The Control of Neutrophil Chemotaxis by Inhibitors of Cathepsin G and Chymotrypsin*

(Received for publication, May 5, 1995)

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Neutrophil chemotaxis plays an important role in the inflammatory response and when excessive or persistent may augment tissue damage. The effects of inhibitors indicated the involvement of one or more serine proteinases in human neutrophil migration and shape change in response to a chemoattractant. Monospecific antibodies, chloromethylketone inhibitors, and reactive-site mutants of α1-antitrypsin and α1-antichymotrypsin were used to probe the specificity of the proteinases involved in chemotaxis. Antibodies specific for cathepsin G inhibited chemotaxis. Moreover, rapid inhibitors of cathepsin G and α-chymotrypsin suppressed neutrophil chemotaxis to the chemoattractants N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) and zymosan-activated serum in multiple blind well assays and to fMLP in migration assays under agarose. The concentrations of antichymotrypsin mutants that reduced chemotaxis by 50% would inactivate free cathepsin G with a half-life of 1.5–3 s, whereas the concentrations of chloromethylketones required to produce a similar inhibition of chemotaxis would inactivate cathepsin G with a half-life of 345 s. These data suggest different modes of action for these two classes of inhibitors. Indeed the chloromethylketone inhibitors of cathepsin G (Z-Gly-Leu-Phe-CMK) and to a lesser extent of chymotrypsin (Cbz-Gly-Gly-Phe-CMK) mediated their effect by preventing a shape change in the purified neutrophils exposed to fMLP. Antichymotrypsin did not affect shape change in response to fMLP even at concentrations that were able to reduce neutrophil chemotaxis by 50%. These results support the involvement of cell surface proteinases in the control of cell migration and show that antichymotrypsin and chloromethylketones have differing modes of action. This opens the possibility for the rational design of anti-inflammatory agents targeted at neutrophil membrane enzymes.

The pathogenesis of lung diseases such as adult respiratory distress syndrome and emphysema is thought to result from an imbalance between leukocyte serine proteinases (such as neutrophil elastase, cathepsin G, and proteinase III) and the proteinase inhibitors of the lung (1–3). There are a variety of these inhibitors, but most important are the serpins, a family of serine proteinase inhibitors, typified by antitrypsin (α1-proteinase inhibitor) and antichymotrypsin (4). Each of the family members has a unique inhibitor specificity, but they all share a similar overall molecular structure (5). Neutrophil chemotaxis is an important component of the inflammatory response, and the recruitment of neutrophils to the lung may play a pivotal role in the pathogenesis of emphysema (6). If neutrophil migration into the lungs is excessive, there will be enhanced delivery and release of proteolytic enzymes, which may overwhelm the native inhibitors causing lung damage and eventually the development of emphysema.

The physiological mechanisms controlling neutrophil chemotaxis are unclear, although Ward and Becker (7) suggested that this process may be under the control of a surface serine proteinase. They showed that the inhibition of this enzyme by organophosphorous compounds reduced neutrophil chemotaxis (8, 9) but were unsure as to the identity of the natural substrate or inhibitor (10). Other workers have reported that chloromethylketone inhibitors of chymotrypsin-like proteinases were able to suppress human neutrophil chemotaxis (11–13) and superoxide production (14) as well as attenuating membrane potential changes in rat neutrophils (15). Furthermore King and co-workers (16) reported that a monoclonal antibody against human neutrophils inhibited superoxide anion generation in response to the chemotactic peptide fMLP by binding to a surface chymotrypsin-like enzyme. Recent work (17) has demonstrated that serine proteinase inhibitors (particularly antichymotrypsin) are able to reduce neutrophil chemotaxis in response to the same chemoattractant. This has led to the suggestion that these agents exert their effects by binding and inhibiting a cognate surface serine proteinase, possibly cathepsin G, involved in receptor-mediated cell activation. This hypothesis is supported by the demonstration that neutrophil elastase, and probably cathepsin G, can bind to the cell membrane after secretion and therefore play a role in cell migration (18). Moreover, the human neutrophil contains isoforms of elastase and cathepsin G that may have differing roles (19). The current study uses inhibitors to assess the role of serine proteinases in modulating the neutrophil chemotactic response. This was achieved by examining the effects of antibody and chloromethylketone enzyme inhibitors along with P1 mutants of antitrypsin and antichymotrypsin, which have a range of inhibitory profiles and thus could be used to probe the specificity of the serine proteinases controlling chemotaxis.

