in the Chediak-Higashi syndrome. J. Clin. Invest. 50: 2645-2652.

Cochrane, C. G., and Janoff, A. 1974. The Arthus reaction: A model of neutrophil and complement-mediated injury. In *The Inflammatory Process*, Vol. III, B. W. Zweifach, L. Grant and R. T. McCluskey, eds. Academic Press, New York.

Comandon, J. 1917. Phagocytose in vitro des hematozoaires du calfat. Comptes rendus hebdomadaires des seances et memoires de la Societe de Biologie 80: 314.

Cornely, H. P. 1966. Reversal of chemotaxis in vitro and chemotactic activity of leukocyte fractions. Proc. Soc. Exp. Biol. Med. 122: 831-835.

DeMeo, A. N., and Andersen, B. R. 1972. Defective chemotaxis associated with a serum inhibitor in cirrhotic patients. New Engl. J. Med. 286: 735-740.

DeShazo, C. V., Henson, P. M., and Cochrane, C. G. 1972. Acute immunologic arthritis in rabbits. J. Clin. Invest. 51: 50-57.

DeShazo, C. V., McGrade, M., Henson, P. M., and Cochrane, C. G. 1972. The effect of complement depletion on neutrophil migration in acute immunologic arthritis. J. Immunol. 108: 1414-1419.

Donaldson, V. H., and Evans, R. R. 1963. A biochemical abnormality in hereditary angioneurotic edema: Absence of serum inhibitor of C'1-esterase. Amer. J. Med. 35: 37-44.

Donaldson, V. H., and Pensky, J. 1970. Some observations on the phylogeny of serum inhibitor of C1 esterase. J. Immunol. 104: 1388-1395.

Edelson, P. J., Stites, D. P., Gold, S., and Fudenberg, H. H. 1973. Disorders of neutrophil function. Defects in the early stages of the phagocytic process. Clin. Exp. Immunol. 13: 21-28.

Estensen, R. D., Hill, H. R., Quie, P. G., Hogan, N., and Goldberg, N. D. 1973. Cyclic GMP and cell movement. Nature (Lond) 245: 458-460.

Fernandez, H., Henson, P., and Hugli, T. E. In press. A single isolation procedure for obtaining both C3a and C5a from activated human serum. J. Immunol.

Forbes, C. D., Pensky, J., and Ratnoff, O. D. 1970. Inhibition of activated Hageman factor and activated plasma thromboplastin antecedent by purified serum C1 inactivator. J. Lab. Clin. Med. 76: 809-815.

Frei, P. C., Baisero, M. H., and Ochsner, M. 1974. Chemotaxis of human polymorphonuclears in vitro. II. Technical study. J. Immunological Methods 5: 375-386.

Gallin, J. I., Clark, R. A., and Frank, M. M. 1975. Kinetic analysis of chemotactic factor generation in human serum via activation of the classical and alternate complement pathways. Clin. Immunol. Immunopathol. 3: 334-346.

Gallin, J. I., Clark, R. A., and Kimball, H. R. 1973. Granulocyte chemotaxis: An improved in vitro assay employing ^{51}Cr-labeled granulocytes. J. Immunol. 110: 233-240.

Gallin, J. I., Durocher, J. R., and Kaplan, A. P. 1975. Interaction of leukocyte chemotactic factors with the cell surface. I. Chemotactic factor-induced changes in human granulocyte surface charge. J. Clin. Invest. 55: 967-974.

Gallin, J. I., and Kirkpatrick, C. H. 1974. Chemotactic activity in dialyzable transfer factor. Proc. Nat. Acad. Sci. USA 71: 498-502.

Gallin, J. I., and Rosenthal, A. S. 1974. The regulatory role of divalent cations in human granulocyte chemotaxis. Evidence for an association between calcium exchanges and microtubule assembly. J. Cell Biol. 62: 594-609.

Gamow, E., and BArnes, F. S. 1974. Chemotactic responses of human polymorphonuclear leukocytes to cyclic GMP and other compounds. Exp. Cell Res. 87: 1-7.

Gewurz, H., Page, A. R., Pickering, R. J., and Good, R. A. 1967. Complement activity and inflammatory neutrophil exudation in man. Studies in patients with glomerulonephritis, essential hypocomplementemia and agammaglobulinemia. Int. Arch. Allergy 32: 64-90.

Gigli, I., Mason, J. W., Coleman, R. W., and Austen, K. F. 1970. Interaction of plasma kallikrein with the C1 inhibitor. J. Immunol. 104: 574-581.

Gigli, I., Ruddy, S., and Austen, K. F. 1968. The stoichiometric measurement of the serum inhibitor of the first component of complement by the inhibition of immune hemolysis. J. Immunol. 100: 1154-1164.

Gitlin, D., and Biasucci, A. 1969. Development of γG, γA, γM, β_{1C}/β_{1A} C1 esterase inhibitor, ceruloplasmin, transferrin, hemopexin, haptoglobin, fibrinogen, plasminogen, α_1-antitrypsin, orosomucoid, β-lipoprotein, α_2-macroglobulin, and prealbumin in the human conceptus. J. Clin. Invest. 48: 1433-1446.

Goetzl, E. J. 1975. Modulation of human neutrophil polymorphonuclear leucocyte migration by human plasma alphaglobulin inhibitors and synthetic esterase inhibitors. Immunol. 29: 163-174.

Goetzl, E. J., and Austen, K. F. 1972a. A method for assessing the in vitro chemotactic response of neutrophils utilizing ^{51}Cr-labelled human leukocytes. Immunol. Commun. 1: 421-430.

Goetzl, E. J., and Austen, K. F. 1972b. A neutrophil-immobilizing factor derived from human leukocytes. I. Generation and partial characterization. J. Exp. Med. 136: 1564-1580.

Goetzl, E. J., and Austen, K. F. 1974a. Stimulation of human neutrophil leukocyte aerobic glucose metabolism by purified chemotactic factors. J. Clin. Invest. 53: 591-599.

Goetzl, E. J., and Austen, K. F. 1974b. Active site chemotactic factors and the regulation of the human neutrophil chemotactic response. In *Antibiotics and Chemotherapy*, Vol. 19, E. Sorkin, ed. S. Karger, Basel.

Goetzl, E. J., Gigli, I., Wasserman, S., and Austen, K. F. 1973. A neutrophil immobilizing factor derived from human leukocytes. II. Specificity of action on polymorphonuclear leukocyte mobility. J. Immunol. 111: 938-945.

Goldstein, I., Hoffstein, S., Gallin, J., and Weissmann, G. 1973. Mechanisms of lysosomal enzyme release from human leukocytes: Microtubule assembly and membrane fusion induced by a component of complement. Proc. Nat. Acad. Sci. USA 70: 2916-2920.

Grinnell, F. 1975. Cell attachment to a substratum and cell surface proteases. Arch. Biochem. Biophys. 169: 474-482.

Harpel, P. C. 1970a. $\overline{C1}$ inactivator inhibition by plasmin. J. Clin. Invest. 49: 568-575.

Harpel, P. C. 1970b. A sensitive, colorimetric method for the measurement of serum C1 inactivator using the substrate N-α-acetyl-L-lysine methyl ester. J. Immunol. 104: 1024-1030.

Harpel, P. C., and Cooper, N. R. 1975. Studies on human plasma $\overline{C1}$ inactivator-enzyme interactions. I. Mechanisms of interaction with C1s, plasmin, and trypsin. J. Clin. Invest. 55: 593-604.

Harpel, P. C., Hugli, T. E., and Cooper, N. R. 1975. Studies on human plasma C1 inactivator-enzyme interactions. II. Structural features of an abnormal $\overline{C1}$ inactivator from a kindred with hereditary angioneurotic edema. J. Clin. Invest. 55: 605-611.

Harpel, P. C., Mosesson, M. W., and Cooper, N. R. 1975. Studies on the structure and function of α_2-macroglobulin and $\overline{C1}$ inactivator. In *Proteases and Biological Control*. E. Reich, D. B. Rifkin, E. Shaw, eds. Cold Spring Harbor Laboratory, Cold Spring Harbor.

Haupt, H., Heimburger, N., Kranz, T., and Schwick, H. G. 1970. Ein beitrag zur isolierung and charakterisierung des $\overline{C1}$-inaktivators aus human plasma. Eur. J. Biochem. 17: 254-261.

Hayashi, H., Koono, M., Yoshinaga, M., and Muto, M. 1969. The role of an SH-dependent protease and its inhibitor in the Arthus-type hypersensitivity reaction, with special reference to chemical mediation of increased vascular permeability and leucocyte emigration. In *Inflammation Biochemistry and Drug Interaction*. A. Bertelli and J. Houck, eds. Excerpta Medica, Amsterdam.

Higgins, G. R., Swanson, V., and Yamazaki, J. 1970. Granulocytasthenia, a unique leukocyte dysfunction associated with decreased resistance to infection. Clin. Res. 18: 209.

Higgs, G. A., McCall, E., and Youlten, L. J. F. 1975. A chemotactic role for prostaglandins released from polymorphonuclear leukocytes during phagocytosis. Brit. J. Pharm. 53: 539-546.

Hill, J. H., and Ward, P. A. 1969. C3 leukotactic factors produced by a tissue protease. J. Exp. Med. 130: 505-518.

Hill, J. H., and Ward, P. A. 1971. The phlogistic role of C3 leukotactic fragments in myocardial infarcts of rats. J. Exp. Med. 4: 885-900.

Hsu, L. S., and Becker, E. L. 1975. Volume changes induced in rabbit polymorphonuclear leukocytes by chemotactic factor and cytochalasin B. Amer. J. Path. 81: 1-14.

Hurley, J. V. 1963. An electron microscopic study of leucocytic emigration and vascular permeability in rat skin. Austral. J. Exp. Biol. Med. Sci. 41: 171-186.

Jensen, J. A., and Esquenazi, V. 1975. Chemotactic stimulation by cell surface immune reactions. Nature (Lond) 256: 213-215.

Johnson, A. M., Alper, C. A., Rosen, F. S., and Craig, J. M. 1971. C1 inhibitor: Evidence for decreased hepatic synthesis in hereditary angioneurotic edema. Science 173: 553-554.

Kagen, L. J., and Becker, E. L. 1963. Inhibition of permeability globulins by C'1-esterase inhibitor. Fed. Proc. 20: 613.

Kaley, G., and Weiner, R. 1971. Effect of prostaglandin E1 on leukocyte migration. Nature: New Biology 234: 114-115.

Kaplan, A. P., Kay, A. B., and Austen, K. F. 1972. A prealbumin activator of prekallikrein. III. Appearance of chemotactic activity for human neutrophils by the conversion of human prekallikrein to kallikrein. J. Exp. Med. 135: 81-97.

Kaplan, A. P., Goetzl, E. J., and Austen, K. F. 1973. The fibrinolytic pathway of human plasma. II. The generation of chemotactic activity by activation of plasminogen proactivator. J. Clin. Invest. 52: 2591-2595.

Kay, A. B., Pepper, D. S., and Ewart, M. R. 1973. Generation of chemotactic activity for leukocytes by the action of thrombin on human fibrinogen. Nature: New Biology 243: 56-57.

Kay, A. B., Pepper, D. S., and McKenzie, R. 1974. The identification of fibrinopeptide B as a chemotactic agent derived from human fibrinogen. Brit. J. Haematol. 27: 669-677.

Kay, A. B., Shin, H. S., and Austen, K. F. 1973. Selective attraction of eosinophils and synergism between eosinophil chemotactic factor of anaphylaxis (ECF-A) and a fragment cleaved from the fifth component of complement (C5a). Immunol. 24: 969-976.

Keller, H. U., and Bessis, M. 1975. Migration and chemotaxis of anucleate cytoplasmic leukocyte fragments. Nature (Lond) 258: 723-724.

Keller, H. G., Borel, J. F., Wilkinson, P. C., Hess, M. W., and Cottier, H. 1972. Re-assessment of Boyden's technique for measuring chemotaxis. J. Immunol. Methods 1: 165-168.

Keller, H. U., and Sorkin, E. 1965. Studies on chemotaxis. I. On the chemotactic and complement-fixing activity of γ-globulins. Immunol. 9: 241-247.

Keller, H. U., and Sorkin, E. 1966. Studies on chemotaxis. IV. The influence of serum factors on granulocyte locomotion. Immunol. 10: 409-416.

Keller, H. U., and Sorkin, E. 1967a. Studies on chemotaxis. V. On the chemotactic effect of bacteria. Int. Arch. Allergy 31: 505-517.

Keller, H. U., and Sorkin, E. 1967b. Studies on chemotaxis. VI. Specific chemotaxis in rabbit polymorphonuclear leucocytes and mononuclear cells. Int. Arch. Allergy 31: 575-586.

Lachmann, P. J., Kay, A. B., and Thompson, R. A. 1970. The chemotactic activity for neutrophil and eosinophil leucocytes of the trimolecular complex of the fifth, sixth, and seventh components of human complement (C567) prepared in free solution by the reactive lysis procedure. Immunol. 19: 895-899.

Landerman, N. S., Webster, M. E., Becker, E. L., and Ratcliffe, H. E. 1962. Hereditary angioneurotic edema. II. Deficiency of inhibitor for serum globulin permeability factor and/or plasma kallikrein. J. Allergy 33: 330-341.

Laurell, A. B., and Martensson, U. 1971. $\overline{C1}$ inactivator protein complexed with albumin in plasma from a patient with angioneurotic edema. Eur. J. Immunol. 1: 146-149.

Leahy, D. H., McLean, E. R., Jr., and Bonner, J. T. 1970. Evidence for cyclic-3', 5'-adenosine monophosphate as chemotactic agent for polymorphonuclear leukocytes. Blood 36: 52-54.

Leber, T. 1891. Die entstehung der entzundung und die wirkung der entzundungerregenden schadlichkeiten. Leipzig, Engelmann.

Lentnek, A. L., Schreiber, A. D., and MacGregor, R. R. 1976. The induction of augmented granulocyte adherence by inflammation. Mediation by a plasma factor. J. Clin. Invest. 57: 1098-1103.

Lepow, I. H., and Leon, M. A. 1962. Interaction of a serum inhibitor of C1-esterase with intermediate complexes of the immune haemolytic system. I. Specificity of inhibition of C1 activity associated with intermediate complexes. Immunol. 5: 222-234.

Levy, L. R., and Lepow, I. H. 1959. Assay and properties of serum inhibitor of C'1-esterase. Proc. Soc. Exp. Biol. Med. 101: 608-611.

Lichtman, M. A., and Weed, R. I. 1972. Alteration of the cell periphery during granulocyte maturation. Relationship to cell function. Blood J. Hematol. 39: 301-315.

McCutcheon, M. 1946. Chemotaxis in leukocytes. Physiol. Rev. 26: 319-336.

McLeod, B., Baker, P., and Gewurz, H. 1975. Studies on the inhibition of C56-initiated lysis (reactive lysis). III. Characterization of the inhibitory activity C567-INH and its mode of action. Immunol. 28: 133-150.

Meier, R., and Schär, B. 1955. Leukocytenmigrations forderung durch antigen-antikorper reaktion in vitro. Experientia 11: 395-396.

Meier, R., and Schär, B. 1958. Ahnlichkeit und unterscheid leu kozytenemigrationsfordender wirkung grampositiver und gramnegativer bakterien. Experientia 14: 366-367.

Menkin, V. 1938. Studies on inflammation. J. Exp. Med. 67: 129-158.

Metchnikoff, E. 1893. Lectures on the comparative pathology of inflammation. Kegan Paul, London.

