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Rat Tryptase: Partial Amino Acid Sequence and Mast Cell Heterogeneity

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RAT TRYPTASE:
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VITA

The author, Zehan Chen, was born in Guangdong, China on August 30, 1957.

In the spring of 1978, Mr. Chen entered South China Agricultural University and received the degree of Bachelor of Science with honors in the spring of 1982. That year, he became an assistant teacher in the University. In January 1984, he entered Cornell University, Ithaca, New York, and received the degree of Master of Science in 1986. Soon after, he entered the Department of Molecular and Cellular Biochemistry at Loyola University of Chicago, Maywood, Illinois. In October 1988, he joined the laboratory of Dr. William H. Simmons where he initiated his research on the amino acid sequence determination and immunohistochemical localization of rat skin tryptase. He performed part of the experiments in the Laboratory of Dr. Lawrence B. Schwartz in the Medical Collage of Virginia, Richmond, Virginia. While in Loyola, he was awarded a Loyola University Basic Science Fellowship. In 1990, he worked as a summer intern on an HTLV project in the laboratory of Dr. Helen Lee in Abbott Laboratories, Illinois.

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PARTIAL AMINO ACID SEQUENCE OF RAT SKIN TRYPTASE

CHAPTER I

INTRODUCTION

Tryptase has been isolated and partially characterized from a variety of species including rat, dog, bovine, and human. The potential roles of tryptase in pathogenesis of different diseases have attracted close attention of many investigators in recent years.

Tryptase is a serine protease which hydrolyses peptide bonds on the C-terminal side of either arginine or lysine. However, tryptase does not exhibit general proteinase activity and has a stronger subsite preference and a higher degree of selectivity toward model substrates than trypsin.

Recently, amino acid sequences of tryptase from mouse, dog, and human have been deduced from the nucleotide sequence of cDNA cloned from mast cell cDNA libraries. The sequences show some basic structural differences between tryptase and trypsin. In this lab, a tryptase has been purified from rat skin and intensively characterized. The rat skin tryptase shows similar inhibitor and substrate specificity to other tryptases. However, rat skin tryptase also appears to have its unique chemical properties.

In this study, partial amino acid sequences of rat skin

tryptase have been determined using the Edman procedure and compared with other tryptase sequences. The results have revealed some profound structural differences and some common features among tryptases. These interesting observations suggest that specific amino acid residues responsible for the functional specificity of tryptases can be identified. The information obtained from this study can be very helpful in designing fulture experiments to explore the structure and function relation of tryptases.

CHAPTER II

LITERATURE REVIEW

1. Biological Function

Tryptase is a trypsin-like serine proteinase found in secretory granules of mast cells (Schwartz *et al.* 1981). Although the precise function of tryptase *in vivo* is not clear, many possible biological and pathological roles have been postulated based on a large number of *in vitro* studies.

There is evidance that tryptase may have anticoagulant effects. Tryptase can hydrolyze fibrinogen and inactivate the thrombin-induced clotting of fibrinogen (Schwartz *et al.* 1985). Tryptase also degrades high molecular weight kininogen (HMWK) (Maier *et al.* 1983). HMWK is known to convert prekallikrein to kallikrein which then can initiate the intrinsic coagulation pathway by activating factor XIIa. By hydrolyzing HMWK, tryptase rapidly inactivates HMWK-dependent coagulation activity. The role of tryptase in the conversion of low molecular weight kininogen to bradykinin, a potent vasodilator, is controversial (Schwartz *et al.* 1986, Proud *et al.* 1988). Although a systematic defect of coagulation does not usually occur, the anticoagulant effect may be important in some local tissue environments such as in mucosa, skin, and lung (Schwartz 1990). However, tryptase was shown to convert prothrombin to thrombin (Kido *et al.* 1985). The potential

biological role of this activity remains to be explained.

Tryptase has been suspected to be a mediator of airway smooth muscle hyperresponsiviness that is characteristic of asthmatic individuals (Caughey 1989, Sekizawa et al. 1989). The evidence showed that dog bronchial smooth muscle contraction was prevented by a tryptase inhibitor. Vasoactive intestinal peptide (VIP) is a potent relaxant of various types of smooth muscles (Matsuzaki et al. 1980, Said 1987). Peptide histidine-methionine (PHM) is also a potent bronchodilating peptide that co-exists with VIP and regulates blood flow and bronchial smooth muscle tone (Itoh et al. 1983, Lundberg et al. 1984, Palmer et al. 1986, Linder et al. 1987). Substance P (SP), on the other hand, is believed to be a potent bronchoconstrictor (Lundberg et al. 1983) which can cause mast cell degranulation in lung (Barrett et al. 1986) and regulate the vasodilator activity of calcitonin gene-related peptide (CGRP), a potent vasodilator in skin (Brain 1988) and lung (Palmer et al. 1987). Tryptase selectively hydrolyzed the bronchodilation peptides VIP, PHM, and CGRP but not the bronchoconstriciton peptide SP (Caughey et al. 1988, Tam & Caughey 1990, Walls et al. 1992). The evidence suggests that tryptase may contribute to smooth muscle contraction by regulating the activity of the neuropeptides.

A potential role for tryptase in peptide prohormone processing has been proposed (Cromlish et al. 1987, Proctor et al. 1991). Adrenocorticotropic hormone (ACTH) is a pituitary

hormone which regulates the growth and function of the adrenal cortex. Human pituitary tryptase selectively cleaves the ATCH(1-39) to form ACTH(1-15) and ACTH(16-39). Atrial natriuretic factor (ANF) is a peptide hormone produced by the heart (Debold 1985) which promotes a reduction of blood volume and pressure and causes a generalized vasodilation. The pro-ANF, which consists of 126 amino acids, can be converted to active ANF by human and bovine tryptase.

Processing of certain enzymes and proteins suggests other potential roles of tryptase. Tryptase was shown to cleave complement protein C3 to generate C3a, a chemotactic factor, in vitro (Schwartz et al. 1983).

A role for tryptase in the degradation of human extracellular matrix has been proposed. Rapid activation of purified prostromelysin or pro-matrix metalloprotease 3 (MMP-3) can result from incubation with physiological concentrations of tryptase (Gruber et al. 1988, 1989). The active stromelysin in turn converts the procollagenase to active collagenase (Vater et al. 1983, Ito et al. 1988). Active stromelysin is also capable of degrading other extracellular matrix components such as type IV and IX collagen, fibronectin, laminin, gelatin, and proteoglycans (Chin et al. 1985, Okada, et al. 1986, Okada et al. 1989). The evidence indicates that tryptase may play an important role in connective tissue turnover such as in the case of rheumatoid arthritis and various fibrotic conditions.

Tryptase was found to hydrolyze the DNA binding protein histone H1 effectively. The observation suggested a possible role for tryptase in the organization of DNA during cell turnover (Harvima et al. 1989). In a recent study (Ruoss et al. 1991), tryptase was implicated as a potent mitogen which strongly stimulates DNA synthesis and cell growth in cultured rat fibroblast and chinese hamster lung cell lines.

Tryptase is stored in an active form in mast cells and is released upon mast cell degranulation (Schwartz 1990b, Vassimon & Rothschild 1990). As a unique marker for the human mast cell (Craig & Schwartz 1989), tryptase has been used as a useful clinical indicator of mast cell activation (Matsson et al. 1991). The specific cellular localization, the abundance in human tissues, and the stability in serum make tryptase a preferred mast cell activation indicator over histamine which has been used traditionally in determination of anaphylaxis. Histamine can be secreted from not only mast cells, but also from basophil. The histamine level in serum declines to baseline within 15-60 minutes while tryptase has a half life in the circulation of about 2 hours (Schwartz et al. 1989).

2. Chemical And Physical Properties

Information regarding tryptase has accumulated rapidly in the past few years. Shortly after the partial purification of a neutral proteinase with characteristics of a tryptase from

human uterine cervix (Ito et al. 1980), a tryptase was purified and characterized from human pulmonary mast cells (Schwartz et al. 1981). Since then, human tryptase has been purified from lung (Smith et al. 1984), pituitary (Cromlish et al. 1987), skin (Harvima et al. 1988), as well as from a human mast cell line established from peripheral blood of a patient with leukemia (Butterfield et al. 1990). Non-human tryptases have been purified from rat peritoneal cavity mast cells (Kido et al. 1985, Lagunoff et al. 1991), rat skin (Braganza & Simmons 1991), dog mastocytoma cells originally isolated from skin tumors (Caughey et al. 1987), and mast cells from bovine liver capsula (Fiorucci et al. 1992).

A total of seven cDNAs and two genes for tryptases have been cloned and sequenced. The first cDNA for tryptase was obtained from a dog mastocytoma cDNA library (Vanderslice et al. 1989). Two cDNAs for human lung tryptases were cloned and sequenced from the same human lung mast cell cDNA library (Miller et al. 1989, 1990). The two lung tryptases exhibited 90% amino acid identity and were named α-tryptase and β-tryptase. After screening a human skin cDNA library, three skin cDNAs for tryptases with over 90% identities among them were sequenced and called human skin tryptase-1, -2, and -3 (Vanderslice et al. 1990). Human skin tryptase-2 is identical to human lung β-tryptase. The gene for human skin tryptase-1 was cloned and sequenced using the human skin tryptase-2 cDNA to screen the genomic library. In mouse, where no tryptase

has been purified, two cDNAs and a gene for tryptase were identified from mouse mast cell lines. Mouse mast cell protease-6 (Reynolds et al. 1991) is identical to mouse mast cell tryptase-1 which has 71% of amino acid identity to mouse mast cell tryptase-2 (Chu 1990). The cDNA sequences provide us with a source for the basic amino acid sequence information about tryptases.

Tryptase is a trypsin-like serine protease that hydrolyzes peptide bonds on the carboxyl side of an arginine or lysine residue. However, tryptase has a more restricted substrate specificity than trypsin. Amino acid residues around the arginine or lysine residues of the substrate greatly affect the rate of substrate hydrolysis by tryptase (Schwartz et al. 1981, Tanaka et al. 1983, Kido et al. 1985, Smith et al. 1984, Cromlish et al. 1987, Braganza & Simmons 1991). Tryptase is inhibited by the serine protease inhibitor di-isopropylfluorophosphate (DFP), trypsin inhibitors such as N-tosyl-L-lysine chloromethylketone (TLCK), and the natural microbial-derived inhibitors leupeptin and antipain. Other trypsin inhibitors such as lima bean trypsin inhibitor (LBTI), soybean trypsin inhibitor (SBTI), and ovomucoid have little or no effect on tryptases. Chymostatin, N-tosyl-L-phenylalanine chloromethylketone (TPCK), pepstatin, and ethylenediamine-tetraacetic acid (EDTA) do not inhibit the enzyme.

Although there are many common properties among tryptases from different sources, they exhibit different responses to

certain inhibitors. Rat skin tryptase, for example, is inhibited by aprotinin (Trasylol) (Braganza & Simmons 1991) while human lung tryptase is not (Smith et al. 1984). The thiol-group inhibitor, p-chloromercuribenzoate (PCMB), inhibits human pituitary tryptase but not human lung, human uterine cervix, rat, or dog tryptase. While human skin tryptase and human lung tryptase are inhibited by benzamidine, human peripheral blood mast cell tryptase is insensitive to the inhibitor. Rat peritoneal mast cell tryptase is sensitive to α-1-antitrypsin which, on the other hand, has little inhibitory effect on rat skin tryptase and other purified tryptases. Rat peritoneal mast cell tryptase was reported to be associated with a unique endogenous Kunitz-type trypsin inhibitor (or basic pancreatic trypsin inhibitor), called trypstatin (Kido et al. 1985, 1988). No associated trypstatin has been detected in other tryptases including rat skin tryptase.

The reported molecular weight of native tryptases varies from 110,000 to 150,000 depending on the source of the enzyme. Tryptase is a tetramer with subunits in the molecular weight range of 30,000 to 38,000. Variation of the reported molecular weights may be due to the extent of glycosylation of the tryptase. Tryptase treated with N-Glycanase (a deglycosylation enzyme) had a subunit molecular weight of 32,400 for human pituitary tryptase and about 27,000 for rat skin tryptase. Putative glycosylation sites detected from the

cDNA-deduced amino acid sequences suggest that different tryptases may have different numbers of glycosylation sites.

Functionally, tryptase share a great deal of their common physical and chemical properties and at the same time exhibit a substantial degree of variations among them. Structurally, despite of the high homology among tryptases, they also reveal a great extent of diversity in the amino acid sequences. The functional and structural properties of a group of similar enzymes will provide the fundamental information necessary for correlating enzyme functions to their structures. For example, chymotrypsin, trypsin and elastase are members of the serine protease family. Their functional properties have been well related to their structural features. Serine 189 in the substrate binding pocket contributes to the specificity of chymotrypsin which requires an aromatic or bulky side chain such as tyrosine, phenylalanine, tryptophan and methionine in the P_1 site of the substrate. The same position occupied by aspartate instead results in a totally different substrate specificity in trypsin. The acidic aspartate residue in trypsin limits its binding only to substrates with basic residues such as lysine and arginine. In elastase, the binding pocket is blocked because of the replacement of two glycine residues in the substrate binding site by two bulkier valine and threonine residues. The structural feature is reflected by the functional specificity of elastase to smaller residues such as alanine, glycine, and serine.

In order to draw a correct correlation, the structure, or amino acid sequence, must be the sequence of the functional enzyme characterized. The data gathered up to date are not sufficient for the correlation. Human skin tryptase-1 cDNA was matched to tryptase purified from human lung based on the 24 amino acids at the N-terminal of the characterized enzyme. However, human skin tryptase-2 cDNA also has 24 identical amino acids at the N-terminal. Thus, the amino acid sequences derived from the cDNAs have not been precisely assigned to any specific human tryptase purified and characterized. Sequencing through the Edman procedure can provide direct correlation of the function of a specific tryptase with its structural properties. Undoubtedly, further exploration of the structural and functional properties of tryptase will lead to a deeper understanding of the role of tryptase in nature and have a great pharmacological significance in the near future.

3. Purpose Of The Project

The main purpose of this project is to investigate the potential structure and function relationship among tryptases. The biological significance of tryptase can be appreciated as a specific component of mast cell granules. Tryptase purified from different sources have demonstrated their common and unique chemical and amino acid sequence properties as seen in the review section above. The observations raise our interest

in preparing to investigate the relation between tryptase functions and their structural features. In order to draw a correct correlation, it is very important to know that the primary structure obtained belongs to the enzyme characterized. To achieve this goal, I tried to obtain as much sequence as possible from rat skin tryptase using the Edman procedure. The sequence obtained here provides some important preliminary information about the structural features of rat skin tryptase and serves as a good starting point for continuing the project in the future.

Specifically, a large amount of tryptase was purified from rat skin. The purity of tryptase was verified by SDS-PAGE and reversed-phase HPLC. The unstable cystine residues in tryptase were converted to stable S-carboxymethylcysteine through an alkylation procedure before the Edman reaction. The alkylated proteins were enzymatically or chemically fragmented. The resulting peptides were subjected to different types of HPLC chromatography including the use of gel permeation and reversed-phase columns. The purified peptides were sequenced by the Edman procedure using an automated sequencer. Rat skin tryptase peptides were compared with other tryptase sequences. The study exhibited some very interesting observations.

CHAPTER III

MATERIALS AND METHODS

A. Materials

Rat skin (Sprague-Dawley), was obtained from other labs or Pel-Freez Biologicals, Rogers, Arkansa. $NaHPO_4$, NaH_2PO_4, NaCl, ethanol, glycerol, acetic acid, and glutaraldehyde were from Mallinckrodt, St. Louis, MO. Ethylenediaminetetraacetic acid (EDTA), agarose-glycyl-glycyl-p-aminobenzamidine gel (PAB), benzamidine.HCl, concanavalin A-agarose gel (Con A), α-methyl-D-mannoside, NaN_3, benzoyl-L-arginine ethyl ester (BAEE), iodoacetic acid, acetonitrile, trifluoroacetic acid, bovine serum albumin fraction V (BSA), and Mark VI protein molecular weight standards were from Sigma Chem. Co., St. Louis, MO. Bradford Reagent for protein assay was from Bio-Rad Laboratories, Richmond, CA. Ammomium sulfate was obtained from Schwarz Mann Biotech. Div. of ICN Biomedicals, Inc., Camgridge, MA. (^{3}H)iodoacetic acid was from Amersham, Arlington Heights, IL. Dialysis tubing was from Bethesda Research Labs, Gaithershurg, MD. Centricon-30 microconcentrator units were from Amicon Division, W.R. Grace & Co., Danvers, MA. Homogeneous gels (12%) and SDS buffer strips were from Pharmacia,. N-Glycanase cloned from

Flavobacterium meningosepticum and expressed in E. Coli. was obtained from Genzyme. Trypsin was from Serva, NJ. Lysylendopeptidase C was from Wako Fine Chem., Japan.

B. Methods

1. Purification Of Rat Skin Tryptase

Sprague-Dawley rats were decapitated and the skins removed and stored at -20 °C until use. Alternatively, rat skin was obtained from Pel-Freez Biological, Rogers, Arkansas. The fur was clipped away as close as possible to the epidermis using an Ostar Professional Animal Grooming Clipper, Model A-5, with a No. 80, Size 40 blade. The fat layer and the thin cutaneous muscle (Hebel & Stromberg 1976) were scraped off using a stainless steel blade. The skins were then rinsed well in tap water followed by distilled water and minced in a Universal No. 3 meat grinder through two cycles. For each purification, about 400 g of skin were used. All subsequent steps were carried out at 4 °C unless otherwise indicated.

The minced skin was weighed and homogenized at a 1:9 (w/v) ratio of the homogenization buffer (Appendix A) using a Waring blender, model PB-5, at full speed for 6-8 times with 20 second rest intervals. The homogenate was filtered through a nylon mesh in a Buchner funnel under vacuum. The volume of the filtrate was recorded and 1 ml was saved for analysis.

The filtrate was brought to 25% $(NH_4)_2SO_4$ saturation by adding 139 g of the salt/l. The solution was allowed to

equilibrate for 4-6 hours. After centrifugation at 13,700 xg for 15 minutes in a Sorvall GSA rotor at 4 °C, the supernatant was brought to 80% $(NH_4)_2SO_4$ by adding 361.5 g of the salt/l quickly (Taylor 1953). The solution was allowed to sit overnight. The solution was then centrifuged at 13,700 xg for 15 minutes. The pellet was resuspended in 100 ml of the dialysis buffer (Appendix A) and dialyzed against two changes of 6 l of the dialysis buffer overnight.

The dialyzed fraction was centrifuged at 23,700 xg in a Sorvall SS-34 rotor for 15 minutes and applied to an agarose-glycyl-glycyl-p-aminobenzamidine (PAB) affinity column (1.5 x 20 cm) equilibrated with the dialysis buffer at a flow rate of 25 ml/h at room temperature. The sample itself was kept on ice during application to the column. The column was then washed with 50 ml of the PAB equilibration buffer (Appendix A) at room temperature and then moved to 4 °C for 1 hour before washing again with another 25 ml of the same buffer. A concanavalin A-agarose (Con-A) column (1 x 10 cm), previously washed with 20 ml of the Con-A activation buffer (see Appendix A) followed by PAB equilibration buffer, was connected to the PAB column. The enzyme was eluted from the PAB column to the Con-A column by applying 40 ml of the PAB elution buffer (Appendix A) at a flow rate of 10 ml/h. After the application, the Con-A column was disconnected from the PAB column and washed with 10 ml of the PAB equilibration buffer. The enzyme was washed off the Con-A column with the Con-A

elution buffer (Appendix A) at a rate of 5 ml/h. The fractions were collected in 1 ml/tube and assayed for enzyme activity. Those fractions containing activity were pooled and concentrated using several Centricon-30 microconcentrator units (Amicon). After concentrating each sample to 50-100 μl, 2 ml of the dialysis buffer was added to each unit and concentrated until 50-100 μl was left in each unit. This step was repeated once. Each unit was then rinsed twice with 100 μl of the dialysis buffer. The final fractions were pooled together and stored at 4 °C. The PAB column was washed with PAB wash buffer (Appendix A) followed by PAB equilibration buffer at room temperature and stored at 4 °C. The Con-A column was washed with the Con-A wash buffer (Appendix A) and stored in Con-A activation buffer at 4 °C.

Enzyme activity was measured by monitoring the hydrolysis of benzoyl-L-arginine ethyl ester (BAEE) at 253 nm (Schwert & Takenaka 1955). The sample cuvette contained 440 μl of the assay buffer (Appendix A), 10 μl of the sample, and 50 μl 10 mM BAEE. The reference cuvette contained 450 μl of the assay buffer and 50 μl of the 10 mM BAEE. The initial rate (0-5 minutes) was measured in a Gilford Response Spectrophotometer. One unit of activity was defined as 1 μmole of substrate hydrolysed per minute. Specific activity is defined as unit activity per mg of protein and calculated according to the following formula:

Specific Activity = $(\Delta A_{253/min})$ (0.5 ml)/(0.9 x mg protein/ml x

ml)

where $\Delta A_{253/min}$ is the rate of BAEE hydrolysis, 0.9 is the absorbance change for total cleavage of BAEE in the cuvette, mg protein/ml is the protein concentration of the sample, and ml is the volume of the sample used in the assay.

Protein concentration was determined using the microassay procedure from Bio-Rad (Bradford 1976). Bovine serum albumin fraction V from Sigma was used as a standard. The standard curve and the sample protein concentration were obtained at A_{595} on the Gilford Response Spectrophotometer.

The purity of the protein was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and reverse phase high performance liquid chromatography (HPLC).

2. SDS-PAGE

SDS-PAGE was performed under reducing conditions on a PhastSystem from Pharmacia according to the manufacturer's manual. Protein samples were concentrated in a Centricon-30 unit to 50 μg/ml or higher. The recovery of tryptase from the Centricon-30 was above 90% in water. The samples were mixed with the sample buffer (Appendix B) at a 1:1 ratio and boiled for 5 minutes before application of the samples to the gels. PhastGel homogeneous 12.5 or PhastGel gradient 10-15 gels were used for the separation. The samples were applied to the gel using size 6/4 sample applicators and separated with the SDS buffer strips using the following program:

SEP. PROGRAM 1

Sample Application Down At					1.2	0 vh
Sample Application Up At					1.3	0 vh
Sep. 1.1	250 v	10 mA	3 w	15 °C		1 vh
Sep. 1.2	250 v	1 mA	3 w	15 °C		1 vh
Sep. 1.3	250 v	10 mA	3 w	15 °C		80 vh

The gel was stained with silver in the development unit using the following program:

DEV. PROGRAM 1

Steps	Solutions	In - Port	Out - Port	Time min	Temp. °C
1	50% Eth, 10% HAc	2	0	2	50
2	10% Eth, 5% HAc	3	0	2	50
3	10% Eth, 5% HAc	3	0	4	50
4	Glutaraldehyde	4	0	6	50
5	10% Eth, 5% HAc	3	0	3	50
6	10% Eth, 5% HAc	3	0	5	50
7	MQ Water	5	0	2	50
8	MQ Water	5	0	2	50
9	Silver Nitrate	6	0	13	40
10	MQ Water	5	0	0.5	30
11	MQ Water	5	0	0.5	30
12	Developer	7	0	0.5	30
13	Developer	7	0	4	30

14	5% HAc	8	0	2	50
15	6% Glycerol	9	0	3	50

Eth stands for ethanol, MQ water for milli Q water, and HAc for glacial acetic acid. The solutions used are given in Appendix B. Protein standards from Sigma included bovine albumin with a molecular weight of 66,000, ovalbumin (43,000), pepsin (34,700), trypsinogen (24,000), β-lactoglobulin (18,400), and lysozyme (14,300). The relative mobility (Rf) of a protein was determined according to the formula: Rf = Distance of protein migration/Distance of tracking dye migration. The Rf values (abscissa) were plotted against the known molecular weights (ordinate) on a semi-logarithmic paper. The molecular weight of a sample was either extrapolated from the determined sample Rf value in a standard curve or calculated from the regression equation derived from the logarithmic values of the molecular weight and the Rf values of the protein standards. A standard was prepared for each gel.

3. Reversed-Phase HPLC

A Varian model 5500 HPLC system was used. The protein and peptides were separated based on their hydrophobicities using butyl- (C4), octyl- (C8), and octadecyl- (C18) columns from Rainin Instrument Co., Inc., Woburn, MA. The samples were dissolved in 6 M quanidine-hydrochloride solution. The

mobile phase was delivered as a linear gradient of increasing acetonitrile in 0.1% trifluoroacetic acid (TFA) at 0.5 ml/min. Solution A contains 0.1% of TFA in water. Solution B contains 0.1% of TFA in acetonitrile. A typical separation program is shown as the following:

Time (min)	Solvent A (%)	Solvent B (%)	Flow Rate (ml/min)
0	100	0	0.5
60	40	60	0.5
62	0	100	0.5
64	100	0	0.5

Protein peaks were detected at 206 nm using a UV mornitor and collected into plastic tubes. The collected fractions were dried by speed vacuum centrifugation.

4. Enzymatic Deglycosylation Of Tryptase

Purified tryptase was concentrated to 2 mg/ml in MQ water using a Centricon-30 unit. Tryptase (3.2 μg) was denatured by boiling for 5 minutes in 20 μl of 100 mM Tris-HCl, pH 8, containing 50 mM β-mercaptoethanol and 0.5% SDS. To the

denatured sample was added 5 μl of 7.5% Nonidet P-40 followed by 5 μl (1.25 units) of recombinant peptide-N-Glycosidase F (N-Glycanase) in a buffer containing 50% glycerol, 0.15 M NaCl, 1 mM EDTA and 80 mM sodium phosphate. The reaction was incubated at 37 °C. After 20 hours, an additional 1.5 μl (0.4 units) of N-Glycanase was added and the reaction was allowed to continue overnight. The reaction was terminated by boiling for 5 minutes. In a parallel control experiment, the N-Glycanase was replaced by the same volume of the enzyme buffer. In another control experiment, the reaction contained all of the components except tryptase which allowed detection of any N-Glycanase on a gel if it occured. The samples were subjected to SDS-PAGE along with Sigma VI molecular weight standards. Gels were developed with silver stain using the Pharmacia PhastSystem as described above.

5. Alkylation of Tryptase

Since cysteine residues are subject to autoxidation, they must be converted to more stable derivatives before sequencing. Iodoacetic acid was used to radiolabel and convert the cysteine residues to a stable S-carboxymethylcysteine (Allen 1986).

The purified tryptase (about 200 μg) was dissolved in a solution containing 6 M quanidine hydrochloride by adding 0.7 g of the salt and 400 μl of a Tris-HCl buffer (Appendix C) and then vortexing well. The protein was reduced by dissolving

0.2 mg of DTT in the solution followed by incubation at room temperature for 1 hour. A 50 μl solution of [^{3}H]-iodo-acetic acid (Appendix C) was added. The solution was capped and sealed in the dark at room temperature for 30 minutes. The reaction was driven to completion by addition of 4.8 mg of DTT to the solution followed by incubation in the dark at room temperature for 1 hour. Non-radioactive iodoacetic acid solution (Appendix C) was then added to the mixture and incubated for another 30 minutes in the dark. Finally, the reaction was terminated by addition of 60 μl of 6 N HCl solution (Appendix C).

The unincorperated iodoacetic acid and other salts were removed from the alkylated enzyme using a 1 x 30 cm Sephadex G-25-80 column. The column was equilibrated in either an ammonium bicarbonate buffer (Appendix C) or a formic acid solution (Appendix C) depending on the digestion method to be used. The column was calibrated using 1 or 5 mg of bovine serum albumin at a flow rate of 30 ml/h. The fractions were collected in about 1 ml/tube. Fractions containing the protein were detected at 280 nm. After the calibration, the alkylation mixture was applied onto the column. The fractions collected were counted for radioactivity to confirm the presence of the sample and then dried by speed vacuum centrifugation before chemical or enzymatic fragmentation.

6. Enzymatic Cleavage Of Tryptase

Trypsin was used to catalyze the hydrolysis of lysyl and arginyl peptide bonds in the alkylated tryptase. Tryptic digestion was conducted at a protein:trypsin molar ratio of 100:1 in a buffer containing 0.1 M NH_4HCO_3 and 0.1 mM $CaCl_2$, pH 8. About 40 μg of the alkylated tryptase was mixed with 0.4 μl of the 1 mg/ml trypsin in 0.1 mM HCl, 10 μl of 1 mM $CaCl_2$, and 90 μl of 0.1 M NH_4HCO_3, pH 8. The reaction was allowed to continue at 37 °C for 16 hours and stopped by speed vacuum centrifugation.

Lysylendopeptidase-C (Lys-C) was used to catalyze the hydrolysis of lysyl peptide bonds in tryptase. The reaction mixture contained 100 μl of 50 mM Tris-HCl, pH 9, 1 μg of Lys-C and 100 μg of the alkylated tryptase. The reaction was allowed to continue at 37 °C for 6 hours and stopped by speed vacuum centrifugation.

7. Separation And Purification Of Peptides

Digestion of tryptase was first evaluated by running a small amount of the sample on 12% SDS-PAGE. Then the peptides were separated and purified by HPLC.