* This work was funded by the Medical Research Council of the United Kingdom, the Wellcome Trust, and the British Lung Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: fMLP, N-formyl-L-methionyl-L-leucyl-L-phenylalanine; CMK, chloromethylketone; pNA, p-nitroanilide; MeO-Suc, methoxysuccinyl; Suc, succinyl; Cbz, carboxybenzoyl; DMK, diazomethylketone; cpf, cells/high power field.
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MATERIALS AND METHODS

Human neutrophil cathepsin G and immunopurified polyclonal anti-cathepsin B antibodies were from Dr. D. Buttle (Strangeways Laboratory, Cambridge, UK), human neutrophil elastase was provided by D. Bruce (Department of Haematology, University of Cambridge), and sheep anti-human cathepsin G and anti-human elastase immunoglobulin were obtained from the Binding Site Ltd. (Birmingham, UK). Cbz-Gly-Gly-Phe-CMK, Tosyl-Lys-CMK, Cbz-Phe-Ala-DMK, and MeSucc-Ala-Ala-Pro-Val-CMK were from Bachem Feinchemikalien AG (Bubendorf, Switzerland), and Z-Gly-Leu-Phe-CMK was from Enzyme System Products. Recombinant methionine (P1) antitrypsin was obtained from Dr. H. P. Schnebli, (Ciba-Geigy, Basel, Switzerland), arginine (P1) antitrypsin and the pVK50 plasmid were from Delta Biotechnology, (Nottingham, UK).

Purification of Anti-cathepsin G Antibodies

Cathepsin G (10 mg) was coupled to cyanogen bromide-activated matrix according to the manufacturer's instructions. The gel (3.5 ml) was then packed into a column at 20 ml/h, washed with 0.05 M Tris, pH 8.0, and 1 ml of anti-cathepsin G antibody was applied at the same flow rate. Immunoaffinity antibody was eluted from the column with 0.2 M glycine, pH 2.5, and fractions of 2 ml were collected into tubes containing Tris to restore neutral pH. The column was reequilibrated with glycine, pH 2.5, and fractions of 2 ml were collected into tubes containing Tris to restore neutral pH. The column was reequilibrated with glycine, pH 2.5, and fractions of 2 ml were collected into tubes containing Tris to restore neutral pH. The column was reequilibrated with glycine, pH 2.5, and fractions of 2 ml were collected into tubes containing Tris to restore neutral pH. The column was reequilibrated with glycine, pH 2.5, and fractions of 2 ml were collected into tubes containing Tris to restore neutral pH.

Construction, Expression, and Purification of Antitrypsin Mutants

Active site mutants of antitrypsin were produced by site-directed mutagenesis and expressed in Saccharomyces cerevisiae strain AB 116 as described previously (20). The recombinant protein was extracted and purified to homogeneity according to the method of Travis et al. (21). Purity was assessed by 10–20% (w/v) SDS-polyacrylamide gel electrophoresis, and total antitrypsin concentration was determined by rocket immunoelectrophoresis (22) with a yeast recombinant methionine (P1) antitrypsin standard that had been calibrated by amino acid analysis. The recombinant P1 antitrypsin mutants migrated as several bands on SDS-polyacrylamide gel electrophoresis; amino-terminal sequence confirmed these to be due to amino-terminal, but not reactive center loop, cleavage. The specific activities of these mutants against bovine α-chymotrypsin were 89%, valine (P1) antitrypsin 67%, and lysine (P1) antitrypsin 81%.

Construction, Expression, and Purification of Antichymotrypsin Mutants

Recombinant antichymotrypsin with leucine, methionine, phenylalanine, and arginine at the P1 (358) position were expressed in Escherichia coli and purified to homogeneity as described previously (23, 24). Purity was confirmed by 10–20% (w/v) SDS-polyacrylamide gel electrophoresis, and total protein concentration was determined by Bradford assay (25) against a recombinant leucine (P1) antichymotrypsin standard that had been calibrated by amino acid analysis. The specific inhibitory activities of leucine, methionine, arginine, and phenylalanine (P1) antichymotrypsin against bovine α-chymotrypsin were 66, 69, 37, and 32%, respectively.