Miller, M. E., and Dooley, R. 1973. Deficient random mobility, normal chemotaxis and impaired phagocytosis in two sibs with pyoderma gangrenosum. Pediat. Res. 7: 137.

Miller, M. E., and Nilsson, U. R. 1970. A familial deficiency of the phagocytosis-enhancing activity of serum related to a dysfunction of the fifth component of complement (C5). New Engl. J. Med. 282: 354-358.

Miller, M. E., Norman, M. E., Koblenzer, P. J., and Schonauer, T. J. 1973. A new familial defect of neutrophil movement. J. Lab. Clin. Med. 82: 1-8.

Miller, M. E., Oski, F. A., and Harris, M. B. 1971. Lazy-leucocyte syndrome. A new disorder of neutrophil function. Lancet i: 665-669.

Mowat, A. G., and Baum, J. 1971. Chemotaxis of polymorphonuclear leukocytes from patients with diabetes mellitus. New Engl. J. Med. 284: 621-627.

Nagaki, K., Hashimoto, C., and Inai, S. 1976. The inactivator of the first component of human complement ($C\bar{1}INA$): The complex formation with plasmin. Int. Arch. Allergy 50: 1-13.

Nagaki, K., Iida, K., and Inai, S. 1974. The inactivator of the first component of human complement. The complex formation with the activated first component of human complement ($C\bar{1}$) or with its subcomponents. Int. Arch. Allergy 46: 935-948.

Nagaki, K., and Inai, S. 1976. Inactivator of the first component of human complement ($C\bar{1}INA$). Enhancement of $C\bar{1}INA$ activity against $C\bar{1}s$ by acidic mucopolysaccharides. Int. Arch. Allergy 50: 172-180.

Nelson, R. D., Quie, P. G., and Simmons, R. L. 1975. Chemotaxis under agarose: A new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. J. Immunol. 115: 1650-1656.

Nilsson, U. R., Mandle, R. J., Jr., and McConnell-Mapes, J. A. 1975. Human C3 and C5: Subunit structure and modifications by trypsin and $C\bar{4}2$-$C\bar{4}23$. J. Immunol. 114: 815-822.

Opferkuch, W., Snyderman, R., and Borsos, T. 1973. Generations of chemotactic activity by immune complexes carrying clustered or nonclustered $C\bar{4}2$ sites. Eur. J. Immunol. 3: 371-372.

Osther, K., and Linnemann, R. 1973a. Measurement of $C\bar{1}$ inactivator (alpha-2-neuraminoglycoprotein) on human blast cells in blast leukaemia. Acta Path. Microbiol. Scand., Section B 81: 271-272.

Osther, K., and Linnemann, R. 1973b. Immunofluorescence measurement of $C\bar{1}$ inactivator (alpha 2 neuraminoglycoprotein) activity of the surface of human carcinoma cells. Acta Path. Microbiol. Scand., Section B 81: 365-372.

Pensky, J., and Lepow, I. H. In press. Isolation of serum inhibitor of C'1a. In *Methods in Immunology and Immunochemistry*. C. A. Williams, M. W. Chase, eds. Academic Press, New York.

Pensky, J., and Schwick, H. G. 1969. Human serum inhibitor of C'1 esterase: Identity with α_2-neuraminoglycoprotein. Science 163: 698-699.

Perillie, P. E., and Finch, S. C. 1964. Quantitative studies of the local exudative cellular reaction in acute leukemia. J. Clin. Invest. 43: 425-430.

Ramseier, H. 1969. Quantitative studies on antigenic recognition. I. Immunological and nonimmunological parameters of the response. J. Exp. Med. 130: 1279-1294.

Ramsey, W. S. 1972a. Analysis of individual leucocyte behavior during chemotaxis. Exp. Cell Res. 70: 129-139.

Ramsey, W. S. 1972b. Locomotion of human polymorphonuclear leukocytes. Exp. Cell Res. 72: 489-501.

Ratnoff, O. D., and Lepow, I. H. 1957. Some properties of an esterase derived from preparations of the first component of complement. J. Exp. Med. 106: 327-343.

Ratnoff, O. D., Pensky, J., Ogston, D., and Naff, G. B. 1969. The inhibition of plasmin, plasma kallikrein, plasma permeability factor, and the Clr subcomponent of the first component of complement by serum C'1 esterase inhibitor. J. Exp. Med. 129: 315-331.

Rebuck, J. W., and Crowley, J. H. 1955. A method for studying leukocytic functions in vivo. Annals of the New York Academy of Sciences 59: 757-794.

Rent, R., Myhrman, R., Fiedel, B. A., and Gewurz, H. 1976. Potentiation of C1-esterase inhibitor activity by heparin. Clin. Exp. Immunol. 23: 264-271.

Rivkin, I., Rosenblatt, J., and Becker, E. L. 1975. The role of cyclic AMP in the chemotactic responsiveness and spontaneous motility of rabbit peritoneal neutrophils. The inhibition of neutrophil movement and the elevation of cyclic AMP levels by catecholamines, prostaglandings, theophylline and cholera toxin. J. Immunol. 115: 1126-1134.

Robineaux, R. 1964. Movements of cells involved in inflammation and immunity. In *Primitive Motile Systems in Cell Biology*. R. D. Allen and N. Kamiya, eds. Academic Press, New York.

Rocklin, R. E. 1974. Products of activated lymphocytes: Leukocyte inhibitory factor (LIF) distinct from migration inhibitory factor (MIF). J. Immunol. 112: 1461-1466.

Rosen, F. S., Charache, P., Pensky, J., and Donaldson, V. H. 1965. Hereditary angioneurotic edema: Two genetic variants. Science 148: 957-958.

Ruddy, S., Austen, K. F., and Goetzl, E. J. 1975. Chemotactic activity derived from interaction of factors D and B of the properdin pathway with cobra venom factor or C3b. J. Clin. Invest. 55: 587-592.

Ruutu, T., Ruutu, P., Vuopio, P., Franssila, K., and Linder, E. 1975. An inhibitor of chemotaxis and phagocytosis in reticulum cell sarcoma. Scand. J. Haematol. 15: 27-34.

Schiffmann, E., Corcoran, B. A., and Wahl, S. M. 1975. N-formylmethionyl peptides as chemoattractants for leucocytes. Proc. Nat. Acad. Sci. USA 72: 1059-1062.

Schiffmann, E., Showell, H. V., Corcoran, B. A., Ward, P. A., Smith, E., and Becker, E. L. 1975. The isolation and partial characterization of neutrophil chemotactic factors from *Escherichia coli*. J. Immunol. 114: 1831-1837.

Schreiber, A. D., Kaplan, A. P., and Austen, K. F. 1973a. Plasma inhibitors of the components of the fibrinolytic pathway in man. J. Clin. Invest. 52: 1394-1401.

Schreiber, A. D., Kaplan, A. P., and Austen, K. F. 1973b. Inhibition of ClINH of Hageman factor fragment activation of coagulation, fibrinolysis, and kinin generation. J. Clin. Invest. 52: 1402-1409.

Shin, H. S., Snyderman, R., Freidman, E., Mellors, A., and Mayer, M. M. 1968. Chemotactic and anaphylatoxic fragment cleaved from the fifth component of guinea pig complement. Science 162: 361-363.

Shin, H. S., Snyderman, R., Freidman, E., and Mergenhagen, S. E. 1969. Cleavage of guinea pig C3 by serum treated endotoxic lipopolysaccharide. Fed. Proc. 28: 485.

Singh, H., Boyd, E., Hutton, M. M., Wilkinson, P. C., Peebles-Brown, D. A., and Ferguson-Smith, M. A. 1972. Chromosomal mutation in bone marrow as cause of acquired granulomatous disease and refractory macrocytic anaemia. Lancet i: 873-879.

Smith, C. W., Hollers, J. C., Bing, D. H., and Patrick, R. A. 1975. Effects of human C1 inhibitor on complement-mediated human leukocyte chemotaxis. J. Immunol. 114: 216-220.

Smith, C. W., Hollers, J. C., Dupree, E., Goldman, A. S., and Lord, R. A. 1972. A serum inhibitor of leukotaxis in a child with recurrent infections. J. Lab. Clin. Med. 79: 878-885.

Snyderman, R., Gewurz, H., and Mergenhagen, S. E. 1968. Interactions of the complement system with endotoxic lipopolysaccharide. Generation of a factor chemotactic for polymorphonuclear leukocytes. J. Exp. Med. 128: 259-275.

Snyderman, R., and Mergenhagen, S. E. 1972. Characterization of polymorphonuclear leukocyte chemotactic activity in serums activated by various inflammatory agents. In *The Biological Activities of Complement*. Proceedings of the Fifth International Symposium of the Canadian Society for Immunology. D. G. Ingram, ed. S. Karger, Basel.

Snyderman, R., Phillips, J., and Mergenhagen, S. E. 1970. Polymorphonuclear leukocyte chemotactic activity in rabbit serum and guinea pig serum treated with immune complexes: Evidence for C5a as the major chemotactic factor. Infect. and Immun. 1: 521-525.

Snyderman, R., Phillips, J. K., and Mergenhagen, S. E. 1971. Biological activity of complement in vivo. Role of C5 in the accumulation of polymorphonuclear leukocytes in inflammatory exudates. J. Exp. Med. 134: 1131-1143.

Snyderman, R., Shin, H. S., and Dannenberg, A. M., Jr. 1972. Macrophage proteinase and inflammation: The production of chemotactic activity from the fifth component of complement by macrophage proteinase. J. Immunol. 109: 896-898.

Snyderman, R., Shin, H. S., Phillips, J. K., Gewurz, H., and Mergenhagen, S. E. 1969. A neutrophil chemotactic factor derived from C'5 upon interaction of guinea pig serum with endotoxin. J. Immunol. 103: 413-422.

Soriano, R. B., South, M. A., Goldman, A. S., and Smith, C. W. 1973. Defect of neutrophil motility in a child with recurrent bacterial infections and disseminated cytomegalovirus infection. J. Pediatr. 83: 951-958.

Spilberg, I., Mandell, B., and Wochner, R. D. 1974. Studies on crystal-induced chemotactic factor. I. Requirement for protein synthesis and neutral protease activity. J. Lab. Clin. Med. 83: 56-63.

Stecher, V. J., and Sorkin, E. 1969. Studies on chemotaxis. XII. Generation of chemotactic activity for polymorphonuclear leucocytes in sera with complement deficiencies. Immunol. 16: 231-239.

Stecher, V. J., and Sorkin, E. 1972. The chemotactic activity of fibrin lysis products. Int. Arch. Allergy 43: 879-886.

Steerman, R. L., Snyderman, R., Leikin, S. L., and Colten, H. R. 1971. Intrinsic defect of the polymorphonuclear leucocyte resulting in impaired chemotaxis and phagocytosis. Clin. Exp. Immunol. 9: 939-946.

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Tainer, J. A., Turner, S. R., and Lynn, W. S. 1975. New aspects of chemotaxis: Specific target-cell attraction by lipid and lipoprotein fractions of *Escherichia coli* chemotactic factor. Amer. J. Path. 81: 401-408.

Taubman, S. B., Goldschmidt, P. R., and Lepow, I. H. 1970. Effects of lysosomal enzymes from human leukocytes on human complement components. Fed. Proc. 29: 434.

Till, G., and Ward, P. A. 1975. Two distinct chemotactic factor inactivators in human serum. J. Immunol. 114: 843-847.

Tse, R. L., and Phelps, P. 1970. Polymorphonuclear leukocyte motility in vitro. V. Release of chemotactic activity following phagocytosis of calcium pyrophosphate crystals, diamond dust, and urate crystals. J. Lab. Clin. Med. 76: 403-414.

Tse, R. L., Phelps, P., and Urban, D. 1972. Polymorphonuclear leukocyte motility in vitro. VI. Effect of purine and pyrimidine analogues: Possible role of cyclic AMP. J. Lab. Clin. Med. 80: 264-274.

Turner, S. R., Campbell, J. A., and Lynn, W. S. 1975. Polymorphonuclear leukocyte chemotaxis toward oxidized lipid components of cell membranes. J. Exp. Med. 141: 1437-1441.

Turner, S. R., Tainer, J. A., and Lynn, W. S. 1975. Biogenesis of chemotactic molecules by the arachidonate lipoxygenase system of platelets. Nature (Lond) 257: 680-681.

Van Epps, D. E., Palmer, D. L., and Williams, R. C., Jr. 1974. Characterization of serum inhibitors of neutrophil chemotaxis associated with anergy. J. Immunol. 113: 189-200.

Van Royen, E. A., Kempes-Van Leeuwen, W., Voss, M., and Pondman, K. W. 1976. Blocking of C1 esterase inhibitor by cold promoted activation of factor VII. In *Protides of the Biological Fluids*. H. Peeters, ed. Pergamon Press, Oxford.

Venge, P., and Olsson, I. 1975. Cationic proteins of human granulocytes. VI. Effect on the complement system and mediation of chemotactic activity. J. Immunol. 115: 1505-1508.

Von Sicherer, O. 1899. Zur chemotaxis der leukocyten in vitro. Central Blatt fur Bakteriologie, Parasitenkunde und Infektionskrankheiten 26: 360.

Walker, J. R., Smith, M. J. H., Ford-Hutchinson, A. W., and Billimoria, F. J. 1975. Mode of action of an anti-inflammatory fraction from normal human plasma. Nature (Lond) 254: 444-446.

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Ward, P. A. 1966. Chemosuppression of chemotaxis. J. Exp. Med. 124: 209-225.

Ward, P. A. 1967. A plasmin-split fragment of C3 as a new chemotactic factor. J. Exp. Med. 126: 189-206.

Ward, P. A., and Becker, E. L. 1967. Mechanisms of the inhibition of chemotaxis by phosphonate esters. J. Exp. Med. 125: 1001-1020.

Ward, P. A., and Becker, E. L. 1968. The deactivation of rabbit neutrophils by chemotactic factor and the nature of the activatable esterase. J. Exp. Med. 127: 693-709.

Ward, P. A., and Becker, E. L. 1970a. Potassium reversible inhibition of leukotaxis by ouabain. Life Sci. 9: 355-360.

Ward, P. A., and Becker, E. L. 1970b. Biochemical demonstration of the activatable esterase of the rabbit neutrophil involved in the chemotactic response. J. Immunol. 105: 1057-1067.

Ward, P. A., and Berenberg, J. L. 1974. Defective regulation of inflammatory mediators in Hodgkin's disease. Supernormal levels of chemotactic factor inactivator. New Engl. J. Med. 290: 76-80.

Ward, P. A., Chapitis, J., Conroy, M. C., and Lepow, I. H. 1973. Generation by bacterial proteinases of leukotactic factors from human serum, and human C3 and C5. J. Immunol. 110: 1003-1009.

Ward, P. A., Cochrane, C. G., and Muller-Eberhard, H. J. 1965. The role of serum complement in chemotaxis of leukocytes in vitro. J. Exp. Med. 122: 327-346.

Ward, P. A., Cochrane, C. G., and Muller-Eberhard, H. J. 1966. Further studies on the chemotactic factor of complement and its formation in vivo. Immunol. 11: 141-153.

Ward, P. A., Cohen, S., and Flanagan, T. D. 1972. Leukotactic factors elaborated by virus-infected tissues. J. Exp. Med. 135: 1095-1103.

Ward, P. A., Data, R., and Till, G. 1974. Regulatory control of complement-derived chemotactic and anaphylatoxin mediators. In *Progress in Immunology II*, Vol. 1. L. Brent and J. Holborow, eds. North-Holland, Amsterdam.

