Monoclonal Antibody Characterization of a Chymotrypsin-like Molecule on Neutrophil Membrane Associated with Cellular Activation

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Abstract

Monoclonal antibody 1-15 (Ab 1-15), is a murine anti-human neutrophil (PMN) IgG_1 that inhibits PMN effector responses to *N*-formyl-met-leu-phe (FMLP) and phorbol myristate acetate. In this study, the effects of Ab 1-15 on PMN membrane-related functions were characterized: Ab 1-15 inhibited PMN superoxide (O_2^-) response to FMLP by 60% ($P < 0.005$) without effect on the onset or duration of O_2^- production. This inhibition of O_2^- response was associated with a significant inhibition of PMN chymotrypsin-like, but not trypsin-like, protease activity. Cell fractionation studies indicated the presence of an Ab 1-15–inhibitible, chymotryptic neutral protease activity in PMN membranes. In studies of Ab 1-15 effects on membrane-related second messenger pathways, Ab 1-15 augmented both FMLP- and isoproterenol-induced intracellular cAMP accumulation, whereas α-chymotrypsin decreased PMN cAMP response to these stimuli. Our data suggest that the function-inhibiting, anti-PMN monoclonal Ab 1-15 defines a PMN chymotryptic enzyme on the membrane surface that is involved in regulation of two membrane-related functions, O_2^- generation and cAMP generation.

Introduction

Neutrophil polymorphonuclear leukocyte (PMN)[1] activation depends on the function of multiple membrane components: in response to peptide stimuli (e.g., the chemotactic factor *N*-formyl-met-leu-phe [FMLP]), activation occurs as a consequence of stimulus binding to receptors on the cell surface (1–4). Immediate, secondary, stimulus-induced changes in PMN metabolism include the activation of phospholipases (5–7), which cause the release of polyphosphoinositides and arachidonic acid, and the activation as well as down-regulation of adenylate cyclase activity (8, 9). These and other early membrane events culminate, after a brief lag period, in detectable increases in important cellular effector functions, including the generation of superoxide anion (10, 11).

To date, many of the molecular mechanisms that couple stimulus exposure to subsequent activation of PMN membrane metabolism remain unknown. Research has been limited by problems confronted in precisely identifying, isolating, and modifying the individual membrane components that are involved in PMN activation. Recently, pertussis toxin, (islet cell activating factor, a highly specific inhibitor of the inhibitory guanine nucleotide regulatory protein [N_i]), has been used to define the significant role played by the N_i in the activation of PMN effector responses (9, 12–14). In a similar fashion, studies using function-inhibiting monoclonal anti–PMN antibodies are beginning to dissect the functions of specific PMN surface antigens (3, 15–19).

In this paper, we describe the ability of a function-inhibiting, PMN-specific monoclonal antibody, monoclonal antibody 1-15 (Ab 1-15), to inhibit PMN membrane–associated chymotryptic activity in conjunction with its inhibition of FMLP-induced superoxide generation. Although the role of proteases in PMN activation has remained controversial for the past two decades (20–26), evidence from many mammalian cell systems now suggests that endogenous membrane proteases and exogenous proteases significantly modify membrane structure and function during the process of cellular activation. Significant protease-induced changes include alterations of receptor expression on platelets (27) and on neuronal cells (28), alteration of cAMP accumulation in platelets, kidney cells, and fibroblasts (29, 30), and alterations in vascular endothelial cell adhesiveness (31). In particular, protease treatment has been shown to alter the function of the regulatory N_i molecule in diverse cell systems (29, 30). Furthermore, exposure to chymotryptic proteases, including neutrophil-derived cathepsin G, has been shown to augment the stimulus-induced effector responses of monocytes, B cells, and neutrophils (32–34).

We previously reported that PMN preincubation with Ab 1-15 inhibits cellular responses to FMLP and phorbol myristate acetate in assays of superoxide generation (19). Ab 1-15 also inhibits PMN degranulation and adhesive responses to FMLP, but, notably has no effect on cell chemotactic response to this peptide. The target PMN surface antigen for Ab 1-15 is an M_r 150,000–180,000 molecule by SDS polyacrylamide gel electrophoresis (PAGE).

In the current investigation, we detail the effects of Ab 1-15 on FMLP-induced superoxide generation, and determine that this inhibitory function is associated with antibody-mediated inhibition of a chymotryptic activity within purified PMN membrane. Although chemical chymotrypsin substrates failed to protect cells from the inhibitory effects of Ab 1-15, it was found that Ab 1-15 acts as an uncompetitive inhibitor of chymotrypsin activity. Because chymotrypsin exposure has been

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1. *Abbreviations used in this paper:* Ab 1-15, monoclonal antibody 1-15; ATEE, *N*-acetyl L-tyrosine ethyl ester; BTEE, benzoyl L-tyrosine ethyl ester; FMLP, *N*-formyl-met-leu-phe; HBSS, Hanks' balanced salt solution; N_i, inhibitory nucleotide regulatory protein; PMN, polymorphonuclear leukocytes; TAME, tosyl L-arginine methyl ester.