Inhibition Kinetics

Active Site Determination of Serine Proteases and Recombinant Inhibitors—The active site titration of bovine α-chymotrypsin and human α-thrombin were performed according to the method of Chase and Shaw (26) using the suicide substrates p-nitrophenyl acetate and p-nitroanilide, respectively. Bovine α-thrombin was used to determine the activity of recombinant antiproteinase and antichymotrypsin using the substrate Suc-Ala-Ala-Pro-Phe-pNA as detailed previously (27). These inhibitors were in turn used to determine the activity of cathepsin G and neutrophil elastase using the substrates Suc-Ala-Ala-Pro-Phe-pNA and MeSucc-Ala-Ala-Pro-Val-pNA, respectively.

Determination of Second Order Association Rate Constants—Equimolar active site concentrations of enzyme and inhibitor were incubated at 20°C as described previously (28). The value of the association rate constant (kass) was determined by nonlinear regression analysis according to the following equation (29).

\[ E = \frac{E_0}{k_{ass} + t + 1} \]  

where E0 is the initial enzyme concentration, and t is time.

All values were determined on 2–3 separate occasions with the results quoted as weighted mean with standard error.

Determination of Pseudo-First Order Association Rate Constants—7-35 fold active site molar excess of inhibitor was incubated with the enzyme for varying time intervals at 20°C. The quantity of free enzyme was then determined by adding reaction buffer containing substrate, and the association rate constant was calculated as described previously (30, 31).

Determination of Association Rate Constants and K1 Values Using Progress Curve Analysis—Kinetic parameters for the interaction of recombinant antiproteinase and antichymotrypsin with human neutrophil elastase were obtained from cathepsin G. The specificity of the antibody was confirmed by radial immunodiffusion.

Isolation of Neutrophils

Venous blood (10 ml) was collected from healthy volunteers into lithium heparin tubes, and the neutrophils were isolated on percoll gradients according to the method of J epsen and Skottun (32). The cells (>95% pure, >95% viable by trypan blue exclusion) were washed, counted, and resuspended at the required concentrations in Hapes-buffered RPMI 1640 medium/bovine serum albumin (2 mg/ml). All reagents were endotoxin-free (≤20 pg/ml) as determined by a Limulus assay. Following isolation, the cells were spherical, indicating that they had not been activated during harvesting.

Neutrophil Chemotaxis in the Multiple Blind Well Assay System

The chloromethylketones and diazomethylketone were dissolved in a minimum quantity of dimethyl sulfoxide and then diluted to the required concentration with Hapes-buffered RPMI 1640 medium. The effect of these inhibitors on chemotaxis was assessed by incubating them with the isolated neutrophils for 30 min at room temperature prior to placing the cells in the chemotactic chamber. Similarly, the effect of the antiproteinase and antichymotrypsin mutants was assessed after incubating with the neutrophils for 30 min, and that of the antibodies was assessed after incubating for 60 min.

The chemotactic response was based on the multiple blind well assay system (34). The lower well contained 27 μl of 10−8 M fMLP or 7% (v/v) zymosan-activated serum as the chemoattractant (35), and the upper chamber was filled with 50 μl of the neutrophil suspension (final concentration, 3 × 106 cells per ml) with or without antibody or inhibitor. The two chambers were separated by a 2.0-μm pore size polycarbonate filter. Negative control wells contained medium but no chemoattractant in the lower chamber. The assay plates were incubated at 37°C for 20 min, and the membrane was then washed, fixed, and stained. The cells were counted from 5 areas of each membrane at 400× magnification, and the mean value was obtained. Each experiment was performed in triplicate, and the mean value of the three membranes was taken as the result. All experiments were repeated on six different cell preparations unless otherwise stated. In the event of the negative control exceeding 5% of the positive control, the membrane was discarded. All values are expressed as mean cells/high power field (cfp) ± S.E., and differences between groups of six subjects were assessed by the Wilcoxon-Signed Rank test. Under these conditions, the chemotactic assay had a control within-batch coefficient of variation of 4.0% (n = 5).