Ward, P. A., and Hill, J. H. 1970. C5 chemotactic fragments produced by an enzyme in lysosomal granules of neutrophils. J. Immunol. 104: 535-543.

Ward, P. A., and Hill, J. H. 1972. Biologic role of complement products. Complement derived leucotactic activity extractable from lesions of immunologic vasculitis. J. Immunol. 108: 1137-1145.

Ward, P. A., Lepow, I. H., and Newman, L. J. 1968. Bacterial factors chemotactic for polymorphonuclear leukocytes. Amer. J. Path. 52: 725-736.

Ward, P. A., and Newman, L. J. 1969. A neutrophil chemotactic factor from human C5. J. Immunol. 102: 93-99.

Ward, P. A., Remold, H. G., and David, J. R. 1970. The production by antigen-stimulated lymphocytes of a leukotactic factor distinct from migration inhibitory factor. Cell. Immunol. 1: 162-174.

Ward, P. A., and Schlegel, R. J. 1969. Impaired leucotactic responsiveness in a child with recurrent infections. Lancet 1: 344-347.

Ward, P. A., and Talamo, R. C. 1973. Deficiency of the chemotactic factor inactivator in human sera with α_1-antitrypsin deficiency. J. Clin. Invest. 52: 516-519.

Ward, P. A., and Zvaifler, N. J. 1971. Complement-derived leukotactic factors in inflammatory synovial fluids of humans. J. Clin. Invest. 50: 606-616.

Ward, P. A., and Zvaifler, N. J. 1973. Quantitative phagocytosis by neutrophils. II. Release of the C5-cleaving enzyme and inhibition of phagocytosis by rheumatoid factor. J. Immunol. 111: 1777-1782.

Wasserman, S. I., Whitmer, D., Goetzl, E. J., and Austen, K. F. 1975. Chemotactic deactivation of human eosinophils by the eosinophil chemotactic factor of anaphylaxis. Proc. Soc. Exp. Biol. Med. 148: 301-306.

Weiss, A. S., Gallin, J. I., and Kaplan, A. P. 1974. Fletcher factor deficiency. A diminished rate of Hageman factor activation caused by absence of prekallikrein with abnormalities of coagulation, fibrinolysis chemotactic activity, and kinin generation. J. Clin. Invest. 53: 622-633.

Weksler, B. B., and Coupal, C. E. 1973. Platelet-dependent generation of chemotactic activity in serum. J. Exp. Med. 137: 1419-1430.

Weksler, B. B., and Hill, M. J. 1969. Inhibition of leucocyte migration by a staphylococcal factor. J. Bact. 98: 1030-1035.

Whur, P., Payne, N. E., and Koppel, H. 1974. Effect of protease and protease inhibitors on the adhesion of Ehrlich ascites tumour cells to plastic. Exp. Cell. Res. 86: 422-425.

Wilkinson, P. C. 1973. Recognition of protein structure in leukocyte chemotaxis. Nature (Lond) 244: 512-513.

Wilkinson, P. C. 1974a. Surface and cell membrane activities of leukocyte chemotactic factors. Nature (Lond) 251: 58-60.

Wilkinson, P. C. 1974b. *Chemotaxis and Inflammation*. Churchill Livingstone, Edinburgh.

Wilkinson, P. C. 1975. Inhibition of leukocyte locomotion and chemotaxis by lipid-specific bacterial toxins. Nature (Lond) 255: 485-487.

Wilkinson, P. C., and McKay, I. C. 1971. The chemotactic activity of native and denatured serum albumin. Int. Arch. Allergy 41: 237-247.

Wilkinson, P. C., and McKay, I. C. 1972. The molecular requirements for chemotactic attraction of leucocytes by proteins. Studies of proteins with synthetic side groups. Eur. J. Immunol. 2: 570-577.

Wilkinson, P. C., O'Neill, G. J., McInroy, R. J., Cater, J. C., and Roberts, J. A. 1973. Chemotaxis of macrophages: The role of a macrophage-specific cytotaxin from anaerobic corynebacteria and its relation to immunopotentiation in vivo. In *Ciba Foundation Symposium on Immunopotentiation*. G. E. Wolstenholme, J. Knight, eds. Associated Scientific Publishers, Amsterdam.

Williams, D., Esquenazi, V., Cirocco, R., and Jensen, J. A. 1976. The chemoattraction of neutrophils by heterologous and homologous cytotoxic sera. J. Immunol. 116: 554-561.

Wissler, J. H. 1972a. Chemistry and biology of the anaphylatoxin related serum peptide system. I. Purification, crystallization and properties of classical anaphylatoxin from rat serum. Eur. J. Immunol. 2: 73-83.

Wissler, J. H. 1972b. Chemistry and biology of the anaphylatoxin related serum peptide system. II. Purification, crystallization and properties of cocytotaxin, a basic peptide from rat serum. Eur. J. Immunol. 2: 84-89.

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Wissler, J. H., Stecher, V. J., and Sorkin, E. 1972a. Chemistry and bi-logy of the anaphylatoxin related serum peptide system. III. Evaluation of leucotactic activity and a property of a new peptide system with classical anaphylatoxin and cocytotaxin as components. Eur. J. Immunol. 2: 90-96.

Wissler, J. H., Stecher, V. J., and Sorkin, E. 1972b. Biochemistry and biology of a leucotactic binary serum peptide system related to anaphylatoxin. Int. Arch. Allergy 42: 722-747.

Wright, D. G., and Gallin, J. I. 1975. Inactivation of the chemotactic molecule C5a by products contained in granules of human polymorphonuclear leukocytes (PMN's) and released during phagocytosis. Fed. Proc. 34: 1019.

Yamamoto, S., Yoshinaga, M., and Hayashi, H. 1971. The natural mediator for PMN emigration in inflammation. II. Common antigenicity of leucoegresin with immunoglobulin G. Immunol. 20: 803-808.

Yoshida, T., Cohen, S., Bigazzi, P. E., Kurasujii, T., and Amsden, A. 1975. Inflammatory mediators in culture filtrates of *Escherichia coli*. Amer. J. Path. 81: 389-400.

Yoshida, K., Yoshinaga, M., and Hayashi, H. 1968. Leucoegresin. A factor associated with leucocyte emigration in Arthus lesions. Nature (Lond) 218: 977-978.

Yoshinaga, M., Yamamoto, S., Kiyota, S., and Hayashi, H. 1972. The natural mediator for PMN emigration in inflammation. IV. In vitro production of a chemotactic factor by papain from immunoglobulin G. Immunol. 22: 393-399.

Yoshinaga, M., Yamamoto, S., Maeda, S., and Hayashi, H. 1971a. The natural mediator for PMN emigration in inflammation. III. In vitro production of a chemotactic factor by inflammatory SH-dependent protease from serum immunoglobulin G. Immunol. 20: 809-815.

Yoshinaga, M., Yoshida, K., Tashiro, A., and Hayashi, H. 1971b. The natural mediator for PMN emigration in inflammation. I. Purification and characterization of leucoegresin from Arthus skin site. Immunol. 21: 281-298.

Zigmond, S. H. 1974. Mechanisms of sensing chemical gradients by polymorphonuclear leukocytes. Nature (Lond) 249: 450-452.

Zigmond, S. H., and Hirsch, J. G. 1972. Effects of cytochalasin B on polymorphonuclear leucocyte locomotion, phagocytosis and glycolysis. Exp. Cell. Res. 73: 383-393.

Zigmond, S. H., and Hirsch, J. G. 1973. Leukocyte locomotion and chemotaxis. New methods for evaluation, and demonstration of a cell-derived chemotactic factor. J. Exp. Med. 137: 387-410.

[1] T_h

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[3] T_c

ARTICLE 1

EFFECTS OF HUMAN $C\bar{1}$ INACTIVATOR ON COMPLEMENT AND NON-COMPLEMENT MEDIATED HUMAN NEUTROPHIL CHEMOTAXIS[1]

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[2]D. Y. Liu was supported as a Predoctoral Trainee (GM-0191108), National Institutes of Health. Taken in part from a dissertation submitted by David Y. Liu to Michigan State University in partial fulfillment of the requirements for the Ph.D. degree.

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ABSTRACT

The presence of purified human $\overline{C1}$ inactivator (ClINH) with either mixed blood leukocytes or isolated polymorphonuclear leukocytes from humans resulted in significant inhibition (44 to 94%) of chemotaxis to human C3a and human or guinea pig C5a. C3-derived chemotactic activity was generated from purified human C3 with either trypsin or $EAC4^{oxy}2$. C5a was generated from purified human C5 with trypsin or isolated from LPS-activated guinea pig serum as the 15,000 molecular weight fraction. When utilizing N-formylmethionyl-phenylalanine as the cytotaxin, ClINH caused substantial enhancement (183% $\pm$ 46) of neutrophil chemotaxis. Well-defined conditions for ClINH participation in either enhancement or inhibition is now clearly established so that the basic mechanism of ClINH function can be further elucidated.

INTRODUCTION

Plasma contains a class of α-globulins which have classically been shown to contain inhibitors of proteolytic enzymes. These protease inhibitors are believed to control the action of several multicomponent enzyme systems that mediate blood coagulation, dissolution of blood clots, kinin production, and complement-associated activities. ClINH possesses a broad inhibitory specificity for many active enzymes functioning within these systems. These include $\overline{C1}$ and the subcomponents $\overline{C1}s$ and $\overline{C1}r$ (1-4), kallikrein (3), PF/dil (3), plasmin (3), Hageman Factor (5), active Hageman Factor fragments (6), and plasma thromboplastin antecedent (5).

Fragments of C3 and C5 (C3a and C5a) have been reported to be chemotactic for polymorphonuclear leukocytes (7,8). The biologically active cleavage products are presumably generated in plasma via activation of complement by LPS, Ag-Ab complexes, zymosan, and other agents (9,10).

Recent reports have demonstrated that ClINH and other plasma inhibitors affect the migration of human neutrophils (11,12) and guinea pig macrophages (13). Smith et al. (11) demonstrated that human ClINH affected the migration of human polymorphonuclear leukocytes in modified Boyden chambers. Results indicated that human ClINH under certain circumstances increased the chemotactic response of human peripheral blood leukocytes in vitro to activated plasma or serum. In preliminary experiments utilizing trypsin-activated C3 as a chemotactic source, ClINH decreased the chemotactic response of human leukocytes.

These studies have been extended in this report to show the effect of ClINH on human neutrophils responding to cytotaxins generated from purified human C3 and C5, and to N-formylmethionyl-phenylalanine. Evidence is presented to show that ClINH causes marked inhibition of the neutrophil chemotactic response to C3a and C5a. Moreover, these results are confirmed by the use of isolated guinea pig C5a generated in whole serum. Additional experiments designed to clarify the conditions of ClINH-mediated enhancement of chemotaxis are also reported here. By use of the potent cytotaxin, N-formylmethionylphenylalanine (14) we present evidence that ClINH can cause a substantial enhancement of the chemotactic response of migrating human neutrophils in a plasma-free system.

MATERIALS AND METHODS

Materials: All chemicals used were reagent or analytical grade and were obtained from the following sources: ATEE[4] from Aldrich Chemical Company, Milwaukee, Wisconsin; BSA, trypsin, and Ficoll (400,000 mol. wt.) from Sigma Chemical Co., St. Louis, Missouri; SBTI from Calbiochem, La Jolla, California; SDS from Pierce Chemical Co., Rockford, Illinois; *Salmonella typhimurium* LPS from Difco Laboratories, Detroit, Michigan; HBSS with phenol red from Grand Island Biological Co., Grand Island, New York; sodium diatrizoate (Hypaque) from Winthrop Laboratories, New York, New York; Sephadex from Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey; DEAE-cellulose from Schleicher and Schuell, Inc., Keene, New Hampshire; Hypatite C from Clarkson Chemical Company, Inc., Williamsport, Pennsylvania; Dowex AG2-X10 from Bio-Rad Laboratories, Richmond, California; and NFMP from Andrulis Chemical Co., Bethesda, Maryland.

Methods: Human C1INH was prepared according to the method of Pensky and Lepow (15). Briefly, this procedure involved ammonium sulfate precipitation of fresh human serum followed by sequential chromatography of the supernatant on Dowex AG2-X10, DEAE-cellulose and Hypatite C (modified hydroxylapatite). C1INH was also purified

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[4]ATEE, N-acetyl-L-tyrosine ethyl ester; BSA, bovine serum albumin; LPS, lipopolysaccharide; Try, trypsin; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; HBSS, Hanks' balanced salt solution; NFMP, N-formylmethionylphenylalanine; PMN, polymorphonuclear neutrophils; C3(Try), trypsin-activated C3; C5(Try), trypsin-activated C5; C3(EAC4), EAC4-treated C3; C3(EAC4^{oxy}2), EAC4^{oxy}2-activated C3; C5a(LPS), LPS-activated guinea pig serum; hpf, high-powered field.

from crude ClINH generously donated by the American National Red Cross, Bethesda, Maryland. The highly purified preparation demonstrated a major and a minor protein band on SDS-polyacrylamide (7% gel) electrophoresis (16) consistent with the band pattern recently reported by Harpel and Cooper (17). Isoelectric focusing (18,19) revealed one broad band when performed in a pH gradient 3.5-10 with an apparent pI of 4.0. The specific activity of the ClINH was 180 inhibitor ATEE units/$OD_{280\ nm}$ (1). The protein content of the ClINH was 330 µg/ml. In addition to the esteratic assay, ClINH was quantitated hemolytically with human complement components according to Gigli et al. resulting in a specific activity of 133 U/µg (4). Monospecific-goat antiserum against ClINH was a generous gift of Dr. Chester Alper, Center for Blood Research, Harvard Medical School, Boston, Massachusetts. This antiserum was utilized for immunochemical quantitation by radial immunodiffusion analysis (20). Antiserum against whole human serum revealed one precipitin arc in the α_2 region in immunoelectrophoresis. Less homogeneous preparations of ClINH (60-100 U/OD) showing three bands in polyacrylamide gel electrophoresis behaved identically to highly purified ClINH in chemotaxis experiments. Unless otherwise noted, ClINH was used in chemotaxis assays at a concentration of 7 ATEE units/ml. Human C3 and C5 were prepared according to published procedures (21,22) and according to Dr. B. F. Tack (personal communication). The Lowry procedure (23) was used to determine protein content with bovine serum albumin as the reference standard. Dilute protein solutions were quantitated spectrophotometrically according to Murphy and Kies (24).

Generation of Cytotaxin: The generation of chemotactic activity from serum or purified complement components was performed in the following manner. One hundred micrograms per milliliter of human C3 was treated with 1% trypsin (w/w) for 90 sec at 30°C, pH 7.4, and the reaction stopped by addition of 3% SBTI. $EAC4^{oxy}2$ was prepared as described by Muller-Eberhard et al. (25). A cell button of 2×10^9 $EAC4^{oxy}2$ was incubated with 1 mg human C3 (1 ml) for 60 min at 37°C. The cells were removed by centrifugation and the supernatants were tested for chemotactic activity. One hundred micrograms per milliliter of human C5 was treated with 1% trypsin for 25 min at 30°C followed by addition of 3% SBTI. Guinea pig C5a was isolated from serum according to the procedure of Snyderman et al. (26). Eight milliliters of guinea pig serum was incubated with 800 µgLPS for 30 min at 37°C, followed by 30 min at 56°C and subsequent chromatography on Sephadex G-75 (5.0 x 10 cm). Eight milliliters of serum without LPS treatment was subjected to the same incubation and gel filtration conditions (sham control).