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shown to modify the function of the N_i molecule in other cell systems, and because the N_i molecule has been shown to play an important role in mediating FMLP-induced cell activation, we examined the effects of Ab 1-15 (a protease inhibitor), and the effects of chymotrypsin on cAMP accumulation in response to two different receptor-dependent stimuli, FMLP and isoproterenol. Ab 1-15 augmented, and chymotrypsin decreased cAMP accumulation in response to either isoproterenol or FMLP stimulation. However, pertussis toxin had no effect on chymotrypsin's inhibition of cAMP response to FMLP, suggesting that this effect is unrelated to N_i molecule activation. Our findings indicate that the Ab 1-15 target antigen/protease is involved in modulating two important events associated with PMN activation, superoxide generation, and inhibition of cAMP accumulation. The precise targets of this activation-associated protease remain to be defined.

Methods

Chemicals. 10 mM phosphate-buffered normal saline, bovine serum albumin (BSA; radioimmunoassay [RIA] grade), horse heart ferricytochrome C (Type III), superoxide dismutase, FMLP, benzoyl-L-tyrosine ethyl ester (BTEE), *N*-acetyl-L-tyrosine ethyl ester (ATEE), tosyl-L-arginine methyl ester (TAME), isoproterenol, crystalline bovine α-chymotrypsin (EC 3.4.21.1), bovine trypsin (EC 3.4.21.4, tosyl phenyl chlormethyl ketone-treated), xanthine, and xanthine oxidase were purchased from Sigma Chemical Co. (St. Louis, MO). 2,6,10,14 tetra methyl pentadecane (Pristane) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Ficoll-Hypaque and dextran were obtained from Pharmacia Fine Chemicals, Inc. (Piscataway, NJ) and Hanks' balanced salt solution (HBSS) with and without calcium and magnesium was obtained from KC Biologicals Inc. (Lenexa, KS). ^{125}I-labeled F(ab')$_2$ fragments of sheep anti-mouse IgG (10 mCi/mg) were purchased from New England Nuclear (Boston, MA). ^{125}I-tyrosine methylester of cAMP was obtained from Becton-Dickinson & Co. (Paramus, NJ). Isobutyl methylxanthine was obtained from Calbiochem-Behring Corp. (La Jolla, CA). Purified pertussis toxin was a gift of Dr. Eric Hewlett (University of Virginia, Charlottesville, VA).

In control studies the solvents used to prepare reagents (ethanol or dimethylsulfoxide) had no effect on cell viability (as assessed by trypan blue exclusion) (35) or cell function in vitro.

Preparation and characterization of Ab 1-15. Purified monoclonal Ab 1-15 was prepared from malignant hybridoma ascites fluid as previously described (19, 36, 37). Cell function assays were performed using either purified IgG or F(ab')$_2$ fragments prepared from IgG by pepsin digestion (38). Some functional studies were performed using Fab' monomer prepared by reduction of F(ab')$_2$ fragments with 2-mercaptoethanol (38). Similarly processed irrelevant IgG$_1$ murine monoclonal antibody was used as control in all studies. Protein determinations were carried out using the method of Lowry et al. (39).

Preparation of cells. Granulocytes were isolated from heparinized venous blood obtained from healthy adult donors by density centrifugation on Ficoll-Hypaque followed by dextran sedimentation and hypotonic lysis of contaminating erythrocytes (40). To avoid cell activation during purification, all washings of cells were performed in Ca^{+2}- and Mg^{+2}-free HBSS at 4°C; complete HBSS (containing Ca^{+2} and Mg^{+2}) was used as final buffer for functional studies. Purity of neutrophil populations was assessed by Wright-Giemsa staining and was > 95%. Viability was determined by trypan blue exclusion and was also > 95%. All PMN preparations were used within 4 h of isolation.

Measurement of superoxide production. O_2^- generation was assayed spectrophotometrically as superoxide dismutase-inhibitable reduction of ferricytochrome C using an extinction coefficient of 2.1×10^4 M^{-1} cm^{-1} (41). One million PMNs in 50 μl HBSS were combined with 500 μl cytochrome C (1.2 mg/ml in HBSS) and 100 μl (500 μg) control or antineutrophil antibody in a cuvette. The volume was brought up to 1,000 μl with HBSS and the mixture was incubated for 2 min at 37°C. 10 μl FMLP (final concentration 5×10^{-7} M) was then added, the solution mixed, and absorbance recorded every minute. For experiments in which the effects of BTEE or ATEE were evaluated, cells were preincubated with various concentrations of these reagents for 2 min at 37°C before starting the assay.

Detection of protease activity in intact PMNs and their subcellular fractions. Chymotrypsin activity was measured by hydrolysis of BTEE as previously described (42). Five million PMNs were suspended in 1 ml 0.00107 M BTEE and incubated on a shaker for 30 min at 37°C. The cells were then pelleted and the absorbance at 256 nm of the supernatant was compared with that of substrate that had not been exposed to PMNs. Trypsin activity was determined using 0.01 M TAME as substrate and readings were taken at 247 nm (43). The effect of antibody on protease activity was determined by incubating cells and substrate in the presence of either 250 μg control antibody or 250 μg Ab 1-15.

For subcellular localization of cell chymotrypsin activity, nitrogen cavitation, and differential centrifugation at 1,000, 27,000, and 105,000 *g* (as described by Klempner et al. [44]) were used to prepare cell fractions enriched, respectively, for nuclei, lysosomal granules, and plasma membrane. Membrane purification was confirmed using PMN surface labeling with ^{3}H-concanavalin A as described by Record et al. (45). In our studies of chymotrypsin activity of PMN fractions, the only modification of the fractionation protocol was the omission of cell pre-exposure to protease inhibitors. Protease activity of solubilized cell components were measured as described below for bovine chymotrypsin.