Neutrophil Chemotaxis under Agarose

Human neutrophils were isolated as described previously and incubated at 5 × 107 cells/ml in Hapes-buffered RPMI 1640 medium with 10% (v/v) fetal calf serum with increasing concentrations of the chloromethylketone or antichymotrypsin at room temperature. The cells were then allowed to migrate under agarose to a chemoattractant (10−7 M fMLP) or buffer (36), which allows the measurement of both chemotaxis and chemokinesis. The value for chemokinesis was then subtracted from that of chemotaxis to give a final result. The coefficient of variation was 9% (n = 6).
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**RESULTS**

The Effect of Anti-cathepsin G, Anti-elastase, and Anti-cathepsin B Antibodies on Neutrophil Chemotaxis

The anti-cathepsin G antibodies reduced the chemotactic response of neutrophils to $10^{-8}$ M fMLP in a dose-dependent manner (Fig. 1) from a control value of 30.5 $\pm$ 3.1 to 11.0 $\pm$ 2.2 cpf at 5.7 µg/ml ($p < 0.025$). The antibody did not affect cell viability (as measured by trypan blue exclusion) or cause neutrophil agglutination (as visualized under light microscopy at 400x magnification), and the inhibition could not be prevented by 5 µg/ml polymyxin B sulfate, a sequester of endotoxin (37). Furthermore, the antibody was able to exert its effect over a range of fMLP concentrations ($10^{-10}$ to $10^{-8}$ M) and was not itself chemotactic when placed in the lower chamber (positive control, $10^{-8}$ M fMLP 44.5 cpf; negative control, 1.3 cpf; 5.7 µg/ml anti-cathepsin G antibody, 1.1 cpf; $n = 2$).

It was possible that the inhibition was nonspecific and resulted from the interaction of the antibody Fc fragment with neutrophil receptors (38). To assess this possibility, the cells were incubated with an immunopurified antibody to the macrophage protein cathepsin B, which is not present in human neutrophils (39). The antibody had no effect on neutrophil chemotaxis (control 24.2 $\pm$ 2.3 cpf; anti-cathepsin G antibody 23.3 $\pm$ 1.9 cpf at 5.7 µg/ml; $n = 4$), whereas similar concentrations of anti-cathepsin G antibody inhibited cell migration by 64%.

Furthermore, polyclonal anti-elastase antibodies also had no effect on the chemotactic response of neutrophils to fMLP, suggesting that the effect was specific to cathepsin G.

The Effect of Chloromethylketones on Neutrophil Chemotaxis

The specific inhibitor of cathepsin G, Z-Gly-Leu-Phe-CMK (40), was highly effective at reducing neutrophil chemotaxis to fMLP. Cell migration was reduced from a control value of 39.5 $\pm$ 4.4 to 3.9 $\pm$ 2.6 cpf (90.2% inhibition; $p < 0.025$) at 55 µM (Fig. 2a). Similarly, Cbz-Gly-Gly-Phe-CMK, an inhibitor of chymotrypsin (41) and to a lesser extent of cathepsin G (40), reduced chemotaxis from a control value of 36.6 $\pm$ 2.7 to 1.5 $\pm$ 1.1 cpf (97.0% inhibition; $p < 0.025$) at 55 µM (Fig. 2a). MeO-Suc-Ala-Ala-Pro-Val-CMK, a specific inhibitor of neutrophil elastase that has no effect on cathepsin G (40, 42), also inhibited neutrophil chemotaxis although less effectively. At 65 µM this chloromethylketone reduced the chemotactic response to fMLP from a control value of 31.8 $\pm$ 6.6 to 14.5 $\pm$ 5.5 cpf (54% inhibition; $p < 0.025$), although lower concentrations (55 µM and below) had no effect (Fig. 2b). None of these agents at concentrations of up to 100 µM had any effect on cell viability as determined by trypan blue exclusion or by lactate dehydrogenase release. Furthermore, none of the chloromethylketones was chemotactic when placed in the lower chamber.

The chloromethylketone inhibitor of trypsin (tosyl-Lys-CMK; Ref. 43), an enzyme that is not found in the neutrophil, had no effect on neutrophil chemotaxis at concentrations of up to 100 µM (control 33.4 $\pm$ 3.1 cpf; 100 µM tosyl-Lys-CMK 29.2 $\pm$ 4.0 cpf; $n = 6$). In addition Cbz-Phe-Ala-DMK, a specific inhibitor of the thiol protease cathepsin B, had no effect on chemotaxis at concentrations up to 100 µM (control 30.8 $\pm$ 4.7 cpf; 100 µM Cbz-Phe-Ala-DMK 26.5 $\pm$ 2.7 cpf; $n = 4$).