Chemotaxis: Granulocyte-rich leukocyte preparations were obtained by dextran sedimentation of heparinized venous blood as previously described (27). The leukocyte-rich plasma was diluted in HBSS and 5 ml aliquots were layered on 4 ml of 6.3% Ficoll - 9.9% Hypaque cushions in siliconized tubes and centrifuged at 800 x G for 30 min at room temperature (28). Cell buttons from this centrifugation containing approximately 95% neutrophils were resuspended in HBSS made 0.5-2% BSA or 10% plasma at 2.0×10^6 cells/ml for use in the modified Boyden assay. Acrylic blind well (.2 ml) chemotaxis

chambers (Neuro Probe, Inc., Bethesda, Maryland) were assembled with Millipore filters (Millipore Corporation, Bedford, Massachusetts) of 3 μm pore size. Following incubation of the chambers the filters were fixed in methanol and stained with Mayer's hematoxylin and eosin. The filters were dehydrated with increasing concentrations of ethanol, cleared with xylene, and mounted on glass slides with Permount. The chemotactic response assessed in triplicate chambers was evaluated by the number of cells accumulating on the lower surface on the filter after a 3 hr incubation at 37°C in an atmosphere containing 5% CO_2 and 95% humidity. Ten 400X fields were counted on each filter and the results expressed as the percentage of positive control. The distribution of cells throughout the filter was counted and plotted as described by Zigmond and Hirsch (29).

The Student's t test was used with $\alpha=.05$ considered significant. Standard deviations ($\pm$ 1 s.d.) were based on the variability of counting ten 400X fields.

RESULTS

Inhibition of C3a-mediated chemotaxis by ClINH: The chemotactic response of human neutrophils to C3(Try) was markedly inhibited when ClINH was added with the neutrophils in the upper compartment of chemotaxis chambers (Figure 1). This inhibitory effect was observed whether mixed blood leukocytes were suspended in 10% homologous plasma (Figure 1A) or isolated PMN were supported in 0.5% BSA (Figure 1B). Under these conditions and in the presence of ClINH the chemotactic responses to C3(Try) were 7% $\pm$ 8 ($\pm$ 1 s.d., $p<.001$) and 56% $\pm$ 6 ($p<.05$) respectively when compared with the cell responses in the

Figure 1. Effect of ClINH on the chemotactic response of neutrophils to C3(Try). A and B columns represent experiments with mixed blood leukocytes (no Ficoll-Hypaque centrifugation) in 10% plasma and PMN in 0.5% BSA in the upper chamber, respectively; A and B columns and brackets are the mean $\pm$ 1 s.d. for five experiments with three different neutrophil donors; Mean control (100%) chemotaxis for A and B was 70 and 32 neutrophils/hpf, respectively; ClINH (7 units/ml), hatched columns; No ClINH, open columns.

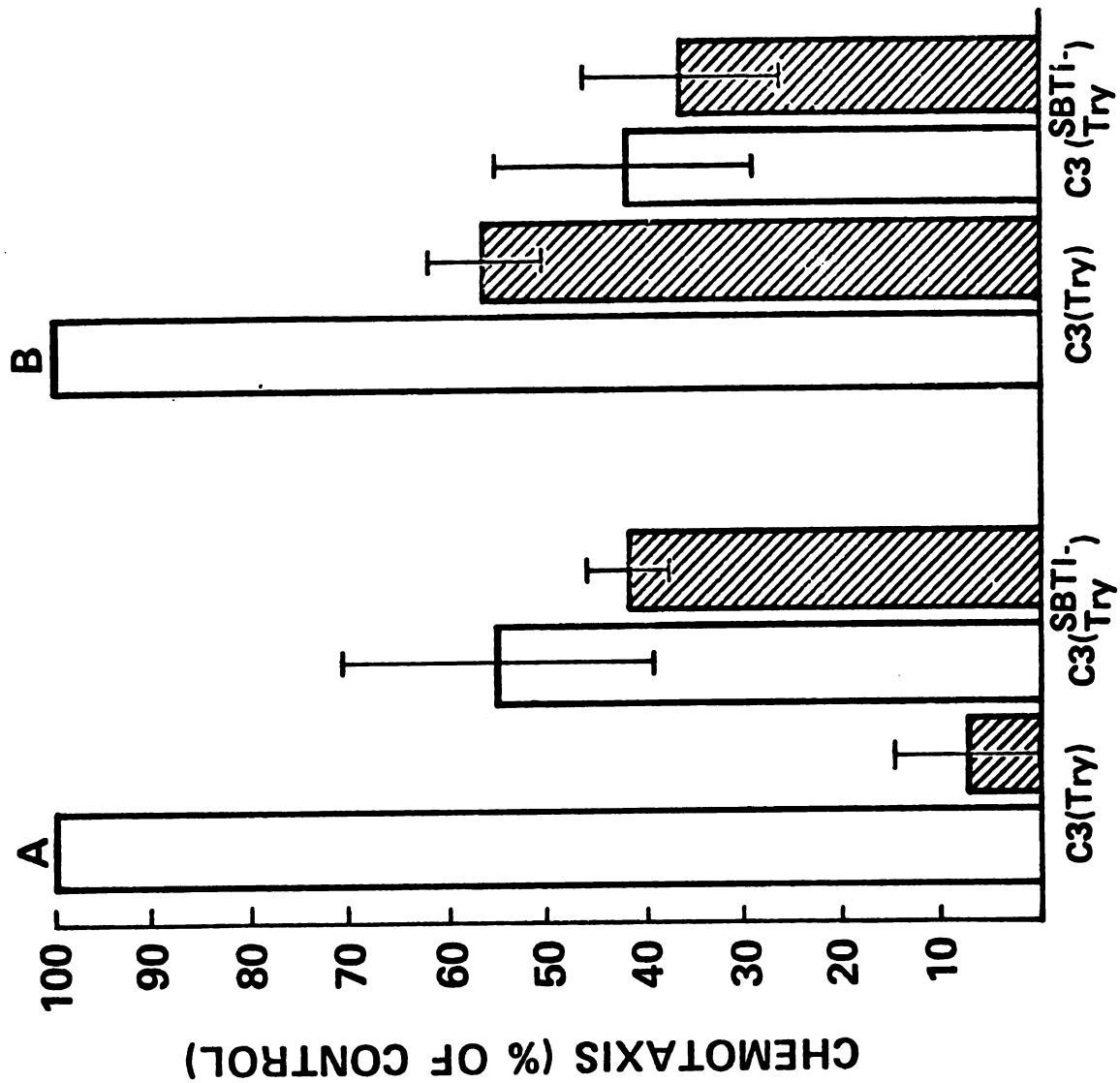

CHEMOTAXIS (% OF CONTROL)
100
90
80
70
60
50
40
30
20
10
A
B
C3(Try)
C3(SBTI-Try)
C3(Try)
C3(SBTI-Try)

Figure 1

absence of ClINH. That trypsin activation of C3 effectively generated chemotactic activity was ascertained by comparison of the PMN response to C3 and SBTI-inactivated trypsin. It should be noted that in the plasma support system ClINH inhibition (7% ± 8 of control) reduced the chemotactic response below ($p<.001$) that obtained with C3 treated with SBTI-inactivated trypsin (41% ± 4).

Substantial inhibition also occurred when $EAC4^{oxy}2$ was used to generate chemotactic activity from C3 (Figure 2). In this instance ClINH decreased the cell response to 35% ± 38 ($p<.01$) of positive control. That $EAC4^{oxy}2$ effectively generates cytotaxin is indicated by control chambers containing EAC4-treated C3. ClINH had no significant effect on this baseline response ($p>.05$).

Upon varying the concentration of ClINH present with the PMN, maximum inhibition occurred at 5 and 7 U/ml and marked inhibition was still seen at 1 U/ml.

Inhibition of C5a-mediated chemotaxis by ClINH: Results shown in Figure 3 indicate that the chemotactic response of neutrophils to human C5(Try) was also inhibited by ClINH. Inhibition was found whether mixed blood leukocytes in 10% plasma (Figure 3A) or isolated PMN in 0.5% BSA (Figure 3B) were used in chemotaxin chambers. Under these conditions and in the presence of ClINH, inhibition of C5(Try)-mediated chemotaxis was 29% ± 25 of control ($p<.001$) and 54% ± 21 of control ($p<.001$) respectively. ClINH did not significantly affect the baseline control ($p>.20$).

Results of confirmatory experiments with guinea pig C5a are shown in Figure 4. The chemotactic response of purified neutrophils

Figure 2. Effect of ClINH on the chemotactic response of neutrophils to C3($EAC4^{oxy}2$). Each column and bracket is the mean $\pm$ 1 s.d. for two experiments with different donors in each experiment; Mean control (100%) chemotaxis was 60 neutrophils/hpf; ClINH (7 units/ml), hatched columns; No ClINH, open columns; C3($EAC4^{oxy}2$) represents $EAC4^{oxy}2$ treatment of human C3; C3 represents treatment of human C3 with EAC4; see Materials and Methods.

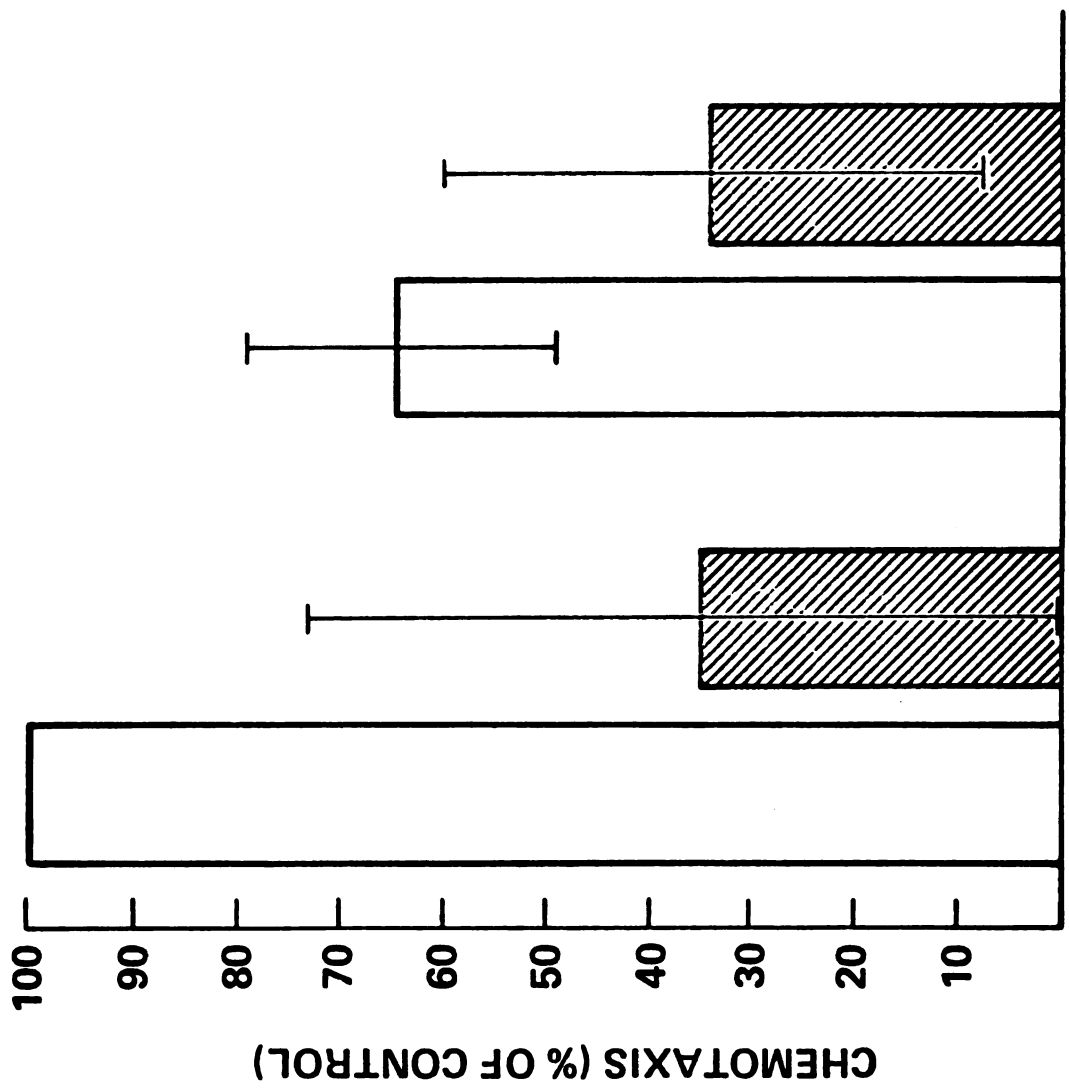
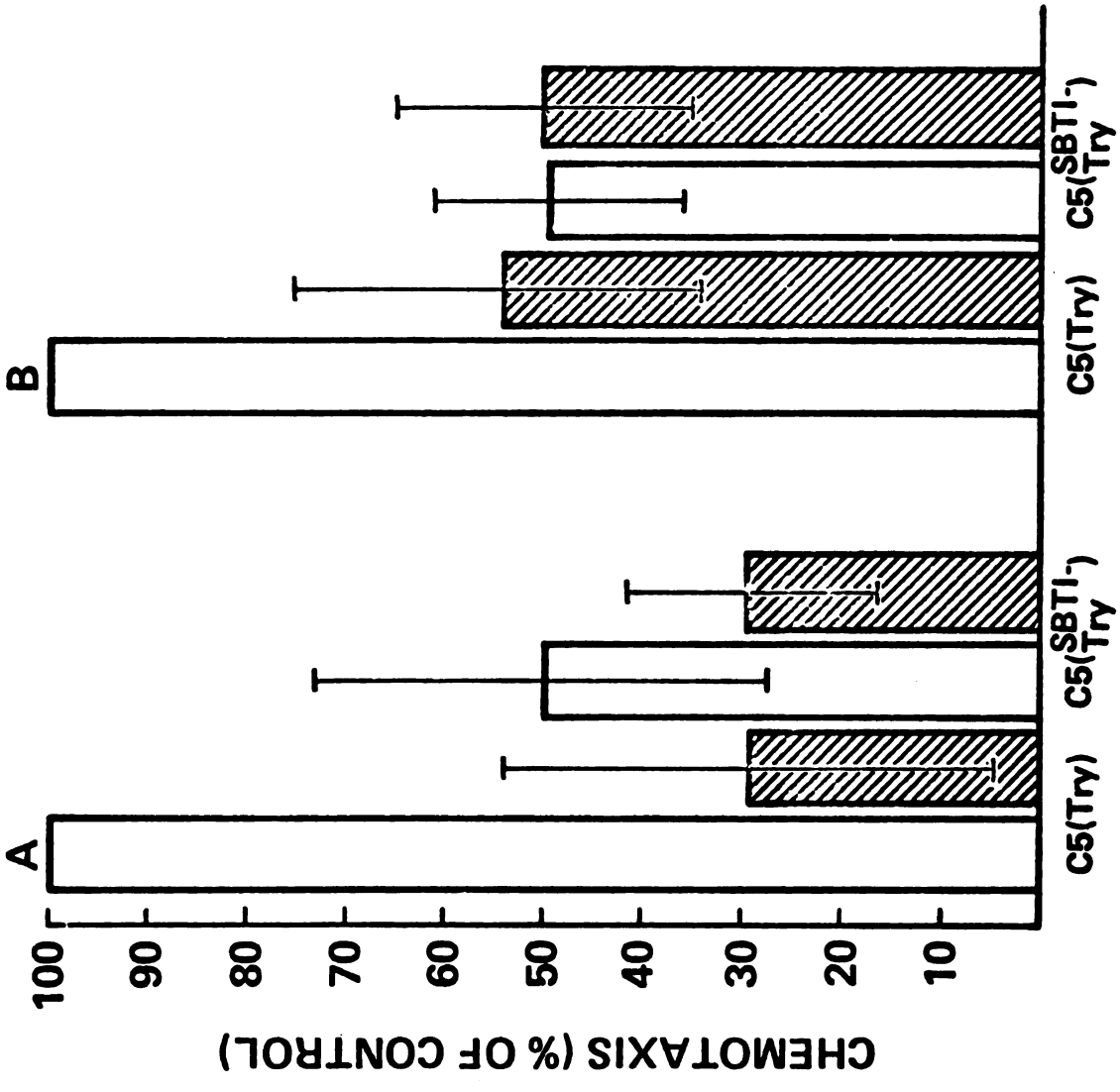

CHEMOTAXIS (% OF CONTROL)
100
90
80
70
60
50
40
30
20
10
C3(EAC4)
C3(EAC4oxy2)

Figure 2

Figure 3. Effect of ClINH on the chemotactic response of neutrophils to human C5(Try). A and B columns represent experiments with mixed blood leukocytes in 10% plasma and PMN in 1% BSA in the upper chamber, respectively; A and B columns and brackets are the mean $\pm$ 1 s.d. for eight experiments with four different neutrophil donors; Mean control (100%) chemotaxis for A and B was 22 and 85 neutrophils/hpf, respectively; ClINH (7 units/ml), hatched columns; No ClINH, open columns.