Characterization of Ab 1-15 interaction with soluble proteases. To determine the effect of Ab 1-15 on protease activities in cell-free systems, chymotrypsin stock of 1 mg/ml in 1 mM HCl was diluted 1:50 with 1 mM HCl just before use. Equal volumes (100 μl) of enzyme and antibody (25–500 μg) were preincubated on ice for 15 min, with shaking every 5 min. 200 μl of chymotrypsin buffer (0.08 M Tris-HCl [pH 7.8] containing 0.1 M $CaCl_2$) were added and the contents were mixed with BTEE substrate. Absorbance at 256 nm was followed every minute for 10 min (43). Trypsin was used at 10 μg/ml freshly diluted in trypsin buffer (0.05 M Tris and 0.01 M $CaCl_2$ [pH 8.1]) from a 1 mg/ml stock. Antibody inhibition studies were carried out as above using the appropriate trypsin buffer and substrate (0.01 M TAME); the spectrophotometer readings were performed every 40 s at 247 nm (43). To confirm specific Ab 1-15 recognition of chymotrypsin-like molecules, Ab 1-15 immunoprecipitation of ^{125}I radiolabeled chymotrypsin (prepared by chloroamine-T method (46) using carrier-free Na ^{125}Iodide [New England Nuclear]) was performed as previously described (19) using a precipitating sheep anti-mouse IgG second antibody (Cappel Laboratories, Cochraneville, PA).

Assay of intracellular cAMP levels. For assay of Ab 1-15 effects on cAMP production by stimulated and unstimulated cells, 10^6 PMN preincubated with control IgG, Ab 1-15, pertussis toxin or medium were added to prewarmed HBSS containing 0.5 mM isobutylmethylxanthine and appropriate stimulus (10 μM isoproterenol or 1 μM FMLP) or control medium. Intracellular cAMP accumulation was measured at 0–5 min by RIA (as previously described [47]) after extraction with TCA. Results were normalized for protein content, and are expressed as femtomoles cAMP per microgram protein or as ΔcAMP accumulation = (experimental − control)/control × 100 (%). In studies of PMN cAMP response with chymotrypsin and/or pertussis toxin, control experiments were run in the presence of equal microgram amounts of BSA.

Data analysis. Differences between experimental groups were tested for statistical significance using *t* test for unpaired data.

Results

Effect of Ab 1-15 on PMN superoxide production. To characterize the effects of Ab 1-15 and its (Fab')$_2$ dimer and F(ab') monomer on cell respiratory burst, PMNs were incubated in the presence of varying concentrations of Ab 1-15 or irrelevant IgG$_1$, and assayed for superoxide production in response to FMLP. Preliminary studies established that in 25–500 μg/ml of control

irrelevant IgG, 0.5 μM FMLP afforded 90–95% of maximal cell superoxide response (12 nmol/10^6 PMN per 10 min) without impairment of cell viability. Subsequent antibody inhibition studies were performed at this concentration of FMLP. In medium containing 500 μg/ml control antibody, superoxide production by 10^6 PMNs was 10.9±3.7 nmol/10 min, whereas PMN exposed to 500 μg/ml of Ab 1-15, gave an O_2^- response of 4.3±1.7 nmol/10 min (mean of three separate experiments). This 60% reduction in superoxide response was significantly different at $P < 0.005$ level. Furthermore, PMN incubation with Ab 1-15 was noted to reduce significantly the maximum rate of cell superoxide production in response to FMLP (1.1 nmol/min in comparison with 2.6 nmol/min for cells incubated in control antibody, $P < 0.01$) without having a significant effect on the onset or duration of cell activation (Fig. 1 *A*). In dose–response studies, the degree of Ab 1-15–mediated inhibition of FMLP-induced superoxide generation was 19% at 0.14 μM (25 μg/ml) and increased to a maximum of 60% at an IgG concentration of 2.8 μM (500 μg/ml) (Fig. 1 *B*). Equimolar concentrations (0.14 μM) of $F(ab')_2$ dimer and F(ab') monomer derived from Ab 1-15 provided significantly greater inhibition of PMN O_2^- production than intact Ab 1-15 IgG (Fig. 1 *C*). Additional experiments showed that Ab 1-15 did not directly stimulate cell superoxide production, and that it was not a scavenger of O_2^- in the cell-free xanthine–xanthine oxidase–generating system (data not shown).

Effect of Ab 1-15 on neutrophil-associated protease activity. We next explored the possibility that the inhibitory effects of Ab 1-15 on superoxide production were secondary to a suppression of cell surface protease activity. These studies were undertaken because Ab 1-15–mediated inhibition of PMN superoxide response resembled PMN inhibition induced by inhibitors of serine proteases (21–23). Intact PMNs were assayed for chymotrypsin- and trypsin-like activity in the presence of either control antibody or Ab 1-15. Both types of enzyme activity were detectable in PMNs incubated with irrelevant IgG_1 monoclonal, (a mean of 2.7±0.6 milliU chymotrypsin activity and 0.8±0.3 milliU trypsin activity/million PMNs). Cell exposure to Ab 1-15 resulted in a 70% decrease in detectable cell-associated chymotrypsin activity (0.8±0.2 milliU, mean±SE of three experiments, $P < 0.01$), while trypsin activity was not significantly diminished (Table I).