Z-Gly-Leu-Phe-CMK (45 µM) suppressed the neutrophil chemotactic response over a range of fMLP concentrations (Fig. 3a). In contrast, similar concentrations of both Cbz-Gly-Gly-Phe-CMK and MeO-Suc-Ala-Ala-Pro-Val-CMK produced a shift in the peak chemotactic response to fMLP.

**Fig. 1.** The effect of anti-cathepsin G antibody on neutrophil chemotaxis to $10^{-8}$ M fMLP. The x axis represents increasing concentrations of antibody, and the y axis the represents the average number of neutrophils counted per high power field. The histogram is mean with standard error bars. The significance of any differences from the control value is indicated.

**Fig. 2.** The effect of Z-Gly-Leu-Phe-CMK, Cbz-Gly-Gly-Phe-CMK (a) and MeO-Suc-Ala-Ala-Pro-Val-CMK (b) on neutrophil chemotaxis to $10^{-8}$ M fMLP. The x axis represents increasing concentrations of inhibitor, and the y axis represents the average number of neutrophils counted per high power field. The histograms are mean with standard error bars. The significance of any differences from the control value is indicated.
The Effect of Antitrypsin Mutants on Neutrophil Chemotaxis—None of the recombinant antitrypsin preparations inhibited neutrophil chemotaxis at concentrations of up to 0.48 μM active site (data not shown). However when the concentration of methionine (P1) antitrypsin was increased to 0.95 μM, there was a 30% reduction in chemotactic response to fMLP (p < 0.05).

Recombinant P1 Antichymotrypsin Mutants

Association Rate Constants of P1 Antichymotrypsin Mutants—The association rate constants of the antichymotrypsin mutants and bovine chymotrypsin, neutrophil elastase, and cathepsin G are shown in Table II. Progress curve analysis allowed the determination of K, values for leucine (P1) antichymotrypsin, methionine (P1) antichymotrypsin, phenylalanine (P1) antichymotrypsin, and arginine (P1) antichymotrypsin with cathepsin G as 62 ± 9 pm, 79 ± 10 pm, 208 ± 11 pm, and 1.9 ± 0.09 nm, respectively.

The Effect of Antichymotrypsin Mutants on Neutrophil Chemotaxis—Those proteins that were efficient inhibitors of cathepsin G were also able to inhibit neutrophil chemotaxis to the peptide fMLP. Leucine (P1) antichymotrypsin produced a concentration-related fall in neutrophil chemotaxis from a control value of 51.5 ± 5.6 to 23.6 ± 3.8 cpf at 0.44 μM active site (Fig. 4). Similarly, phenylalanine (P1) antichymotrypsin produced a concentration-related fall in neutrophil chemotaxis from a control value of 36.8 ± 2.5 to 16.2 ± 2.0 cpf at 0.44 μM active site, and methionine (P1) antichymotrypsin reduced the chemotactic response from 41.4 ± 1.9 to 18.8 ± 2.0 cpf at 0.44 μM active site (Fig. 5).

There was no effect on cell viability, and the proteins were not themselves chemoattractants. Furthermore the effects were not reduced by the endotoxin sequeser polymyxin B, and the inhibition was apparent over a range of fMLP concentrations (data not shown). Arginine (P1) antichymotrypsin had no effect on the chemotactic response at active site concentrations (against human α-thrombin) of up to 1.5 μM when a slight but significant (p < 0.025) fall was observed (control 32 ± 3.6 cpf; arginine (P1) antichymotrypsin 1.5 μM active site 26.6 ± 3.4 cpf; n = 6).

The inhibitory effect of antichymotrypsin was also apparent with a second chemotactrant, zymosan-activated serum. Using this agent, the peak chemotactrant response was observed at a dilution of 7% (v/v). Leucine (P1) antichymotrypsin reduced the control value from 38.6 ± 6.0 to 30.6 ± 7.2 cpf at 0.22 μM and to 23.8 ± 3.3 cpf at 0.44 μM active site. Arginine (P1) antichymotrypsin had no effect at active site concentrations of up to 1.5 μM (control 24.0 ± 4.4 cpf; arginine (P1) antichymotrypsin 1.5 μM active site 29.4 ± 6.5; n = 4).