CHEMOTAXIS (% OF CONTROL)
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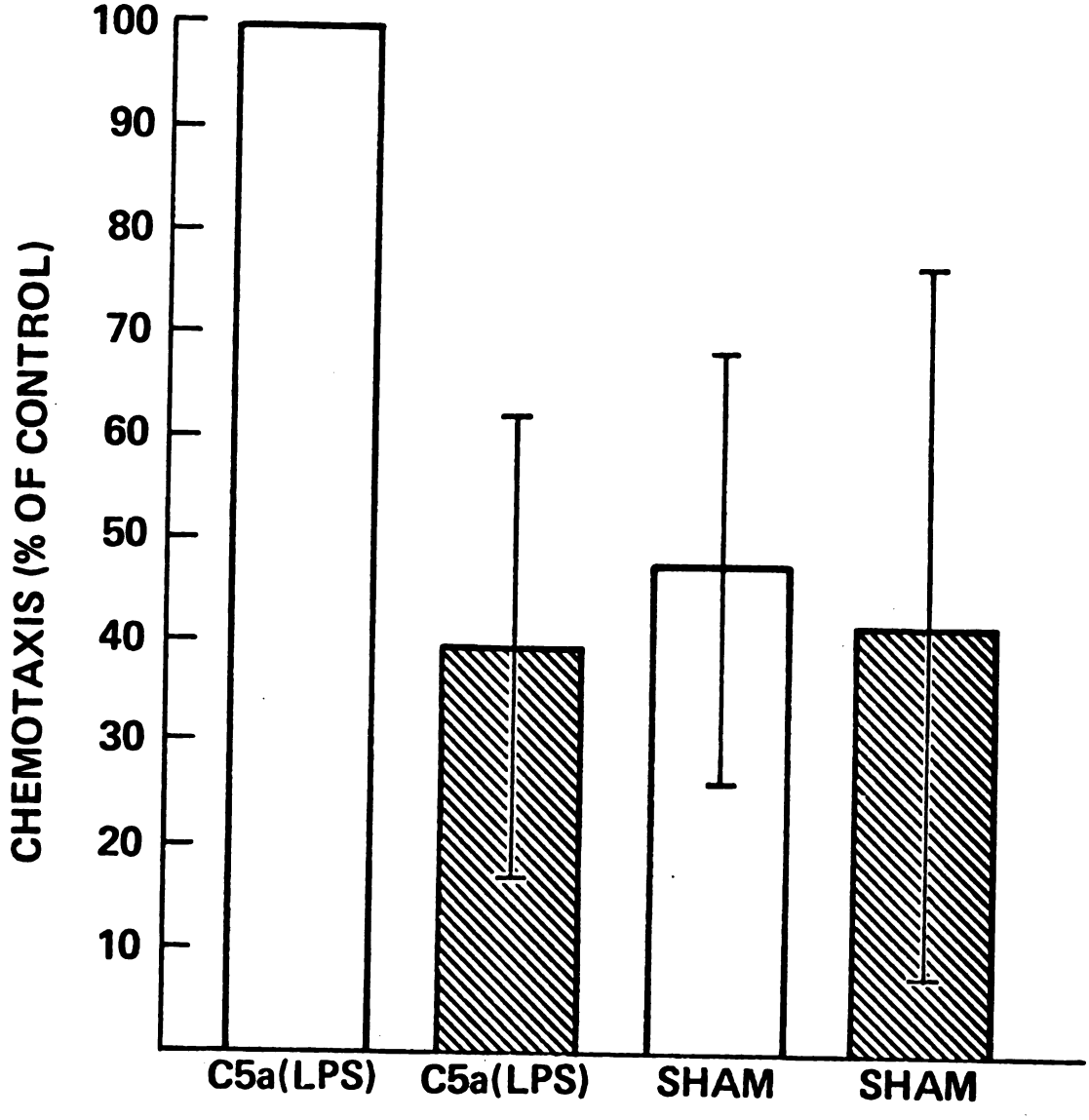
A
B
C5(Try)
C5(SBTI-Try)
C5(Try)
C5(SBTI-Try)

Figure 3

Figure 4. Effect of ClINH on the chemotactic response of neutrophils to guinea pig C5a. Each column and bracket is the mean $\pm$ 1 s.d. for three experiments with different donors in each experiment; Mean control (100%) chemotaxis was 23 neutrophils/hpf; ClINH (7 units/ml), hatched columns; No ClINH, open columns; PMN were suspended in 2% BSA; Sham represents 15,000 molecular weight fractions from G-75 Sephadex gel filtration of guinea pig serum previously heated at 37°C for 30 min and then 56°C for 30 min; see Materials and Methods.


CHEMOTAXIS (% OF CONTROL)
100
90
80
70
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40
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20
10
C5a(LPS)
C5a(LPS)
SHAM
SHAM

Figure 4

to 0.2 ml of the C5a preparation (3 µg/ml protein) was significantly inhibited (40% ± 22 of control, $p<.001$) by ClINH. The 15,000 molecular weight fraction of untreated guinea pig serum (sham control) was not significantly affected by ClINH ($p>.20$). Another preparation of highly purified ClINH obtained as a generous gift from Dr. Jack Pensky (Department of Medicine, Case Western Reserve School of Medicine) gave similar results.

The dose response of ClINH-mediated inhibition of C5(Try) chemotaxis was similar to that found with C3(Try), i.e., maximum inhibition (96%) at 5 and 7 U/ml with substantial inhibition (40%) observed at 1 U/ml.

Cell distribution of ClINH-mediated inhibition of C3(Try) chemotaxis: A comparison of the migrating cell distribution in micropore filters in the absence and presence of ClINH was performed (Figure 5). Mixed blood leukocytes responding to C3(Try) at 45', 90', and 135' were quantitated in 10 micron increments. The resulting cell distributions clearly show that at each time interval (45', 90', or 135') ClINH was associated with a greater number of cells near the top of the filter. In addition ClINH was associated with shorter migration distances at all time intervals. The migration distance of the two leading neutrophils (29) at 45', 90', and 135' in the absence of ClINH was 100 µm, 120 µm, and 130 µm respectively. The penetration distance in the presence of ClINH was diminished to 70 µm, 80 µm, and 90 µm respectively.

Figure 5. Distribution of leukocytes through the Millipore filter. Each point represents the mean of 4 fields counted, 2 on each of 2 duplicate filters; Chemotactic substance in the lower compartment was C3(Try); Incubation time, 45 min, ■—■—■ ; 90 min, ▲—▲—▲ ; 135 min, ●—●—● ; No ClINH in upper compartment, - - - - ; ClINH (7 units/ml), ————.

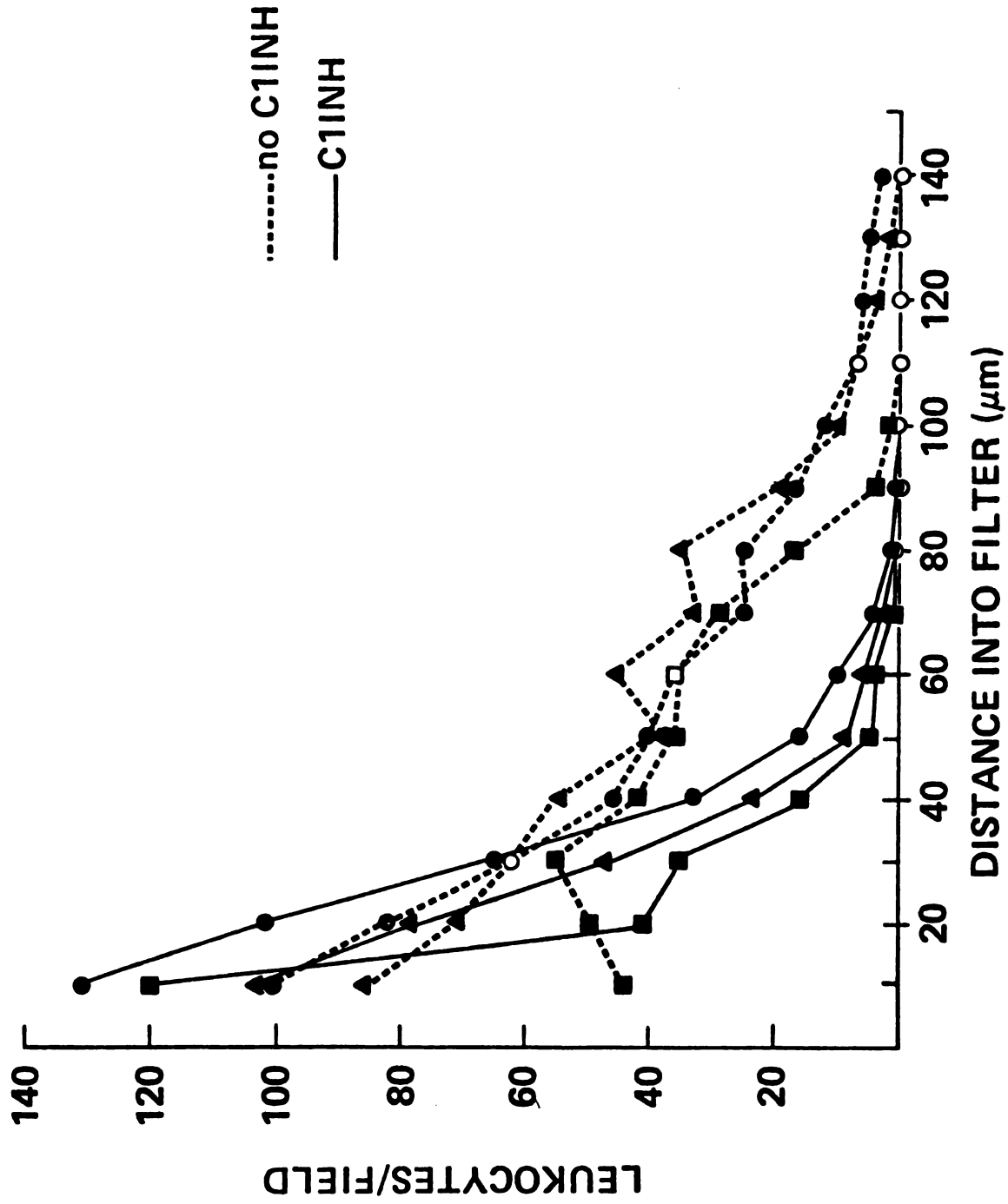
.....no C1INH
—C1INH
LEUKOCYTES/FIELD
140
120
100
80
60
40
20
20
40
60
80
100
120
140
DISTANCE INTO FILTER (μm)

Figure 5

Effect of ClINH on the chemotactic response of PMN to NFMP: The effect of ClINH on the PMN chemotactic response to N-formyl-methionylphenylalanine is shown in Figure 6. After establishing that potent chemotactic activity for human neutrophils was demonstrable with 10^{-6}M NFMP, ClINH was placed with the PMN in chemotaxis chambers and the response quantitated. Figure 6 is the result of five experiments utilizing four cell donors. It can be seen that ClINH caused a marked (183% $\pm$ 46 of control, $p<.001$) enhancement of chemotaxis (Figure 6A). Similar results were also obtained with the preparation of ClINH donated by Dr. Jack Pensky.

The concentration of ClINH causing significant enhancement was ascertained. ClINH at 8, 6, 4, and 2 U/ml were added with the PMN in chambers of cells responding to 10^{-6}M NFMP. A sharp decline in enhancement activity to baseline levels occurred between 8 and 6 U/ml of ClINH.

In order to evaluate the reversibility of ClINH-mediated enhancement, ClINH was incubated 30'/37°C with the PMN which were then washed 3X with HBSS and assayed in chemotaxis chambers. The results shown in Figure 6B indicate that a 2.7-fold enhancement ($p<.01$) of chemotaxis was diminished to levels insignificantly ($p>.20$) different from baseline levels due to the washing procedure.

Cell distribution of ClINH-mediated enhancement of NFMP chemotaxis: An analysis of the distribution of migrating neutrophils in the absence and presence of ClINH in micropore filters is shown in Figure 7. PMN responding to NFMP at 90', 135', and 210' were quantitated in 10 micron increments. The distribution profiles

Figure 6. Effect of ClINH on the chemotactic response of neutrophils to NFMP. A and B columns and brackets are the mean $\pm$ 1 s.d. for five experiments with four different neutrophil donors; Mean control (100%) chemotaxis for A and B was 36 and 13 neutrophils/hpf, respectively; ClINH (7 units/ml), hatched columns; No ClINH, open columns; PMN were suspended in 2% BSA: columns B3 represent experiments which utilized PMN that had been preincubated 30'/37°C with ClINH and washed 3X with HBSS.