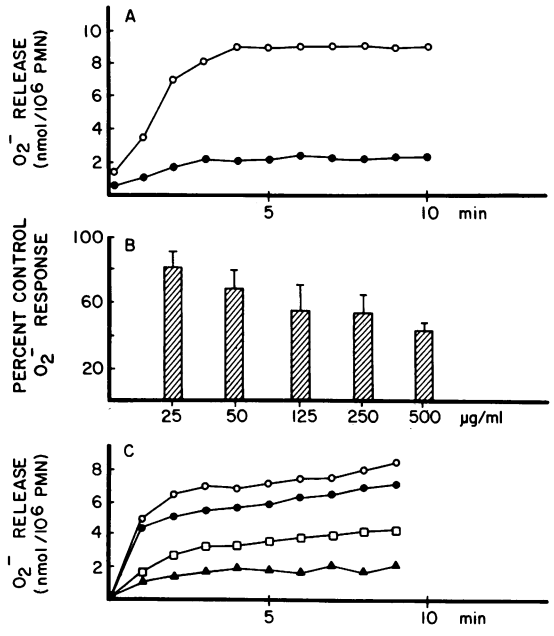

Figure 1. Effects of Ab 1-15 on PMN superoxide response. *A*, onset and duration of O_2^- generation in FMLP-stimulated PMN exposed to 500 μg/ml control IgG_1 (*open circles*) or Ab 1-15 (*solid circles*). *B*, dose–response of Ab 1-15–mediated inhibition of O_2^- production in response to fmet-leu-phe. *C*, effects of different forms of Ab 1-15 in equimolar concentrations. *Open circles*, control IgG; *solid circles*, IgG Ab 1-15; *open squares*, $F(ab')_2$; *solid triangles*, F(ab'). Data points for *A* and *C*, means of duplicate determinations from one of three similar experiments. Data in *B*, means±SE for three to five experiments at each antibody concentration.

To examine the subcellular localization of PMN proteases inhibited by Ab 1-15, subcellular fractions of PMN were prepared by nitrogen cavitation and differential sedimentation (without the use of protease inhibitors). Table II summarizes the distribution of protein recovery and marker enzymes. Pellet 1 (1000 *g*), containing nuclei and undisrupted cells, showed a mixture of all marker enzymes. Pellet 2 (27,000 *g*) was enriched for the lysosomal enzyme marker, lysozyme, but also contained a significant amount of the membrane markers alkaline phosphatase and ^{3}H-Con A. By contrast, pellet 3 (105,000 *g*) was significantly enriched ($P < 0.05$) for alkaline phosphatase and ^{3}H-Con A but had no detectable lysozyme activity, indicating relatively pure membrane fractionation. Chymotrypsin-like activity (BTEE hydrolysis at pH 7.8) was demonstrated in all recovered fractions, including the washed and resuspended membrane-containing pellet 3. Of note, the mean chymotrypsin-like activity of pellet 3 (0.16 mU/mg protein) was significantly greater ($P < 0.05$) than the activity of its supernatant (0.04 mU/mg protein). Chymotrypsin-like activity in the membrane was completely inhibited (100%) by 100 μg/ml Ab 1-15, whereas equivalent enzyme activities diluted from the resuspended pellet 2 (containing granules) and the cytosolic fraction were only partially inhibited by the same concentration of antibody (56 and 59%, respectively).

Effect of chymotrypsin synthetic substrates on inhibition of superoxide production by Ab 1-15. We subsequently examined whether chymotrypsin-specific substrates could block the Ab 1-15–mediated inhibition of O_2^- production by FMLP-stimulated PMN. It was first confirmed that concentrations ≥ 10 μM BTEE and ≥ 50 μM ATEE were themselves effective inhibitors of PMN superoxide production (23). In our attempts at substrate protection of membrane protease activity, PMN were exposed to

Table I. Ab 1-15 Effects of Trypsin- and Chymotrypsin-like Protease Activities of Intact Unstimulated PMN

PMN co-incubation	Chymotrypsin-like* activity	Trypsin-like‡ activity
	mU/10^6 PMN	*mU/10^6 PMN*
HBSS	2.5±0.9	0.7±0.2
Ab 1-15 (250 μg/ml)	0.8±0.2 (−70%)§	0.6±0.2 (−19%)
Control IgG_1 (250 μg/ml)	2.7±0.6	0.8±0.2

* Assay for chymotrypsin-like activity as hydrolysis of BTEE by 5×10^6 PMN over 30 min at 37°C.
‡ Assay for trypsin-like activity as hydrolysis of TAME as above.
§ Significant difference from control ($P < 0.05$) for three experiments.

Table II. Distribution of Marker Enzymes and Chymotrypsin-like Activity among Subcellular Fractions of Cavitated PMN

Fraction	Total cell protein recovered	Lysozyme	Alkaline phosphatase	^{3}H-Con A recovered	Chymotrypsin-like activity	Inhibition of chymotrypsin-like activity by Ab 1-15
	%	*U/μg protein*	*mU/μg protein*	*cpm/μg protein*	*mU/μg protein*	%
Pellet 1, (nuclei, debris, undisrupted PMN)	31±14	219±33	125±6	11.5	0.21±.09	NT
Pellet 2, (lysosome)	13±5	267±72	144±2	8.0	0.23±.03	56%
Pellet 3, (membrane, ER)	2±1	Below detection	302±46*	37.7	0.16±.04‡	100%
Supernatant 3, (cytosol)	55±18	Below detection	Below detection	1.9	0.04±.01	59%