The Effect of Inhibitors of Cathepsin G on Neutrophil Migration under Agarose—There has been some debate about the ability of the multiple blind well assay system to differentiate between neutrophil chemotaxis and random movement, chemokinesis. Inhibitors of cathepsin G were therefore assessed, by a different observer, for their effect on neutrophil migration to fMLP under agarose. The peak leading front response was apparent at 10⁻⁹ M fMLP and was reduced in a dose-related manner by inhibitors of cathepsin G and chymotrypsin. Z-Gly-Leu-Phe-CMK reduced the leading front from a control value of 8.3 ± 5.6 to 7.2 ± 5.3 mm at 1 μM, 3.7 ± 0.3 mm at 10 μM, 2.7 ± 0.7 mm at 50 μM, and 1.7 ± 0.3 mm at 100 μM. Similarly Cbz-Gly-Gly-Phe-CMK reduced the control value from 8.3 ± 0.7 to 7.0 ± 0.1 mm at 1 μM, 4.7 ± 0.3 mm at 10 μM, 4.3 ± 0.4 mm at 50 μM, and 1.7 ± 0.3 mm at 100 μM. Plasma antichymotrypsin-reduced neutrophil chemotaxis from the control value to 7.0 ± 0.6 mm at 0.1 μM, 6.7 ± 0.3 mm at 0.3 μM and 5.7 ± 0.3 mm

![Graph](https://via.placeholder.com/150)
at 0.5 μM. All values are based on results of cells isolated from 3 individuals.

The Effect of Inhibitors of Cathepsin G on Neutrophil Polarization—Z-Gly-Leu-Phe-CMK abolished neutrophil polarization at 100 μM in a dose-dependent manner (Fig. 5). Cbz-Gly-Gly-Phe-CMK was less effective at inhibiting neutrophil shape change and at higher concentrations activated the cells inducing polarization above the value of the negative control. Anticytptotrysin had no effect on polarization at concentrations of 0.5 μM (Fig. 5).

### Table I

| Inhibitor          | M(P1)AT | V(P1)AT | K(P1)AT | R(P1)AT |
|--------------------|---------|---------|---------|---------|
| Bovine chymotrypsin| 3.6 ± 0.1 × 10^6 (2.5) | 1.0 ± 0.6 × 10^6 (50) | 2.6 ± 0.3 × 10^4 (25) | 3.5 ± 0.2 × 10^5 (50) |
| Elastase           | 1.9 ± 0.02 × 10^2 (0.1) | 1.1 ± 1.7 × 10^3 (0.5) | 7.1 ± 0.7 × 10^3 (10)^* | 1.8 ± 0.04 × 10^2 (100)^* |
| Cathepsin G        | 1.6 ± 0.08 × 10^4 (1.4)^# | <1.1 × 10^2 (100)^# | <1.7 × 10^3 (0.1)^# | 2.9 ± 0.3 × 10^3 (1.0)^# |

The association rate constants (s⁻¹) for P1 antichymotrypsin mutants with bovine α-chymotrypsin, neutrophil elastase, and cathepsin G L, M, R, and F represent leucine, methionine, arginine, and phenylalanine (P1) antichymotrypsin, respectively. The active site values of L(P1)ACh, M(P1)ACh, and F(P1)ACh were determined against bovine α-chymotrypsin and that for R(P1)ACh against thrombin. The association rate constants were determined under second order conditions apart from those highlighted ‡, which were determined using progress curves, and those marked *, which were determined under pseudo-first order conditions using 7–28 fold excess of inhibitor over enzyme. The enzyme concentration is given in brackets, and the values for second order kinetics and progress curves are quoted as weighted mean with standard error. Pseudo-first order kinetics are quoted with standard deviation. All assays were performed at 20°C. The correlation co-efficient of M(P1)ACh and neutrophil elastase, determined under pseudo-first order conditions was 0.97.

### Table II

| Inhibitor          | L(P1)ACh | M(P1)ACh | R(P1)ACh | F(P1)ACh |
|--------------------|----------|----------|----------|----------|
| Bovine chymotrypsin| 5.5 ± 0.1 × 10^3 (6.7) | 4.6 ± 0.1 × 10^3 (6.7) | 4.6 ± 0.1 × 10^4 (50) | 3.0 ± 0.1 × 10^5 (50) |
| Elastase           | <1 × 10^5 (100)^* | 6.6 ± 0.7 × 10^4 (100)^* | <1 × 10^5 (20)^* | <5 × 10^5 (100)^* |
| Cathepsin G        | 8.1 ± 0.3 × 10^5 (1.0)^# | 1.0 ± 0.1 × 10^6 (1.0)^# | 5.0 ± 0.2 × 10^4 (1.0)^# | 4.5 ± 0.15 × 10^5 (1.0)^# |

**FIG. 4. The effect of leucine, methionine, and phenylalanine (P1) antichymotrypsin on neutrophil chemotaxis to 10⁻⁸ M fMLP.** The histograms are mean with standard error bars for six experiments. The significance of any differences from the control value is indicated.