Separation of the tryptic peptides was carried out using a C18 column. The sample was dissolved in 6 M quanidine-HCl solution and injected into the HPLC. The mobile phase contained a linear gradient of increasing acetonitrile from 0% to 60% in 60 minutes. The peaks from the separation were collected in separate tubes, labeled and centrifuged under

vacuum. Some peaks which had overlap shoulders were separated again using a C8 column. The acetonitrile concentration was increased from 10% to 20% over 20 minutes.

Peptides from the Lys-C digest were separated using a gel permeation column (Bio-Sil TSK-125 from Bio-Rad). Samples were dissolved in 6 M quanidine-HCl. The mobile phase contained 6 M quanidine-HCl and 10 mM potassium phosphate, pH 6. Peptide peaks were collected in separate plastic tubes. Peptides separated by the gel permeation column were subject to a second purification using a C4 hydrophobic column. The acetonitrile gradient was increased from 0% to 60% in 30 minutes. The fractions were dried by speed vacuum centrifugation.

8. Amino Acid Sequence Determination

Reverse-phase HPLC purified peptides were sequenced by the Edman procedure using an ABI 477A automated sequencer equipped with on-line PTH analyzer 120A.

CHAPTER IV

RESULTS

1. Purification Of Rat Skin Tryptase

A large amount of tryptase had to be purified from rat skin in order to satisfy the requirements of the project. A total of 2,000 μg of tryptase was isolated from 22 purifications. A typical purification result is shown in Table 1.

In the purification procedure, a high salt concentration (0.5 M NaCl) was used to facilitate extraction of tryptase from rat skin. Precipitation of protein in the crude extract at 25-80% ammonium sulfate saturation removed large amounts of contaminating proteins. As observed in most of the purifications, the enzyme activity measured at this step was lower than expected. It may be due to either the elimination of other non-tryptase BAEE activity in this step or interference of residual ammonium sulfate after an overnight dialysis. The salt did not significantly interfere with the binding of tryptase to the PAB column at room temperature.

In one purification, the fat and the cutaneous muscle layers associated with the skin were not removed prior to homogenization to see if this affected the ability to obtain

Fractions	Volume (ml)	Prot.Conc. (mg/ml)	Tot.Prot. (mg)	Act. (U/ml)	Sp.Act. (U/mg)	Tol.Act. (U)	Yield (U%)	Pur.Fact. (Fold)
Crude Extract	1530	2.23	3412	0.085	0.038	130.1	100	1
$(NH_4)_2SO_4$ 25-80%	95	9.81	932	0.216	0.022	20.5	16	0.6
PAB/Con A Columns	2.7	0.032	0.085	4.943	154	13.2	10	4053

Table 1. Purification Of Rat Skin Tryptase.

purifed enzyme. The presence of components from the fat and muscle layers seriously decreased the flow rate of the PAB affinity column step. In the subsequent purifications, these layers were removed as in the original published procedure. The PAB/Con-A step gave a purification factor of about 4,000-fold and yielded a recovery of BAEE hydrolyzing activity of about 10%. The result is consistant with the previous report in which the calculated recovery based on cleavage of the specific substrate D-Ile-Pro-Arg-pNA was 35%, giving an enzyme preparation purified 14,000 fold (Braganza & Simmons 1992). A typical preparation from about 300-400 g skin tissue yielded about 80-90 μg of purified tryptase. The procedure was used successfully a few times to obtain about 150 μg of highly purified tryptase in one purification from a larger amount of skin. In one isolation, over 230 μg of tryptase was purified using the procedure. However, scaling up this procedure for the preparation of larger amounts of tryptase was difficult.

The purity of tryptase was checked with SDS-PAGE and reversed-phase HPLC before it was used for sequencing or antibody production. Figure 1 shows a broad band of the purified tryptase on a SDS-PAGE gel. The molecular weight was determined to be 31,000 ± 1,000 (Figure 2). The broad band is due to different extents of glycosylation of the enzyme (see below). Reversed-phase HPLC of the enzyme (Figure 3) on a C4 hydrophobic column showed a single

Figure 1. SDS-PAGE Of Purified Tryptase. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Pharmacia PhastSystem. Purified rat skin tryptase (0.7 μg) was separated on a 12.5% homogeneous gel under denaturing and reducing conditions. The positions of the molecular weight standards are indicated next to the sample. The gel was developed with silver stain.

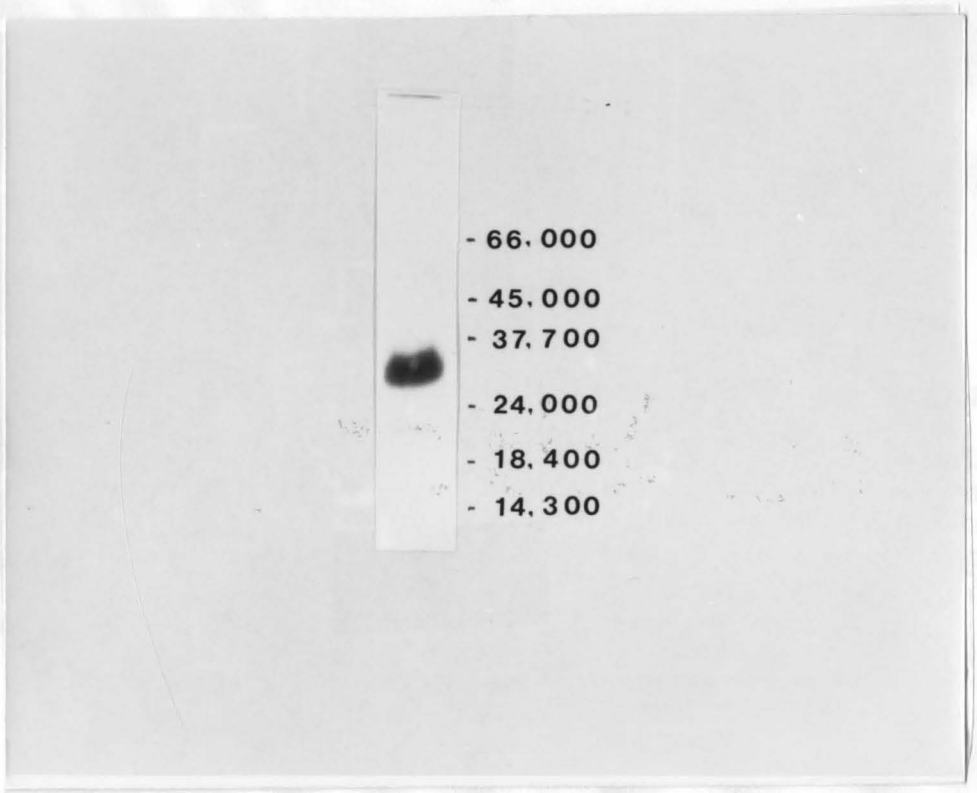

- 66.000
- 45.000
- 37.700
- 24.000
- 18.400
- 14.300

Figure 2. Molecular Weight Of Tryptase By SDS-PAGE. Purified rat skin tryptase was separated on a 12.5% homogeneous gel using a Pharmacia PhastSystem. Six molecular weight standards on the indicated molecular weight are shown in the open circles. The molecular weight of the standards at a common logarithm scale are plotted against the corresponding Rf values as defined in the text. An average Rf value for the broad tryptase band is shown in the solid circle.

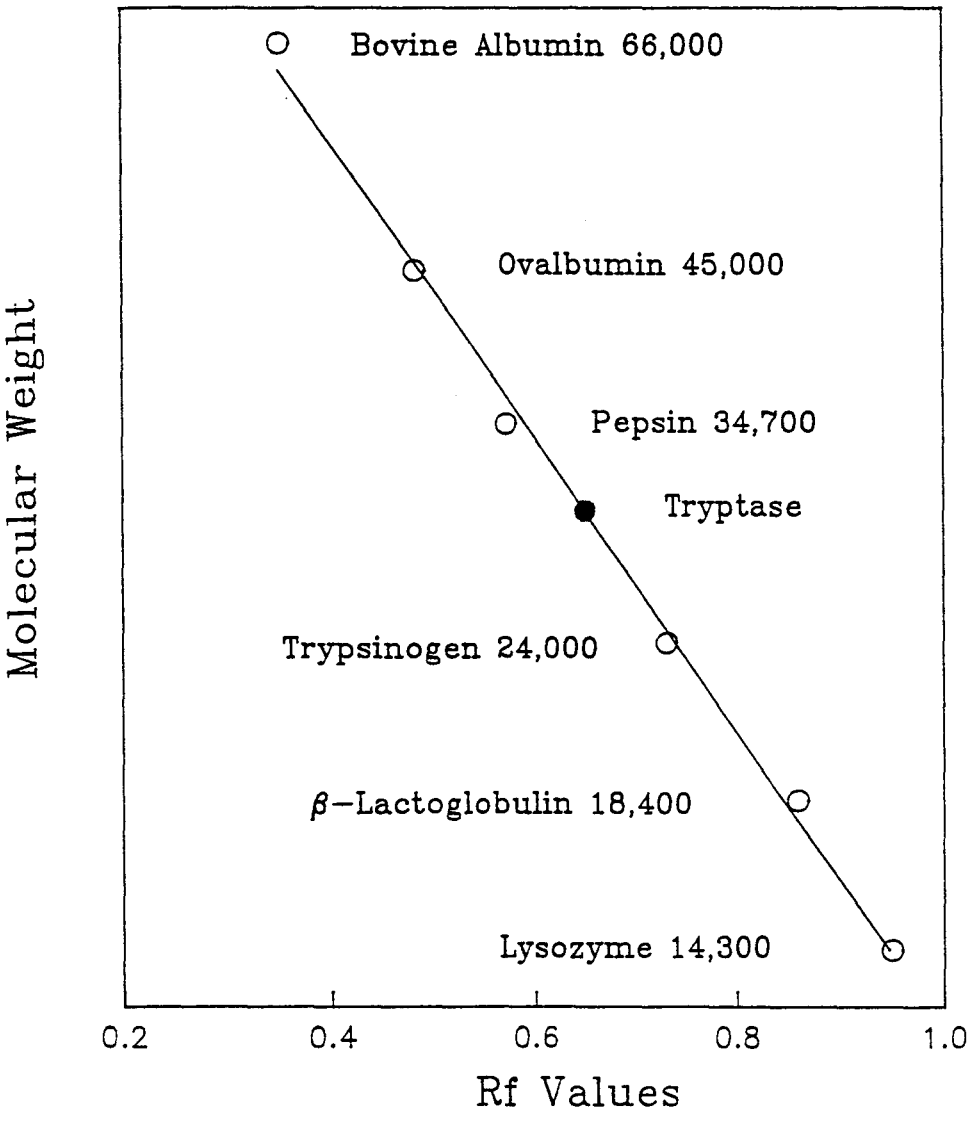
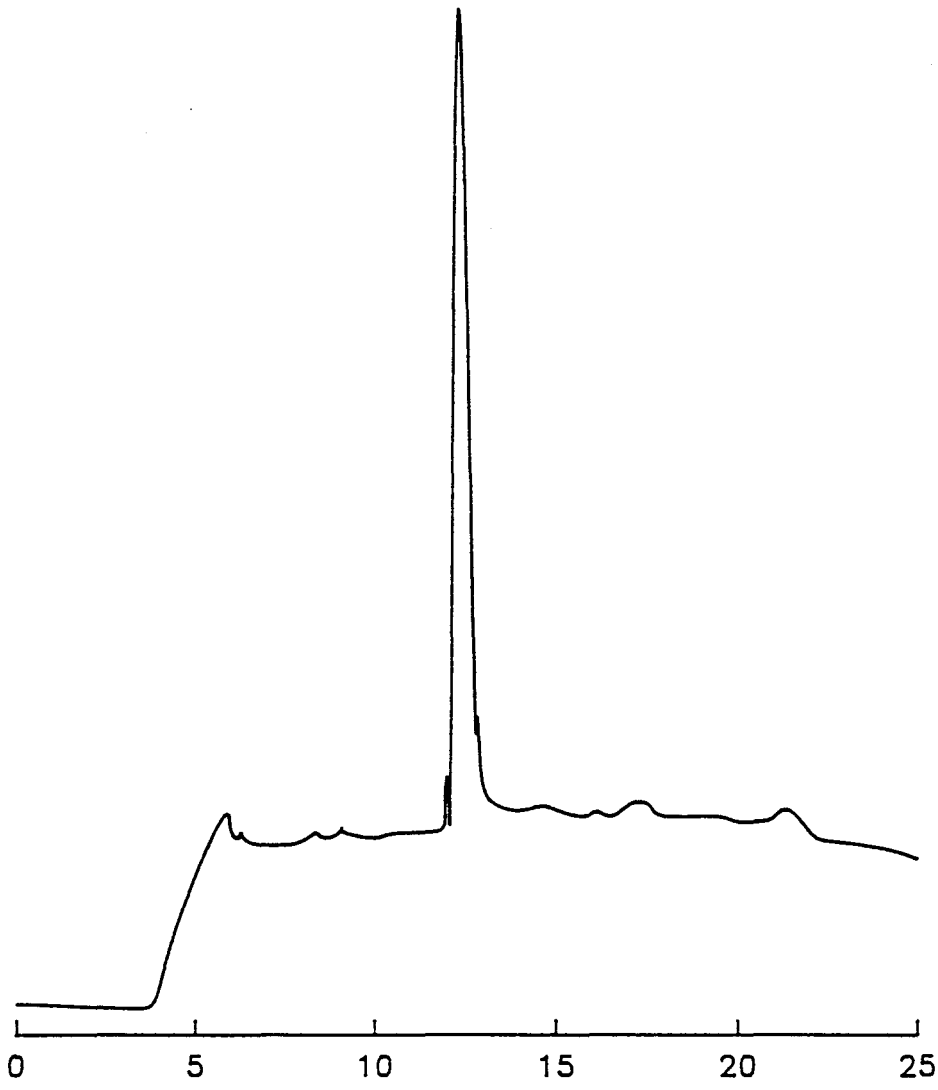

Bovine Albumin 66,000
Ovalbumin 45,000
Pepsin 34,700
Tryptase
Trypsinogen 24,000
β–Lactoglobulin 18,400
Lysozyme 14,300
Molecular Weight
0.2
0.4
0.6
0.8
1.0
Rf Values

Figure 3. Reversed-Phase HPLC Of Purified Tryptase. Purified rat skin tryptase was subjected to reversed-phase HPLC chromatography using a C4 column. The separation conditions are shown below.

Column: Aquapore Butyl (C4)
Mobile Phase: A: 0.1% TFA in H_2O
B: 0.08% TFA in CH_3CN
Gradient: 30-50% B, 20 minutes, linear
Flow Rate: 0.75 ml/min
Detection: UV, 206 nm
Sample: 4 μg tryptase


0
5
10
15
20
25
Minutes

peak under the specified conditions. A control experiment run prior to the sample revealed an identical base line. Subsequent N-terminal amino acid sequence analysis using the Edman degradation method revealed a single sequence from the purified tryptase.

2. Deglycosylation Of Tryptase

An enzymatic deglycosylation reaction was performed to evaluate the extent and heterogeneity of glycosylation of tryptase. N-Glycanase (Peptide-N^4-(N-acetyl-β-glucosaminyl) asparagine amidase) (Plumer et al. 1984) catalyzes the hydrolysis of Asn-linked oligosaccharide at the β-aspartyl-glycosylamine bond between the innermost GlcNAc and the asparagine residue. The N-Glycanase releases all common classes of Asn-linked oligosaccharide such as high-mannose, complex and hybrid oligosaccharide including sialylated, phosphorylated, or sulfated sugar chains (Maley et al. 1989). Figure 4 shows that N-Glycanase modified the properties of tryptase as observed by SDS-PAGE. Lane 1 is the tryptase treated with 1.65 units of N-Glycanase under the conditions described in Methods. Lane 2 is the untreated tryptase. Molecular weight standards are indicated in the figure. The untreated tryptase appeared as a broad band on the gel. This type of appearance is not uncommon for glycosylated proteins. The presence of N-Glycanase reduced the broad band to a sharp single band suggesting that

Figure 4. SDS-PAGE Of N-Glycanase Treated Tryptase. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a Pharmacia PhastSystem. N-Glycanase-treated and untreated tryptase samples were separated in a 12.5% homogeneous PhastGel under denaturing and reducing conditions. The positions of the molecular weight standards are indicated next to the samples. The gel was developed in silver stain. Lane 1 is the treated tryptase (0.4 μg). Lane 2 is the untreated tryptase (0.4 μg).

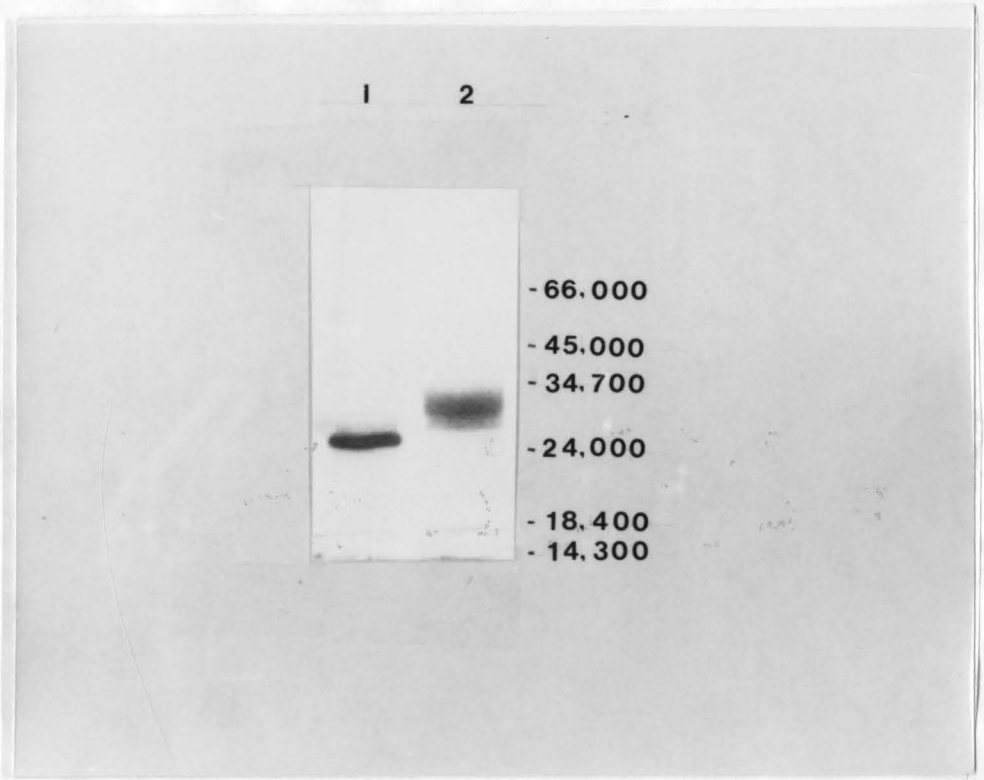
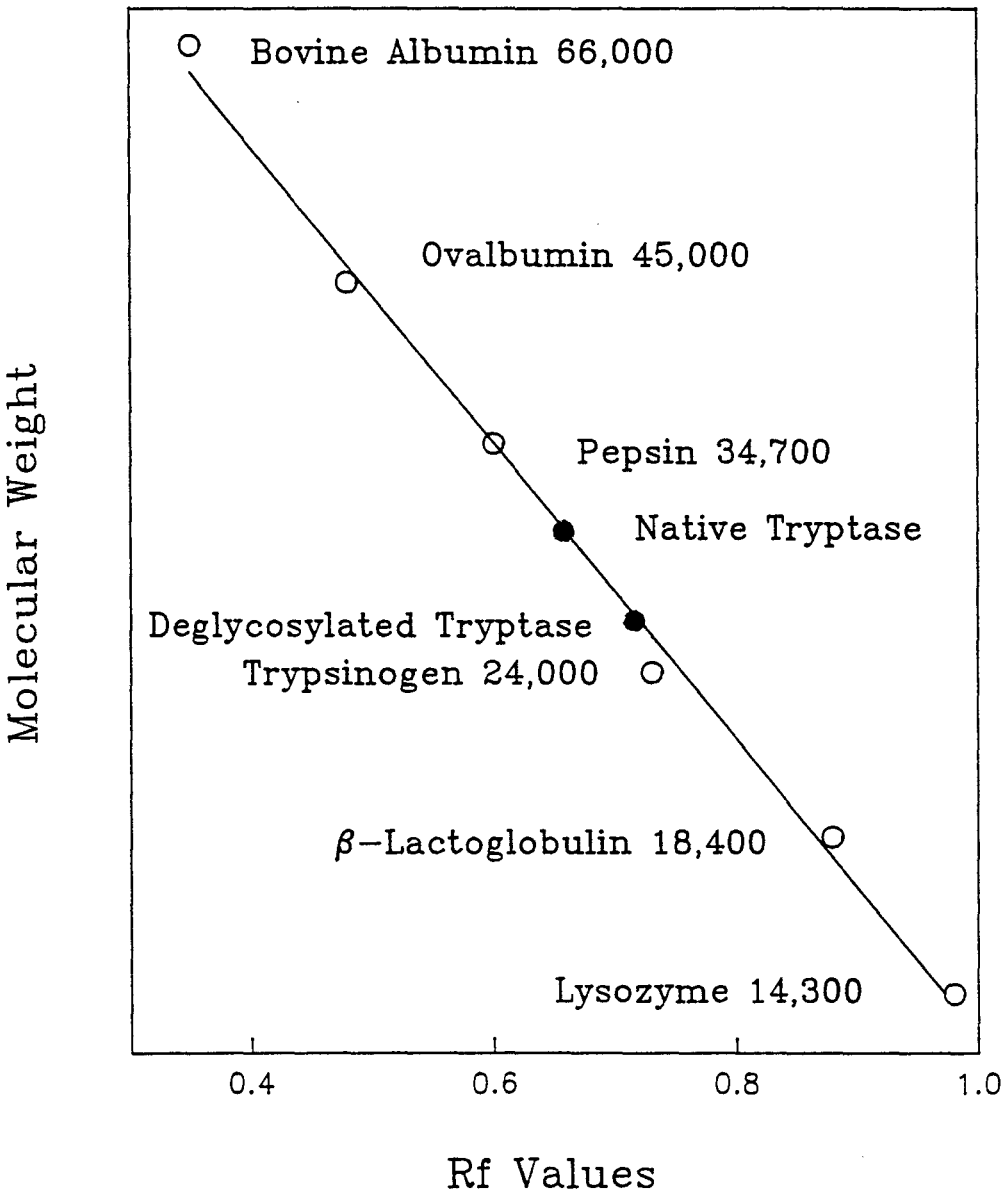

1
2
-66.000
-45.000
-34.700
-24.000
-18.400
-14.300

Figure 5. Molecular Weight Change Following Deglycosylation. Tryptase was enzymatically deglycosylated by incubation with N-Glycanase and subjected to SDS-PAGE as shown in Figure 4. The molecular weight standards are shown in the open circles. The molecular weights of the standards on a common logarithm scale are plotted against the corresponding Rf values as defined in the text. Native tryptase and N-Glycanase treated tryptase samples are shown in the solid circles. The deglycosylated tryptase shows an apparent reduction of its molecular weight by about 4,000 daltons.


Bovine Albumin 66,000
Ovalbumin 45,000
Pepsin 34,700
Native Tryptase
Deglycosylated Tryptase
Trypsinogen 24,000
β–Lactoglobulin 18,400
Lysozyme 14,300
Molecular Weight
0.4
0.6
0.8
1.0
Rf Values

tryptase is glycosylated through asparagine linkages. The fact that tryptase was purified with a concanavalin A-agarose column is consistant with this observation. N-Glycanase in a control, which contained 1.65 units of N-Glycanase and all of the other reaction components except tryptase, was not detected under the above experimental conditions. The molecular weight of tryptase was reduced by about 4,000 daltons by N-Glycanase (Figure 5).

3. Alkylation And Peptide Purification

Before fragmentation of tryptase, cystine residues of the enzyme were converted to stable S-carboxymethylcysteine using (^{3}H)iodoacetic acid as described in Methods. Residual salts were removed from the alkylated protein through a Sephadex G-25-80 column. The column was calibrated with bovine serum albumin (BSA) in NH_4HCO_3 buffer. Two separate BSA samples, 5 mg and 1 mg, were applied to the column. In both cases, the protein peaks fell into the same fraction numbers. Consistent result of the same calibration was obtained in another column equilibrated with formic acid. Calibration was performed every time prior to desalting the alkylated protein. The protein was very well separated from the salts in the columns under the conditions used. The major salt peak did not come out until after 16 ml of the solvents passed through the column as indicated by precipitation of the salt. Fractions were counted in a β-counter to confirm the presence of the

alkylated protein. A typical alkylation result is presented in Figure 6. The calibration curve indicated that BSA came out at fraction number 8 and 9. Results of radioactivity measurement precisely located the highest DPM values of the alkylated and radiolabeled tryptase in the same fraction numbers.

After alkylation, the protein was subjected to enzymatic fragmentation. The cleavage of tryptase by enzymatic methods results in a mixture of peptide fragments which must be separated and purified before determination of their amino acid sequence. Analytical polyacrylamide gel electrophoresis was performed in 12% gel containing 8 M urea. Based on the results, a fractionation method was selected for separation of the peptides. Three surface chemistries (butyl-, octyl-, and octadecyl-) were used in an attempt to separate the peptides according to their different hydrophobicities. After sufficient pilot testing, larger amount of the samples was separated using a selected column and specific condition which provided the best resolution.

Peptides from the tryptic digest were separated on a C18 column (OD102, Rainin). Figure 7 shows results of this separation. All identifiable peaks were collected in separate plastic tubes. The shoulder of a peak was collected in a separate tube to minimize any possible

Figure 6. Desalting Of Alkylated Tryptase On A Sephadex G-25-80 Column. A G-25-80 Sephadex column was equilibrated in 9% formic acid. A typical calibration curve for 1 mg BSA is shown in the open circles. The absorbance values of BSA at 280 nm are plotted against the fraction numbers. Elution of alkylated tryptase is detected by the radioactivity in the fractions. The fraction numbers are plotted against the DPM values shown in the solid circles.