Subcellular fractions were prepared by nitrogen cavitation of intact PMN and differential sedimentation at 1000 g (pellet 1), 27,000 g (pellet 2), and 105,000 g (pellet 3). For each fraction,, marker enzymes for cell organelles and chymotrypsin activity were assayed by standard techniques (43, 57, 58). In studies of Ab 1-15–mediated inhibition, 80 mU each of chymotryptic activity from pellet 2, pellet 3 and supernatant 3 were incubated with substrate (BTEE) in the presence of 100 μg/ml of Ab 1-15 or control IgG. NT = not tested. Mean values±standard error of the mean are reported from three experiments. The data for ^{3}H-Con A labeling and enzyme inhibition are the means of two experiments. * Significantly greater than alkaline phosphatase activity of pellet 2, $P < 0.05$. ‡ Significantly greater than chymotrypsin activity of supernatant 3, $P < 0.05$.

noninhibitory concentrations of either ATEE (5–25 μM) or BTEE (1–5 μM) before exposure to minimal inhibitory concentrations of Ab 1-15 (25–125 μg/ml, titrated for each cell donor) or the equivalent amount of control monoclonal. It was found that PMNs exposed to buffer, then exposed to control IgG, yielded a mean of 8.40±0.7 nmol O_2^-/10 min in the presence of 0.5 μM FMLP (mean of three experiments). By contrast, buffer-treated cells added to Ab 1-15 produced only 6.10±1.0 nmol O_2^-/10 min, a 27% inhibition in O_2^- release ($P < 0.05$). Cell preexposure to ATEE did not significantly alter the effect of Ab 1-15: ATEE pretreated cells exposed to control IgG produced 8.84±1.0 nmol O_2^-/10 min, whereas ATEE pretreated cells exposed to Ab 1-15 released 5.25±1.0 nmol O_2^-/10 min. Although somewhat greater in magnitude, the Ab 1-15–mediated inhibition (mean = 41%) of superoxide release from ATEE pretreated cells was not significantly different from Ab 1-15 inhibition (27%) of medium pretreated cells from the same donors. Furthermore, it was apparent that concentrations of substrate in 35–100 fold excess of antibody concentration did not block the inhibitory effect of Ab 1-15 on O_2^- production. Results with BTEE were comparable: Ab 1-15 mediated a mean 40% inhibition of O_2^- production by cells preincubated with buffer vs. a 56% inhibition for cells preincubated with BTEE.

Effect of Ab 1-15 on highly purified pancreatic proteases. The effect of Ab 1-15 on the activity crystalline bovine chymotrypsin and trypsin was examined next. Compared with the effects of control monoclonal antibody, preincubation of bovine chymotrypsin with Ab 1-15 (250 μg/ml) resulted in a mean 57% decrease in the enzyme's ability to hydrolyze its substrate. In three separate experiments, the specific activity of bovine chymotrypsin was 44±12 U/mg in control IgG, whereas in the presence of Ab 1-15, specific activity decreased to 19±6 U/mg ($P < .001$). In parallel experiments, an inhibitory effect on trypsin activity was not detected (691±123 U/mg in control IgG vs. 617±99 U/mg in Ab 1-15, NS). Lineweaver-Burk analysis of the effects of 100 μg/ml Ab 1-15 on bovine chymotrypsin activity indicated that this antibody modified both the V_{max} and K_m of the enzyme (Fig. 2). The pattern of inhibition, (a parallel upward shift of the kinetic plot) suggests an uncompetitive inhibition of the enzyme by Ab 1-15. The K_I of Ab 1-15 for chymotrypsin was 39 μM.

Immunologic cross-reactivity of Ab 1-15 for chymotrypsin-like peptides was demonstrated by specific immunoprecipitation of bovine α-chymotrypsin (Fig. 3). Denaturation of bovine chymotrypsin at 100°C for 5 min abolished Ab 1-15 recognition of the enzyme in the immunoprecipitation reaction.

Effects of Ab 1-15 and chymotrypsin on intermediate pathways of PMN activation. We next examined the effects of Ab 1-15 versus those of chymotrypsin on PMN cAMP response. Because earlier observations demonstrated that protease treatment of other cell lines significantly alters their cAMP response to stimulation (29, 30), we examined antibody and enzyme effects on PMN cAMP response to two receptor-dependent stimuli, FMLP and isoproterenol. Neither Ab 1-15 nor chymotrypsin had a significant effect on cAMP levels in unstimulated PMN, however PMN preincubation with Ab 1-15 significantly enhanced ($P < 0.05$) cAMP accumulation after cell stimulation with either isoproterenol (10 μM) or FMLP (1 μM) (51 and 35%, respectively, Fig. 4). By contrast, exposure of neutrophils to chymotrypsin (10 μg/ml) before challenge significantly inhibited their cAMP response (by 19, [isoproterenol] and 31% [FMLP], $P < 0.05$, Fig. 5). PMN preincubation with pertussis toxin (500 ng/ml) reduced the chymotrypsin-mediated inhibition of PMN cAMP response to isoproterenol but had no effect on chymotrypsin's inhibition of cAMP response to FMLP (Fig. 5). Neither Ab 1-15 preincubation nor chymotrypsin exposure altered cAMP response to the adenylate cyclase activator, forskolin (data not shown).