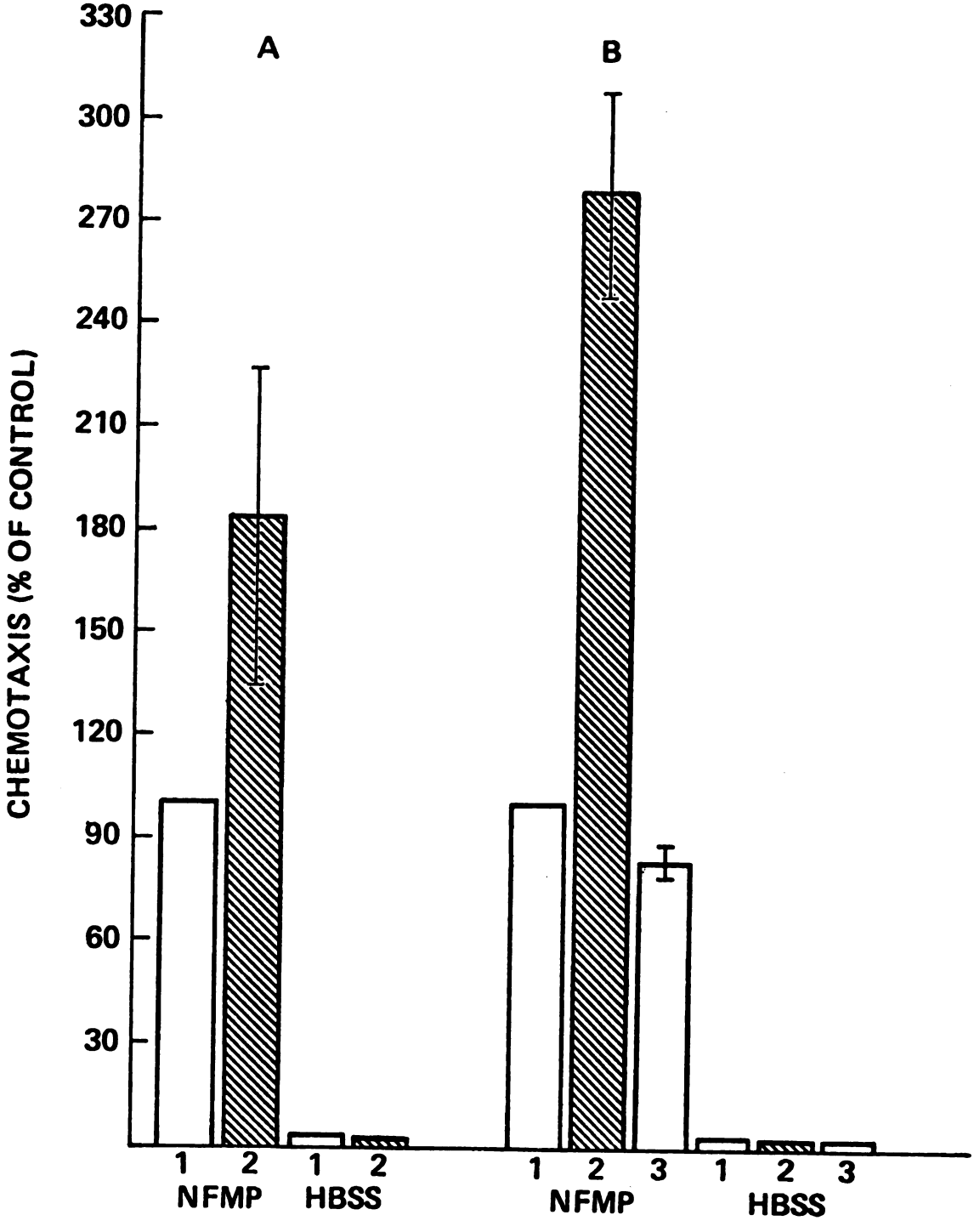
330
300
270
240
210
180
150
120
90
60
30
CHEMOTAXIS (% OF CONTROL)
A
B
1 2 1 2
NFMP HBSS
1 2 3 1 2 3
NFMP HBSS

Figure 6

Figure 7. Distribution of PMN through the Millipore filter. Chemotactic substance in the lower chamber was NFMP; Each point represents the mean of 4 fields counted, 2 on each of 2 duplicate filters; Incubation time, 90 min, ■—■—■ ; 135 min, ▲—▲—▲ ; 210 min, ●—●—● ; No ClINH in upper compartment, - - - - ; ClINH (7 units/ml), ————.

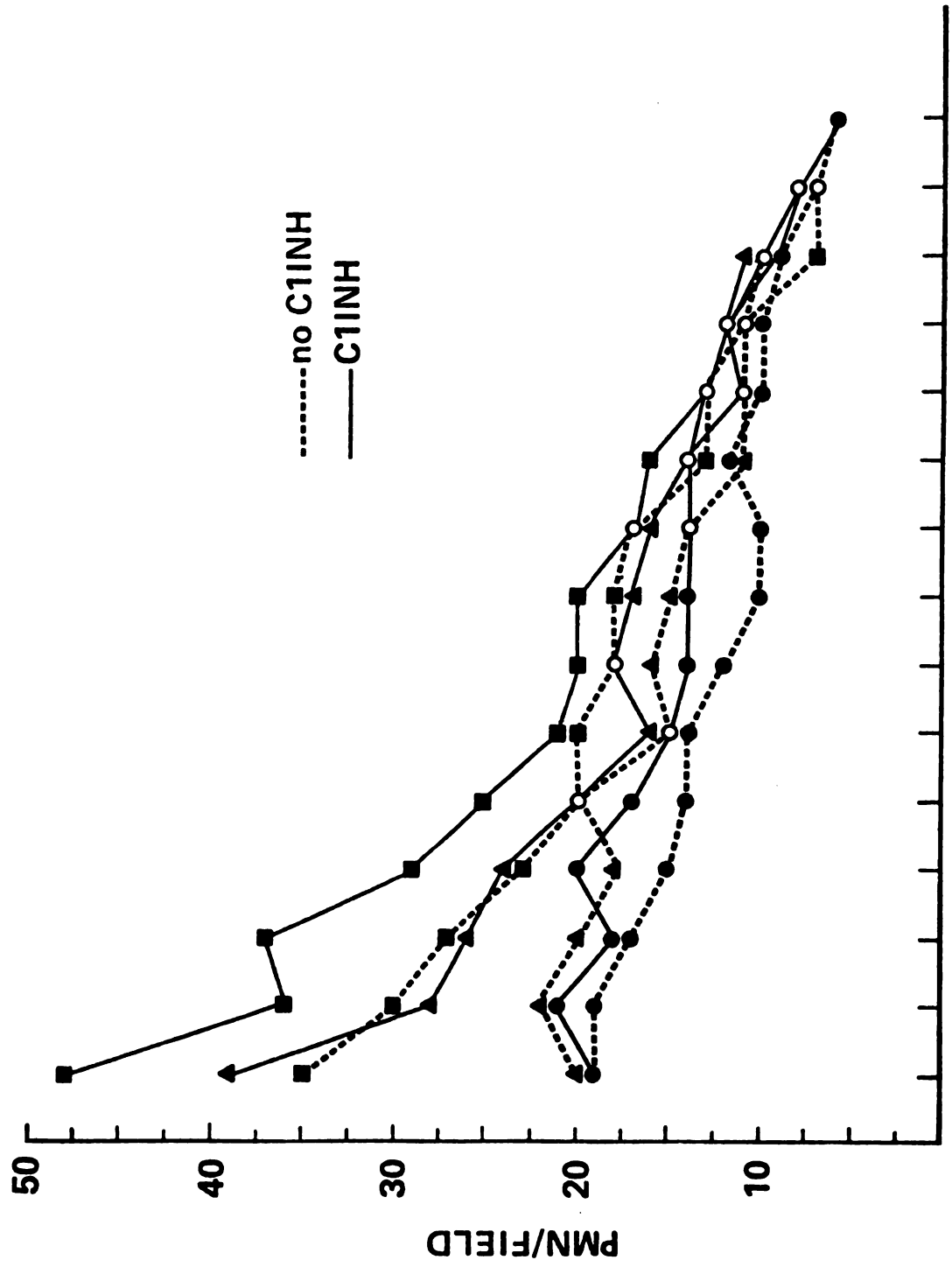
no C1INH
C1INH
PMN/FIELD
50
40
30
20
10
20
40
60
80
100
120
140
DISTANCE INTO FILTER (μm)

Figure 7

revealed a significantly higher number of cells in a zone nearest the upper surface at earlier time intervals (90' and 135') when ClINH was present with PMN. The cell distributions also suggest that at each time interval the number of PMN throughout the filter was increased in the presence of ClINH.

DISCUSSION

Considering the data in this report and in previous reports, the following interpretation seems plausible: human ClINH at physiologic serum concentrations (4) alters the response of human neutrophils to several cytotaxins by a direct effect on the cell. The effect does not require other leukocytes since it occurs with a purified neutrophil population and it occurs in the absence of other plasma or serum factors. However, the nature of the effect seems to depend on the specific cytotaxin and on the conditions under which the cell encounters ClINH and/or cytotaxin.

ClINH and other plasma protease inhibitors have been shown to affect leukocytes. Smith et al. (11) reported that human ClINH enhances the chemotactic response of human leukocytes to LPS, AgAb complex and zymosan-activated plasma and inhibits the response to C3(Try) but does not by itself influence spontaneous motility. Goetzl (12) found that ClINH causes a transient, slight enhancement of the neutrophil response to trypsin-activated C5 without any effect on random migration. Remold and Rosenberg (13) described an effect of ClINH on the migration of guinea pig macrophages. These workers showed that ClINH enhances the response of macrophages to migration inhibitory factor. Hollers et al. (30) demonstrated that

ClINH prevents and reverses the "deactivation" of human neutrophils caused by zymosan-activated plasma filtrate. These data may ultimately be related to the role of cell-associated serine esterases in cell function. Indeed, much data support a role for esterases in such cell functions as histamine (31), SRS-A (31) and ECF-A (32) release from human lung fragments, serotonin release from platelets (33), erythrophagocytosis (34), neutrophil chemotaxis (35), lysosomal enzyme release (36), and baby hamster kidney cell attachment to a substratum (37). Becker (35) described the activation of a cell-bound esterase essential for rabbit neutrophil chemotaxis as the result of a direct action of cytotaxins on the cell. However, a direct association between the effects of the naturally occurring plasma protease inhibitors and cell-associated serine esterases remains to be demonstrated. Preliminary studies undertaken to test such a possibility (Patrick and Becker, unpublished results) failed to demonstrate any effect of ClINH on the action of or the activation of esterase 1 on rabbit neutrophils.

The chemotactic response of human neutrophils to C3(Try) and NFMP was not only analyzed in terms of the number of cells that had migrated to the bottom of the filter but also in terms of the cell distribution throughout the filter. At early time intervals (45'-135') a significantly higher number of cells in the presence of ClINH migrated into the filter. Furthermore, neutrophils responding to C3(Try) exhibited a shallower penetration in the presence of ClINH than in the absence of ClINH. PMN responding to NFMP in the presence of ClINH demonstrated higher levels distributed throughout the filter than in the absence of ClINH. Such an analysis (29)

confirmed the results seen when counting the number of neutrophils that had migrated through the filter.

The possibility was considered that subtle variations in the chemical structures of the cytotaxins might significantly contribute to those conditions associated with ClINH participation in cellular responses. There exist chemical differences within the C3a molecule depending on the method of generation. Generation of C3a by $EAC4^{oxy}2$ cleavage of C3 is well documented (7,45,46). Budzko and Muller-Eberhard (47) reported the close similarity in the molecular weights and amino acid compositions of C3a(Try) and C3a($EAC4^{oxy}2$) with a difference in their C-terminal amino acid residues. Based on electrophoretic studies Bokisch and associates (7) concluded that the smooth muscle contracting and chemotactic activities of the C3a fragment are a function of different chemical groups within the fragment. Extended treatment of C3a with trypsin resulted in a fragment retaining chemotactic but not anaphylactic activity and caused the originally basic fragment to become negatively charged. In view of these differences, both trypsin and $EAC4^{oxy}2$ generated cytotaxins were examined. The negative effect of ClINH on the cell response to C3($EAC4^{oxy}2$) was just as pronounced as that seen with C3(Try).

Chemical differences within the C5a molecule associated with the method of generation are not clearly understood. Human serum, activated by LPS or immune complexes (48), contains chemotactic C5a without anaphylactic activity. Treatment of guinea pig C5 with $EAC\overline{1423}$ results in a chemotactic and anaphylactic fragment (49). After prolonged trypsin treatment, human C5 retains its chemotactic

(8) activity but not its spasmogenic activity (50). It would seem, therefore, that differences in experimental procedure, especially during trypsin utilization, could result in C5a molecules with distinct characteristics. Nilsson et al. (46) recently reported evidence supporting this possibility. Species differences in the chemical nature of C5a have only been documented for anaphylactic and not chemotactic activities. It seems feasible that trypsin treatment of human C5 and LPS-activation of guinea pig serum could result in two functionally similar but chemically different molecules. However, the inhibitory effect of ClINH on the neutrophil response to C5(Try) was just as pronounced as that observed with guinea pig C5a (LPS-generated).

The chemotactic potency of a number of small N-formylmethionyl peptides has been found to correlate with differences in chemical structure (14). There appeared to be requirements for N-acylation, methionine, and minimum size, and a degree of specificity for hydrophobic residues was suggested. Future investigations may permit inferences on the dependency of ClINH effects (enhancement or inhibition) on such well-defined chemical differences.

Still other experimental conditions give rise to important consequences associated with ClINH. Hollers et al. (30), by maintaining ClINH with the neutrophils during or after chemotactic deactivation, achieved marked prevention or complete reversal of this phenomenon. This clearly demonstrates the significance of understanding the exact conditions under which ClINH and cytotaxin encounter the cell. Goetzl (12) reported a slight enhanced neutrophil chemotactic responsiveness to C5(Try) at the highest concentration

of ClINH employed (100 μg/ml). This is not necessarily contradictory to results reported in this study considering the significant differences in conditions under which the cells were treated. Also, a direct comparison of the specific activity of ClINH (12) with that utilized in these studies cannot be made.

It is possible that C5a is involved in ClINH-mediated enhancement of the neutrophil response to activated plasma. Other plasma factors or conditions more like those utilized by Goetzl may be required in order for enhancement to occur. It is also possible that other naturally occurring cytotaxins participate in the enhancement phenomenon. Kallikrein (51), plasminogen activator (52), the trimolecular complex $\overline{C567}$ (53), and $\overline{C3B}$ (54) have all been reported to be chemotactic for neutrophils. Wissler and associates (55) have described a chemotactic peptide system in mammalian serum that is related to anaphylatoxin. All of these cytotaxins will be especially prevalent in the plasma activated by LPS, zymosan, or immune complexes. Kallikrein may not be as relevant since ClINH interacts directly with kallikrein to inhibit its neutrophil chemotactic activity (56).

The findings presented in this report provide further evidence to support the concept that ClINH participates in the mechanisms of directional migration of human neutrophils without a concomitant effect on spontaneous migration. Human ClINH at physiologic concentrations inhibits the chemotactic response of neutrophils to the chemotactic fragments of C3 and C5 and enhances the response to N-formylmethionylphenylalanine without participation of other plasma substances or other cell types.

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REFERENCES

1. Levy, L. R., and I. H. Lepow. 1959. Assay and properties of serum inhibitor of C'1-esterase. Proc. Soc. Exp. Biol. Med. 101:608.
2. Lepow, I. H., and M. A. Leon. 1962. Interaction of a serum inhibitor of C1-esterase with intermediate complexes of the immune hemolytic system. I. Specificity of inhibition of C1 activity associated with intermediate complexes. Immunol. 5:222.
3. Ratnoff, O. D., J. Pensky, D. Ogston, and G. B. Naff. 1969. The inhibition of plasmin, plasma kallikrein, plasma permeability factor, and the C1r subcomponent of the first component of complement by serum C1 esterase inhibitor. J. Exp. Med. 129:315.
4. Gigli, I., S. Ruddy, and K. F. Austen. 1968. The stoichiometric measurement of the serum inhibitor of the first component of complement by the inhibition of immune hemolysis. J. Immunol. 100:1154.
5. Forbes, C. D., J. Pensky, and O. D. Ratnoff. 1970. Inhibition of activated Hageman factor and activated plasma thromboplastin antecedent by purified serum $\overline{C1}$ inactivator. J. Lab. Clin. Med. 76:809.

6. Schreiber, A. D., A. P. Kaplan, and K. F. Austen. 1973. Inhibition by $C\bar{1}INH$ of Hageman factor fragment activation of coagulation, fibrinolysis, and kinin generation. J. Clin. Invest. 52:1402.