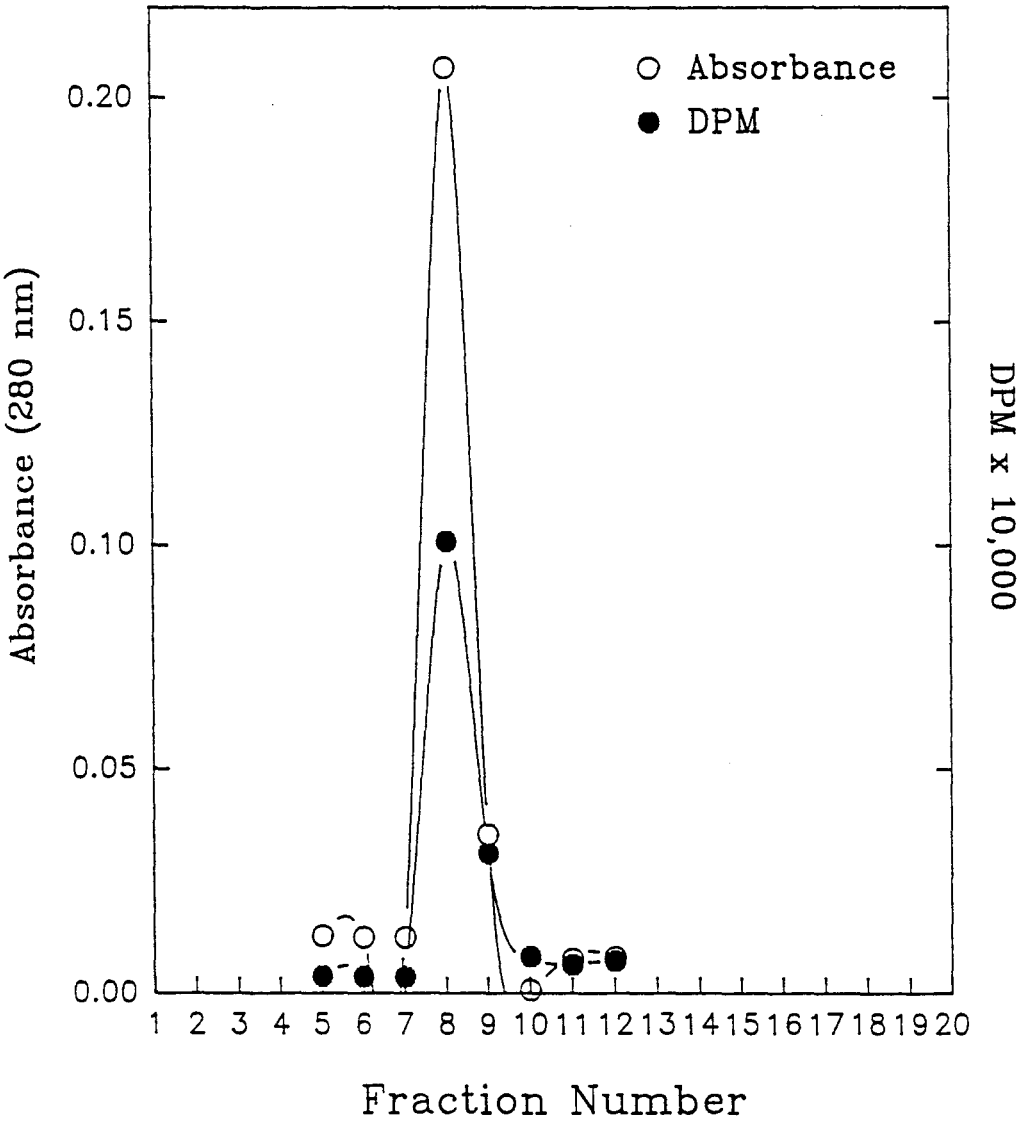
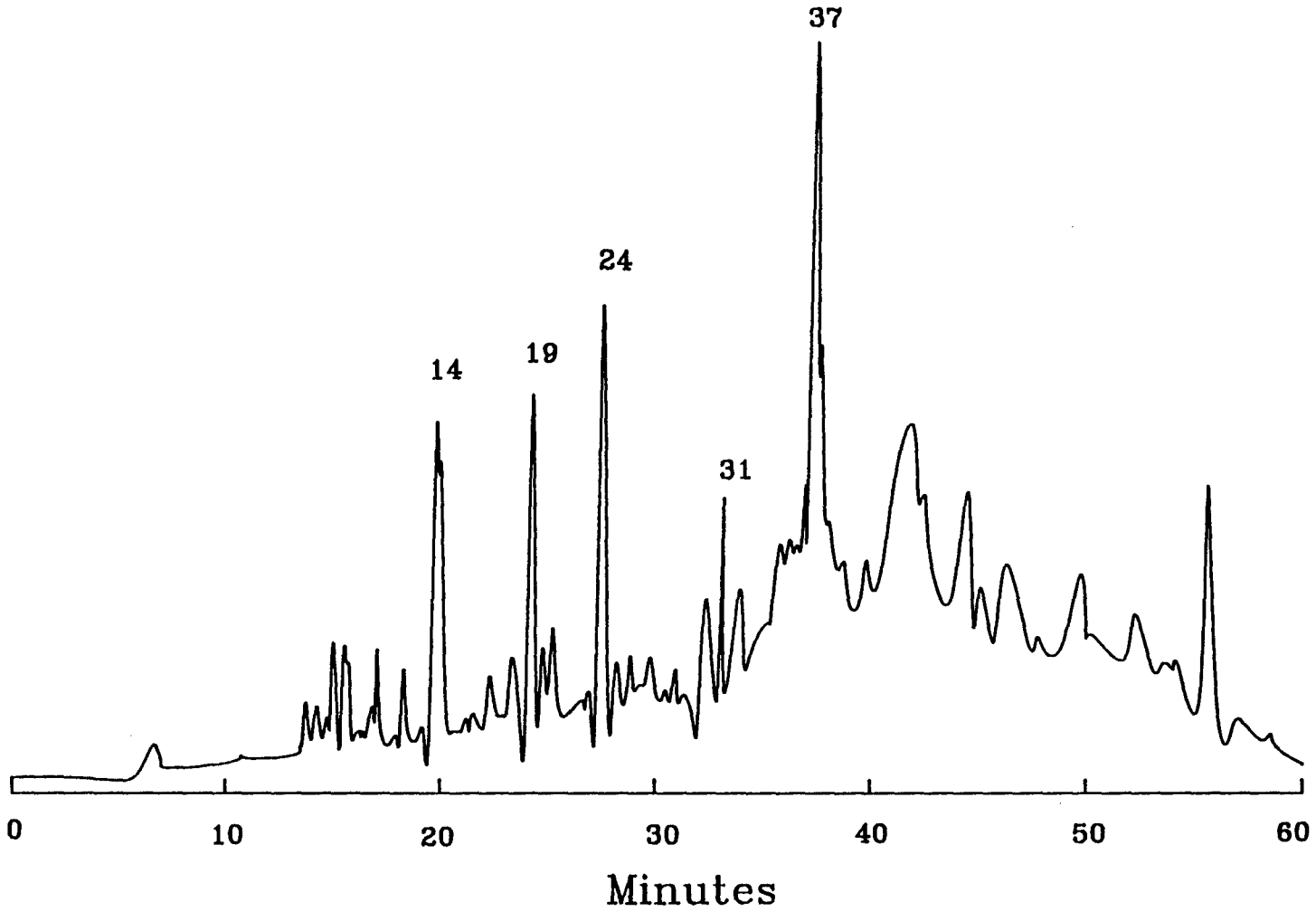

Absorbance
DPM
0.20
0.15
0.10
0.05
0.00
Absorbance (280 nm)
DPM x 10,000
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Fraction Number

Figure 7. Tryptic Digest Of Tryptase. Rat skin tryptase fragments from a tryptic digest were separated by reversed-phase HPLC using a C18 column on a Varian Model 5500 system. All identifiable peaks were collected in separate plastic tube. Peak number 19, 24, 31, and 37 were sequenced. The conditions of the separation were as the follows.

Column: OD102 (C18)
Mobile Phase: A: 0.1% TFA in H_2O
B: 0.08% TFA in CH_3CN
Gradient: 0-60% B, 60 minutes, linear
Flow Rate: 0.5 ml/min
Detection: UV, 206 nm
Samples: 20 μg tryptic peptides


37
24
14
19
31
0
10
20
30
40
50
60
Minutes

overlap of the peaks. Peaks number 19, 24, 31, and 37 were successfully sequenced without further purification.

The strategy for separation of the Lysylendopeptidase-C (Lys-C) digest was different. The digest was first subjected to gel permeation chromatography (Figure 8) to give three peaks containing peptides of different sizes. Peak 1 was then subjected to reversed-phase HPLC using a C4 column (Figure 9). Only one peak was observed which was subjected to amino acid sequence determination.

4. Peptide Sequences

The sequence of a total of 119 amino acids were determined from 6 peptides (Figure 10). Direct sequencing of the purified tryptase produced a sequence of 37 amino acids for the amino-terminus (N-T) indicating that the N-terminus is not blocked. The unambiguous sequence indicated that the tryptase was purified to homogeneity. A blank cycle appeared at position 21, which was followed by aspartate and threonine. The site for an N-glycosidic linkage has the general structure: Asn-(X)-Ser or Thr, where X can be a variety of amino acid residues. Asn provides the attachment point for a glycosyl chain which often contains terminal sialic acid residues, galactose, mannose, and N-acetylglucosamine. The hydrophilic glycosyl groups in the glycosylated Asn residue often prevent extraction of the Asn anilinothiazolinone derivative during Edman sequencing.

Figure 8. Gel Permeation Chromatography. The Lysylendopeptidase-C (Lys-C) digest was separated by HPLC using a gel permeation column. Three broad peaks are identified. The separation conditions are shown below.

Column: Bio-Sil TSK-125, 60 cm L x 7.5 mm ID
Mobile Phase: 6 M quanidine HCl
10 mM potassium phosphate, pH 6
Flow Rate: 0.5 ml/min
Detection: UV, 226 nm
Samples: Lys-C digest

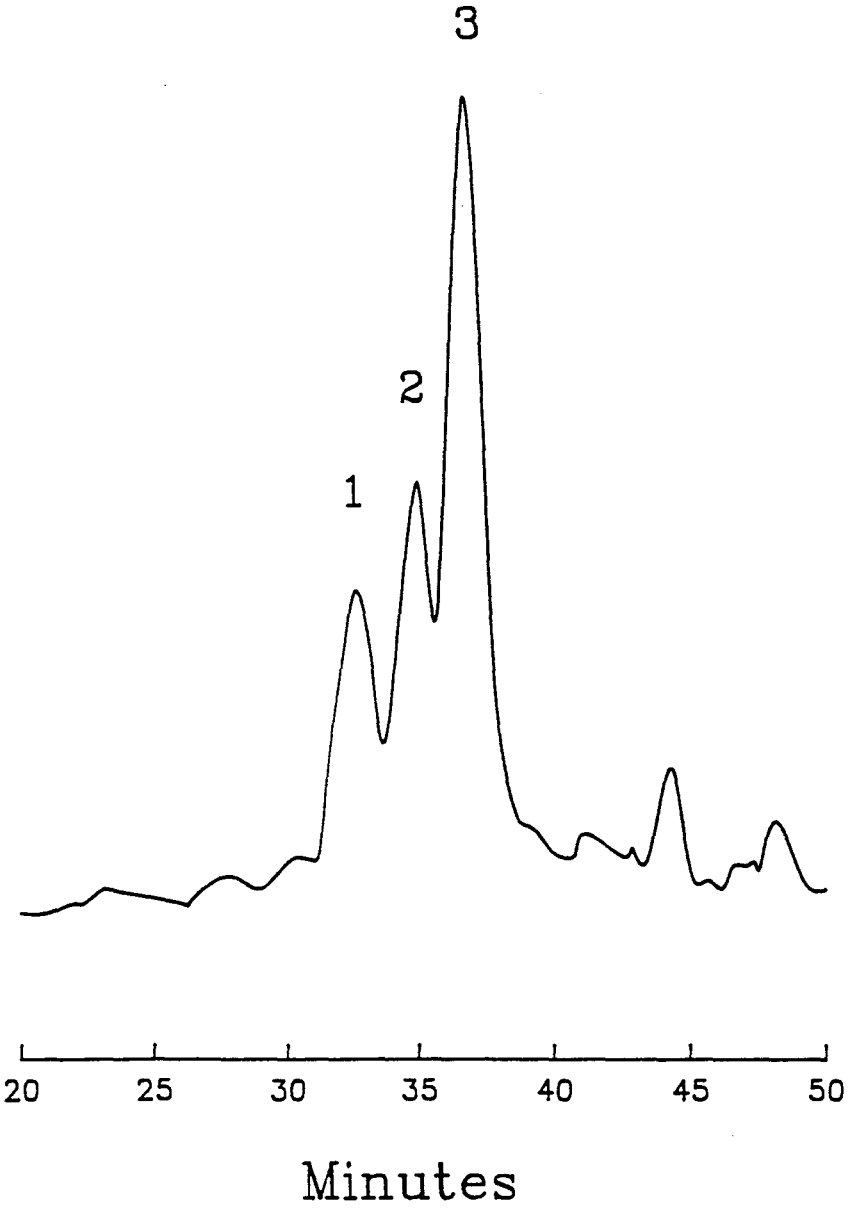
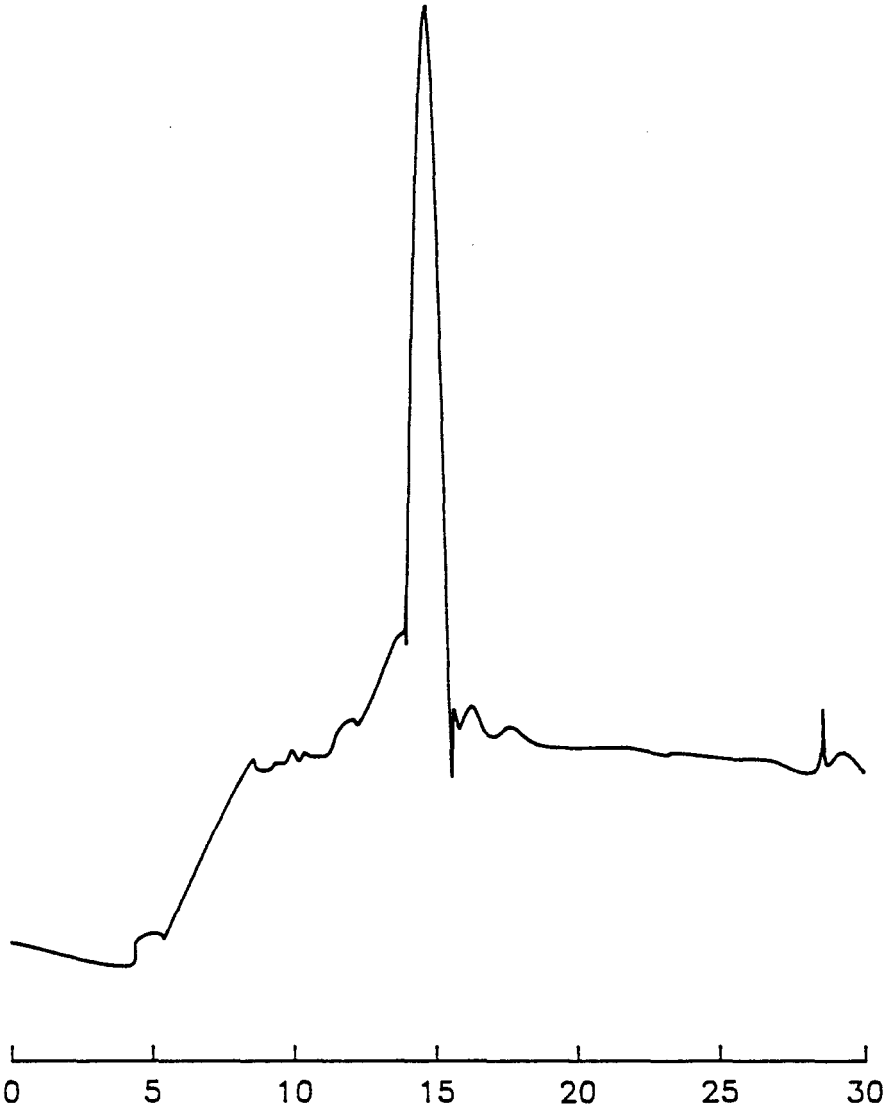

3
2
1
20
25
30
35
40
45
50
Minutes

Figure 9. Separation By C4 Reversed-Phase Column. Peak number 1 from the gel permeation chromatography was subjected to reversed-phase HPLC using a C4 column. A single peak was identified. The separation conditions are shown below.

Column: BU300 (C4)
Mobile Phase: A: 0.1% TFA in H_2O
B: 0.08% TFA in CH_3CN
Gradient: 25-50% B, 25 minutes, linear
Flow Rate: 0.5 ml/min
Detection: UV, 206
Sample: peak number 1 of the Lys-C digest from gel permeation chromatography


0
5
10
15
20
25
30
Minutes

The glycosylated residue is not normally identifiable through a regular sequencing procedure. The above observation together with the fact that the tryptase was purified from concanavalin A column and deglycosylated by N-Glycanase strongly suggest that the blank cycle could be an asparagine residue in the putative glycosylation site.

The Lys-C peptide was sequenced through 23 amino acids. A blank cycle was also present in this peptide at the sixth position. The presence of Ile and Thr after this cycle suggests the presence of another glycosylation site in tryptase.

A total of 59 amino acid residues were determined from peptides Try-19, Try-24, Try-31, and Try-37 from the tryptic digest. Peptide Try-24 has an unidentified residue.

Figure 10. Peptide Sequences. Purified peptides from rat skin tryptase were sequenced by the Edman procedure using an ABI 477A automated sequencer with on-line PTH analyzer 120 A. Sequences of six peptides are presented in the figure. N-terminal: amino terminal sequence obtained by sequencing purified tryptase directly; Lys-C: the peptide of Lysylendopeptidase-C digest after C4 reversed-phase column purification; Try-19, Try-24, Try-31, and Try-37: peptides of the tryptic digest from peak number 19, 24, 31, and 37 after C18 reversed-phase column chromatography; X: blank cycle representing potential glycosylated Asn residue in the sequence; Z: undetermined amino acid residue.

PEPTIDES SEQUENCED

1. N-Terminal
   Ile-Val-Gly-Gly-Gln-Glu-Ala-Ser-Gly-Asn-Lys-Trp-Pro-Trp-Gln-Val-Ser-Leu-Arg-Val-X-Asp-Thr-Tyr-Trp-Met-His-Phe-Cys-Gly-Gly-Ser-Leu-Ile-Asn-Pro-Glu

2. Lys-C
   Leu-Thr-Asn-Pro-Val-X-Ile-Thr-Ser-Asn-Val-His-Thr-Val-Ser-Leu-Pro-pro-Ala-Ser-Glu-Thr-Phe

3. Try-19
   Gly-Leu-Asn-Thr-Gly-Asp-Asn-Val-His-Ile-Val-Arg

4. Try-24
   Val-Asp-Met-Leu-Cys-Ala-Gly-Asn-Glu-Gly-His-Asp-Ser-Cys-Z-Gly-Asp-Ser-Gly-Gly-Pro

5. Try-31
   Val-Thr-Tyr-Tyr-Leu-Asp

6. Try-37
   Val-Glu-Asp-Thr-Trp-Leu-Gln-Ala-Gly-Val-Val-Ser-Trp-Gly-Glu-Gly-Cys-Ala-Gln-Pro-Asn

CHAPTER V

DISCUSSION

Partial Amino Acid Sequence Of Rat Skin Tryptase.

The sequence of 119 amino acids determined for rat skin tryptase account for about 45% of the total primary sequence of the enzyme. Among all tryptases, rat skin tryptase is the first to be sequenced to this extent by the Edman procedure. As mentioned in the literature review section, amino acid sequences of other tryptases have all been derived from cDNA sequences. Although precise assignment of any of the amino acid sequences to a functionally active tryptase remains to be clarified, the primary sequences still provide a wealth of information for analysis of the potential structural properties of tryptases. The six peptides from rat skin tryptase were aligned to the corresponding regions of other tryptase sequences as shown in Figure 11 according to the alignment data generated by GCG (Devereux et al. 1984) and others (Johnson & Barton 1992). Tryptase numbering system is used. The homology between each peptide and its corresponding portion in other tryptase sequences is summarized in Table 2. References to the tertiary structures is made based on computer models of serine proteases (Greer 1990) and tryptase (Johnson & Barton 1992).

Figure 11. Tryptase Sequence Alignment. Peptide sequence of rat skin tryptase are compared with the amino acid sequences of bovine trypsin and other tryptases. Sequence alignment is constructed based on the earlier presentation (Chen et al. 90) and a recent report (Johnson & Barton 92). Amino acid positions of tryptase are indicated beneath the first digit of each number. BT, bovine trypsin; HLTα, human lung tryptase α (Miller et al. 89); HLTβ, human lung tryptase β (Miller et al. 90), which is identical to human skin tryptase 2 (Vanderslice et al. 90); HST1, human skin tryptase 1; HST3, human skin tryptase 3; DMT, dog mastocytoma tryptase (Vanderslice et al. 89); MMCT1, mouse mast cell tryptase 1 (Chu 90); MMCT2, mouse mast cell tryptase 2, which is identical to mouse mast cell protease 6 (Reynolds et al. 91); ♥, identifies the catalytic site histidine, aspartate, and serine; ♦, points to the substrate binding site aspartate; and *, highlights the putative glycosylation sites in small letters.

```
      1          11         *          31         41 ♥       51         61         71         81
BT    IVGGYTCGANTVPYQVSLN  SGY  HFCGGSLINSQWVVSAAHCYKSGIQVRLGEDNINVVEGN   EQFISASKSIVHPSYN
HLTα  IVGGQEAPRSKWPWQVSLRVRDRYWMHFCGGSLIHPQWVLTAAHCLGPDVKDLATLRVN SGTHLYYQDQLLPVSRIMVHPQFY
HLTβ  IVGGQEAPRSKWPWQVSLRVHGPYWMHFCGGSLIHPQWVLTAAHCVGPDVKDLAALRVQLREQHLYYQDQLLPVSRIIVHPQFY
HST1  IVGGQEAPRSKWPWQVSLRVHGPYWMHFCGGSLIHPQWVLTAAHCVGPDVKDLAALRVQLREQHLYYQDQLLPVSRIIVHPQFY
HST3  IVGGQEAPRSKWPWQVSLRVRDRYWMHFCGGSLIHPQWVLTAAHCVGPDVKDLAALRVQLREQHLYYQDQLLPVSRIIVHPQFY
DMT   IVGGREAPGSKWPWQVSLRLKGQYWRHICGGSLIHPQWVLTAAHCVGPNVVCPEEIRWQLREQHLYYQDHLLPVNRIVMHPNYY
MMCT1 IVGGHEASESKWPWQVSLRFKLNYWIHFCGGSLIHPQWVLTAAHCVGPHIKSPQLFRVQLREQYLYYGDQLLSLNRIVVHPHYY
MMCT2 IVGGQEAHGNKWPWQVSLRAndtYWMHFCGGSLIHPQWVLTAAHCVGPDVADPNKVRVQLRKQYLYYHDHLMTVSQIITHPDFY
RST   IVGGQEASGNKWPWQVSLRVxdtYWMHFCGGSLINPQ
            ♥          *         111        121        131        141        151        161
BT    SNTLNNDIMLIKLKSAASLNSRVASISLPTSCA  SAGTQCLISGWGNTKSS GTSYPDVLKCLDAPILSDSSCKSAYP
HLTα  IIQTGADIALLELEEPVnisSRVHTVMLPPASETFPPGMPCWVTGWGDVDNDEPLPPPFPLKQVKVPIMENHICDAKYHLGAYT
HLTβ  TAQIGADIALLELEEPVKVSSHVHTVTLPPASETFPPGMPCWVTGWGDVDNDERLPPPFPLKQVKVPIMENHICDAKYHLGAYT
HST1  TAQIGADIALLELEEPVnvsSHVHTVTLPPASETFPPGMPCWVTGWGDVDNDERLPPPFPLKQVKVPIMENHICDAKYHLGAYT
HST3  TAQIGADIALLELEEPVnvsSHVHTVTLPPASETFPPGMPCWVTGWGDVDNDERLPPPFPLKQVKVPIMENHICDAKYHLGAYT
DMT   TPENGADIALLELEDPVnvsAHVQPVTLPPALQTFPTGTPCWVTGWGDVHSGTPLPPPFPLKQVKVPIVENSMCDVQYHLGLST
MMCT1 TAEGGADVALLELEGPVnvsTHIHPISLPPASETFPPGTSCWVTGWGDIDNDEPLPPPYPLKQVKVPIVENSLCDRKYHTGLYT
MMCT2 IVQDGADIALLKLTNPVnisDYVHPVPLPPASETFPSGTLCWVTGWGNIDNGVNLPPPFPLKEVQVPIIENHLCDLKYHKGLIT
RST               STNPVxitSNVHTVSLPPASETF                                              GLNT
        171        181       ♦  191♥          *        211        221        231        241
BT    GQ    ITSNMFCAGYLEGGKDSCQGDSGGPVVC  SGK LQ GIVSWGSGCAQKNKPGVYTKVCNYVSWIKQTIASN
HLTα  GDDVRIIRDDMLCAG  NSQRDSCKGDSGGPLVCKVngtWLQAGVVSWDEGCAQPNRPGIYTRVTYYLDWIHHYVPKKP
HLTβ  GDDVRIVRDDMLCAG  NTRRDSCQGDSGGPLVCKVngtWLQAGVVSWGEGCAQPNRPGIYTRVTYYLDWIHHYVPKKP
HST1  GDDVRIVRDDMLCAG  NTRRDSCQGDSGGPLVCKVngtWLQAGVVSWGEGCAQPNRPGIYTRVTYYLDWIHHYVPKKP
HST3  GDDVRIVRDDMLCAG  NTRRDSCQGDSGGPLVCKVngtWLQAGVVSWGEGCAQPNRPGIYTRVTYYLDWIHHYVPKKP
DMT   GDGVRIVREDMLCAG  NSKSDSCQGDSGGPLVCRVRGVWLQAGVVSWGEGCAQPNRPGIYTRVAYYLDWIHQYVPKEP
MMCT1 GDDFPIVHDGMLCAG  NTRRDSCQGDSGGPLVCKVKGTWLQAGVVSWGEGCAQPNKPGIYTRVTYYLDWIHRYVPEHS
MMCT2 GDNVHIVRDDMLCAG  NEGHDSCQGDSGGPLVCKVEDTWLQAGVVSWGEGCAQPNRPGIYTRVTYYLDWIHHYVPKDF
RST   GDNVHIVRVDMLCAG  NEGHDSCZGDSGGP    VEDTWLQAGVVSWGEGCAQPN       VTYYLD
```

	N-Ter (37)	Lys-C (23)	Try-19 (12)	Try-24 (21)	Try-31 (6)	Try-37 (21)	Overall (119)
BT	68	26	0	64	33	67	43
HLTα	86	78	58	80	100	90	82
HLTβ	84	70	67	80	100	90	81
HST1	84	74	67	80	100	90	82
HST3	86	74	67	80	100	90	82
DMT	73	52	75	80	83	86	75
MMCT1	76	61	58	75	100	90	77
MMCT2	92	78	92	95	100	100	93

Table 2. Peptide Sequence Homology. Peptide sequences from rat skin tryptase were compared with the corresponding portion of the amino acid sequence of other tryptases. The table gives the percentage of the identity of each peptide from rat skin tryptase to the corresponding regions in other tryptases. The overall identity is also indicated. The number of amino acids in each peptide is indicated in a parentheses. BT, bovine trypsin; HLTα, human lung tryptase α (Miller et al. 89); HLTβ, human lung tryptase β (Miller et al. 90), which is identical to human skin tryptase 2 (Vanderslice et al. 90); HST1, human skin tryptase 1; HST3, human skin tryptase 3; DMT, dog mastocytoma tryptase (Vanderslice et al. 89); MMCT1, mouse mast cell tryptase 1 (Chu 90); MMCT2, mouse mast cell tryptase 2, which is identical to mouse mast cell protease 6 (Reynolds et al. 91); N-Ter, N-terminal amino acid sequence from rat skin tryptase; Lys-C, the peptide from lysylendopeptidase-C digest; Try-19, Try-24, Try-31, and Try-37, the peptides of peak number 19, 24, 31, and 37 respectively from the tryptic digest.

The structural differences between tryptases and trypsin has been described in a recent paper (Johnson & Barton 1992). The homology between tryptases and trypsin is about 49% with 20-21 additional amino acids for tryptases. Rat skin tryptase peptides (Chen et al. 1990) show a low overall identity (43%) to trypsin. Some regions are highly conserved among trypsin and tryptases. The catalytic site H44, D91, and S194 residues (based on tryptase numbering system) are present in all tryptases and trypsin. So is the substrate binding D188, which is characteristic of trypsin-like rather than chymotrypsin-like serine proteases. However, tryptase does show some marked differences from trypsin. The most distinct feature of tryptases is the two insertions on either side of the active site cleft compared with trypsin. There are four more amino acid residues in tryptase than in trypsin between residues 20 and 26 (Figure 11). There is also a 9 amino acid insertion located between 164 and 174. Both of the insertion sites are involved in inhibitor binding region of serine protease. These two insertions may contribute to the higher substrate selectivity of tryptase as well as to the resistance of tryptase to many trypsin inhibitors. Another unique feature of tryptases is the extra number of tryptophan residues and proline patches. Tryptase has five more tryptophan residues than does trypsin. Three dimensional modeling of tryptase (Johnson & Barton 1992) based on trypsin structure reveals that four of the five tryptophan residues

not found in trypsin, W12, 14, 126, and 206, form a cluster on the opposite side of the active site. There is another hydrophobic stretch between L139 and L145 which includes the proline patches along one edge of the tryptase model (Johnson & Barton 1992). The sequences obtained from the rat skin tryptase peptides account for four of the tryptophan residues unique to tryptase. Three of them, W12, 14, and 25, are located in the N-terminal peptide. The other one, W206, is located in the Try-37 peptide. Tryptophan, leucine, and proline residues in tryptase seem to have arisen from mutation rather than insertion. Tryptase is a tetramer. The hydrophobic regions may play a role in the interaction between the subunits.

Tryptase is a mediator of mast cell granule and released together with heparin from mast cell during degranulation (Pearce 1989). Human lung tryptase activity is stabilized by heparin (Schwartz & Bradford 1986). The observations mentioned suggest that tryptase somehow interacts with heparin. Binding to heparin requires that tryptase have sufficient surface positive charges (Blankenship et al. 1990). Amino acid sequences show that tryptase has 6 more lysine and arginine, and 7-10 more histidine residues than trypsin does. These basic residues are found on the surface of the molecule indicating that they may play a role in heparin binding. Human tryptase has been shown to bind to heparin at neutral pH as well as at acidic pH. The pKa for histidine is 6, which

suggest that histidine may not be an important factor for heparin binding at neutral pH. However, histidine may play a more important role in heparin binding inside mast cell granules which has a pH value of 5.6 (DeYong et al. 1987). Since the pI values for lysine and arginine are 9.7 and 10.8 respectively, they are believed to have a major role in extracellular tryptase binding to heparin at neutral pH. In spite of the potential role of the basic residues, charges alone should not be over emphasized. Trypsin has a net charge of +5 in comparison to -4 to -10 for tryptase due to more total acidic residues. Further evidence is needed to identify the heparin binding sites. Rat skin tryptase, which has an overall 92% homology to MMCT-2, does not show evidence of heparin binding in vitro (Braganza & Simmons 1991).

Another difference of tryptase from trypsin is that all tryptases identified are glycosylated, which is not the case in trypsin. A number of putative glycosylation sites are found in all tryptases in Figure 11. Enzymatic deglycosylation of the purified tryptases showed the molecular weight of tryptase shifted to a smaller size after incubation with deglycosylation enzymes as shown in the results above and else where (Cromlish et al. 1987, Braganza & Simmons 1991). Like other tryptases, rat skin tryptase peptides significantly differ from trypsin with only from 0 to 68% homology between the corresponding regions (Table 2).