**FIG. 5. The effect of Z-Gly-Leu-Phe-CMK, Cbz-Gly-Gly-Phe-CMK, and plasma antichymotrypsin on neutrophil polarization to 10⁻⁸ M fMLP.** The curves are representative of the results obtained on cells from three subjects. A, negative control; B, positive control (10⁻⁸ M fMLP); C, Z-Gly-Leu-Phe-CMK 100 μM; D, Z-Gly-Leu-Phe-CMK 50 μM; E, Z-Gly-Leu-Phe-CMK 10 μM; F, Z-Gly-Leu-Phe-CMK 1 μM; G, Cbz-Gly-Gly-Phe-CMK 100 μM; H, plasma antichymotrypsin 0.5 μM.

### DISCUSSION

Despite the obvious importance of neutrophil migration, little is known about the mechanisms available to control or reduce this response at a site of inflammation after the initiating insult has been removed. We have shown that inhibitors of cathepsin G (antibodies, chloromethylketones, and active site mutants of antichymotrypsin) are able to attenuate neutrophil migration in vitro. The chloromethylketones and anticytptotrysin that retard neutrophil chemotaxis were also rapid inhibitors of bovine α-chymotrypsin.

Immunopurified antibodies to cathepsin G were able to retard neutrophil chemotaxis to the chemoattractant fMLP, presumably by binding to this enzyme on the surface of the neu-
trophils. The results of King et al. (16) also support the hypothesis that cathepsin G plays a role in neutrophil activation; antibodies against a chymotrypsin-like enzyme on the surface of neutrophils were able to inhibit superoxide anion production induced by FMLP. The absence of an effect with anti-elastase and anti-cathepsin B antibodies suggested a specific role for cathepsin G in mediating the cellular response to FMLP. In order to confirm this role for cathepsin G, synthetic chloromethylketone inhibitors of neutrophil elastase, cathepsin G, chymotrypsin and trypsin were assessed for their ability to attenuate neutrophil chemotaxis. Those that inhibited predominantly cathepsin G (Z-Gly-Leu-Phe-CMK) and chymotrypsin (Cbz-Gly-Gly-Phe-CMK) were potent inhibitors of chemo-migration or chemokinesis (24). Z-Gly-Leu-Phe-CMK reduced chemotaxis by 50% at a concentration of 0.48 mM (20) and 4.1 M−1 s−1, respectively (40). The concentrations of chloromethylketones used here were similar to those used by other workers to inhibit chemotaxis (11–13) and attenuate antibody-dependent cellular cytotoxicity (44). Strikingly, the reduction in chemotaxis by both Cbz-Gly-Gly-Phe-CMK and MeOSuc-Ala-Ala-Pro-Val-CMK was mitigated by increasing the concentration of the chemoattractant. This suggests that these agents were not irreversible inhibitors of chemotaxis. In contrast, the effect of Z-Gly-Leu-Phe-CMK, which is significantly more efficient in inactivating cathepsin G than chymotrypsin (51.2 M−1 s−1 and 3.0 M−1 s−1, respectively (40)), was apparent over a range of FMLP concentrations. The results with synthetic inhibitors led to the assessment of variants of the naturally occurring protease inhibitors, antitrypsin, and antichymotrypsin, on neutrophil chemotaxis.

Active site mutants were used to probe the specificity of the neutrophil surface enzyme involved in the chemo tactic response. The results were assessed using active concentrations of proteins as this allowed a more useful comparison between proteins and avoided inaccuracies and distortions when using preparations with different specific activities. Recombinant antitrypsin preparations had no effect on neutrophil chemotaxis at active site concentrations of up to 0.48 μM (20 μg/ml). Indeed the concentration of methionine (P1) antitrypsin had to be raised to 0.98 μM (40 μg/ml) before there was a significant fall in the chemotactic response. Nevertheless this response still occurred at physiological concentrations of antitrypsin found within the plasma or at sites of inflammation (45). Clearly the inhibition of neutrophil elastase alone (which was efficiently obtained with valine (P1) antitrypsin) was insufficient to inhibit the chemotactic response at concentrations up to 0.48 μM.