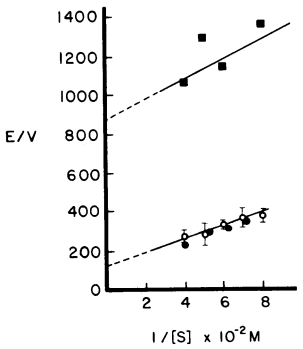

Figure 2. Lineweaver-Burk plot of Ab 1-15–mediated inhibition of bovine α-chymotrypsin. 2 μg enzyme (0.12 U) was incubated with varying concentrations of the synthetic substrate BTEE in the absence (*open circles*) or presence of antibody. 100 μg/ml Ab 1-15 (*solid squares*, $r = 0.75$) altered maximum velocity (V_{max}) and substrate affinity (K_m) whereas control IgG (*solid circles*, $r = 0.96$) did not. The apparent K_I for Ab 1-15 was 39.3 μM.

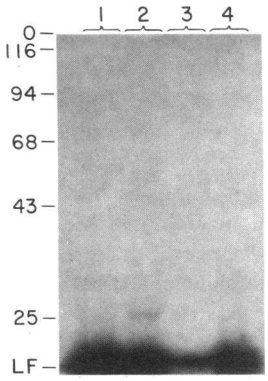

Figure 3. Immunoprecipitation of ^{125}I-labeled chymotrypsin with Ab 1-15. 100 μg of purified IgG (Ab 1-15 or control) was incubated with 20 μg of precleared ^{125}I-labeled chymotrypsin for 2 h at 37°C. Immune complexes were recovered using 100 μg precipitating sheep anti-mouse antibody (18 h at 4°C), then washed three times, and separated by SDS PAGE. Autoradiography reveals immunoprecipitation of ^{125}I-chymotrypsin by Ab 1-15 (lane *2*) but not irrelevant IgG_1 (lane *3*). Neither antibody bound heat denatured (100°C for 5 min) enzyme (lanes *1* and *4*).

Discussion

The results of this study provide a biochemical mechanism for the inhibitory effects of Ab 1-15, an anti-neutrophil monoclonal antibody. Prior studies have demonstrated that PMN exposure to Ab 1-15 inhibits cell effector responses to FMLP and phorbol ester, and that this antibody immunoprecipitates a single 180,000-mol-wt component of PMN membrane (19). In the current study, the presence of Ab 1-15 was found to reduce FMLP-induced PMN O_2^- production in a dose-dependent manner. The rate of O_2^- generation was reduced, without effects on onset or duration of O_2^- response. Furthermore, $F(Ab')_2$ dimer and Fab' monomer of Ab 1-15 reproduced the effects of the intact Ab 1-15 IgG_1 molecule, indicating that the function-inhibiting effects of the antibody are related to its antigen-combining site, and are less likely due to cross-linking of PMN surface antigens with consequent preactivation of the cell.

The Ab 1-15–mediated inhibition of chemotactic factor–induced PMN superoxide response was incomplete, with a 60% maximum reduction in superoxide response at the highest concentrations of Ab 1-15. This raises the possibility that a specific subpopulation of PMN binds and is inhibited by Ab 1-15, while another subpopulation, not bound by Ab 1-15, continues to respond normally to FMLP stimulation. However, flow cytometry studies with fluorescein-conjugated antibody have indicated

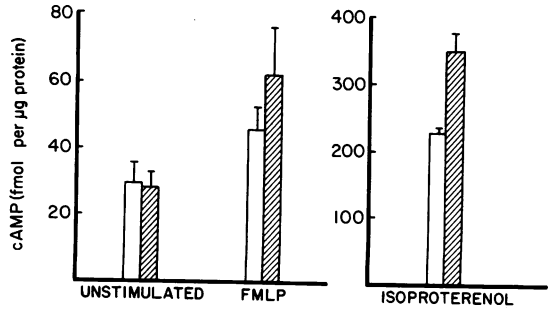

Figure 4. Ab 1-15 effects on stimulated PMN cAMP response. Intracellular cAMP accumulation by 10^6 PMN in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine was measured at 2 min for cells incubated with buffer, 10^{-6} M FMLP, or 10^{-5} M isoproterenol. cAMP accumulation was significantly enhanced ($P < 0.05$ and $P < 0.001$, respectively) for FMLP- and isoproterenol-stimulated cells pretreated with Ab 1-15 (25 μg/10^6 PMN) (*shaded bars*) vs. control IgG (*open bars*). Data are mean±SE for three to five experiments.