While the differences between tryptase and trypsin is

remarkable, the distinction among tryptases is also significant. Rat skin tryptase peptide sequences and other tryptase sequences have revealed a great extent of homology as well as differences. The common and unique functional properties of tryptases may be the result of the structural similarities and differences among them. Like other tryptases, rat skin tryptase peptides have revealed the conserved residues such as the catalytic site residue S194, substrate binding site residue D188, and the tryptophan residues specific to tryptase but not to trypsin, W12, W14, W25, and W206. Residues between D188 and C200 located next to the active site of serine protease are highly conserved among tryptases. This region show 100% amino acid identity among tryptases except HLT-α which has a 92% identity. The stretch from W206 to N222 starts from one side opposite to the active site, passes through the interior of the molecule, and reaches the region on the other side near the substrate binding site according to the computer models (Johnson & Barton 1992). Yet, this long stretch reserves about 100% amino acid identity among all of the tryptases including rat skin tryptase. The highly homologous regions sampled above may reflect the underlying structural identity which determines the functional similarities among tryptases.

The unique functional properties of tryptases may be related to their structural variations. Within the two insertions, residues 20-26 and 164-174, high degree of amino

acid variation among the tryptases is apparent. In the insertion between residues 20 and 26 on one side of the active site clef, for instance, the amino acid at position 23 among the tryptases varies significantly. HST-1 and HLT-β have a hydrophobic proline residue at the position. HST-3 and HLT-α have basic amino acid residues, lysine and arginine instead. While MMCT-1 and DMT have asparagine or glutamine with an amide group, both RST and MMCT-2 have a more aliphatic threonine with a hydroxyl group. Tolerance to this type of mutational change would mean that the position either has little biological significance to the enzyme or defines certain functional specificity for tryptase. Location of the position in the inhibitor binding region tends to support the latter. The amino acids in the other insertion region (164-174) also revealed some variations among tryptases from different species. For example, all human tryptases have alanine at position 166 while mouse, dog, and rat tryptases have leucine instead. While all the human and the dog tryptases have an arginine at position 173, tryptases in mouse and rat have either proline or histidine. Glycine 215 is a well documented substrate binding site residue which is replaced by a valine in elastase (Shotton & Watson 1970). The glycine residue in HLTα is replaced by an aspartate.

Another obvious difference among the tryptases is the location and number of the putative glycosylation sites. All human tryptases have a putative glycosylation site at position

203 while rat, mouse, and dog tryptases do not. A highly conserved putative glycosylation site is located at position 102 in all tryptases except HLT-β. Both of the two positions are on the opposite side of the active site according to the three dimensional models proposed for serine proteases (Greer 1990) and tryptases (Johnson & Barton 1992). Another putative glycosylation site which does not exist in other tryptases is located at position 21 in MMTC-2 and RST. This position appears to be in a region near the substrate binding site. The functional importance of the glycosylation sites in tryptases remain unclear.

The results have revealed some interesting structural properties of tryptases. The structural characteristics of tryptase provide very encouraging and helpful information for future exploration of the structure and function relationship of the enzyme.

PART II

RAT MAST CELL HETEROGENEITY

CHAPTER VI

INTRODUCTION

The pace of mast cell research has been exceptionally rapid during the past several years because of the potential biochemical and clinical importance. The most recent advances in mast cells are summarized in the review section. Mast cells are localized immune cells which have been implicated in the pathogenesis of many human disorders. Different mast cell types have been described in rodent and human tissues. Understanding mast cell heterogeneity is very important for identification of the precise role of each mast cell type in different disease states. In rats, mature mast cells are grouped under two major subsets, the connective tissue mast cells and mucosal mast cells, based on their dye-binding properties. Alcian blue identifies both the mucosal mast cell type which contains chondroitin sulfate proteoglycans, and the connective tissue mast cell type, which contains heparin and chondroitin sulfate proteoglycans. Safranin stains proteoglycan and thereby selectively stains the connective tissue mast cell type. In general, mature cells that remain blue after the alcian blue/safranin double stain would belong to the mucosal mast cell type; those which counterstain red are of the connective tissue mast cell type. However, immature connective tissue mast cells stain with alcian blue,

but poorly, if at all, with safranin. Therefore, the dye-binding properties of mast cells have limitations as discriminating markers of the two mast cell types. Recently, mast cell granular enzymes have been used successfully as markers in the identification of mast cell subsets. Monospecific and monoclonal antibodies against rat mast cell chymase I and chymase II were used to distinguish the connective tissue mast cell from the mucosal mast cell type respectively. Anti-chymase I antibody identified connective tissue mast cell in skin, tongue, intestinal serosa and submucosa, and lung pleural membrane. Anti-chymase II antibody identified mucosal mast cells as the predominant type in mucosa of small intestine and stomach. Chymase II positive mast cells also were detected in other tissue locations such as around bronchi, thymus, liver, and submucosa of stomach.

In humans, mast cells have also been classified based on their neutral protease content. One type contains only tryptase; the other contains tryptase, chymase, mast cell carboxypeptidase and a cathepsin G-like protease. Based on tissue distribution, the type with only tryptase corresponds more closely to the rat mucosal mast cell type while the other type corresponds more closely to the connective tissue mast cell type in rat.

In the current study, rat tryptase is shown to be located only in a subpopulation of the safranin-positive, chymase I-positive mast cells of the connective tissue type. The

obvious distinction of tryptase distribution in human mast cell population and rat mast cell population may reflect an interesting evolutionary aspect of the two species.

CHAPTER VII

LITERATURE REVIEW

It's been more than 100 years since mast cells were first described (Ehrlich 1878, Schwartz & Huff 1991). This chapter is designed to briefly summarize the most recent advances in our knowledge about mast cells and to elicit an appreciation of the significance of mast cell research.

1. Biological And Pathological Roles

Thanks to the advance in cell biology, biochemistry, and molecular biology techniques, mast cell research has gained momentum in the past few years. Due to the large number of publications, it is impossible to cover all the details reported. In this section, the biological and pathological roles of mast cells are reviewed to help understand mast cells and their important roles in health and diseases. Mast cells are granulocytic immune cells that are frequently found at the interface of the internal and external environments. Degranulation occurs when mast cells are stimulated by antigens via IgE molecules on the cell surface (Segal et al. 1977) or other endogenous and exogenous secretogogue such as hormones, neurofactors, cytokines, and physical stimuli such as heat, cold, or trauma (Bernstein & Lawrence 1990, Galli 1990). The biologically active mediators released from the

granules interact with the local environment in a unique way and facilitate the initiation of some normal or pathological events.

Mast cells in the airway. The role of mast cells in bronchial asthma has been well established (Maron 1991, Schwartz & Huff 1991). Brochial asthma, a manifestation of immediate hypersensitivity, is a disease characterized by increase in responsiveness of bronchial smooth muscle, cellular infiltration, and glandular tissue secretion. Mast cells are present in abundance in bronchial and alveolar (Fox et al. 1981, Schulman et al. 1982, Irani et al. 1986). Release of mediators from the effector cells in these locations can directly affect the activity of the respiratory tract. When challenged with endobronchial allergen (Wenzel et al. 1988) or oral aspirin (Bosso et al. 1989), mast cell activation occured as indicated by elevated plasma levels of tryptase and histamine. In cases where airway obstruction occurs, mast cell activation and degranulation also occur (Broide et al. 1991, Jarjour et al. 1991). In allergic rhinitis, the immediate response correlates with mast cell degranulation (Sedgwick et al. 1991). Following this mast cell mediated immediate response, eosinophils are attracted to the airway and cause airway injury during the late-phase allergic reaction. Mast cells are also involved in experimentally induced nasal allergic response (Walden et al. 1988). It was reported that mast cells participate in

pulmonary fibrosis when rats are challenged with bleomycin or asbestos (Goto et al. 1984, Wagner et al. 1984). When mast cells were co-cultured with fibroblast and mesothelial cells, two predominant and yet morphologically distinct neighboring cell types, and mast cell degranulation was induced by 48/80, subsequent induction of mitogenesis occurred in both fibroblasts and mesothelial cells (Druvefors & Norrby 1988). The observation supports the role of mast cells in fibrosis. However, whether the mast cells cause pulmonary fibrosis or result from the fibrosis remains unclear. The observation that pulmonary fibrosis could be induced in mast cell-deficient mice suggested the possibility that the increase of mast cell number might be the result of pulmonary fibrosis (Mori et al. 1991).

Mast cells in gastric system. The participation of mast cells in gastrointestinal infections has been documented. Mast cells are found in large number in intestinal mucosa (Enerback 1966, Fox et al. 1985). Parasitic infection dramatically increases mast cell population in the mucosal area in rodents (Mayrhofer 1979a, 1979b, Mayrhofer & Bazin 1981, Stevens et al. 1986). After roundworm infection, rat mucosal mast cells degranulate and result in elevated levels of secreted chymase II (Woodbury et al. 1984), a serine protease which may enhance vascular permeability, suggesting that mast cell activity may play an important role in resisting parasite infection. Mast cells have been found to be responsible for the acute gastric

damage and restraint stress-induced gastric ulceration (Guth & Kozbur 69, Andre et al. 85, Galli et al. 85). Compound 48/80 causes mast cell degranulation leading to severe gastric lesions (Franco et al. 1959, Cho et al. 1979, Ogle et al. 1980, Takeuchi et al. 1986). Ethanol is known to cause mast cell-dependent augmentation of acute gastric mucosal damage (Wallace et al. 1982, Galli et al. 1985). Sodium cromoglycate and FPL-52694 are agents known to inhibit mast cell degranulation and are called mast cell stabilizers (Takeuchi et al. 84, Goossens et al. 1987). These agents have been shown to inhibit gastric damage resulting from changes in acid secretion and gastric motility induced by compound 48/80, ethanol, restraint stress and other compounds which cause mast cell degranulation (Ogle & Lau 1980, Canfield & Curwain 1983, Takeuchi et al. 1984, Takeuchi et al. 1986, Goossens et al. 1987, Wallace et al. 1988, Beck et al. 1989). In rats pretreated with the two mast cell stabilizers, the number of mast cells were significantly reduced in the ethanol treated rats. Correspondingly, both agents significantly reduced ethanol-induced damage in a dose dependent manner (Beck et al. 1989).

Mast cells and tumors. There is an increasing amount of evidence for mast cell interaction with tumor cells (Hartveit 1981, Hartveit et al. 1984, Parwaresch et al. 1985, Ionov 1989, Flynn et al 1991). Mast cells have been found at tumor sites (Dabbous 1980, Van den Hooff et al. 1983, Woolley 1984,

Roche 1985). Degranulation of mast cells at these sites suggests that mast cell-tumor cell interactions might enhance invasive growth of human and animal tumors (Csaba et al. 1961, Hartveit & Sandstad 1982, Farnoush & Mckensie 1983, Norrby & Enstrom 1984). Previous studies had shown that extensive extracellular matrix degradation was associated with tumor invasion and metastasis (Yamanishi et al. 1972, Hashimoto et al. 1973, Liotta et al. 1980). In terms of release of collagenolytic activity, tumor-derived fibroblast-like cells appeared to be more responsive to mast cell products than did normal fibroblasts (Dabbous et al. 1986). The mast cell factors responsible for stimulating collagenolytic activity were apparently not histamine or heparin. In more recent studies (Gruber et al. 1988, 1989), tryptase was shown to have a potential role in matrix degradation via activation of prostromelysin to stromelysin which in turn degrades extracellular matrix components and activates latent collagenase. Studies using mast cell-stabilizing compounds provided support for mast cell involvement in tumor metastasis (Dabbous et al. 1991). Rat pretreated with FPL 55618 which prevents mast cell degranulation, caused a 70% inhibition of tumor growth at the primary site. After 23 days without treatment, tumor growth of the rats accelerated and reached a similar tumor size to that of control animals. Numerous mast cells were detected around the tumor periphery and often showed signs of degranulation. In contrast to the untreated

animals, FPL 55618 treated animals showed numerous intact mast cells within the tumor mass. In contrast to *in vivo* experiments, FPL 55618 had no direct effect on the rate of rat mammary adenocarcinoma proliferation *in vitro*. Soluble mast cell product, on the other hand, significantly increased the rate of tumor cell proliferation. These observations demonstrated the potential importance of mast cell and tumor cell interaction in the growth and invasive properties of tumor cells *in vivo*. In IgE-dependent mast cell activation, the immediate release of preformed mast cell mediators is followed by synthesis and sustained release of large quantities of newly formed tumor necrosis factor alpha (Gordon & Galli 1991). Mast cells have been implicated in angiogenesis in tumor sites (Messler *et al*. 1976, Fraser & Simpson 1983, Starkey *et al* 1988, Crowle & Starkey 1989). Tumor necrosis factor is mitogenic for fibroblasts (Dayer *et al*. 1985, Young *et al*. 1987) and capable of stimulating angiogenesis *in vivo* (Kessler *et al*. 1976, Frater-Schroder *et al*. 1987, Leibovish *et al*. 1987). Fibroblast can in turn influence mast cell differentiation (Levi-Schaffer *et al*. 1986) and granule maturation (Davidson *et al*. 1983). Mast cell granules can actually be transferred into fibroblasts via phagocytosis (Nicolson 1984, Norrby & Enstrom 1984) and transgranulation (Greenberg & Burnstock 1983), a process in which mast cells establish a special cell contact with other cell types and release its granule into the contact cell. The

specific role of mast cells in tumor growth and metastasis remain an interesting area of investigation.

Mast cells and the nervous system. Interaction of mast cells with the nervous system has been widely reported (Olsson 1968, Ibrahim 1974, Dropp 1976, Theoharides 1990, Johnson & Krenger 1992). In the brain, greater than 98% of the mast cells are located in the thalamus (Goldsehmidt et al. 1984). The connective tissue coverings of the central nervous system are also rich in mast cells (Orr 1988, Ferrante et al. 1990, Dimlich et al. 1991). In the peripheral nervous system, mast cells are located in the autonomic nerve endings in tissues such as skin (Heine & Forster 1975, Alving et al. 1991), thymus and spleen (Williams & Felten 81, Weihe et al. 1989, Muller & Weihe 1991), intestine (Newson et al. 1983, Stead et al. 1987, Stead et al. 1989), mesentery (Skofitsch et al. 1985), and lung (Bienenstock et al. 1988, Alving et al. 1991).

Several studies have suggested that mast cells and nervous tissues may interact with each other bidirectionally (Barrett 1991). There is evidence that the nervous system can influence the growth, differentiation, and degranulation of mast cells. When co-cultured with mast cells, glial cells show its ability to support mast cell growth in vitro (Seeldrayer et al. 1989, Ryan & Huff 1990). The glial cells are known to secrete nerve growth factor (Lindholm et al. 1987, Houlgatte et al. 1989) which can affect mast cell development. In cell culture, addition of nerve growth factor

increases the number and size of mast cells (Aloe 1977, 1988). When spleen cells pretreated with nerve growth factor were injected into the brains of developing rats, the cells differentiated into mast cells (Aloe & DeSinone 1989). While the ability of noradrenaline and acetylcholine to stimulate mast cell degranulation is still controversial (Mammaioini et al. 1975, Fantozzi et al. 1978, Kazimierczak et al. 1980, Alm & Bloom 1981, Masini et al. 1985, Botana et al. 1987), many neuropeptides have been shown to activate mast cells. Somatostatin induces degranulation of rat peritoneal mast cells and human skin mast cells (Theoharides et al. 1978, 1980, Piotrowski & Foreman 1985, Renold et al. 1987). Substance P (Erjavec et al. 1981, Fewtrell et al. 1982, Shibata et al. 1985, Ebertz et al. 1987), endorphins (Yawasaki et al. 1982, Shanahan et al. 1984), neurotensin (Lazarus et al. 1977, Sydbom 1982), opioid peptides (Casale et al. 1984), vasoactive intestinal peptide (Piotrouski & Foreman 1985), calcitonin gene-related peptide (Fremann 1987), neurokinin (Wallengren & Hakanson 1987), prostaglandin D2 (Levi-Schaffer & Shalit 1989), and neuropeptide Y (Arzubiaga et al. 1991) are all capable of causing mast cell degranulation, perhaps through the β-adrenergic receptors (Marquarett & Wasserman 1982, Lowman et al. 1988, Arbabian et al. 1989) and muscarinic cholinergic receptor (Masini et al. 1983) on the surface of mast cells.

Mast cells can also influence nervous tissue. Histamine,

a mast cell mediator, is a neurotransmitter capable of regulating synaptic transmission (Steinbusch & Muller 1984, Villena et al. 1986, Weinreich & Undem 1987, Christian et al. 1989, Wada et al. 1991) and inducing electrophysiological changes in vitro (Green et al. 1988, Janiszewski et al. 1990). Immunological regulation of synaptic transmission has been observed in isolated guinea pig autonomic ganglia (Weinreich et al. 1987) and in patients with allergic disease (Casale 1983). Mast cells can affect the content of 5-hydroxytryptamine, a neurotransmitter, in rodent sensory ganglia (Kai & Keen 1985), and rat intestinal motor (Bueno et al. 1991). Serotonin, vasoactive intestinal peptide, and somatostatin-like molecules secreted by mast cells of brain and other tissues may also modulate the neural activities in the nervous system (Cutz et al. 1978, Goetzl et al. 1985, Lambracht-Hall et al. 1990).

Besides serving as sources of neurotransmitters, mast cells can also regulate local neuronal activities through modification or processing of other neural peptides by mast cell neutral proteases. As mentioned earlier, tryptase selectively hydrolyzes potent bronchodilators such as VIP, PHM, and CGRP but not the bronchoconstrictor SP.

Although the mechanism of mast cell/nervous system interaction has not been resolved (Marshall et al. 1989, Bienenstock et al. 1991), evidence for such interactions have been uncovered in many neural disorders such as CNS

inflammatory demyelination (Mokhtarian & Griffin 1984, Griffin & Mendoza 1986), systemic mastocytosis (Rogers et al. 1986, Iriarte et al. 1988, Schwartz et al. 1987), neurofibromatosis (Isaacson 1976, Riccardi & Eichner 1986, Claman 1987, Riccardi 1987, Johnson et al. 1989, Riccardi, 1989), and in peripheral nerve disease (Barnes et al. 1990, Kiernan 1990). For example, increased mast cell degranulation was detected in experimental allergic encephalomyelitis (EAE), a model of CNS demyelination (Bo et al. 1990, Krenger et al. 1991). Mast cell degranulation was also observed in experimental allergic neuritis (Powell et al. 1983, Izumo et al. 1985, Seeldrayers et al. 1989). The precise role of mast cell in these disorders remain to be defined.

Mast cells in the cardiovascular system. Involvement of mast cells in the cardiovascular system has gained increasing attention in the past several years. The role of mast cells in atherogenesis (Kovanen 1991) and angiogenesis (Rakusan et al. 1990) have been investigated. The molecular events in coronary atherosclerosis are characterized by the local accumulation of LDL-derived cholesterol in the coronary intima, the innermost layer of the arterial wall (Smith 1974, Richardson et al. 1989, Movanen 1990). In the early stage, macrophages in the intima absorb cholesterol from modified LDL and become foam cells filled with cholesterol ester droplets (Fogelman et al. 1988, Hoff et al. 1990). Cholesterol can accumulate in the foam cells and then extend from its

intracellular position to form a soft extracellular cholesterol core (Smith 1974, Guyton et al. 1990) which develops into atherosclerotic lesions (McGill 1968, Stary 1987, Stehbens 1987). However, studies have shown that macrophages do not endocytose LDL unless the LDL molecules are first modified (Goldstein et al. 1979, Steinberg et al. 1989), a process which may involve mast cells.

Mast cells have been detected in the arterial intima (Pollak 1957, Pouchlev et al. 1966, Kamio et al. 1979). An extensive autopsy study of human from birth to age 39 showed that twice as many mast cells were present in the arterial intima of both early and advanced atherosclerotic lesions compared to non-disease intima (Stary 1990). The role of mast cells in atherosclerosis has been proposed based on both in vitro and in vivo experimental results. After degranulation of mast cells, heparin released from the granules binds to the positively charged domains of apolipoprotein B and E on LDL (Kokkonen & Kovanen 1987a, 1989, Kovanen & Kokkonen 1991). Mast cell granular proteases, mainly chymase and carboxypeptidase A, degrade the apolipoproteins on the LDL surface (Kokkonen & Kovanen 1985, Kokkonen et al. 1986). This results in modified LDL molecules binding to the mast cell granule remnant which are ultimately phagocytosed by macrophages located nearby (Lindahl et al. 1979, Baggiolini et al. 1982, Kokkonen & Kovanen 1987b, Lindstedt et al. 1992). Under normal circumstances, prevention of cholesterol

accumulation in arterial intima can be facilitated by high density lipoprotein (HDL) (Kovanen 1990). When mast cells are activated, however, HDL molecules are also modified by mast cell mediators and are then unable to remove cholesterol from the foam cells (Kovanen 1991). Mast cells have been implicated in cardiac hypertrophy (Rakusan et al. 1990). A significantly higher percentage of mast cells was found close to arteriolar portions of coronary capillaries than one would expect from their even distribution along the capillary wall. The increase in cardiac mass and formation of new capillaries in hypertrophic rat hearts induced by pressure overload is accompanied by increasing number of mast cells (Rakusan & Campbell 1991).

Mast cells in other diseases. Involvement of mast cells in arthritis has been described extensively (Mican et al. 1990, Gruber 1991). The number of mast cells are greatly increased in synovial tissue of patients with rheumatoid arthritis (Crisp et al. 1984, Godfreg et al. 1984, Irani et al. 1987, Bridge et al. 1991) and in rat adjuvant arthritis (Gryfe et al. 1971). In one study, the severity of antigen-induced arthritis following adjuvant injection was compared in mast-cell deficient mice and normal mice with mast cells (Malone et al. 1988, Van den Broek et al. 1988). The results indicated that mast cells can dictate the acute inflammatory events and potentiate chronic changes of cartilage degradation. When purified arthritogenic peptide was injected

into rat knee, acute mast cell degranulation occurred (Caulfield et al. 1988). The event was followed by inflammation, fat necrosis, and fibrin deposition. Tryptase released from mast cell granules may play an important role on chemotaxis, cartilage and bone turnover, and synovial fibrosis.

The importance of mast cells in skin has been well recognized (Lawrence et al. 1987, Lowman et al. 1988, Rothe et al. 1990, Church et al. 1991, Tainsh et al. 1991). Increased number of mast cells have been observed in the pathogenesis of many skin disorders such as atopic dermatitis (Mihm et al. 1976), scleroderma (Nishioka et al. 1987), progressive sclerosis (Hawkins et al. 1985), neurofibromatosis (Isaasson 1976, Riccardi 1981, 1987), bullous pemphigoid (Wintroub et al. 1978), abnormal wound healing such as keloid and hypertrophic scar (Smith et al. 1987), striae distensea (Sheu et al. 1991), and urticaria (Schwartz 1991). The pathways in fibrotic disorders may rely on the interaction between mast cells and fibroblasts. One possible cause of the fibrotic disease is the increase in collagen synthesis by fibroblasts (Botstein et al. 1982). Initially, factors secreted by T lymphocytes such as IL-3 can induce the growth and differentiation of immature mast cells from bone marrow cells. However, the progress from the immature cells to mast cell-committed progenitors depends on stem cell factors produced by fibroblasts (Ashman et al. 1991). Mast cells in turn can

affect the growth of fibroblasts. Co-culture of bone marrow-derived mast cells with mouse 3T3 fibroblasts resulted in the loss of the fibroblast contact inhibition (Dayton et al. 1989). Tryptase was shown to enhance proliferation of rat and hamster-derived fibroblasts (Ruoss et al. 1991). The action of mast cells was linked to macrophage, T cells, endothelial cells, and platelet (Claman 1989). In lesions of early striae distensae during puberty, sequential changes of elastolysis is accompanied by mast cell degranulation, followed by an influx of activated macrophages, which endocytose the fragmented elastic fibers (Sheu et al. 1991). Mast cells are present in ovary (Jones et al. 1980, Krishna & Terranova 1985, Shinohara et al. 1987, Schmidt et al. 1988, Drishna & Terranova 1991), uterus (Massey et al. 1991, Tainsh et al. 1991), and placenta (Purcell & Hanahoe 1991). Variations in the number and extent of degranulation of mast cells in ovary have been shown to correspond to the oestrous cycle (Schmidt et al. 1988, Krishna & Terranova 1991). Uterine mast cells show a significant negative correlation with years of post-menopause (Drudy et al. 1991). The functional response of uterine mast cells to an embryo-derived histamine releasing factor led to the proposal of a possible role of mast cell in embryo implantation and menstruation (Finn 1986, Cocchiara et al. 1988). Mast cells in the proximity of placenta also led to speculation about their involvement in pathological process during pregnancy and parturition (Purcell & Hanahoe 91).

Mast cells were identified in the arterial wall of rat adrenal gland and found to modulate both vascular and secretory responses in the intact adrenal gland (Hinson et al. 1988). Adrenocorticotropic hormone (ACTH) is known to increase steroidogenesis and cause vasodilation in adrenal gland. It has been demonstrated that ACTH stimulates adrenal blood flow and corticosterone secrection by its action on mast cells (Hinson et al. 1991).

In the eye, mast cells found in the conjunctiva were found to be responsible for ocular anaphylaxis (Allansmith et al. 1981, 1985, 1989).

Oral erythema multiform is usually caused by Herpes simplex virus or various drugs. A recent study revealed that mast cell numbers in the oral mucosa of patients with erythema multiform lesions were higher than those in the control subjects, suggesting that mast cell degranulation occurs in the most intensely inflamed area (Ruokonen 1992). Participation of mast cells in other oral diseases is also reflected by the increase in mast cells in oral lichen planus (Jontell et al. 1986) and gingivitis (Zachrisson 1968). Tobacco smoke has been shown to cause release of preformed mediators from canine mast cells (Thomas et al. 992).

An increase in renal tissue mast cell number was detected in fulminant hepatitis patients with hepato-renal syndrome (Yoshimura & Mori 1991). This suggests a role for renal tissue mast cells in the onset of acute renal failure in liver

diseases. The role of mast cell in systemic anaphylaxis and mastocytosis is well documented (Schwartz et al. 1987). Elevation of mast cell activation correlates with the severity of anaphylaxis (Matsson et al. 1991, Yunginger et al. 1991, Van der Linden et al. 1992). In systemic mastocytosis, liver, spleen, and lymph nodes may be infiltrated by mast cells and lead to hepatosplenomegaly and enlarged lymph nodes (Metcalfe 1991). Extensive involvement of mast cells may result in liver fibrosis, portal hypertension, and abdominal ascites. In vitro studies showed that mast cells enhanced the spontaneous and T-mitogen-induced proliferation of spleen and lymph node cells (Gushchin et al. 1991).

The involvement of mast cells in the various biological and pathological situations reviewed above has revealed the functional significance of mast cells in health and disease. Investigation of the specific roles of different mast cell sub-types, although in its juvenile stage, has been considered very important in the search for more definite answers to the questions that remain.

2. Mediators

Understanding the mechanism of mast cell action and mast cell heterogeneity relies, to a great extend, on the knowledge of mast cell granular mediators. Mast cell mediators are generally divided in two groups, preformed mediators and newly generated mediators (Bernstein & Lawrence 1990, Gordon et al.

1990, Schwartz & Huff 1991). The following table lists the major mediators present in mast cell granules.

Preformed	Newly Formed
Histamine	PGD2
5-HT	Thromboxane
Dopamine	LTB4
Heparin	LTC4
Condroitin sulfate	LTD4
Tryptase	LTE4
Chymase	PAF
Cathepsin G	Cytokines
Carboxypeptidase	TNF-α
VIP	IL-1,2,3,4,5,6,10
SLM	MIP-1α
	MIP-1β
	MCAF
	TCA3
	GM-CSF
	TGF-β
	IFγ
	SCF

Abbreviations used above are: 5-hydroxytryptamine (5-HT);

vasoactive intestinal peptide (VIP); somatostatin-like molecules (SLM); prostaglandin D2 (PGD2); leukotrienes (LT); tumor necrosis factor α (TNF-α); Interleukins (IL); macrophage inflammatory proteins (MIP); monocyte chemotactic and activating factor (MCAF); granulocyte/macrophage-colony stimulating factor (GM-CSF); transforming growth factor (TGF); interferon (IF); stem cell factor (SCF); platelet activating factor (PAF).

Biogenic amines such as histamine, serotonin, and dopamine can be secreted from mast cells (Enerback 1966, Jenkinson et al. 1970, Weitzman et al. 1985, Lambracht et al. 1990). Histamine in lung serves as a potent spasmogen which can cause bronchoconstriction and vasoconstriction. As a vasodilator in affecting local vascular permeability, histamine must act via the interaction with cell-specific receptors H1 and H2 (Schwartz 1991).