7. Bokisch, V. A., H. J. Muller-Eberhard, and C. G. Cochrane. 1969. Isolation of a fragment (C3a) of the third component of human complement containing anaphylatoxin and chemotactic activity and description of an anaphylatoxin inactivator of human serum. J. Exp. Med. 129:1109.

8. Ward, P. A., and L. J. Newman. 1969. A neutrophil chemotactic factor from human C5. J. Immunol. 102:93.

9. Forsgren, A., R. H. McLean, A. F. Michael, and P. G. Quie. 1975. Studies of the alternate pathway in chelated serum. J. Lab. Clin. Med. 85:904.

10. Snyderman, R., H. Gewurz, and S. E. Mergenhagen. 1968. Interactions of the complement system with endotoxin lipopolysaccharide. Generation of a factor chemotactic for polymorphonuclear leukocytes. J. Exp. Med. 128:259.

11. Smith, C. W., J. C. Hollers, D. H. Bing, and R. A. Patrick. 1975. Effects of human C1 inhibitor on complement-mediated human leukocyte chemotaxis. J. Immunol. 114:216.

12. Goetzl, E. J. 1975. Modulation of human neutrophil polymorphonuclear leucocyte migration by human plasma alpha-globulin inhibitors and synthetic esterase inhibitors. Immunol. 29:163.

13. Remold, H. G., and R. D. Rosenberg. 1975. Enhancement of migration inhibitory factor activity by plasma esterase inhibitors. J. Biol. Chem. 250:6608.

14. Schiffman, E., B. A. Corcoran, and S. M. Wahl. 1975. N-formylmethionyl peptides as chemoattractants for leucocytes. Proc. Nat. Acad. Sci. USA 72:1059.

15. Pensky, J., and I. H. Lepow. In press. Isolation of serum inhibitor of C'1a. *Methods in Immunology and Immunochemistry.* Vol. IV, Edited by C. A. Williams and M. W. Chase, Academic Press, New York.

16. Stoklosa, J. T., and H. W. Latz. 1974. Molecular weight determinations of proteins by polyacrylamide gel electrophoresis with sodium dodecyl sulfate in just the sample solution. Biochem. Biophys. Res. Commun. 58:74.

17. Harpel, P. C., and N. R. Cooper. 1975. Studies on human plasma $\overline{C1}$ inactivator-enzyme interactions. I. Mechanisms of interaction with $\overline{C1s}$, plasmin, and trypsin. J. Clin. Invest. 55:593.

18. Eder, J. 1972. Isoelectric focusing of antibodies in polyacrylamide gels. J. Immunol. Methods 2:67.

19. Righetti, P., and J. W. Drysdale. 1971. Isoelectric focusing in polyacrylamide gels. Biochem. Biophys. Acta 236:17.

20. Mancini, A., A. O. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochem. 2:235.

21. Nilsson, U. R., and H. J. Muller-Eberhard. 1965. Isolation of β_{1F} globulin from human serum and its characterization as the fifth component of complement. J. Exp. Med. 122:277.

22. Nilsson, U. R., R. H. Tomar, and F. B. Taylor. 1972. Additional studies on human C5: Development of modified purification method and characterization of the purified product by polyacrylamide gel electrophoresis. Immunochem. 9:709.

23. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265.

24. Murphy, J. B., and M. W. Kies. 1960. Note on spectrophotometric determination of proteins in dilute solutions. Biochem. Biophys. Acta 45:382.

25. Muller-Eberhard, H. J., M. J. Polley, and M. A. Calcott. 1967. Formation and functional significance of a molecular complex derived from the second and fourth component of human complement. J. Exp. Med. 125:359.

26. Snyderman, R., H. S. Shin, J. D. Phillips, H. Gewurz, and S. E. Mergenhagen. 1969. A neutrophil chemotactic factor derived from C5 upon interaction of guinea pig serum with endotoxin. J. Immunol. 130:413.

27. Smith, C. W., J. C. Hollers, E. Dupree, A. S. Goldman, and R. A. Lord. 1972. A serum inhibitor of leukotaxis in a child with recurrent infections. J. Lab. Clin. Med. 79:878.

28. English, E., and B. R. Andersen. 1974. Single step separation of red blood cells, granulocytes, and mononuclear leukocytes on discontinuous density gradients of Ficoll-Hypaque. J. Immunol. Methods 5:249.

29. Zigmond, S. H., and J. G. Hirsch. 1973. Leukocyte locomotion and chemotaxis. New methods for evaluation, and demonstration of a cell-derived chemotactic factor. J. Exp. Med. 137:387.

30. Hollers, J. C., C. W. Smith, D. Y. Liu, and R. A. Patrick. 1976. The effects of Cl inhibitor on chemotactic deactivation of human neutrophils. Fed. Proc. 35:429.

31. Orange, R. P., M. A. Kaliner, and K. F. Austen. 1971. The immunological release of histamine and slow-reacting substance of anaphylaxis from human lung. III. Biochemical control mechanisms involved in the immunologic release of the chemical mediators. In *Biochemistry of the Acute Allergic Reactions*. Edited by K. F. Austen and E. L. Becker. Blackwell Scientific Publications, Oxford.

32. Wasserman, S. I., E. J. Goetzl, M. Kaliner, and K. F. Austen. 1974. Modulation of the immunological release of the eosinophil chemotactic factor of anaphylaxis from human lung. Immunol. 26:677.

33. Henson, P. M. 1970. Mechanisms of release of constituents from rabbit platelets by antigen-antibody complexes and complement. I. Lytic and nonlytic reactions. J. Immunol. 105:476.

34. Pearlman, D. S., P. A. Ward, and E. L. Becker. 1969. The requirement of serine esterase function in complement-dependent erythrophagocytosis. J. Exp. Med. 130:745.

35. Becker, E. L. 1972. The relationship of the chemotactic behavior of the complement-derived factors, C3a, C5a, and $\overline{C567}$, and a bacterial chemotactic factor to their ability to activate the proesterase 1 of rabbit polymorphonuclear leukocytes. J. Exp. Med. 135:367.

36. Becker, E. L., and H. J. Showell. 1974. The ability of chemotactic factors to induce lysosomal enzyme release. II. The mechanism of release. J. Immunol. 112:2055.

37. Grinnell, F. 1975. Cell attachment to a substratum and cell surface proteases. Arch. Biochem. Biophys. 168:474.

38. Berenberg, J. J., and P. A. Ward. 1973. Chemotactic factor inactivator in normal human serum. J. Clin. Invest. 52:1200.

39. Walker, J. R., M. J. H. Smith, A. W. Ford-Hutchinson, and F. J. Billimoria. 1975. Mode of action of an anti-inflammatory fraction from normal human plasma. Nature 254:444.

40. Goetzl, E. J., and K. F. Austen. 1972. A neutrophil-immobilizing factor derived from human leukocytes. I. Generation and partial characterization. J. Exp. Med. 136:1564.

41. Rocklin, R. E. 1974. Products of activated lymphocytes: Leukocyte inhibitory factor (LIF) distinct from migration inhibitory factor. J. Immunol. 112:1461.

42. Venge, P., and I. Olsson. 1975. Cationic proteins of human granulocytes. VI. Effects on the complement system and mediation of chemotactic activity. J. Immunol. 115:1505.

43. Horwitz, D. A., and M. A. Garrett. 1971. Use of leukocyte chemotaxis in vitro to assay metidators generated by immune reactions. I. Quantitation of mononuclear and polymorphonuclear leukocyte chemotaxis with polycarbonate (Nucleopore) filters. J. Immunol. 106:649.

44. Kay, A. B., H. S. Shin, and K. F. Austen. 1973. Selective attraction of eosinophils and synergism between eosinophil chemotactic factor of anaphylaxis (ECF-A) and a fragment cleaved from the fifth component of complement (C5a). Immunol. 24:969.

45. Dias da Silva, W., J. W. Eisele, and I. H. Lepow. 1967. Complement as a mediator of inflammation. III. Purification of the activity with anaphylatoxin properties generated by interaction of the first four components of complement and its identification as cleavage products of C3. J. Exp. Med. 126: 1027.

46. Nilsson, U. R., R. J. Mandle, Jr., and J. A. McConnell-Mapes. 1975. Human C3 and C5: Subunit structure and modifications by trypsin and $\overline{C42}$ - $\overline{C423}$. J. Immunol. 114:815.

47. Budzko, D. G., V. A. Bokisch, and H. J. Muller-Eberhard. 1971. A fragment of the third component of human complement with anaphylatoxin activity. Biochem. 10:1166.

48. Gallin, J. I., R. A. Clark, and M. M. Frank. 1975. Kinetic analysis of chemotactic factor generation in human serum via activation of the classical and alternate complement pathways. Clin. Immunol. Immunopathol. 3:334.

49. Shin, H. S., R. Snyderman, E. Freidman, A. Mellors, and M. M. Mayer. 1968. Chemotactic and anaphylatoxic fragment cleaved from the fifth component of guinea pig complement. Science 162:361.

50. Cochrane, C. G., and H. J. Muller-Eberhard. 1968. The derivation of two distinct anaphylatoxin activities from the third and fifth components of human complement. J. Exp. Med. 127:371.

51. Kaplan, A. P., A. B. Kay, and K. F. Austen. 1972. A prealbumin activator of prekallikrein. III. Appearance of chemotactic activity for human neutrophils by the conversion of human prekallikrein to kallikrein. J. Exp. Med. 135:81.

52. Kaplan, A. P., E. J. Goetzl, and K. F. Austen. 1973. The fibrinolytic pathway of human plasma. II. The generation of chemotactic activity by activation of plasminogen proactivator. J. Clin. Invest. 52:2591.

53. Lachmann, P. J., A. B. Kay, and R. A. Thompson. 1970. The chemotactic activity for neutrophil and eosinophil leucocytes of the trimolecular complex of the fifth, sixth, and seventh components of human complement ($\overline{C567}$) prepared in free solution by the reactive lysis procedure. Immunol. 19:895.

54. Ruddy, S., K. F. Austen, and E. J. Goetzl. 1975. Chemotactic activity derived from interaction of factors $\overline{D}$ and B of the properdin pathway with cobra venom factor or C3b. J. Clin. Invest. 55:587.

55. Wissler, J. H., V. J. Stecher, and E. Sorkin. 1972. Biochemistry and biology of a leucotactic binary serum peptide system related to anaphylatoxin. Int. Arch. Allergy 42:722.

56. Goetzl, E. J., and K. F. Austen. 1974. Active site chemotactic factors and the regulation of the human neutrophil chemotactic response. *Antibiotics and Chemotherapy*, Vol. 19. Edited by E. Sorkin. S. Karger, Basel.

ARTICLE 2

EFFECTS OF $\bar{Cl}$ INHIBITOR ON CHEMOTACTIC DEACTIVATION OF HUMAN NEUTROPHILS[1]

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Condensed title: Effects of $\bar{Cl}$ Inhibitor on Chemotactic Deactivation of Neutrophils

[1]This work was presented in part at the annual meeting of the Federation of the American Societies for Experimental Biology, Anaheim, California, April 1976.

ABSTRACT

$C\bar{1}$ inhibitor has been shown to enhance and inhibit neutrophil chemotactic responsiveness under appropriate conditions by a direct effect on human leukocytes. To further define the nature of this effect, we have tested the influence of $C\bar{1}$ inhibitor on another cytotaxin mediated phenomenon, chemotactic deactivation. In the present study, a 10% zymosan-activated plasma filtrate was used as both the deactivating agent and the cytotaxin. The human leukocytes used in these experiments contained greater than 93% neutrophils and were obtained by Ficoll-Hypaque centrifugation. Neutrophils which were deactivated by incubating with zymosan-activated plasma filtrate for 30 min and extensively washed showed a mean chemotactic index of 13% of control values ($p<.001$). The presence of $C\bar{1}$ inhibitor during the incubation of neutrophils with zymosan-activated plasma filtrate resulted in significant prevention of deactivation ($p<.01$), increasing the mean response to 57% of control values, whereas complete reversal of deactivation ($p<.001$) was obtained by adding $C\bar{1}$ inhibitor to previously deactivated and washed neutrophils. The results of this study lend support to the conclusion that $C\bar{1}$ inhibitor can directly affect the chemotactic response of neutrophils. Furthermore, its ability to reverse deactivation, which has hitherto been considered irreversible, may be relevant to the understanding of chemotactic responsiveness.

INTRODUCTION

The chemotactic response of neutrophils in vitro is influenced not only by the specific chemotactic stimulus but by other naturally

occurring factors which may inhibit or enhance through an influence on the stimulus or the cell's response to the stimulus. One of these factors is $C\bar{1}$ inhibitor (ClINH).[2] This protease inhibitor has been shown to alter the response of human neutrophils to certain chemotactic factors (1,2). Its action seems to be directly on the cell rather than through some plasma intermediate, and it has no apparent effect on cells in the absence of chemotactic factors.

In an effort to characterize more completely the influence of ClINH on the response of human neutrophils to cytotaxins, we tested its possible influence on chemotactic deactivation. Deactivation has been described as an alteration of the cells in a high concentration of certain cytotaxins, e.g., immune complex activated serum (3), zymosan activated serum (3), $\overline{C567}$ (4), C5a (5,6), kallikrein (6) or $\overline{C3B}$ (7). The altered response is seen as a decrease in the number of cells penetrating the filter in a Boyden-type chemotaxis chamber, and it seems to reflect a change in the cells since repeated washing does not reverse the effect. Though the exact mechanism is not understood, an expending of a proenzyme (proesterase 1) necessary for migration has been proposed (4).

The results presented in this report show a profound influence of ClINH on deactivation.

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[2]Abbreviations used in this paper: ClINH, $C\bar{1}$ inhibitor; ATEE, N-acetyl-L-tyrosine ethyl ester; ZAPF, zymosan activated plasma filtrate; HBSS, Hank's balanced salt solution; PMN, human neutrophils.

METHODS

Human $C\bar{1}$ inhibitor (ClINH) was prepared and evaluated for purity and activity as described by Pensky and Lepow (8). The specific activities of the ClINH were 180 inhibitor ATEE units/$OD_{280\ nm}$ (9) and 133 U/μg (10). The highly purified preparation upon immunoelectrophoretic analysis revealed one precipitin arc with α_2 mobility against anti-whole human serum. Isoelectric focusing demonstrated one band, and SDS gel electrophoresis demonstrated a major and minor band pattern consistent with the pattern recently reported by Harpel and Cooper (11). Unless otherwise noted, ClINH was used at 7 ATEE U/ml in all experiments.

Zymosan activated plasma filtrate (ZAPF) was prepared by incubating zymosan (Nutritional Biochemicals Corporation, Cleveland, Ohio) 10 mg/ml with fresh human plasma for 30 min at 37°C, diluting this mixture 10-fold with Hank's balanced salt solution (HBSS), and then removing the zymosan particles by centrifugation and filtration through a 0.22 μm pore size filter (Millipore Corporation, Bedford, Massachusetts).

Neutrophil enriched cell suspensions were obtained by Ficoll-Hypaque centrifugation. Leukocytes and plasma from heparinized dextran sedimented whole human blood were diluted with an equal volume of HBSS and layered in 17 mm diameter siliconized tubes above a single Ficoll-Hypaque solution consisting of 10 parts of 33.9% Hypaque (Winthrop Laboratories, New York, New York) and 24 parts of 9% Ficoll (Sigma Chemical Co., St. Louis, Missouri). The tubes were centrifuged at 800g for 30 min at 25°C. The leukocytes remaining in the cell button at the bottom of the tubes after

the upper layers were removed by aspiration contained greater than 98% granulocytes of which approximately 95% were neutrophils (PMN). Consistently less than 2% of the leukocytes were blood monocytes or lymphocytes. No platelets could be seen in these preparations, and the RBC to PMN ratio was approximately 5:1. The PMN viability was greater than 98% as determined by eosin exclusion.