Active site mutants of antichymotrypsin, the cognate inhibitor of cathepsin G, were then assessed for their ability to inhibit chemotaxis. The kinetic analysis confirmed that those mutants, which were efficient inhibitors of cathepsin G and chymotrypsin (leucine, methionine, and phenylalanine (P1) antichymotrypsin) were also able to attenuate neutrophil chemotaxis to the peptide FMLP. Once again, this occurred at concentrations well below those obtained in the plasma or at sites of inflammation (46). The importance of inhibitor specificity was confirmed by arginine (P1) antichymotrypsin, which has a 10-fold lower association rate constant and forms a 10-fold less stable complex (as determined by the Kᵣ value) with cathepsin G than the other active site mutants. This protein had only a small effect on chemotaxis at concentrations over 3-fold greater than those required by the other mutants to reduce the chemotactic response by over 50% (Fig. 4). The effect of inhibitors of cathepsin G on neutrophil migration was confirmed in assays under agarose. Such assay systems also take into account random migration or chemokinesis.

Previous work has shown that the neutrophil surface serine protease has a Kᵣ value for a synthetic substrate closer to chymotrypsin than cathepsin G (24), suggesting that the enzyme, although cathepsin G-like, is not cathepsin G. The present data, however, suggest that the enzyme involved in the control of chemotaxis is unlikely to have a specificity identical to chymotrypsin, as methionine (P1) antitrypsin is able to inhibit chymotrypsin more efficiently than the antichymotrypsin mutants and yet has little effect on chemotaxis until higher concentrations of inhibitor are used. The association rate constants reported here allow the determination of half time for the inhibition of cathepsin G at a given inhibitor concentration. For the antichymotrypsin mutants tested, there was a correlation between a short half-life for the inhibition of cathepsin G and a reduction in the chemotactic response. The concentrations of antichymotrypsin mutants that reduced chemotaxis by 50% would inactivate cathepsin G with a half-life of approximately 1.5–3 s. The correlation between a short half-life for inactivation of cathepsin G and the inhibition of chemotaxis could not be extended across different classes of inhibitors. Z-Gly-Leu-Phe-CMK reduced chemotaxis by 50% at a concentration that would inactivate cathepsin G with a half-life of 345 s. This is significantly longer for the same inhibition of chemotaxis by the antichymotrypsin mutants and suggests that the enzyme may not be cathepsin G or that, by virtue of their size, chloromethylketones are more able to inactivate membrane bound cathepsin G than larger proteins. A third possibility is that the uncharged chloromethylketones are able to cross the cell membrane and inhibit an intracellular cathepsin G that is released from intracellular granules during chemotaxis. The differential effects of antichymotrypsin and the chloromethylketone inhibitors is underscored by their effect on neutrophil shape change in response to FMLP. The cathepsin G inhibitor Z-Gly-Leu-Phe-CMK abolished neutrophil shape change in a dose-dependent manner, Cbz-Gly-Gly-Phe-CMK, a less potent inhibitor, had less effect, whereas antichymotrypsin had no effect at all. Thus chloromethylketone inhibitors mediate their effect, at least partly, by blocking cell shape change in response to a chemoattractant, but the point of action of antichymotrypsin occurs subsequent to polarization.

The results suggest that the serpins antitrypsin and antichymotrypsin can play an important role in modulating neutrophil migration by interacting with cathepsin G or a chymotrypsin-like enzyme on the surface of the neutrophil. This contrast with the effect of antichymotrypsin on neutrophil superoxide anion production, which appears to be independent of the active site (24) and may be mediated by enzyme-inhibitor complex formation (47). Interestingly, both complexed (48, 49) and cleaved (50, 51) serpins are able to stimulate neutrophil chemotaxis. Thus native and cleaved proteins may interact to modulate neutrophil migration at sites of inflammation, the former reducing chemotaxis during periods of health, and the latter promoting cell migration to sites of inflammation.

Acknowledgments—We wish to thank Dr. H. P. Schnebli for providing the methionine (P1) antitrypsin standard and the Department of Biochemistry, University of Cambridge for performing N-terminal sequencing and amino acid analysis on the recombinant proteins.

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