Mast cell proteoglycans includes highly sulfated heparin and less sulfated condroitin (Stevens et al. 1988, Thompson et al. 1988). When degranulation occurs, the proteoglycans are released together with granular enzymes. Heparin and chondroitin have a stabilizing effect on these enzyme activities (Schwartz & Bradford 1986, Alter et al. 1987) and on growth factor activities (Kellen & Lindahl 1991, Ruoslahti & Yamaguchi 1991). Heparin also has well known anticoagulant activity (Metcalfe et al. 1981). Mast cell proteoglycans may play an important role in initiating binding of LDL and HDL

molecules in atherosclerosis (Kovanen 1991).

Tryptase and chymase are neutral serine proteases unique to mast cells (Schwartz 1990). The possible roles of tryptase are discussed above. Chymase converts angiotensin I to angiotensin II at a rate 4 times faster than does angiotensin-converting enzyme (Wintroub et al. 1984). Other postulated roles for chymases include involvement in basement membrane degradation (Briggaman et al. 1984), glandular mucus production (Sommerhoff et al. 1989), and bradykinin degradation (Reilly et al. 1982). Cathepsin G is a neutral serine endopeptidase found in neutrophils and monocytes. A cathepsin G-like protease has also been found using immunocytochemical and enzyme histochemical techniques in mast cells (Schechter et al. 1990). Carboxypeptidase A is also localized in mast cell secretory granules (Goldstein et al. 1989). This enzyme preferentially removes amino acids from the C-terminus of peptides which have terminal aromatic residues or leucine. Rat mast cell carboxypeptidase A is similar to bovine pancreatic carboxypeptidase A (Everitt et al. 1980, Schwartz et al. 1982). As discussed previously, mast cells also contain preformed peptides such as VIP (Cutz et al. 1978), and SLM (Goetzl et al. 1985).

Newly formed mast cell mediators include various cytokines (Gorden et al. 1990), and arachidonic acid metabolites (Peters et al. 1984). Since the discovery of IL-4 and TNF-α in mast cells (Brown et al. 1987, Young et al.

1987), many cytokines have been found in activated mast cells. These include IL-1, IL-3, and IL-6. Transcripts for IL-2, IL-5, IL-10, MIPs, MCAF, GM-CSF, TGF-β, IFγ, and SCF are also found in the activated mast cells. Many of the cytokines can affect mast cell growth and development. IL-3, for instance, can stimulate IL-3 dependent progenitors derived from bone marrow to progress to mast cell-committed progenitors (Huff & Justus 1988). These progenitors then migrate to fibroblast-rich sites possibly by binding to laminin through their cell surface laminin receptors (Thompson et al. 1989). SCF, on the other hand, can directly stimulate proliferation of the committed progenitors and induce granulation of mast cells (Tsai et al. 1991). IL-4, and IL-10 are co-factors for proliferation and differentiation of mast cells (Hamaguchi et al. 1987, Gordon et al. 1990). In contrast to the other cytokines, GM-CSF and IFγ can suppress proliferation of mast cells (Bressler et al. 1989, Burd et al. 1989). The biological significance of some mast cells' ability to release both negative and positive effectors remains unclear.

Mast cells can incorporate exogenous arachidonic acid into neutral lipids and phospholipids and store these lipids in membranes and cytoplasmic lipid bodies (Dvorak et al. 1983, Peters et al. 1984). When mast cells are activated, arachidonic acid is release from the lipids and metabolized to PGD2 and thromboxanes through the cyclooxygenase pathway or to leukotrienes and lipoxins through the lipoxygenase pathways

(Schwartz 1987, Serofim & Austin 1987). PGD2 is a vasoconstrictor which is released from not only mast cells (Robinson 1988) but also macrophages (MacDermot et al. 1984), platelet (Oelz et al. 1977), and CNS neurons to promote sleep (Hayaishi 1991). LTB4 is a potent chemotactic factor for neutrophils, eosinophils, monocytes, and basophils. Its ability to attract neutrophils is as strong as complement C5a (Ford-Hutchinson et al. 1980). LTC4, LTD4, and LTE4 are bronchoconstrictors and vasoconstrictors. In lung where there are few LTC4 receptors, LTC4 must be converted to LTD4 and LTE4 to mediate its effects on bronchoconstriction. LTD4-receptor antagonists have been shown to attenuate allergic-induced bronchoconstriction (Griffen et al. 1983). PAF, a phosphotidylcholine analog, can cause aggregation of platelets and dilation of blood vessels (Kerdel & Soter 1989).

3. Mast Cell Heterogeneity

Mast cell are not a homogeneous cell population. The heterogeneity of mast cells is reflected by their responses to different stimuli, the content of secretory granules, and their phenotypes.

Responses to stimuli. Mast cells from different sources were found to have different functional responses to IL-3. The growth and differentiation of mast cells from intestinal mucosa depend on IL-3 while those from connective tissues are mostly independent of Il-3 (Razin et al. 1984).

However, the dependency of mast cells on IL-3 depends on the microenvironment and is interchangeable (Valent et al. 1991). When IL-3 dependent mouse mast cells were co-cultured with fibroblasts, they exhibited a phenotypic change toward IL-3-independent mast cells (Levi-Schaffer et al. 1986). Data for different responses of MC_T (human mast cells containing tryptase only) and MC_{TC} (human mast cells containing both tryptase and chymase) to cytokines were derived from tissue culture and in vivo observations. When cord blood mononuclear cells are co-cultured with mouse 3T3 fibroblasts, about 80-90% of the resulting mast cells are the MC_{TC} phenotype (Furitsu et al. 1989). It has been known that SCF secreted from fibroblasts can stimulate development and growth of rodent connective tissue mast cells (CTMC). Addition of T lymphocyte factors to the culture did not alter the result of MC_{TC} cells. In rodent cell culture, T lymphocyte factors can reverse the CTMC type to mucosal mast cells (MMC). These observations suggest that, like rodent CTMC, human MC_{TC} type is not T lymphocyte dependent.

Mast cells from different sources often respond differently to specific agents. Secretogogues such as the calcium ionophore A23187, 48/80, basic peptides (polylysine, polyarginine), VIP, SP, somatostatin, bradykinin, endorphins, morphine sulfate, and anaphylatoxin C5a can stimulate degranulation of IL-3-independent mast cells while IL-3-dependent mast cells such as those isolated from human lung

respond to A23187 but none of the others (Church et al. 1982, Bernstein & Lawrence 1990). Disodium cromoglycate which is widely used for treatment of allergic asthma can inhibit degranulation of mast cells from human intestinal lung mucosa (Flint et al. 1985, Schmulzler et al. 1985, Befus et al. 1987) but can not inhibit degranulation of mast cells from human skin (Cleggs et al. 1985). In contrast to human, disodium cromoglycate induces degranulation of connective mast cells but not mucosal mast cells in rodent (Pearce 1986, Breninstock 1988). Mast cells from different sources respond to nerve growth factor (NGF) differently. Proliferation and differentiation of rat mast cells from cultured blood cells and spleen cells were enhanced by NGF (Aloe 1977, 1988, Aloe & DeSimone 1989). Mucosal mast cells, on the other hand, did not respond to NGF (Johnson & Krenger 1992). Mast cells in the gut appear to be ready to defend against parasitic infection (Stead et al. 1987, Arizono et al. 1990). When brain tissues were infected by the larval stage of the cestode taenia solium, brain mast cell numbers did not increase as the gut mast cells had (Johnson & Krenger 1992).

Differences in mediator content. The mediator content of mast cells also reflects mast cell heterogeneity. Heterogeneity of proteoglycan content has long been recognized in different mast cells. Heparin has been found predominantly in connective tissue mast cells (Yurt et al. 1977) while condroitin E has been found mainly in mucosal mast cells

(Eliakim et al. 1986, Gilead et al. 1987). The distribution of chymase and tryptase varies depending on mast cell type and species (Schwartz et al. 1981a, Woodbury & Miller 1982, Irani et al. 1986). Carboxypeptidase A has been found in both human skin and human lung mast cells (Goldstein et al. 1989, Reynolds et al. 1989) but occurs only in CTMC in the rat (Everitt et al. 1980, Schwartz et al. 1982). Production of eicosanoids also reflects mast cell heterogeneity (Kewus et al. 1982, Heavey et al. 1988, Church et al. 1989, Robinson et al. 1989). PGD2 is produced predominantly by rat peritoneal mast cells but is consistently produced by both human skin and lung mast cells. LTC4 and LTB4 are produced at a level significantly higher in mucosal mast cells than in connective tissue mast cells in the rat. Human lung mast cells produce approximately nine times more leukotrienes than do skin mast cells. The leukotrienes are mainly LTD4 and LTE 4 in human lung mast cells as opposed to LTC4 in skin mast cells. Serotonin is present in mouse peritoneal mast cells (Weitzman et al. 1985) and brain mast cells (Lambracht et al. 1990) but not intestinal mast cells (Enerback 1966). Dopamine has been found in bovine skin mast cells (Jenkinson et al. 1970). Protease contents of the two mast cell types are also different. Cathepsin G, for example, was detected in CM_{TC} but not in MC_T using immunoassay techniques (Meier et al. 1985). Carboxypeptidase was found at high concentration in MC_{TC} type from human skin mast cells but in negligible amounts in MC_T

type from lung mast cells based on the activity of enzymatic hydrolysis of angiotensin (Goldstein et al. 1987).

Different phenotypes. Besides differences in stimuli response and mediator content discussed above, mast cells exhibit additional phenotypic heterogeneity (Galli 1990). Mast cells have been found in different species such as in human, other mammals, fish and amphibians (Michels 1963). Marked morphological differences exist between mast cells from different species as well as between mast cells in different anatomical locations within a species. The differences are defined based the properties of histochemical stains which discriminate mucosal type mast cell from connectives tissue type mast cells (Enerback 1966). In the rodent, mucosal type mast cells tend to be smaller and contain fewer granules than connective tissue type mast cells (Gleich et al. 1986). In the human, mast cells have subpopulations comparable, but less well defined, than rodent connective tissue type mast cells and mucosal type mast cells (Otsuka et al. 1985, Eliakim et al. 1986). Ultrastructural evidence from cytoplasmic granules reveal striking characteristics unique to either the human MC_T or the MC_{TC} (Craig et al. 1988, 1989, Dvorak et al. 1989). Under the electronmicroscope, mast cell cytoplasmic granules exhibit a variety of substructural patterns termed scrolls and crystals (or lattice features). Mast cell granules from human lung are more uniform in shape and have lattice features. Human skin mast cell granules, on the other hand, have a less

well defined shape and show discrete scrolls. Immunoelectronmicroscopic analysis indicate that the scroll feature is characteristic of MC_T mast cells which contains only tryptase while the lattice or grating feature is characteristic of MC_{TC} type mast cells which contain both tryptase and chymase. Unlike human mast cell granules which have intricate lamellae and scroll work, rodent mast cell granules show a more amorphous appearance under the electron microscope (Dvorak et al. 1983).

Data on morphology, responses to specific stimuli, and mediator content reveal a marked degree of diversity in mast cell populations. Various mechanisms have been proposed to account for mast cell heterogeneity (Galli 1990). The mechanisms of cellular maturation and differentiation, changes associated with functional activation, and acquisition of molecules derived from other cell types may contribute to phenotypic heterogeneity of mast cells by acting alone or in combination with the other mechanisms. Whatever the mechanisms, the demonstration of different mast cell subpopulations continue to elicit great interest due to its clinical significance.

4. Classification Of Mast Cells

Histochemistry Methods. Heterogeneity of mast cells poses the need for meaningful and reliable methods to identify

distinct subsets of mast cells which may have functional differences. As discussed above, mast cells are defined based on their content of large, electron-dense cytoplasmic granules made up of sulfated proteoglycans and other mediators. Mediators are part of the electron-dense granules of mast cells. However, many of the mediators are not unique to mast cells and are not considered proper markers for mast cells. Prostaglandin, thromboxanes, leukotrienes, and a varieties of cytokines can be products of other cell types. So can the biogenic amines and neuropeptides. Cathepsin G can be found in neutrophil and monocytes while carboxypeptidase A can be found in other cell types in the pancreas. The mediators unique to mast cells granules are tryptase and chymase.

Proteoglycans are also part of the electron-dense granules which consist of many glycoaminoglycans, a chain of disaccharide repeating units containing an amino sugar derivative such as glucoamine or galactoamine, covalently linked to a core protein. The intracellular proteoglycans found in mast cell granules contain the highly sulfated heparin and/or the less sulfated condroitin sulfate E (Stevens et al. 1988, Thompson et al. 1988). The number of carboxylate and sulfate groups in the disaccharide units determine the overall negative charge density of a specific type of proteoglycan. The average number of sulfate residues in each disaccharide repeating unit is 2.5 in heparin and 1.5 in chondroitin sulfate E.

These differences in charge are the basis for histochemical techniques which utilize metachromatic dye staining. These techniques were first used to identify mast cell subsets in rodents (Enerback 1966). Metachromatic dyes are cation dyes such as toluidine O, alcian blue and safranin O. When certain tissue elements are stained with one of the dyes, the color of the dye changes from blue to a purple or reddish violet color, i.e. the absorbance of the dye changes from a higher wavelength to a lower wavelength in the reaction. The change in color is called metachromasia. The tissue elements capable of inducing metachromasia are called chromotropes and are usually polyanions such as heparin and chondroitin. The dyes resulting in metachromasia are called metachromatic dyes. The chemical theory of metachromasia has been discussed (Schubert & Hamberman 1956). Metachromatic dyes contain chromophobic groups which exist in two tautomeric forms in a resonance system. Each tautomeric form has its own energy level for the π electrons and absorbs light of a characteristic wavelength. A reversible shift in the equilibrium between the two tautomeric forms of the dye results in a color change. It has been found that polymerization of metachromatic dyes results in a shift of the equilibrium of the molecular species in the resonance system. Chromotropes with their negative charges close together can bind cationic metachromatic dyes through salt bridges. The binding brings the dye molecules into close contact which

initiates polymerization. These shifts the peak absorption spectra to a shorter wavelength resulting in metachromasia.

Alcian blue 8 GX is a metachromatic dye containing four isothiouronium groups (Scott 1972). Alcian blue differs from other proteoglycan stains in that it does not stain nucleic acids and has a high solubility even in the presence of high salt concentrations. At pH 2.5-2.7, alcian blue stains both sulfated and non-sulfated proteoglycans, giving a blue color. At pH 1, only the sulfated glycosaminoglycans are stained (Pearse 1968). Safranin O is a metachromatic dye used to stain highly sulfated proteoglycans such as heparin. The less sulfated condroitin sulfate does not bind safranin O well. Together with alcian blue, safanin has been used to identify rodent mast cell types based on their metachromatic staining properties (Enerback 1966).

Utilizing those properties of the chromotropes and metachromatic dyes, Enerback and others demonstrated two types of mast cells in rodent (Enerback 1966, Miller & Walshaw 1972). In these experiments, mast cells in different tissues were fixed, and stained first with alcian blue and then with safranin stain. It was found that mast cells in the intestinal lamina propria were stained blue with the alcian blue but remained blue upon subsequent staining with safranin. On the other hand, mast cells in the skin and peritoneal cavity turned red after double staining indicating staining by

safranin. These observations resulted in the classification of rodent mast cells into two subtypes, the mucosal type mast cells (MMC) from the intestinal mucosal and the connective tissue type mast cell (CTMC) from skin and peritoneal lavage. Subsequent experiments found that these two types of mast cells appeared to exhibit differences in many aspects including the response to cytokines and various pharmacological agents as well as mediator content as discussed above. The terms MMC and CTMC refer to the phenotype rather than the tissue location of mast cells because each of them may be found both in mucosal and connective tissue sites.

Histochemical techniques relying on the metachromatic dyes has limitations which can not be over emphasized. As observed earlier (Combs et al. 1965), immature CTMC cells exhibit MMC staining properties presumably due to their lack of heparin. In contrast to rodent mast cells which have distinct proteoglycan content in the majority of the two mast cell subtypes, human mast cells isolated from both skin and lung have heparin proteoglycans as measured (Metcalfe et al. 1979, Metcalfe et al. 1980, Eliakim et al. 1986, Stevens et al. 1988, Thompson et al. 1988). The presence of heparin in the two potentially different types of mast cells abolishes the discriminating basis for the metachromatic staining techniques in human.

Immunohistochemistry. Immunohistochemical techniques

have become the tools of choice in the recent years to identify subtypes of mast cells in the human and rodent (Irani et al. 1986, Gibson et al. 1987). The techniques uses tryptase and chymase of mast cell granules as the preferred markers. Trypsin-like activity was originally demonstrated in human mast cells by histochemical techniques (Glenner & Cohen 1960, Chiu & Lagunoff 1972). Tryptase has been found to be the principal enzyme accounting for the trypsin-like activity in human mast cells (Schwartz et al. 1981a). The amount of tryptase found in human mast cells (10-35 pg/cell) accounts for 20-50% of the total protein in the cells (Schwartz et al. 1981b). In contrast to mast cells, only a negligible amount of the enzyme (<0.04 pg/cell) was measured in human basophils. No detectable amount of tryptase has been measured in other cell types in normal skin, lung bowel, and eosinophils, neutrophils, monocytes, or lymphocytes from peripheral blood. Sequential staining with alcian blue followed by either immunoperoxidase or immunofluorescence using a monoclonal murine anti-tryptase antibody showed a one-to-one correlation between anti-tryptase-positive cells and alcian blue-positive cells, which means that all cells with tryptase are mast cells as demonstrated by the metachromatic property (Schwartz 1985). Results from electron microscopy showed tryptase-positive stain in immature mast cells which have newly formed granules (Craig et al. 1989). Evidence also indicated that tryptase appears in cultured human fetal liver mast cell granules prior

to the acquisition of their metachromatic properties (Irani & Schwartz 1990). Thus, tryptase has been chosen as a highly selective and specific marker for human mast cells. In dispersed human foreskin mast cells, a substantially lower amount of chymase (94.5 pg/cell) was found in comparison to tryptase in the same cell type (Irani et al. 1986). Based on the specific location of the enzymes, tryptase and chymase have been convincingly applied to classification of mast cell subtypes in humans.

The presence of chymase activity in rat mast cells was also demonstrated some time ago by histochemical techniques (Benditt & Arase 1959). Two types of chymase have been located in rat mast cell granules (Miller et al. 1990). Chymase I is found exclusively in CTMC-type and chymase II in MMC. The amount of chymase I (24-30 pg/cell) accounts for about 25% of the total proteins in the CTMC type cell (Schwartz et al. 1981). An equivalent amount of chymase II (26 pg/cell) was found in cultured MMC (Haig et al. 1982).

Using human lung, skin, and small intestine fixed in Carnoy's solution, Irani's group (Irani et al. 1986) demonstrated two distinct human mast cell types using a polyclonal rabbit anti-chymase and a monoclonal murine anti-tryptase antibody. First, they performed single staining in adjacent tissues. The results showed that all mast cells stained tryptase-positive. However, only skin, intestinal submucosal, and peribronchial mast cells, but not intestinal

mucosal or lung alveolar mast cells, stained chymase positive. These results indicated the presence of different mast cells based on their protease content. Second, in order to confirm that those cells with chymase also contain tryptase, simultaneous double labelling experiments were performed by incubating the two antibodies simultaneously with the same sections. Then chymase-positive cells were stained blue using an alkaline phosphatase reaction followed by staining of tryptase-positive cells using peroxidase. The results showed that all cells that first stained blue turned dark brown following the subsequent stain suggesting that chymase truly co-resides with tryptase in the same cell (Irani et al. 1989). Finally, in order to demonstrate the presence of two distinct mast cell types in the same tissue section, sequential double labeling experiments were performed. Sections were first incubated with the anti-chymase antibody, and developed to give clusters of brown color which happen to interfere with the subsequent stain. The sections were subsequently incubated with the anti-tryptase antibody followed by development of the alkaline phosphatase blue stain. Cells containing chymase remained brown while those with tryptase but without chymase were stained blue. The results confirmed the existence of the two types of mast cells. Human mast cells were therefore divided into two subtypes, the MC_T type which contains tryptase only, and the MC_{TC} type which contains both tryptase and chymase. Using a newly developed monoclonal

murine anti-chymase antibody and the murine anti-tryptase antibody, Irani et al. confirmed their previous findings in a varieties of human tissues (Irani et al. 1989, 1990).

Electron microscopic evidence also provided strong support for the classification of the two human mast cell types. Ultrastructural studies using double immunogold staining procedures showed that all granules in MC_{TC} cells contained both tryptase and chymase and that the MC_T cell granules had only tryptase (Craig et al. 1988). Subsequent studies based on enzyme activity assay, mediator contents, and the response of the MC_T and MC_{TC} types to cytokines showed differences between the two types of human mast cells. Synthetic substrates are also available for detection of tryptase and chymase activities in tissue sections (Osman et al. 1989). The experiments likewise showed tryptase activity in all mast cells in human intestine while chymase activity was detected only in the submucosal mast cells. Mast cells with chymase activity also demonstrated tryptase activity. Thus, the intestinal mucosal mast cells are predominantly MC_T type while the submucosal mast cells are MC_{TC} type. These observations confirm the distinction between the two mast cell types based on immunohistochemical results.

The two different mast cell types have been related to different diseases. In patients with congenital disorders affecting T cell functions or with AIDS and combined immunodeficiency, a significant decrease in the MC_T type was

detected from the analysis of gastrointestinal tissues of the patients while the MC_{TC} type was virtually not affected (Irani et al. 1987a). In synovium of rheumatoid arthritis subjects, where heavy lymphocyte infiltration occurs, a mixture of MC_T and MC_{TC} types were found while usually only MC_{TC} type is present in the normal synovium (Irani et al. 1987b). These observation indicate that activity of MC_T cells may be lymphocyte factor dependent. The significance of the immunohistochemical techniques is also demonstrated by the ability to distinguish the two subtypes in immature human mast cells. Coupled with electron microscopy, Craig's group observed that the amount of MC_T and MC_{TC} found in immature mast cells in various tissues are parallel to those found in the counter part of the mature mast cells (Craig et al. 1989).

In rat, immunohistochemical techniques have targeted two different forms of rat chymases found in rat mast cells, chymase I and chymase II (Gibson et al. 1987, Huntley et al. 1990). In the experiments, polyclonal monospecific rabbit anti-chymase I and monoclonal mouse anti-chymase II were used. Adjacent sections of tissues from various sources were stained individually with anti-chymase I, anti-chymase II, and metachromatic dye such as toluidine blue or alcian blue and safranin O. Populations of stained cells were counted and compared to the adjacent sections. A correlation was then drawn between the sections. The results provided high correlation of the chymase I-positive cells with the CTMC type

defined by the metachromatic properties and the chymase II-positive cells with MMC. Although some overlap of chymase I- and chymase II-positive cells were observed, the population of the cells is very small. A minor population of the chymase I-positive cells were shown to be MMC type in both nonmucosal and mucosal tissues. The results suggest that granule protease phenotyping can be an alternative techniques in identifying mast cell subsets in rodent system.

Although tryptase has been purified from rat tissues, no information is available regarding its distribution among rat mast cells.

5. Purposes Of The Project

As reviewed above, mast cells play an important role in health and diseases. However, investigation of the specific mechanisms involved in mast cell functions has been complicated by the existence of mast cell heterogeneity. Rat is one of the major sources of our knowledge about mast cell functions. Thus it is very important to identify heterogeneity of rat mast cells for interpretation of data and comparison to the human system. Successful identification of human mast cell types suggested the possibility that rat mast cell types could be distinquished in a similar way. Demonstration of chymase I-positive and chymase II-positive rat mast cell types provided a basic reference for detecting rat mast cell types based on the contents of their neutral

proteases.

A tryptase has been purified and characterized from rat skin in our lab. It was our goal to raise a polyclonal anti-rat tryptase antibody and localize the enzyme in rat skin, lung, peritoneal lavage cells, and small intestinal mucosa. To do this, antibody specificity would first be evaluated by ELISA and Western Blot using the purified tryptase as antigen. Immunohistochemistry techniques would be used to identify mast cells containing tryptase and/or chymase. Mast cell distribution in the tissues would be evaluated with the metachromatic dyes, alcian blue and safranin O. Single staining with alcian blue, anti-tryptase, and anti-chymase I antibodies would provide data for distribution of total mast cells, tryptase-containing mast cells, and chymase I-containing mast cells, respectively, in the tissues. Double staining with metachromatic dye followed by anti-tryptase or anti-chymase I would confirm the existence of the proteases in the metachromatic mast cells. At the same time, it would allow correlation between the CTMC and MMC types with tryptase-positive or chymase I-positive cells. In order to verify if tryptase and chymase I co-reside in the same cell, simultaneous double labeling would be performed by labeling the tissue sections with both anti-tryptase and anti-chymase I antibodies simultaneously followed by developing the corresponding colors one after the other. As a confirmation of the existence of two distinct mast cell types, tissue

sections would be labeled with the anti-tryptase antibody first followed by color development. Then the sections will be labeled a second time with anti-chymase I antibody to confirm the distinct types of mast cells based on the presence of the enzymes.

CHAPTER VIII

MATERIALS AND METHODS

A. Materials

Alcian blue 8GX, safranin O, diaminobenzidine, 3-amino-9-ethylcarbazole, H_2O_2 (30%), and peroxidase-conjugated rabbit anti-goat IgG were obtained from Sigma Chemical Co., St. Louis, MO, Molecular weight standards were from Pharmacia, Piscataway, NJ and peroxidase-conjugated swine anti-goat IgG from Boehringer Mannheim, Indianapolis, IN. Rabbit IgG anti-chymase I had been made specific for chymase I by adsorption with chymase II and affinity purification with chymase I (Gibson & Miller 1986). Tryptase used for immunization and various immunochemical procedures was purified to homogeneity from rat skin as described (Braganza & Simmons 1991). Lung, skin and peritoneal cells were from normal Sprague Dawley animals; bowel was taken 14 days after infection of Lewis rats with Nippostrongylus brasiliensis (3000 organisms/animal) as described previously (Befus & Bienenstock 1979) to amplify the number of mucosal mast cells in the intestine. Samples of lung, skin and bowel fixed in Carnoy's fluid for 24 hours and then transferred to absolute ethanol. Tissues were embedded in paraffin. Sections of lung and bowel tissues were 4 μ, while those for skin was 5 μ. Rat peritoneal cells were

collected from the lavage with PBS solutions (Appendex E) and subjected to cytocentrifugation. Each slide contained 2 x 10^4 viable cells.

B. Methods

1. Western Blot

Protein extracts were prepared from rat skin (10 g), lung, (2.3 g), bowel (2.5 g), and peritoneal cavity cells (4 X 10^7 cells). In the case of skin, cutaneous muscle and fat layer were removed and the fur was clipped away as close as possible to the dermis. The lung and bowel tissues were rinsed well with tap water followed by MQ water. All tissues were cut into small pieces before extraction. The skin tissue was homogenized at a 1:9 (w/v) ratio in the homogenization buffer (Appendix D) using a Polytron homogenizer at full speed for three 15 second on-off intervals. The lung and bowel tissues were homogenized at 1:5 (w/v) ratio in the same buffer. Peritoneal lavage cells were ultrasonicated for three 20 second pulses on ice. The homogenate was centrifuged at 18,000 xg for 40 minutes at 4 °C. The supernatants from the tissue extracts were stored at -20 °C after assaying for tryptase activity. The skin and peritoneal lavage cell extracts were subjected to TCA precipitation. Extract (500 μl) was mixed with an equal volume of 20% TCA (Appendix D) and kept on ice for 5 minutes. The proteins were precipitated by centrifugation at 4 °C for 10 minutes. The pellet was rinsed

with 500 µl of acetone twice. After air drying, the pellet was resuspended in 5 µl of the SDS-PAGE sample buffer (Appendix B).

The crude extracts were assayed for tryptase and chymase activities. The tryptase assay reaction contained 850 µl of the tryptase assay buffer (Appendix A), 50 µl of the crude extract, and 100 µl of 10 mM BAEE (Appendix A). The reaction was monitored at 253 nm for 5 minutes. The amount of tryptase was estimated using a specific activity of 160 units/mg protein in the formula presented in Part I. The chymase reaction contained 500 µl of the chymase assay buffer (Appendix D), 33 µl of the crude extract, and 467 µl of the 1 mM BTEE (Appendix D). The reaction was monitored at 256 nm for 5 minutes.

Protein concentrations of the crude extracts were determined using protein assay reagent A and B from Pierce Chemical Company, Rockford, Illinois. The assay reagent consisted of 1:50 ratio of reagent A:B. Each microtiter well contained 10 µl of the sample and 200 µl of the reagents. Five BSA concentrations of 25, 100, 200, 300, and 500 µg/ml were used as standards. Color development was allowed to continue at 37 °C for 30 minutes. Protein concentrations were measured at 562 nm on a Micoplate Reader. The standard protein concentrations were plotted against the absorbance values. Sample protein concentrations were determined using the regression equation derived from the standard curve.