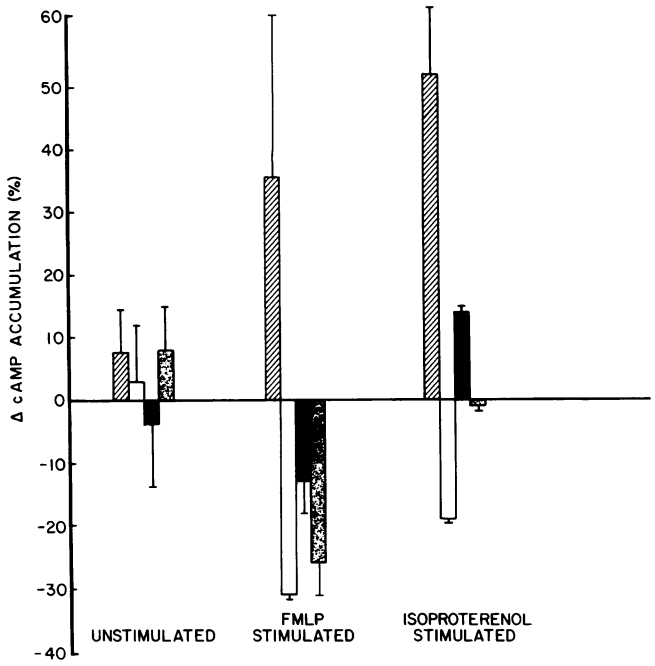

Figure 5. Effects of Ab 1-15 vs. chymotrypsin on stimulated cAMP response. Shown are the net effects of PMN exposure to Ab 1-15 (25 μg/ml, *striped bars*), α-chymotrypsin (10 μg/ml, *open bars*), pertussis toxin (1 h preincubation in 500 ng/ml, *solid bars*) and pertussis toxin preincubation followed by chymotrypsin exposure (*stippled bars*) on 2 min cAMP accumulation by resting and FMLP (10^{-6} M) and isoproterenol (10^{-5} M)-treated cells. *Bars,* mean percentage change from the cAMP levels of control cells incubated in irrelevant IgG_1 or BSA (as appropriate) for three experiments. *Lines,* SEM. Ab 1-15 significantly enhanced ($P < 0.001$ for isoproterenol, $P < 0.05$ for FMLP) and chymotrypsin significantly depressed ($P < 0.05$) stimulated cAMP response. Preincubation with pertussis toxin was found to offset the effects of chymotrypsin on response to isoproterenol but not its effects on response to FMLP.

that > 90% of purified PMN bind Ab 1-15 (Sawyer, J. A., C. A. Peck, and C. H. King, unpublished data). An alternative explanation for the incomplete inhibition of PMN response is that the target inhibited by Ab 1-15 is only partly responsible for activation and/or regulation of superoxide response to FMLP. To resolve this issue we defined an enzymatic function for the membrane antigen bound by Ab 1-15 and compared Ab 1-15's effects with those of other inhibitors of PMN activation.

We chose to examine the effects of Ab 1-15 on protease activity because of the pattern of antibody-mediated inhibition of superoxide response. Like the protease inhibitors tosyl-phenylalanyl-chlormethyl ketone and aprotinin, Ab 1-15 partially reduced PMN superoxide production and enzyme release in response to FMLP (21, 23). Unlike calcium antagonists and calmodulin inhibitors (48), Ab 1-15 did not prolong the lag period of superoxide production. Furthermore, Ab 1-15 decreased secretory responses without inhibiting chemotactic response, in a manner similar to promoters of membrane fluidity such as butanol and pentanol (49). Our results demonstrate that Ab 1-15 reduces significantly the chymotrypsin-like activity of intact PMN, as well as the chymotrypsin-like activity of membrane-enriched PMN fractions. Interference with cellular enzyme did not appear to be mediated through a direct interaction with the enzyme's active site, as excess (but noninhibitory) amounts of

chymotrypsin substrate failed to protect neutrophils from the effects of Ab 1-15 on superoxide production. However, our results with bovine chymotrypsin indicate that Ab 1-15 acts as a noncompetitive inhibitor of enzyme activity. In our previous studies, we have found comparable levels of Ab 1-15–mediated inhibition whether cells were preincubated with Ab and then washed, or simply incubated with antibody at the time of stimulation (19). These findings suggest that under normal conditions enzyme activity susceptible to Ab 1-15 inhibition is active (20) at the time of stimulation, and is not activatable, i.e., elicited during the process of cell activation (23). The observed 2.7 milliU of chymotrypsin activity for one million PMN is equivalent to $\sim 10^6$ enzyme molecules per unstimulated cell, if a catalytic constant of 25 s^{-1} (equal to the k_{cat} of α-chymotrypsin for BTEE) is assumed (50). This estimate corresponds to the mean value of 6×10^5 antigen sites/cell measured in antibody binding saturation studies (51). Adding Ab 1-15 after the onset of FMLP-induced O_2^- generation did not significantly inhibit this cell function. The kinetics of Ab 1-15 antigen movement suggest rapid surface turnover of the Ab 1-15 target molecule: ^{125}I-labeled Ab 1-15 bound to cells is internalized within 5 min at 37°C, however, the total number of surface binding sites on the PMN remains constant, even after cell activation with FMLP or calcium ionophore A23187 (51). Because failure to inhibit superoxide release under these conditions is not due to a lack of antigen targets, it appears that the Ab 1-15 antigen target may be involved only in the initiation of PMN superoxide generation and not its perpetuation.

The anti–chymotrypsin activity of Ab 1-15 demonstrated for PMN was further confirmed in studies with purified chymotrypsin enzyme. Ab 1-15 significantly inhibited the function of bovine α-chymotrypsin and specifically immunoprecipitated this enzyme in its undenatured form. The availability of a specific anti–protease antibody offers an opportunity to reexamine the controversial role of membrane proteases in PMN activation. Early studies of phosphonate ester–mediated inhibition of PMN chemotaxis suggested that PMN protease is essential for cellular activation (20). Further studies using protease inhibitors, whether macromolecular (aprotinin, soybean trypsin inhibitor) or micromolecular (synthetic ester) apparently confirmed the prostimulatory function of PMN chymotrypsin-like proteases (21–23, 25). In these studies, chymotrypsin-specific inhibitors, such as tosyl phenyl chlormethyl ketone, and chymotrypsin substrates (such as BTEE), demonstrated significantly greater inhibition of PMN functions than other protease inhibitors (21, 23). However, it appears that some of the effects of protease inhibitors on intact cells may be nonspecific. In most studies, protease inhibitors have been used in high concentration (0.1–1.0 mM), making it possible that inhibitor-mediated effects are due to several nonspecific factors, including inhibition of nonproteolytic esterases, changes in membrane fluidity, inhibitor-mediated protein alkylation or inhibitor-mediated phosphorylation (reviewed in reference 26). Results of PMN inhibition studies using macromolecular protease inhibitors (which are excluded from the cell) have been variable; one study of three highly purified soybean peptides showed an inverse correlation between protease inhibition by these peptides and their inhibition of PMN effector responses (26).