PMN chemotactic responsiveness was determined by a modified Boyden technique using blind-well chemotaxis chambers (Neuroprobe, Inc., Bethesda, Maryland). A Millipore filter of 3 μm pore size was used, and the chambers were loaded with 2×10^4 PMN/mm^2 of exposed filter surface. The chemotactic response was evaluated by counting the number of PMN accumulating on the lower surface of the filter after 3 hours of incubation at 37°C in an atmosphere containing 5% CO_2 and 95% humidity. Ten 400X microscope fields were counted on each filter and the averages of triplicate determination in each experiment were expressed as a percent of control values. This index of chemotactic responsiveness was obtained by the following formula:

$$\% \text{ of control} = \frac{\text{experimental count - background count}}{\text{control count - background count}} \times 100$$

where background = counts from chambers containing control cells in the upper compartment and 10% plasma in the lower compartment, and control = counts from chambers containing control cells in the upper compartment and ZAPF in the lower.

Control values and background values would therefore be defined as 100% and 0%, respectively. The comparison of experimental results to control values in terms of percent response was necessary

because of the variability in cell counts due, in part, to the use of plasma and cells from different human donors or even from the same donor in experiments performed on different days. However, the differences in cell counts from triplicate chambers within any one experiment did not exceed 25%. The results in Table 1 were analyzed statistically, employing the Student's t test.

PMN chemotactic deactivation was accomplished by incubating 3.0×10^7 PMN/ml in ZAPF for 30 min at 37°C and then washing the cells 3 times in HBSS. These cells were then adjusted to a concentration of 2×10^6 PMN/ml with 10% fresh plasma and were assayed for chemotactic responsiveness. Cells that were incubated with 10% plasma instead of ZAPF but were otherwise treated identically served as controls and are referred to in the text as "control cells."

RESULTS

Various concentrations of ZAPF and PMN were incubated together for 30 min at 37°C to determine the experimental conditions necessary for deactivation with this cytotaxin. There was no apparent effect on the level of deactivation exerted by varying the cell concentration from 2×10^7 to 6×10^7 PMN/ml in incubation mixtures with ZAPF. A standard concentration for all further experiments of 3×10^7 PMN/ml was chosen on the basis of technical convenience. However, the efficacy of deactivation was dose-dependent on ZAPF. The results of the experiment illustrated in Figure 2 indicate that PMN incubated with undiluted ZAPF showed the lowest level of chemotactic responsiveness as compared to control values (16%) or the

Figure 1. Experimental procedure.

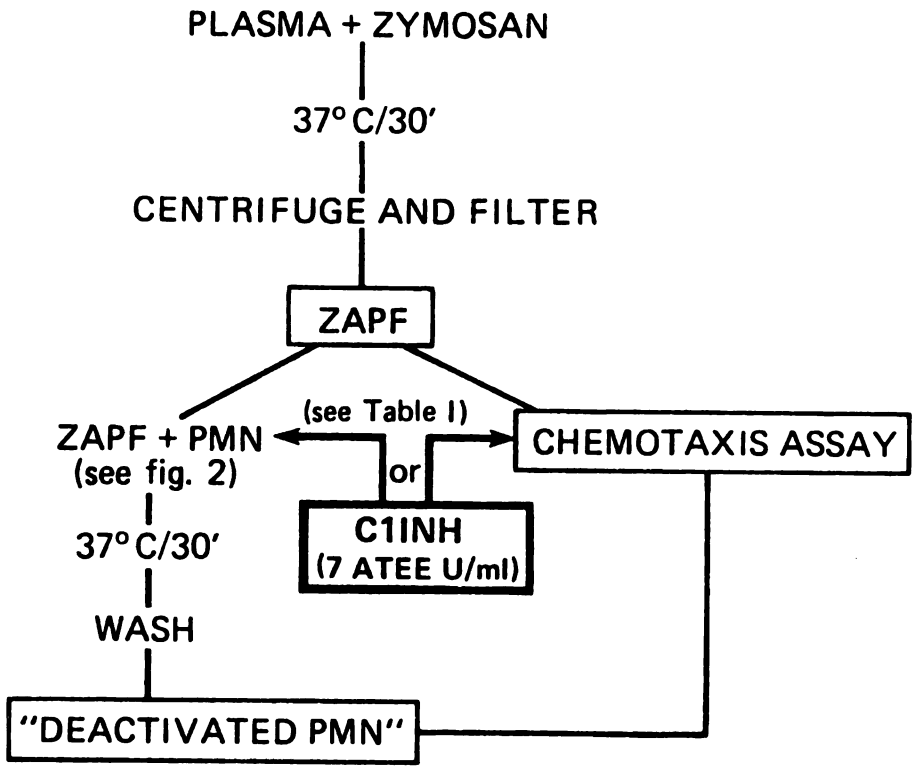
PLASMA + ZYMOSAN
37° C/30'
CENTRIFUGE AND FILTER
ZAPF
ZAPF + PMN
(see fig. 2)
(see Table I)
or
CHEMOTAXIS ASSAY
37° C/30'
C1INH
(7 ATEE U/ml)
WASH
"DEACTIVATED PMN"

Figure 1

Figure 2. Effect of various concentrations of ZAPF in pre-incubation mixtures on the chemotactic response of neutrophils. The notation 1:1 indicates no dilution. Control is same as group 2, Table 1.

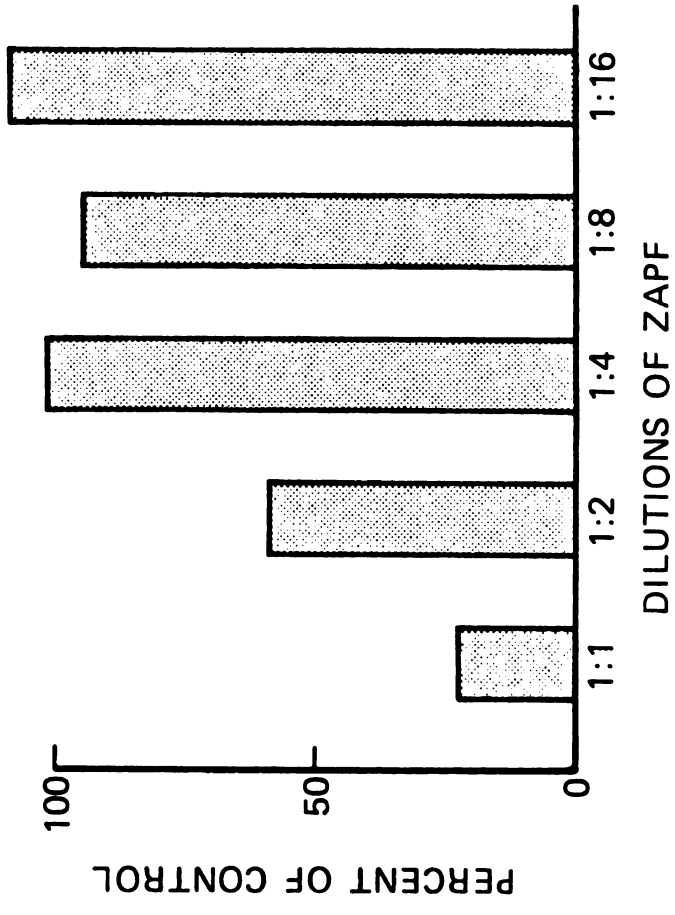

Figure 2

greatest degree of deactivation. The deactivating effects of ZAPF were abolished at a dilution of 1:4 or higher. Undiluted ZAPF was used in all subsequent experiments.

The effect of ClINH on the deactivation phenomenon was tested by adding ClINH to previously deactivated cells during the chemotaxis assay or to the deactivating mixture (Figure 1). The results of these experiments are summarized in Table 1. Briefly, when ClINH was added to the PMN-ZAPF mixtures during the deactivation step and then removed along with the ZAPF by washing the cells prior to the chemotaxis assay, there was significant partial prevention ($p<.01$) of deactivation (group 4 vs. 3). Further experiments showed no change in the degree of prevention of deactivation by varying ClINH concentrations between 7 and 22 U/ml; however, there was no prevention of deactivation seen with ClINH at 5 U/ml. If deactivated cells were tested for chemotactic responsiveness in the presence of ClINH, deactivation was completely ($p<.001$) reversed (group 5 vs. 3), having a chemotactic index which was not significantly ($p>.4$) different from that of control cells (group 2 vs. 5).

DISCUSSION

Diminished chemotactic responsiveness induced by preincubation of polymorphonuclear leukocytes with chemotactic substances is a characteristic of rabbit neutrophils (3), and human neutrophils (6), eosinophils (10,11), and mononuclear cells (6). This altered response has been termed "deactivation" (3). Previous results (1) from this laboratory showed that addition of highly purified ClINH to the media containing human neutrophils effects significant

Table 1

Effects of C1 Inhibitor on Chemotactic Deactivation of Neutrophils

Group	Cell Compartment*	Lower Compartment	% of Control ± s.d.†	n§
1	(PMN+P)P$^{\Psi}$	P	0	18
2	(PMN+P)P	ZAPF	100	18
3	(PMN+ZAPF)P	ZAPF	13.3±25.4	18
4	(PMN+ZAPF+ClINH)P	ZAPF	56.6±33.5	5
5	(PMN+ZAPF)ClINH+P	ZAPF	122.7±20.6	5

Groups 2 and 3, $p<.001$; 3 and 4, $p<.01$; 3 and 5, $p<.001$; 2 and 5, $p<.4$.

*Cells were preincubated 30 min at 37°C with substances in parentheses and washed 3X in Hank's solution.

†Control - group 2.

§Number of experiments done in triplicate.

$^{\Psi}$P - 10% fresh plasma in Hank's solution.

changes (enhancement) in the chemotactic responses of the cells to zymosan-activated plasma (ZAPF). Results reported here show that ClINH can directly alter chemotactic deactivation of human PMN to ZAPF.

Chemotactic deactivation of human neutrophils was partially prevented by ClINH (Table 1, group 4). In addition, ClINH apparently effects alterations of the "deactivated" state such that the phenomenon becomes reversible (Table 1, group 5).

Phosphonate and aromatic amino acid esters have been reported by Ward and Becker (3) to partially prevent deactivation of rabbit neutrophils by cytotaxins derived from activated rabbit serum. Since ClINH and phosphonate inhibitors are both known to irreversibly alter the active site of serine esterases, it seemed plausible to hypothesize an interaction of ClINH with the esterase 1 associated with rabbit neutrophil chemotaxis (4). However, experiments designed to directly test this hypothesis (Patrick and Becker, unpublished results) failed to show that ClINH affected the action of or the activation of esterase 1 on rabbit neutrophils.

Since a human corollary to rabbit esterase 1 has not been demonstrated, no direct comparison between the effect of phosphonate inhibitors on rabbit neutrophil deactivation and the effect of ClINH on human neutrophil deactivation can be made at this time.

That reversal of deactivation occurs when ClINH is present with the deactivated neutrophils during the chemotaxis assay strongly suggests a role for ClINH in the facilitation of a chemotactically responsive population of neutrophils. Furthermore, ClINH must be acting at some step in the mechanisms for chemotactic responsiveness since it does not increase spontaneous motility and since the simultaneous presence of cytotaxin is necessary for the effects of ClINH to be expressed (1,2).

These data suggest an interpretation of the previous observations that ClINH enhances the chemotactic response of neutrophils to zymosan-activated plasma (1). If deactivation eventually occurs during the response of cells to a chemotactic gradient and ClINH prevents or reverses this, then the responsiveness of the cells

might be maintained at higher levels. This would be reflected in a larger number of cells responding to ZAPF and migrating through the micropore filter.

Chemotactically deactivated leukocytes are not at the present time well characterized with regard to biochemical and cellular functions. A recent report (12) indicated that stimulation of the hexose monophosphate shunt by chemotactic factor was unaffected in deactivated human neutrophils. It is hoped that the effects of ClINH may allow for a functional characterization of chemotactic deactivation which, in turn, may lead to a better understanding of the chemotactic response of granulocytes.

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REFERENCES

1. Smith, C. W., J. C. Hollers, D. H. Bing, and R. A. Patrick. 1975. Effects of human C1 inhibitor on complement-mediated human leukocyte chemotaxis. J. Immunol. 114:216-220.
2. Goetzl, E. J. 1975. Modulation of human neutrophil polymorphonuclear leukocyte migration by human plasma alpha-globulin inhibitors and synthetic esterase inhibitors. Immunol. 29:163-174.
3. Ward, P. A., and E. L. Becker. 1968. The deactivation of rabbit neutrophils by chemotactic factor and the nature of the activatable esterase. J. Exp. Med. 127:693-709.

4. Ward, P. A., and E. L. Becker. 1970. Biochemical demonstration of the activatable esterase of the rabbit neutrophil involved in the chemotactic response. J. Immunol. 105:1057-1067.

5. Becker, E. L. 1972. The relationship of the chemotactic behavior of the complement-derived factors, C3a, C5a, and $\overline{C567}$, and a bacterial chemotactic factor to their ability to activate the proesterase 1 of rabbit polymorphonuclear leukocytes. J. Exp. Med. 135:376-387.

6. Goetzl, E. J., and K. F. Austen. 1974. Active site chemotactic factors and the regulation of the human neutrophil chemotactic response. In *Antibiotics and Chemotherapy*, Vol. 19. E. Sorkin, ed. S. Karger, Basel. 19:218-232.

7. Ruddy, S., K. F. Austen, and E. J. Goetzl. 1975. Chemotactic activity derived from interaction of factors D and B of the properdin pathway with cobra venom factor or C3b. J. Clin. Invest. 55:587-592.

8. Pensky, J., and I. H. Lepow. In press. Isolation of serum inhibitor of C'1a. In *Methods in Immunology and Immunochemistry*, Vol. IV. C. A. Williams and M. W. Chase, eds. Academic Press, New York.

9. Levy, L. R., and I. H. Lepow. 1959. Assay and properties of serum inhibitor of C'1-esterase. Proc. Soc. Exp. Biol. Med. 101:608-611.

10. Gigli, I., S. Ruddy, and K. F. Austen. 1968. The stoichiometric measurement of the serum inhibitor of the first component of complement by the inhibition of immune hemolysis. J. Immunol. 100:1154-1164.

11. Harpel, P. C., and N. R. Cooper. 1975. Studies on human plasma $\overline{C1}$ inactivator-enzyme interactions. I. Mechanisms of interaction with $\overline{C1s}$, plasmin, and trypsin. J. Clin. Invest. 55: 593-604.

12. Wasserman, S. I., D. Whitmer, E. J. Goetzl, and K. F. Austen. 1975. Chemotactic deactivation of human eosinophils by the eosinophil chemotactic factor of anaphylaxis. Proc. Soc. Exp. Biol. Med. 148:301-306.

13. Clark, R. A. F., J. I. Gallin, and A. P. Kaplan. 1975. The selective eosinophil chemotactic activity of histamine. J. Exp. Med. 142:1462-1476.

14. Goetzl, E. J., and K. F. Austen. 1974. Stimulation of human neutrophil leukocyte aerobic glucose metabolism by purified chemotactic factors. J. Clin. Invest. 53:591-599.