Western blot was performed as described (Towbin et al. 1979, Bollag & Edelstein 1991). The protein samples were separated in SDS homogeneous 12.5 PhastGel using PhastSystem. Each lane was loaded with 20 μg of the protein extracts or purposely over-loaded with 30 μg of the extracts to evaluate any nonspecific binding activity of the antibody. Two identical gels were run simultaneously under the same condition. The samples were separated using the following program:

Separation Program 2

Sample Appl. Down At					2.1	1 vh
Sample Appl. Up At					2.1	10 vh
Sep. 2.1	250 v	10 mA	3 W	15 °C		60 vh
Sep. 2.2	50 v	0.1 mA	0.5 W	15 °C		0 vh

After the separation, one gel was subject to coomassie blue stain using the following development program:

Development Program 2

Steps	In-port	Out-prot	Time (min)	Temp. (°C)
2.01	4	0	10	50
2.02	2	0	5	50
2.03	2	0	8	50
2.04	2	0	10	50
2.05	9	0	5	50

where in-port 4 connects to the stain solution (Appendix D); in-port 2 connects to the destain solution (Appendix D); in-port 9 connects to the preservative solution (Appendix D); and out-port 0 connects to the waste collection bottle.

The companion gel was used for blotting. A nitrocellulose membrane (0.45 μm), six pieces of blotting paper, and the gel were soaked in a PhastTransfer buffer (Appendix D) for 5 minutes. Then, the gel was clamped to a mounting block and separated from the plastic gel backing. The nitrocellulose membrane was carefully laid on the gel in order to remove the gel from the plastic backing. The transfer sandwich consisted of 3 pieces of the wetted blotting paper at the bottom, the nitrocellulose membrane with the gel attached to the upper side, and, on the top of the sandwich, another 3 pieces of the blotting paper soaked in the transfer buffer. A semi-dry electrophoretic transfer was performed on a PhastTansfer system using the following program:

Separation Program 3

Sample Appl. Down At					3.0	0 vh
Sample Appl. Up At					3.0	0 vh
Sep. 3.1	20 v	25 mA	1 W	15 °C		5 vh

Blotting one gel requires 15-20 minutes. A longer time of 30-40 minutes may be necessary when blotting two gels simultaneously. After transfer, the nitrocellulose membrane was pealed off the gel carefully, rinsed in the transfer buffer and dried briefly between 2 pieces of the 3 mm paper.

The nitrocellulose membrane was blocked in 12 ml of the blocking buffer (Appendix D) in a Seal-A-Meal plastic bag for 1 hour with gentle rocking. After blocking, the membrane was rinsed briefly with TBS (Appendix D) three times and incubated with the goat anti-rat tryptase antibody overnight at 4 °C with gentle rocking. The antibody concentration was 5 μg/ml in 0.5% BSA/TBS solution (Appendix D). After washing with TBS for 10 minutes three times at room temperature, the membrane was incubated with 12 ml of the second antibody solution (Appendix D) for 2 hours at room temperature and washed again with TBS for 10 minutes for three times. When the color was fully developed in the AEC/H_2O_2 solution (Appendix D), the reaction was terminated by rinsing the membrane with tap water briefly followed by rocking in MQ water for 30 minutes. Finally, the membrane was dried on 3 mm paper and

photographed.

2. Tissue Section Preparation

Skin, bowel, and lung tissues from Sprague Dawley rats were fixed in Carnoy' solution (Appendix E) and embedded in paraffin wax by Dr. G. Newlands and Dr. S.S. Craig in the Medical College of Virginia. The bowel tissue was obtained from N. Brasiliensis-infected animals. Sections of lung and bowel (4 μm) and skin (5 μm) were prepared.

Rat peritoneal lavage cells were obtained by a modification of the method of Saeki (Saeki 1964). Animals were decapitated with an animal guillotine, and exsanguinated. Fifteen milliliters of PBS/Heparin buffer (Appendix E) were injected into the peritoneal cavity. The abdominal wall was massaged gently and the body was turned around a few times. The fluid was collected by cutting along the midventral line of the abdomin and flipping the opening downward into a funnel. Peritoneal lavage cells from two rats (30 ml) were spun down at 1,000 rpm in a swinging rotor for 10 minutes at room temperature and resuspended in 5 ml of PBS/DNase buffer (Appendix E). Viable cell concentration was determined in the following manner. Cells were first stained with Trypan at a 1:1 ratio of cell/trypan (v/v) in which intact cells remained colorless while dead cells turned blue. The number of viable cells was counted under a microscope using a 25 grid counting chamber. The concentration of the peritoneal lavage cells was

calculated using the following formula: $N \times D \times 10^4 = C$ where N is the number of the viable cells counted in the 25 grid area; D is the total dilution factor of the cell solution; and C is the final concentration in number of viable cells/ml. About 2×10^4 peritoneal lavage cells were adhered to each slide by centrifugation at 600 rpm for 6 minutes at room temperature using a cytospin centrifuge.

Before staining, the cytospin slide with peritoneal lavage cells was fixed in Carnoy's solution at room temperature for 15 minutes and washed in H_2O for 5 minutes. The paraffin embedded sections were dewaxed and rehydrated before staining. The sections were first dewaxed in three changes of xylene, 5 minutes each. Then, they were submerged through two changes of 100% ethanol for 5 minutes and 3 minutes respectively. Endogenous peroxidase was inactivated by incubation of the sections with a H_2O_2/methanol solution (Appendix E) for 30 minutes. Finally, the sections were rehydrated through 95%, 80%, 70%, and 50% ethanol for 5 minutes each, and then in MQ water for 3 minutes.

3. Histochemical Stain

Alcian blue was used to identify mast cells in the tissues. A rehydrated section was stained in 0.1 ml of 0.5% of alcian blue solution (Appendix F) for 30 minutes at room temperature. The alcian blue was rinsed off the section gently with MQ water. A drop of 90% glycerol from a needle

was laid on each section and then covered with a piece of the cover glass. If a second stain were to follow, the cover glass was placed lightly on top of the section. For long term storage, excess glycerol was pressed out of the cover glass and wiped away with a piece of Kimwipe.

Safranin O was used to stain the connective tissue type mast cells. A rehydrated tissue section was stained with 0.1 ml of a 0.1% safranin solution (Appendix F) for 5 minutes at room temperature. After rinsing off the safranin with MQ water, the section was mounted either to glycerol when a second stain was required or to permount for long term storage.

Alcian blue/safranin double stain was used to evaluate the distribution of the CTMC and MMC types of mast cells in the tissues. A tissue section was first stained with alcian blue. After rinsing off the alcian blue a few times with MQ water, the section was mounted in 90% glycerol and lightly covered with a piece of the cover glass. Different fields in the section were photographed under a microscope. The slide was soaked in water at 37 °C for 10-30 minutes to remove the cover glass. Following a second safranin stain, the section was dried thoroughly in air and mounted to permount. Photomicroscopy was performed on the same fields taken after the alcian blue stain.

4. Immunohistochemical Stain

Single Stain A goat anti-rat tryptase antibody generated in Dr. L.B. Schwartz' lab was used to localize tryptase in lung, skin, bowel, and peritoneal lavage cells. A series of dilutions from a stock of 29 mg/ml was used to titer the goat anti-rat tryptase antibody. The stock was diluted to 29, 23, 20, 16, 15, 10, 5, 1, and 0.5 μg/ml in PBS/BSA solution (Appendix F). The rehydrated tissue sections were blocked for non-specific binding of the second antibody with 0.1 ml of the normal serum from the same species as the second antibody for 2-3 hours at room temperature. Then the sections were washed in MQ water for 2 minutes and incubated with the anti-tryptase IgG at the above dilution overnight at 4 °C in a moisture chamber. The next day, the sections were rinsed gently with MQ water and washed through 3 changes of TTBS (Appendix F) for 5 minutes each and then with MQ water for 2 minutes. A 1:50 dilution of the peroxidase-conjugated swine anti-goat IgG was incubated with the sections for 1 hour at room temperature. Washing the sections through 3 changes of TTBS for 5 minutes each and one change of water was sufficient to rinse the unbound IgG away before the color reaction. Reddish brown color for the stained cells appeared after incubation of a section with 0.1 ml of the AEC/H_2O_2 solution (Appendix F) for 7 minutes at room temperature. The sections were then rinsed with water and mounted in 90% glycerol. The optimal concentration was determined from the titration experiments and used for all of the other experiments. In the anti-rat

tryptase stain experiments, a goat anti-rat chymase I antibody was used as a positive control for all the tissues tested. preimmune goat IgG which was collected before the goat was primed with rat tryptase provided a negative control for the anti-tryptase experiments. Both the positive and negative experiments used the same procedure described above except that the anti-tryptase IgG was replaced with either the anti-chymase in the positive or the preimmune goat IgG in the negative controls.

A polyclonal rabbit anti-rat chymase I monospecific antibody was used to locate rat chymase I in the tissues. A stock solution of 80 μg/ml was diluted into 1.6, 3.2, and 10 μg/ml and titered for its optimal concentration on rat lung, skin, bowel, and peritoneal lavage cell preparations. A 1:50 dilution of peroxidase-conjugated goat anti-rabbit IgG was used as a second antibody.

Double Staining. Alcian blue/tryptase sequential stain combined the single staining procedure for the two stains together. The rehydrated sections were stained with alcian blue first. After performing photomicroscopy, the section were stained for rat tryptase. Cells which contained tryptase changed their color from blue after the alcian blue stain to brown following the tryptase stain using AEC. Finally, the sections were mounted on 90% glycerol and the same fields were photographed.

Safranin/tryptase sequential stain must be performed by

staining the tissue with safranin first and then staining for tryptase. The red color from the safranin stain disappeared during the subsequent tryptase stain. Likewise, the tryptase staining procedure somehow prevented the tissue from getting stained by safranin. Unlike in the alcian blue/tryptase stain, safranin/tryptase stain used DAB/H_2O_2 (Appendix F) as substrate for the color development. Cells stained red by safranin would turn dark brown after the DAB/H_2O_2 reaction if the cells contained tryptase. The disappearance of the red safranin color in fact provided easy identification of the positive cells in the second stain.

Alcian blue/chymase I sequential stain basically followed the same procedure as the alcian blue/tryptase stain except that after alcian blue stain, the sections were blocked with normal goat serum for 3 hours. The primary antibody used was the rabbit anti-rat chymase I IgG. The second antibody was a 1:50 dilution of peroxidase-conjugated goat anti-rabbit IgG. AEC/H_2O_2 was used as the substrate for the color reaction.

Simultaneous Double Labeling With Anti-Tryptase And Anti-Chymase I Antibodies. The prepared sections were blocked with normal swine serum at room temperature for 3 hours. After rinsing with MQ water, the sections were incubated with 5 μg/ml of the goat anti-tryptase antibody and 3.2 μg/ml of the rabbit anti-chymase I antibody simultaneously at 4 °C overnight. The antibodies were washed away through 4 changes of TTBS, 5 minutes each, and one change of MQ water. A 1:50

dilution of alkaline phosphatase-conjugated swine anti-goat IgG was incubated for 1 hour with the sections in the presence of 0.1% of normal rabbit serum. The normal rabbit serum prevented swine anti-goat IgG from binding nonspecifically to the rabbit anti-rat chymase I. The sections were washed through 3 changes of TTBS and one change of MQ water again, and then developed in the Naphthol/Fast Blue solution in the dark until the blue stained cells were clearly seen under the light microscopy. Tryptase-positive cells which stained blue were counted and photographed. To identify chymase I-positive cells, the sections were incubated with peroxidase-conjugated goat anti-rabbit IgG at room temperature for 1 hour and then developed in AEC/H_2O_2 solution. Blue cells which contained tryptase turned dark brown indicating that chymase I resides in the same cells as does tryptase. Photomicroscopy of the same fields was repeated. Four controls were run in parallel to the samples. Instead of using both antibodies, the first one contained only goat anti-rat tryptase antibody without the rabbit anti-chymase I antibody. This control would verify whether the second peroxidase-conjugated goat anti-rabbit IgG might cross-react with the goat anti-tryptase antibody. The second control was the opposite to the first. It contained only the rabbit anti-chymase I antibody without the goat anti-tryptase antibody. This control would reveal any nonspecific binding of the alkaline phosphatase-conjugated swine anti-goat IgG to the rabbit anti-chymase I antibody in the presence of

0.1% normal rabbit serum. The third control contained neither one of the two primary antibodies but MPC-11, a murine myeloma IgG, kappa, which has no known antigenic specificity. Any nonspecific binding of either one of the secondary antibodies to IgG would be detected under this control. In the fourth control, the two primary antibodies were replaced by PBS/BSA to see if the secondary antibodies may bind nonspecifically to the tissues.

Sequential Double Labeling With The Anti-Tryptase and Anti-Chymase I Antibodies. Tissue sections were blocked with normal swine serum for 3 hours and rinsed with MQ water briefly. The first overnight reaction contained 5 μg/ml of goat anti-tryptase antibody in the PBS/BSA solution incubated with the sections at 4 °C. After washing with three changes of TTBS, 5 minutes each, and one change of MQ water the next day, the sections were incubated with peroxidase-conjugated swine anti-goat IgG at room temperature for 1 hour. The color reaction was developed in AEC/H_2O_2 solution as described above. Then, the slides were mounted lightly in 90% glycerol and photographed. The cover glasses were removed in MQ water at 37 °C. In the second overnight reaction, the tissues were incubated with 3.2 μg/ml of the rabbit anti-chymase I antibody. The next day, the antibody was washed away with three changes of TTBS and one change of MQ water again. After an one-hour incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG, the sections were washed with TTBS and

MQ water and then developed in the Naphthol/Fast Blue solution for 15 minutes in the dark. Photomicroscopy of the same fields were then performed. Tryptase-positive cells were stained reddish brown by AEC. The color deposit somehow blocked the subsequent stain. As a result, any cell staining blue after the second stain indicated the presence of chymase I alone in the cell.

5. Photomicroscopy And Calculations

Photomicroscopy was performed with a Zeiss Ultraphot microscope (Carl Zeiss, Inc., Thornwood, N.Y.) using Kodak Ektachrome 64T films. For reflected light photomicroscopy, a didydium filter and a TIFFEN 49 mm, 82 °C filter were used. For phase contrast, only the didydium filter was used. The pictures were taken at either 160 x magnification using a 40 x oil lens or 250 x using a 63 x oil lens.

Assessment of tissue surface area was obtained with a Zeiss Videoplan apparatus. In lung sections, only the peribronchial areas were measured. In the bowel sections, more than a dozen of the randomly selected areas were measured and positive cells counted. In skin, the area of a whole section was usually included. Mast cell concentrations were determined as described previously (Irani et al. 90):

$$\text{Mast Cells/mm}^3 = [\text{Mast Cells/mm}^2] \times [10^3/(10\ \mu\text{m} + 4\ \mu\text{m})]$$

where 10 μm was used as an estimate of the average mast cell diameter and 4 μm was the tissue thickness for lung and bowel;

5 μm was used for the thickness of skin.

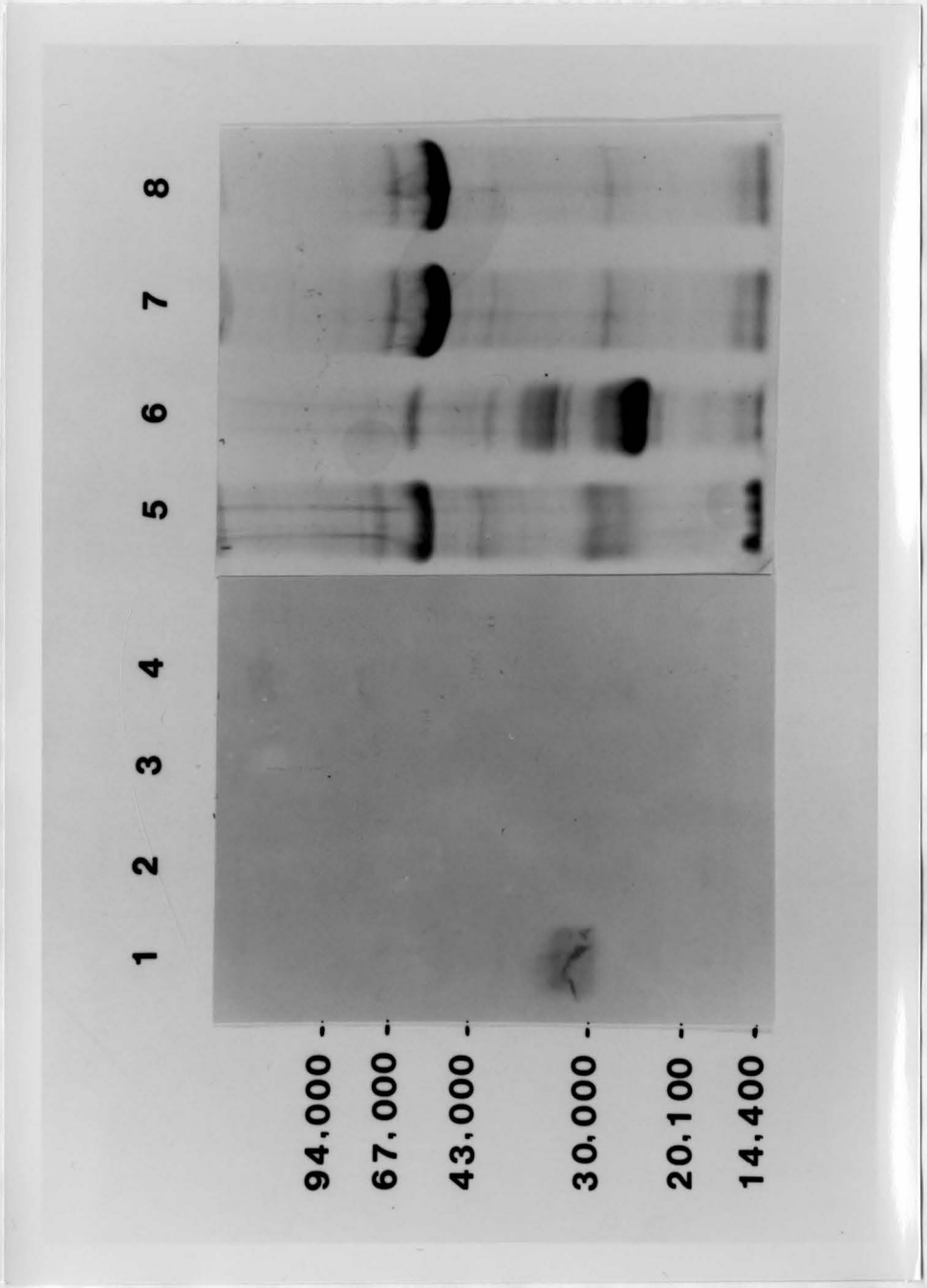
CHAPTER IX

RESULTS

1. Specificity Of Goat Anti-Rat Tryptase Antibody

To examine the specificity of the anti-tryptase IgG, Western blots were performed as shown in Figure 12. Lanes 1-4 show the results of the Western blot; lanes 5-8 show the Coomassie blue stain of an identically prepared gel run in parallel. Lanes 1 and 8 each contained 20 μg of the initial extract from rat skin and 100 ng of purified tryptase; respective lanes 2 and 7 were identical except for lack of purified tryptase. Lanes 3 and 6 contained 20 μg of the crude protein extract from rat peritoneal lavage cells. Lanes 4 and 5 contained 20 μg of the crude extract from rat lung. Amounts of tryptase in these extracts was estimated based on the specific activity of the enzyme purified from skin and the enzymatic activity in each extract using benozyl-arginine ethyl ester. In each case there was less than 1 ng of tryptase applied to the gel. The sensitivity of the Western blot technique for purified tryptase was approximately 50 ng; approximately 500 ng tryptase were needed for detection by Coomassie blue. Accordingly, tryptase was detected immunologically in lane 1, but not in lanes 2, 3, and 4. The results do show a lack of binding to

Figure 12. Specificity Of Goat IgG Anti-Rat Tryptase. A Western blot (lanes 1-4) and Coomassie blue-stained polyacrylamide gel (lane 5-8) containing extracts of skin (lanes 1, 2, 7 and 8, 20 μg protein/lane), peritoneal cells (lanes 3 and 6, 20 μg protein/lane) and lung (lane 4 and 5, 20 μg protein/lane). Purified rat tryptase was included in lanes 1 and 8 (100 ng/lane). Molecular weight standards used included phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), α1-trypsin inhibitor (20,100), and α-lactalbumin (14,400) (Pharmacia).


1 2 3 4 5 6 7 8
94.000 -
67.000 -
43.000 -
30.000 -
20.100 -
14.400 -

the numerous other proteins present in each tissue extract, indicating a high degree of specificity for tryptase.

2. Distribution Of Mast Cells And Tryptase Positive Cells

The distributions of connective tissue (red) and mucosal (blue) types of mast cells by staining with alcian blue and safranin, and tryptase-positive cells by immunohistochemistry were determined in comparable sections of skin, bowel, and lung and in cytospin preparations of peritoneal cells. In lung, mast cells were detected primarily in peribronchial tissue, 53% being of the connective tissue type (Table 3). In contrast to the situation in human lung, few mast cells were detected in the rat lung alveolar wall. In skin, most of the mast cells were located in the dermis and in the layer under the adipose tissue, and in each case were 81% connective tissue type. In the bowel mucosa from N. brasiliensis-infected animals, mucosal (safranin-negative) mast cells were abundant, accounting for >99% of the mast cells. Mast cells obtained by peritoneal lavage accounted for 5-10% of the cells, and over 95% were of the connective tissue type (safranin-positive).

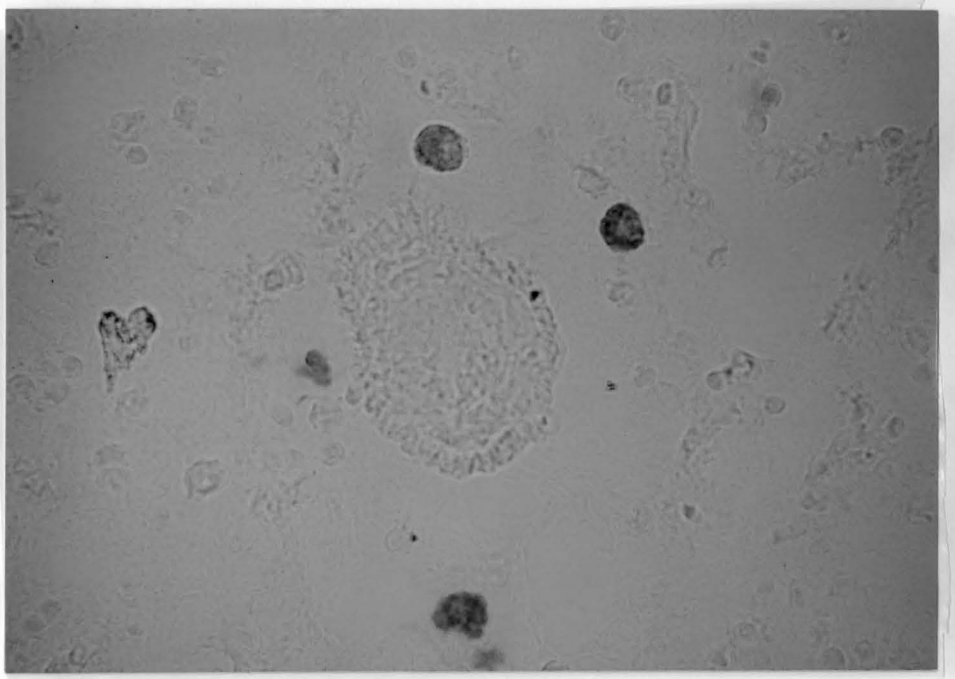
Tryptase-containing cells also were assessed in lung, skin bowel and peritoneal cells using goat IgG anti-tryptase antibody. No staining was observed using preimmune goat IgG as a negative control. Tryptase-containing cells were most abundant in lung, where they were distributed in a

peribronchial location. Among peritoneal cells they accounted for only 20% of the cells. In skin, tryptase-positive cells were detected in the dermis and below the hypodermis, but at concentrations that appeared to be less than for mast cells detected histochemically. In bowel mucosa there were essentially no tryptase-positive cells.

3. Rat Tryptase Is Localized In Mast Cells

To determine whether tryptase-containing cells were mast cells and only mast cells, sequential staining with Alcian blue and anti-tryptase antibody was performed. For example, in a section of rat lung, among 4 Alcian blue-stained mast cells (Figure 13A), two stained positive for tryptase (Figure 13B). In these double labelling experiments, the concentration of mast cells in peribronchial tissue was about 15×10^3 cells/mm^3, 63 ± 8% contained tryptase (Table 3). In rat skin, two Alcian blue-positive mast cells are shown in Figure 13C, one of which stains positive for tryptase (Figure 13D). In general, tryptase-positive mast cells stained less intensely in skin than in lung. Tryptase-positive mast cells accounted for only 7 ± 4% of the 882 ± 136 mast cells/mm^3 in the dermis (Table 3). A cluster of 7 mast cells obtained from the

Figure 13. Sequential Staining With Alcian Blue And Anti-Tryptase Antibody. Tissue sections from lung (A, B), skin (C, D), a cytospin of peritoneal cells (E, F), and bowel (G, H) were stained first with alcian blue (A, C, E, G) and then with goat IgG anti-tryptase/peroxidase (brown) (B, D, F, H). Identical fields for each cell source are shown.