As a means of defining a function-associated PMN protease, Ab 1-15 offers several distinct advantages. In contrast to low molecular weight protease inhibitors, the Ab 1-15, because of its size and target specificity, has effects that are probably limited to surface structures. In addition, its interaction with proteins is not covalent. In micromolar concentrations, the antibody alters different aspects of FMLP- and PMA-induced cellular response without affecting cell activation by opsonized particles or by activated complement (19). In our inhibition studies, we excluded the nonspecific effects of solvents, pH, and osmolarity by dissolving Ab 1-15 in cell buffer and by using a similarly processed, isotype-matched antibody in all control assays. The cell inhibitory effects of Ab 1-15 are not due to heterologous desensitization, as antibody exposure alone did not activate superoxide or degranulation responses (19), neither did it significantly alter resting cAMP levels or membrane potential (King, C. H., and C. S. H. Goralnik, unpublished data).

Three distinct chymotryptic proteases of low molecular weight (20–25,000) have been isolated from the granules of human neutrophils (52, 53). Furthermore, Tsung et al. have described a large ($\sim$ 200,000-mol-wt) chymotryptic protease that copurifies with membranes of rabbit PMN (24). Our previous immunoprecipitation data, indicating Ab 1-15 recognition of a 180,000-mol-wt PMN surface molecule (19), and our present cell fractionation and enzyme inhibition data suggest that Ab 1-15 recognizes an analogous high molecular weight protease within purified human PMN membranes. Although this membrane protease is the most likely target for Ab 1-15–mediated inhibition when cells are intact, we noted that Ab 1-15 also partially inhibits the chymotryptic activity associated with cell granule fractions and cytosol. It is not known whether this latter effect also contributes to Ab 1-15–mediated inhibition of cellular function.

In other cells, exposure of cell membranes to chymotrypsin provides differential enhancement or depression of the function of the GTP-dependent N proteins (29, 30). In PMN, pertussis toxin, an inhibitor of the N_i molecule, inhibits FMLP-induced PMN activation in assays of chemotactic and secretory response (9, 12–14). The latter finding suggests that the N_i molecule is an important intermediary in FMLP receptor–mediated cell stimulation. We sought, therefore, to determine whether the chymotryptic activity recognized by Ab 1-15 might act to stimulate cells by enhancing the function of the N_i molecule during the early stages of membrane activation. We hypothesized that if the Ab 1-15–identified protease served to activate the N_i molecule, then antibody-mediated inhibition of this protease could reduce different N_i-dependent PMN functional responses to FMLP stimulation, such as superoxide anion generation and degranulation, as previously demonstrated (19). Additionally, protease inhibition might also enhance cAMP response to the beta-adrenergic agonist isoproterenol through a reduction of isoproterenol's N_i activation (54) and a consequent decrease in N_i-mediated effects on adenylate cyclase. We found that Ab 1-15 did indeed enhance cAMP accumulation in response to isoproterenol and to FMLP, and by contrast, that chymotrypsin treatment significantly reduced cAMP response to both of these surface receptor–dependent stimuli. However, when cells were treated with pertussis toxin to block the function of the N_i molecule, the inhibitory effect of chymotrypsin on cAMP response to FMLP remained unchanged. The net effect of chymotrypsin on the response of toxin-treated PMN to isoproterenol was < 5%, suggesting a merely additive offsetting (by chymotrypsin) of the enhancing effect of pertussis toxin. Although Lad et al. (9) have implicated the N_i molecule as mediator in the FMLP-mediated reduction of forskolin-stimulated adenylate cyclase activity, Verghese et al. (55) have recently demonstrated that in the intact

cell, FMLP-mediated alternation in cAMP levels do not appear to depend on N molecule regulation. Rather, this down-regulation of cAMP accumulation appears to depend on calcium-dependent events that modify phosphodiesterase activity. Taken together, these findings suggest that multiple membrane events are associated with ligand occupancy of the FMLP receptor, and that certain of these events, (probably excluding N_i molecule activation,) are the consequence of the proteolytic function of a membrane-associate chymotryptic enzyme. Although recent evidence has suggested an association of the Ab 1-15 protease activity with the FMLP receptor (50), Ab 1-15 also inhibits PMN response to phorbol ester (a surface receptor–independent stimulus binding directly to protein kinase C [56]) and augments cAMP response to isoproterenol. Therefore, the function of the Ab 1-15 antigen/protease is not uniquely associated with the function of the FMLP receptor, nor are its effects limited to a single facet of cell effector response.

In summary, our results demonstrate that molecules on the neutrophil surface, which enzymatically and immunologically resemble chymotrypsin, are involved in cellular response to FMLP, isoproterenol, and phorbol ester. Their function appears to be the alteration and activation of second messenger pathways situated in the PMN membrane. Further analysis of the role of Ab 1-15 antigen in membrane pathways of PMN activation will allow a better definition of the mechanisms of stimulus-response coupling in neutrophil activation.

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