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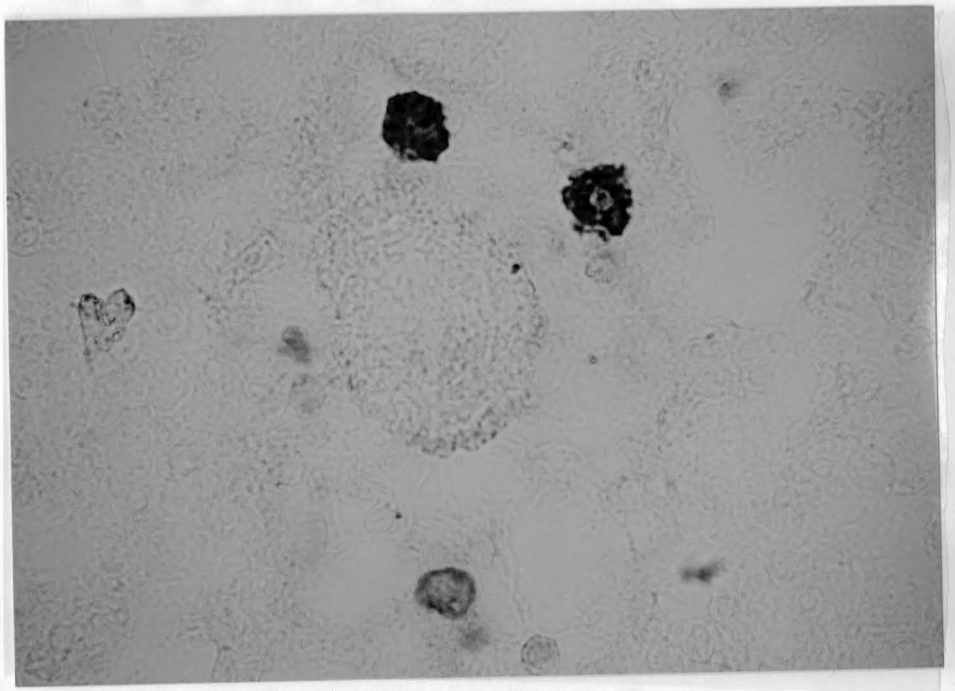

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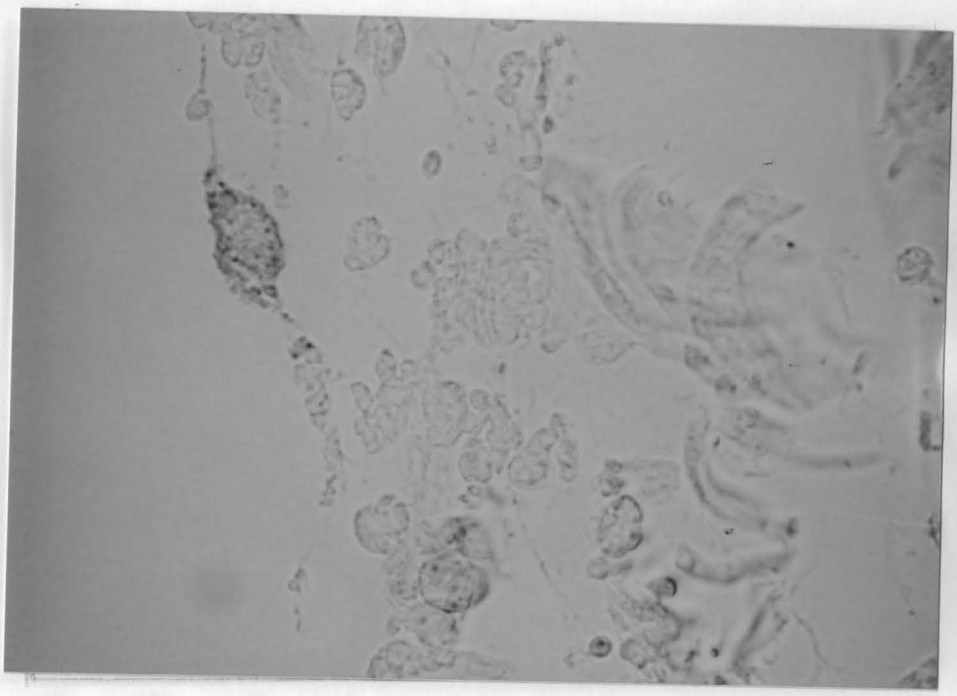

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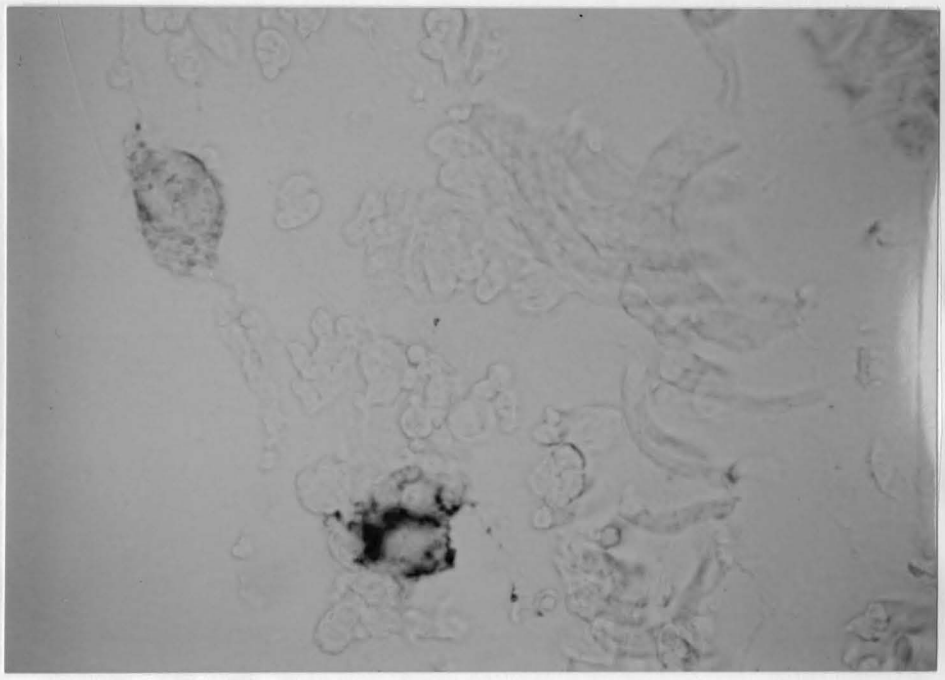

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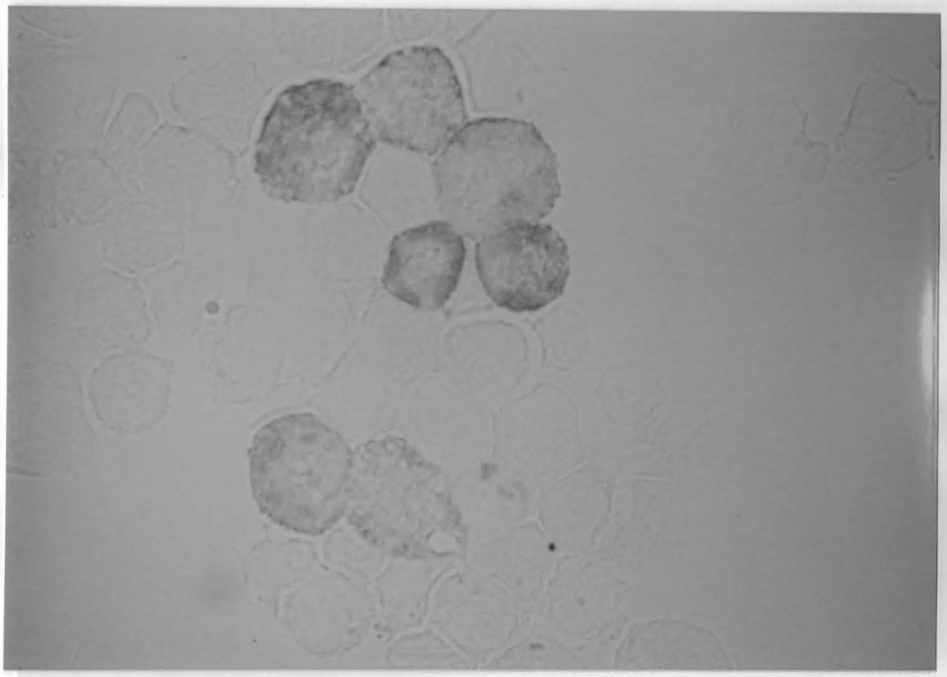

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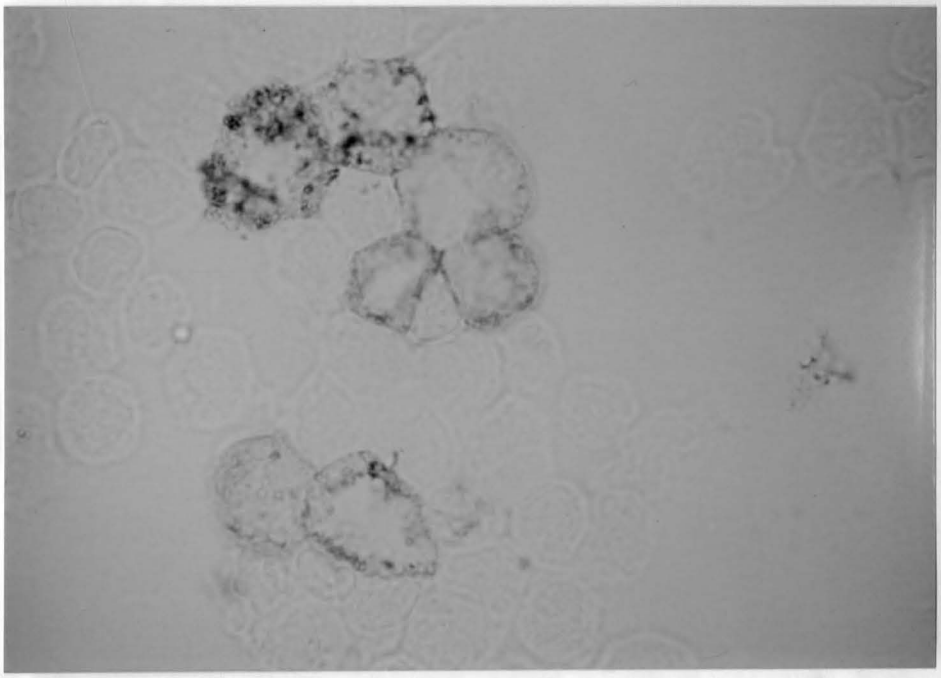

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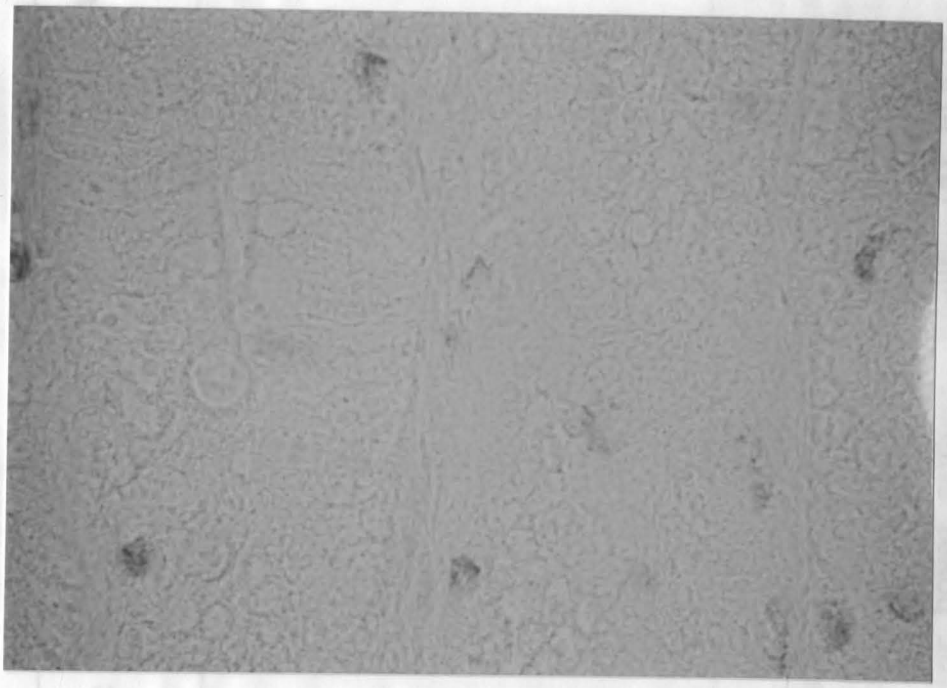

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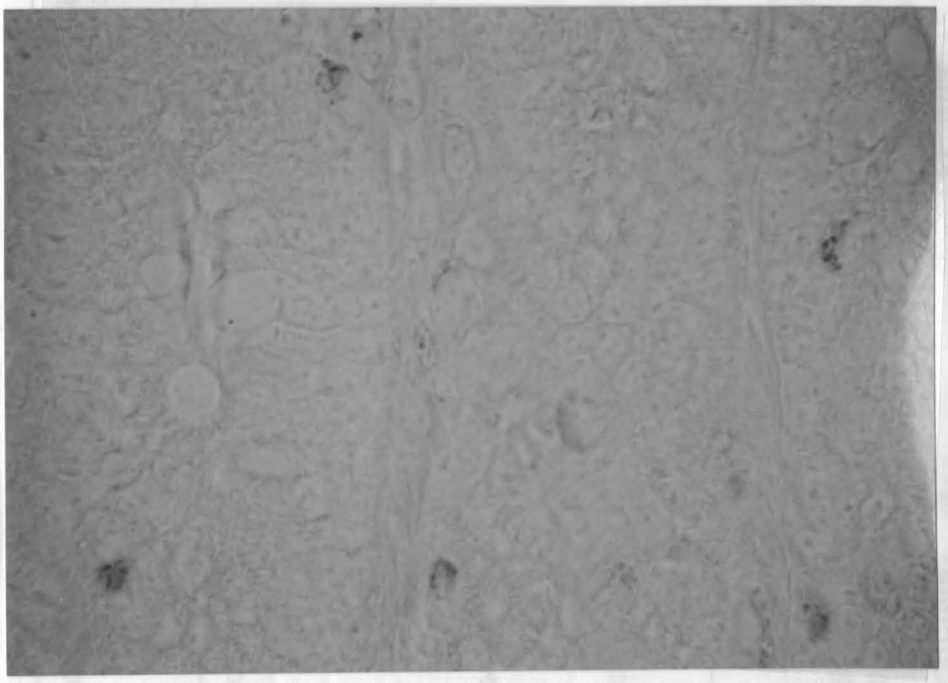


H

Cell Source	MC/mm^3 ± SD (n)	%Safranin$^-$MC %Safranin$^+$MC ± SD (n)	Tryptase$^+$ x 100 Alcian Blue$^+$ ± SD (n)	Tryptase$^+$ x 100 Safranin$^+$ ± SD (n)
Peribronchus	14,867 ± 3966 (6)	47/53 ± 19 (4)	63 ± 8 (6)	94 ± 10 (4)
Dermis	882 ± 136 (6)	19/81 ± 8 (6)	7 ± 4 (6)	6 ± 2 (4)
Bowel Mucosa	32,264 ± 22,219 (4)	100/0 ± 0 (4)	ND (4)	ND (4)
Peritoneal Cells	NA	5/95 (*)	19 ± 6 (12)	20 ± 8 (12)

Table 3. Distribution Of Tryptase-Positive Mast Cells. The results of sequential alcian blue/tryptase and safranin/tryptase stains are summarized in the table. MC, mast cell; SD, standard deviation; n, number of tissue sections used; ND, not detectable; NA, not applicable; *, a total of 80 Safranin$^-$/Alcian Blue$^+$ mast cells among 1600 Safranin$^+$/Alcian Blue$^+$ mast cells. Areas of tissues examined were 2.69 mm^2 for peribronchus, 39.9 mm^2 for dermis, and 17.0 mm^2 for bowel mucosa. Sequential staining with alcian blue and safranin was used to determine %Safranin$^-$ and %Safranin$^+$ mast cells.

peritoneal lavage is shown in Figure 13E, three of which contain tryptase (Figure 13F). The intensity of tryptase staining was comparable to mast cells in skin. Mast cells accounted for 8% of the total cells, 19% of which contained tryptase (Table 3). In rat bowel mucosa from N. brasiliensis-infected animals, essentially none of the mast cells (32,264 ± 22,219 cells/mm^3) (Table 3) that stained blue (Figure 13G), showed detectable tryptase (Figure 13H). Bowel submucosa was not examined. The high standard deviation for the concentration of mast cells in the bowel reflect the uneven distribution of these mast cells in the cross sections of the bowel. Of 591 tryptase-positive cells examined in lung, skin, and peritoneal lavage cells, all stained with Alcian blue, implicating mast cells as the major source of this enzyme among the cells and tissues examined.

Sequential staining with Alcian blue and anti-chymase I antibody showed that 60% of the Alcian blue cells in lung are chymase I-positive, over 85% of the Alcian blue cells are chymase I-positive in skin, 98% of the Alcian blue cells are chymase I-positive among peritoneal cells and none of the Alcian blue cells in bowel mucosa were chymase I-positive.

4. Rat Tryptase Resides In The Safranin-Positive CTMC Cells

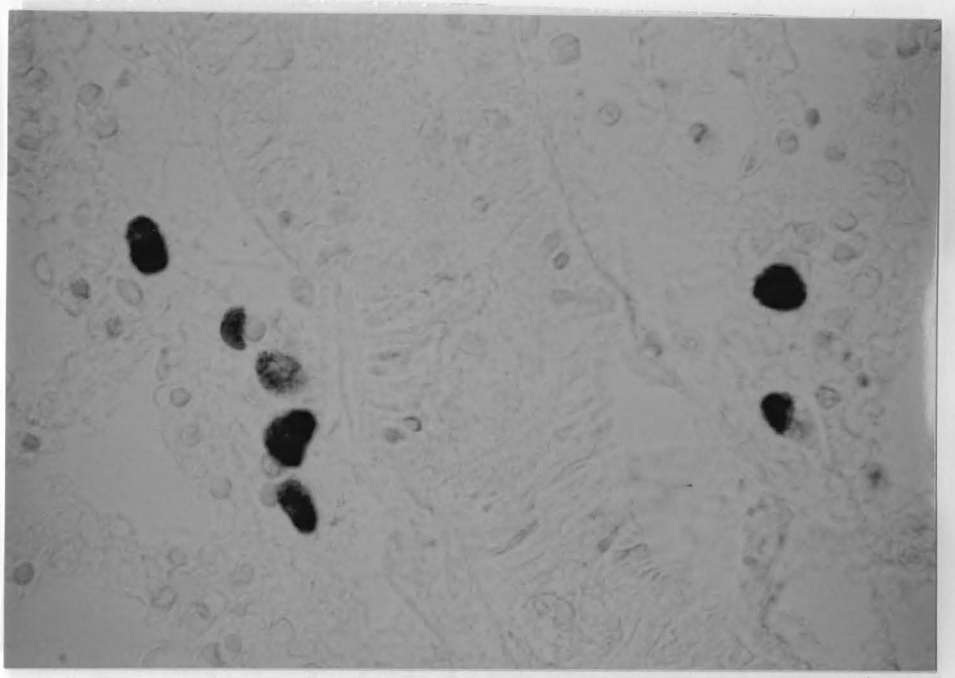
To determine the distribution of tryptase-positive mast cells among safranin-positive and safranin-negative mast cells, sequential staining was performed, first with safranin

and then with anti-rat tryptase. The results revealed that all tryptase-positive cells were safranin-positive, but not all safranin-positive cells contained tryptase. For lung, Figure 14A shows 7 safranin-positive mast cells, six of which contained tryptase (Figure 14B). In rat lung, over 90% of the safranin-positive cells are tryptase-positive (Table 3). By contrast, in skin, 6 safranin-positive mast cells were shown in Figure 14C, only 1 of which was tryptase-positive (Figure 14D). Only about 6% of the safranin-positive cells in skin contained tryptase (Table 3). Bowel mucosa lacked safranin and anti-tryptase stainable cells. In peritoneal cells, Figure 14E shows numerous safranin-positive mast cells, most of which contained no detectable tryptase (Figure 14F). Tryptase-positive cells account for only 19% of the rat peritoneal connective tissue mast cells (Table 3).

5. Distribution Of Tryptase And Chymase I In Rat Mast Cells

Simultaneous double-labeling experiments were performed to determine whether tryptase-positive cells are also chymase I-positive. Figure 15A shows a section of lung which had 6 blue tryptase-positive cells, all of which

Figure 14. Sequential Staining With Safranin And Anti-Tryptase Antibody. Tissue sections from lung (A, B) and skin (C, D), and a cytospin of peritoneal cells (E, F) were stained first with safranin (red) (A, C, E) and then washed and stained with goat IgG anti-tryptase/peroxidase (brown). Identical fields for each cell source are shown. The red safranin stain was removed by the washing procedure. All fields are shown under light microscopy except E, for which phase contrast was used to show stained mast cells along with unstained cells. This makes the red cells appear brown.



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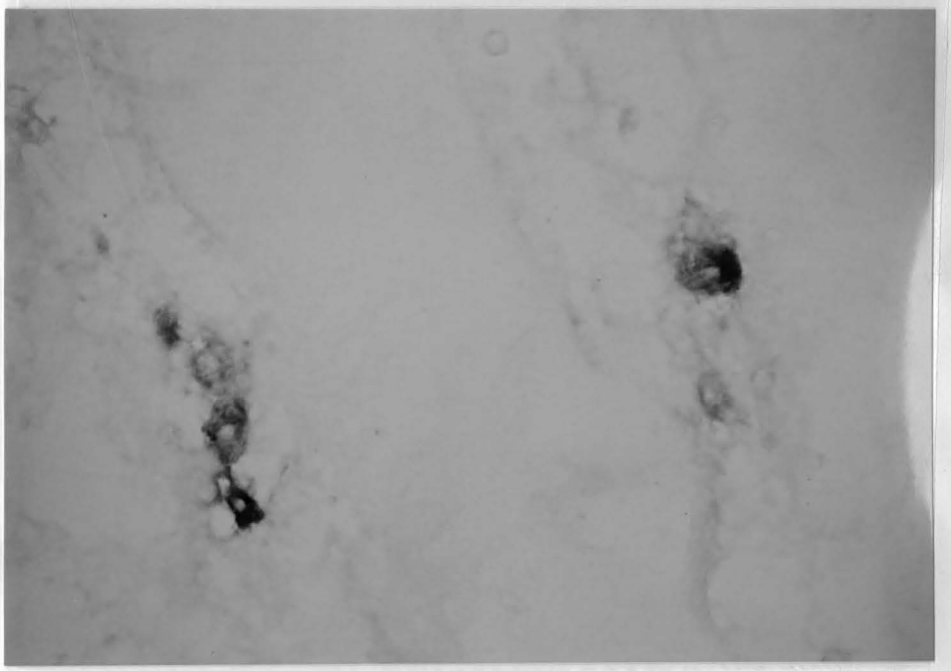

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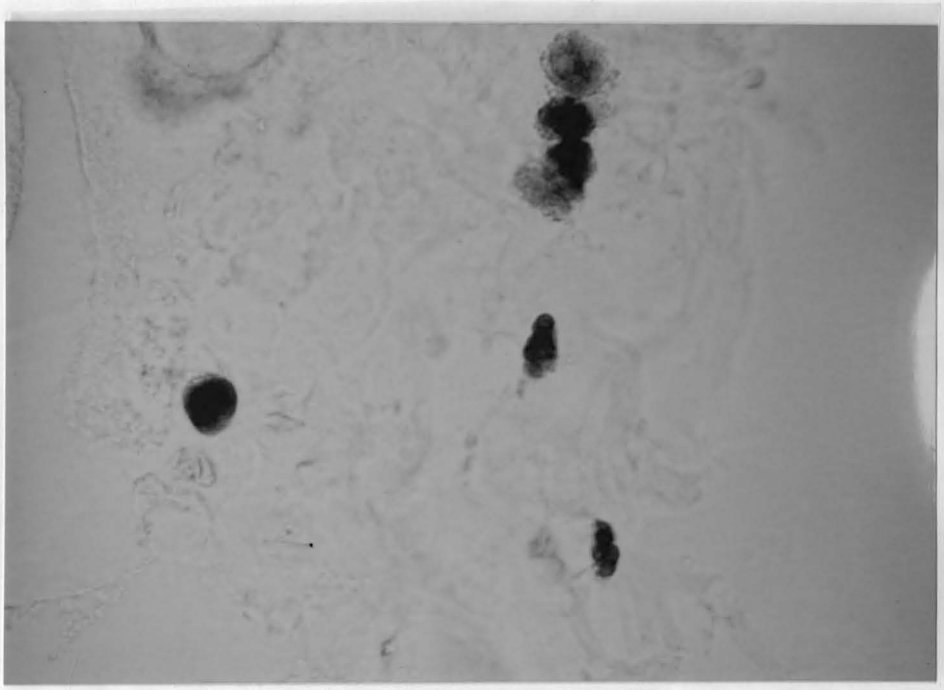

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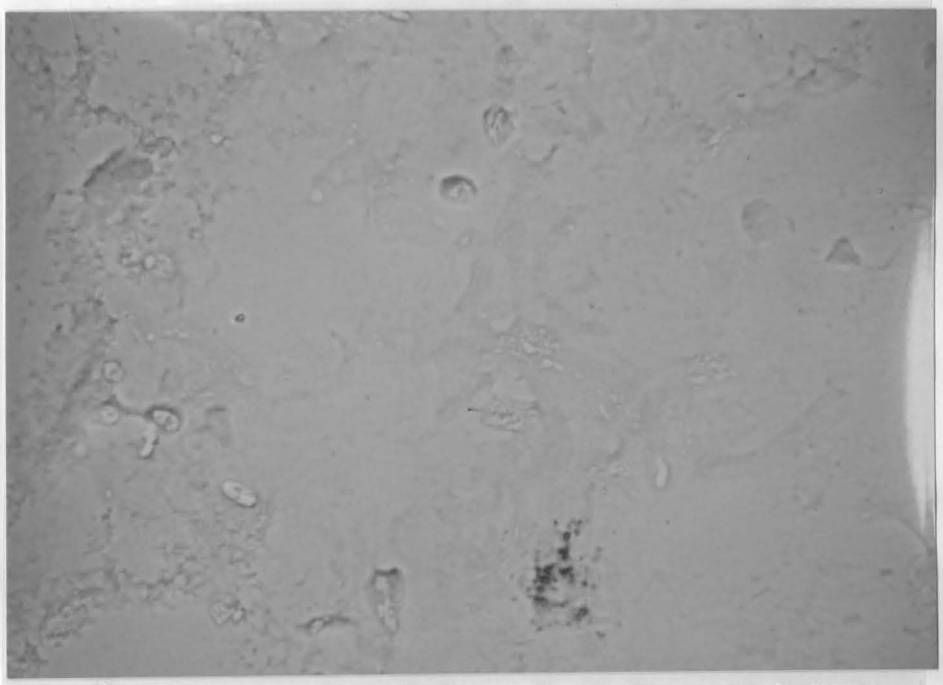

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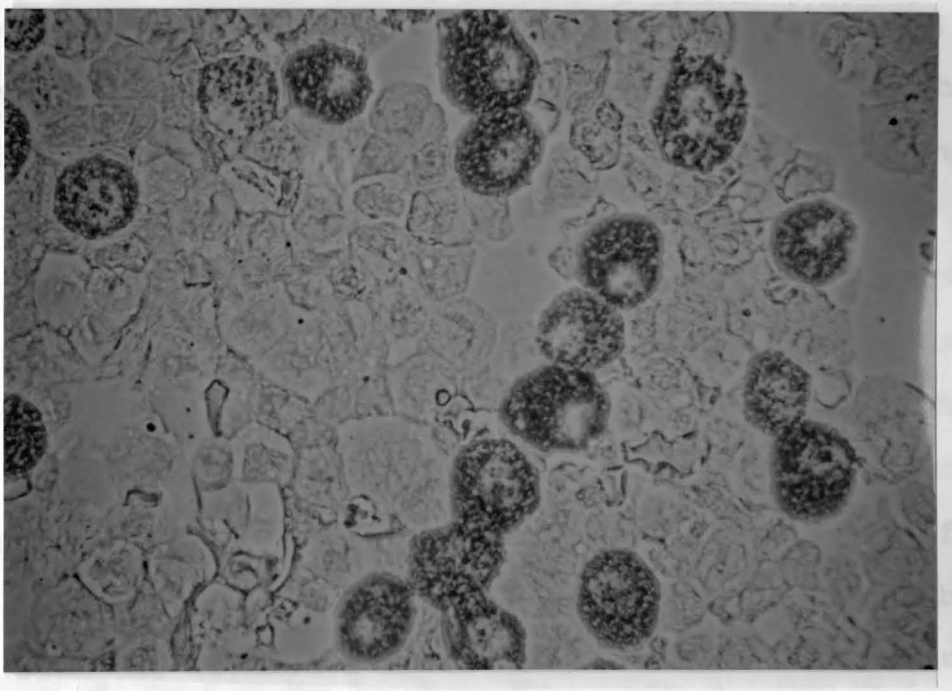

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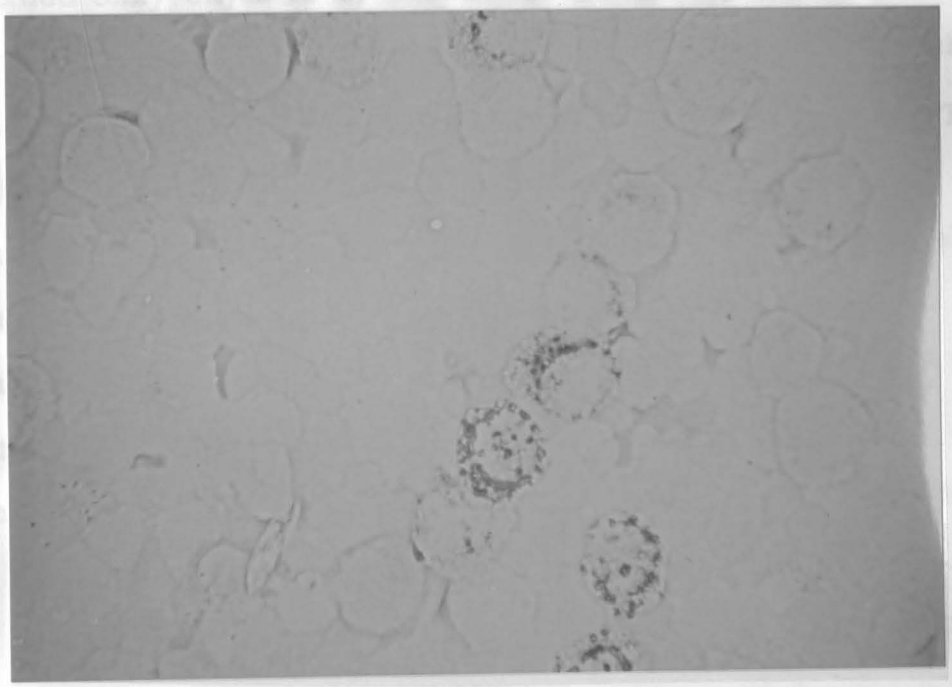
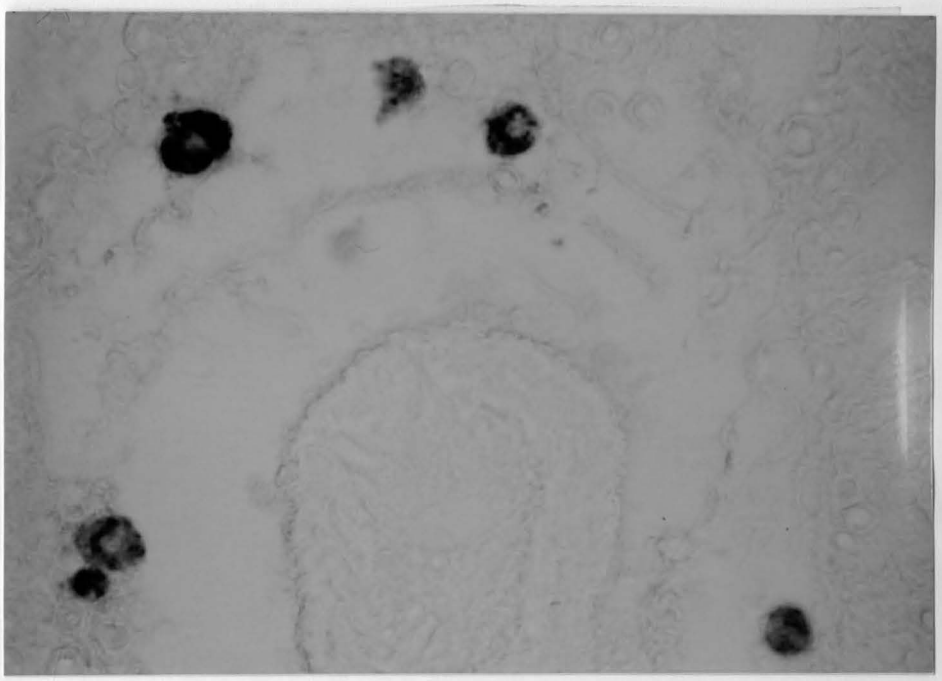


F

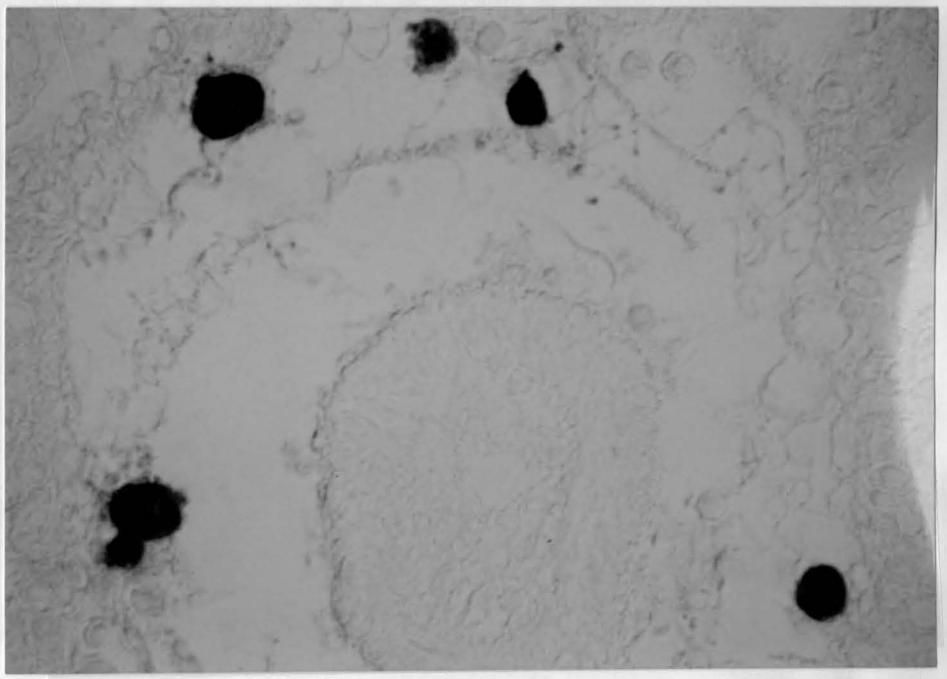
became dark brown (Figure 15B), indicating the co-localization of tryptase and chymase I in these cells. Of the lung mast cells which contained either tryptase, chymase I, or both, 77% contained both proteases, 4% contained only tryptase and 19% contained only chymase I (Table 4). Whether the small population of cells (4%) that stained for tryptase and not chymase I truly reflect chymase I-negative cells is not completely certain, because low levels of staining for chymase I may not produce sufficient brown staining to overcome the blue stain from tryptase. Figure 15C shows a section of skin which contained one blue tryptase-positive cell that became brown with the chymase I stain (Figure 15D). Among the mast cells in skin which contained tryptase and chymase I, 96% contained only chymase I, 4% contained both proteases while no cells contained tryptase only (Table 4). Figure 15E shows a section of peritoneal cells which had one blue tryptase-positive cell that became brown with the chymase I stain (Figure 15F) as well as numerous chymase I-positive cells in which tryptase was not detected. Among the mast cells containing tryptase and chymase I in peritoneal lavage fluid, 15% contained both protease, 85% contained chymase I alone, and none contained tryptase without chymase I (Table 4).

To better ascertain the distribution of two distinct mast cell types which contain either chymase I or tryptase

Figure 15. Simultaneous Double Labeling With Anti-Tryptase And Anti-Chymase I Antibodies. Sections of lung (A, B) and skin (C,D), and a cytospin of peritoneal cells (E, F) (phase contrast) were incubated simultaneously with goat IgG anti-tryptase and rabbit IgG anti-chymase I antibodies. Sections were developed first with alkaline phosphatase-conjugated swine anti-goat IgG (blue) (A, C, E) and then with peroxidase-conjugated goat anti-rabbit IgG (brown) (B, D, F). Blue tryptase-positive cells also containing chymase I turn brown; Those with tryptase but not chymase I remain blue; Those containing chymase I and not tryptase would appear brown, and never blue.



A



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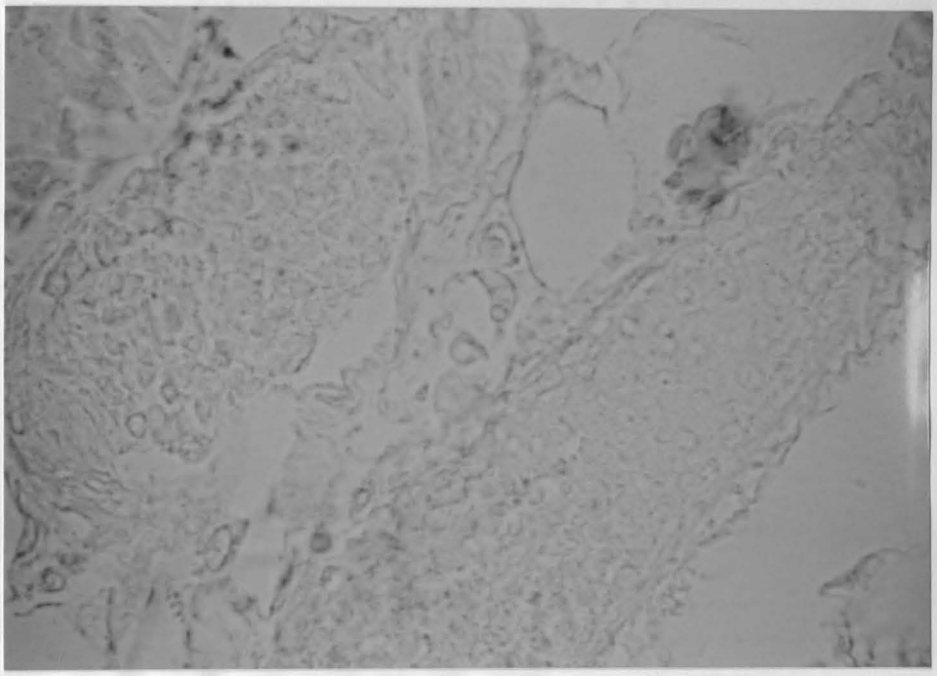
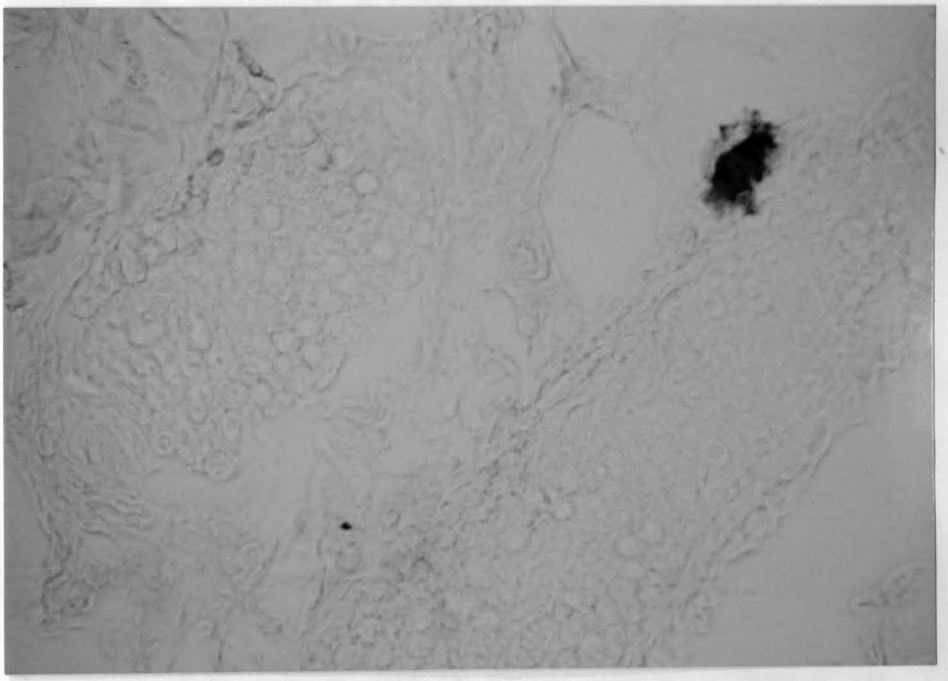


C



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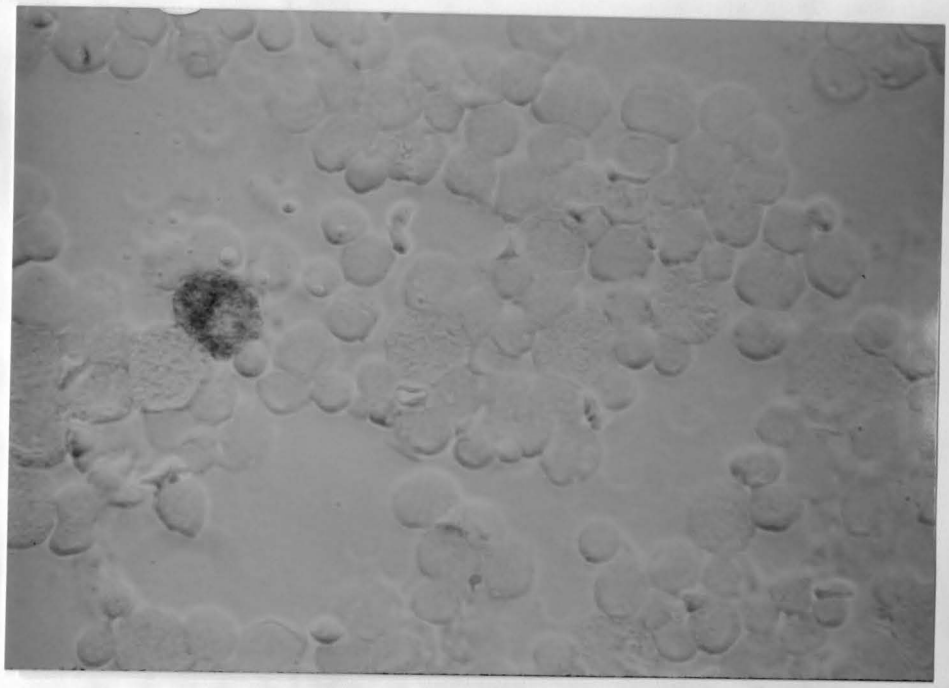

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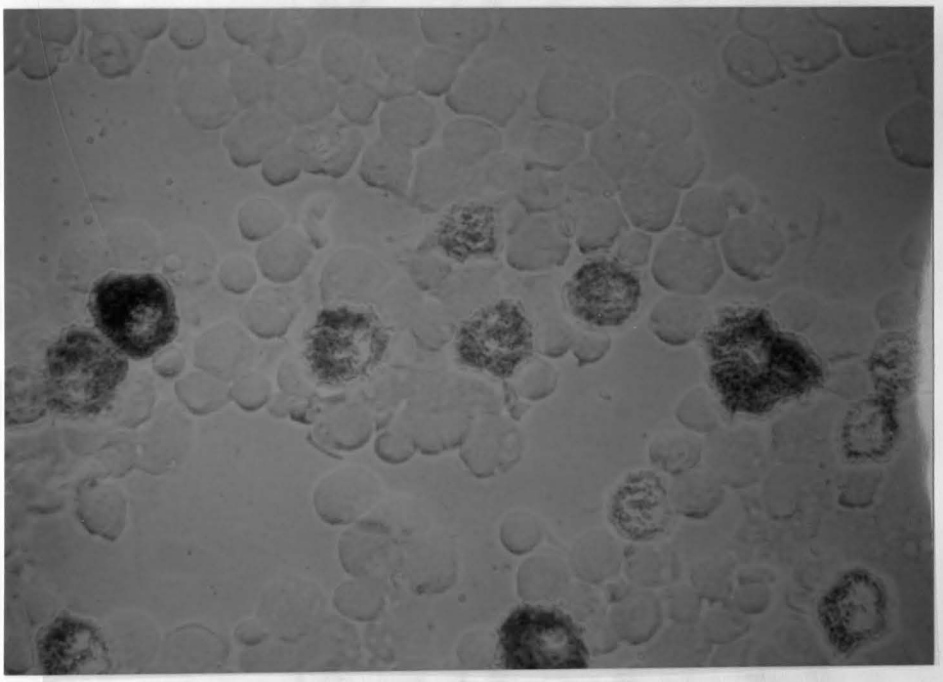


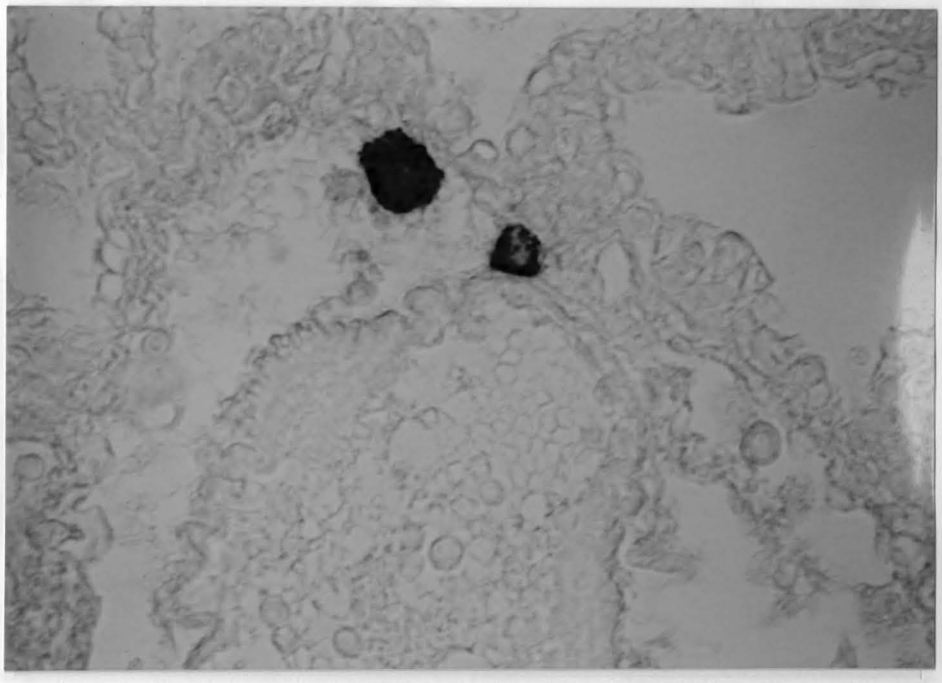
F

Cell Source	Cells* (n)	Tryptase$^+$ x 100 Chymase I$^+$ ± SD	Tryptase$^+$ x 100 Chymase I$^-$ ± SD	Tryptase$^-$ x 100 Chymase I$^+$ ± SD
Peribronchus	45-60 (5)	77 ± 2	4 ± 2	19 ± 7
Dermis	159-299 (7)	4 ± 1	0	96 ± 1
Peritoneal Cells	110-300 (12)	15 ± 2	0	85 ± 3

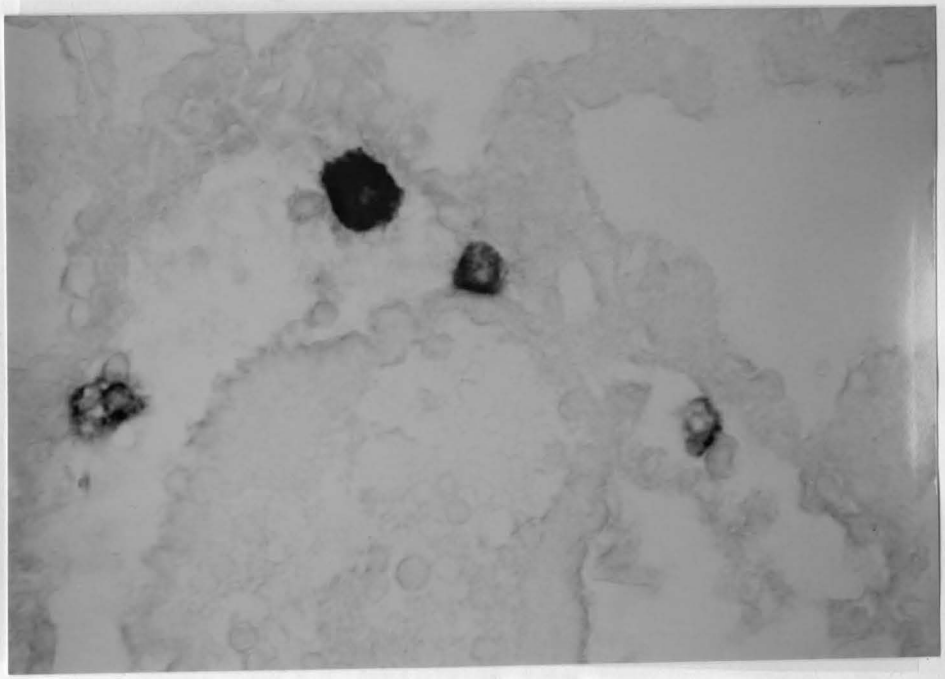
Table 4. Simultaneous Labeling Of Tryptase And Chymase I. This table shows the results of the simultaneous double labeling of mast cells with anti-tryptase and anti-chymase I antibodies. SD, standard deviation; n, number of tissues used in the experiment; *, number of cells counted per section or slide.

in a single section, sequential double-labelling experiments were performed. With this technique, deposition of the brown peroxidase-based stain with the first primary antibody blocks subsequent staining of those cells with the second primary antibody linked to alkaline phosphatase. This same phenomenon has been observed previously for staining of human mast cells (Irani et al. 1986, 1989). In lung, Figure 16A shows 2 brown tryptase-positive cells. These cells remain brown while 2 others stain blue with the anti-chymase antibody (Figure 16B). By sequential staining, tryptase-positive cells in lung accounted for 84 ± 7% of the chymase I-positive cells (Table 5). In skin, a section is shown that contains one tryptase-positive cell (Figure 16C) and one chymase I-positive cell (Figure 16D). Tryptase-positive cells in skin account for 7 ± 1% of the chymase I-positive cells (Table 5). In peritoneal cells, Figure 16E shows 5 tryptase-positive cells; subsequent staining with anti-chymase I antibody reveals an additional 9 chymase I-positive cells (Figure 16F). About 13 ± 7% of the chymase I-positive cells in peritoneal lavage fluid are also tryptase-positive (Table 5).

Figure 16. Sequential Double Labeling With Anti-Tryptase And Anti-Chymase I Antibodies. Sections of lung (A, B) and skin (C, D), and a cytospin of peritoneal cells (E, F) were incubated first with goat IgG anti-tryptase and developed with peroxidase-conjugated swine anti-goat IgG (brown) (A, C, E). Sections and cytospin were then incubated with rabbit IgG anti-chymase I antibody and developed with alkaline phosphatase-conjugated goat anti-rabbit IgG (blue) (B, D, F). Cells with tryptase stained brown and remained brown; those with chymase I became blue only if tryptase was not present. Panel E was photographed under phase contrast.



A



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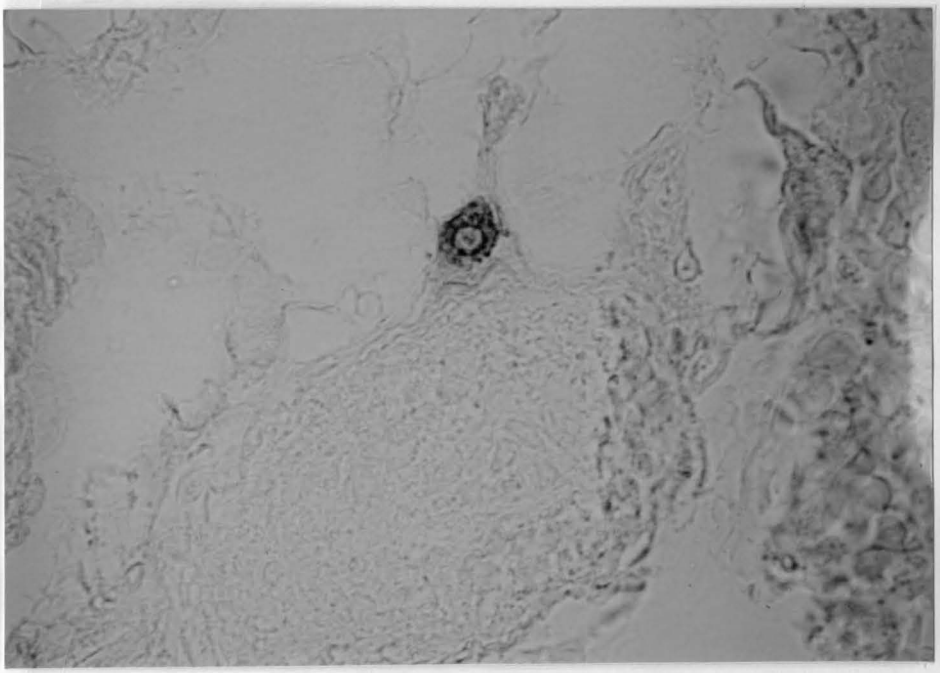

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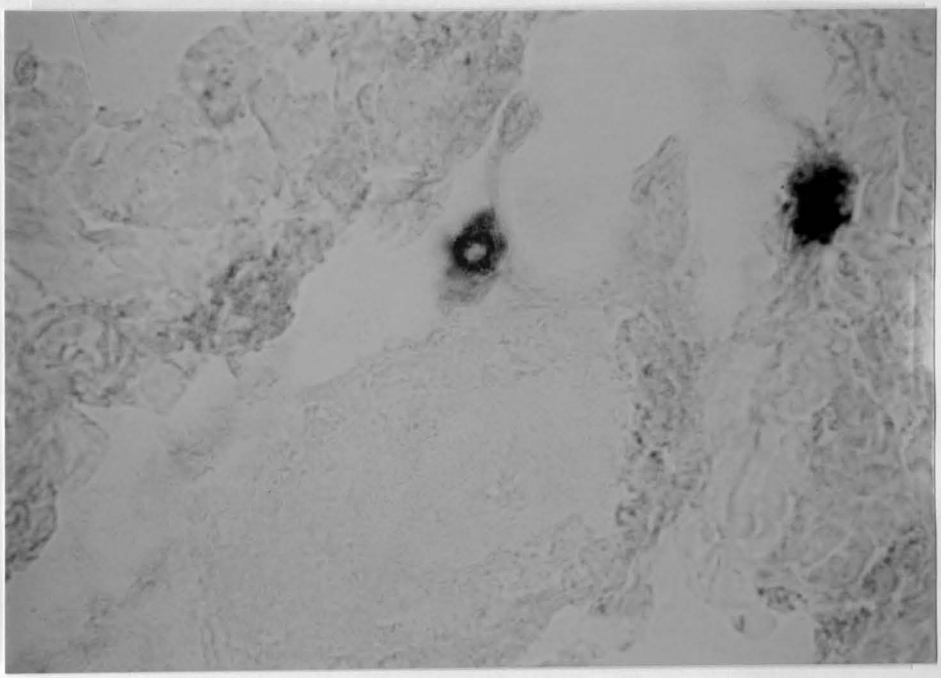

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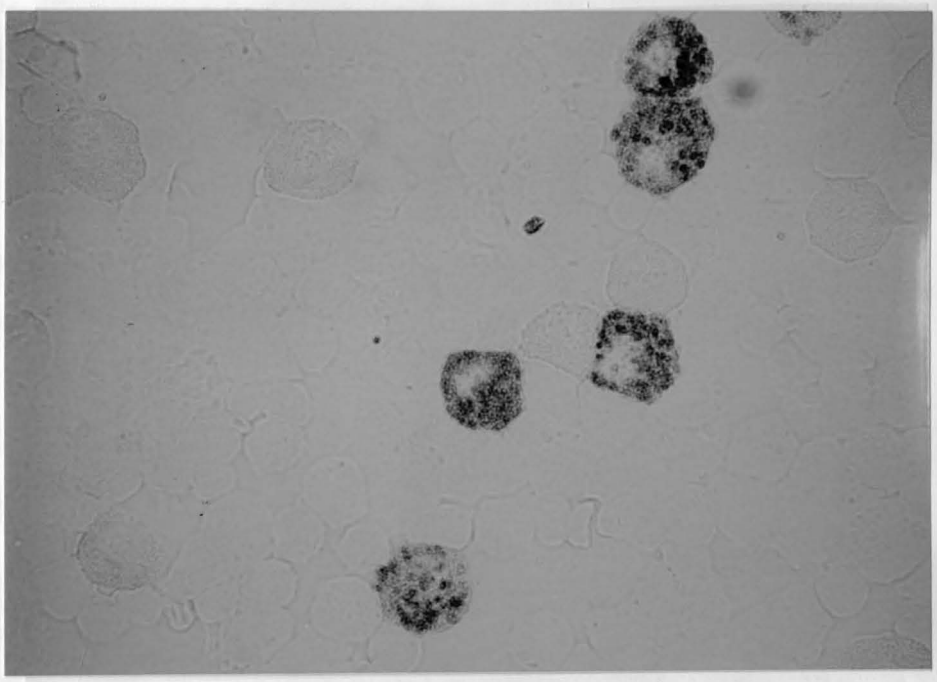

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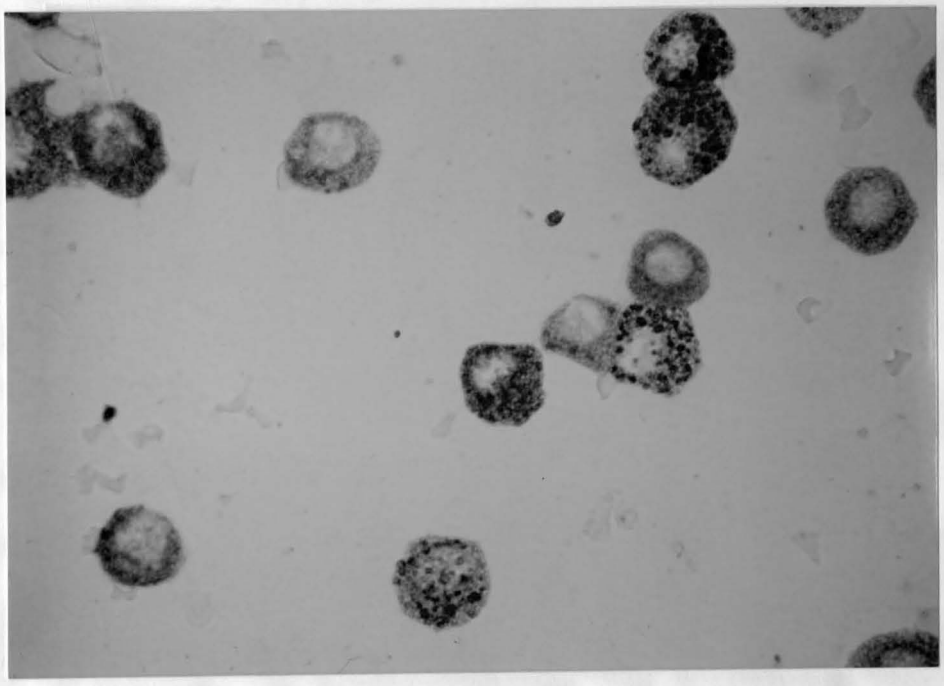

F

Cell Source	Cells* (n)	%Tryptase$^+$ / %Chymase I$^+$ ** ± SD
Peribronchus	40-50 (4)	84/16 ± 7
Dermis	156-412 (4)	7/93 ± 9
Peritoneal	105-264 (12)	13/87 ± 7

Table 5. Sequential Labeling Of Tryptase And Chymase I. The results of sequential double labeling of mast cells with anti-tryptase and anti-chymase I are summarized in the table. SD, standard deviation; n, number of tissues used in the experiments; *, number of cells counted per section or slide; **, refers to ChymAse I$^+$/Tryptase$^-$ mast cells.

CHAPTER X

DISCUSSION

Rat Mast Cell Heterogeneity.

Although rat tryptase has been purified from skin (Braganza & Simmons 1991) and peritoneal cells (Kido et al. 1985), its cellular localization has never been formally determined in this species. In the current study, a goat polyclonal IgG antibody prepared against purified rat skin tryptase was used to determine the distribution of tryptase in rat skin, lung, bowel mucosa and peritoneal cells. The antibody was produced in high titer, as determined by ELISA, and was specific, as determined by Western blotting with extracts from lung, skin and peritoneal cells. Although the level of endogenous tryptase (approximately 0.003% of total protein) was too low for detection by this technique, exogenously added tryptase, but no other proteins, were detected. By a variety of histochemical and immunohistochemical techniques, tryptase was localized exclusively in mast cells, but only in a subset of the connective tissue type of mast cell.

Localization to mast cells was demonstrated by sequential staining with alcian blue and anti-tryptase antibody. Only the blue mast cells were stained by the antibody. However,

although about 50% of mast cells in lung contained tryptase, most in skin, bowel and peritoneal cells did not contain tryptase. Before examining the distribution of tryptase among mucosal and connective tissue mast cells, the distributions of these two mast cell types were assessed histochemically, by sequential staining with alcian blue and safranin. Mucosal mast cells stain blue; mature connective tissue mast cells stain red. This sequential staining technique showed that lung tissue contained similar numbers of both mast cell types. Skin and peritoneal mast cells were almost exclusively of the connective tissue type, while those in the bowel mucosa were almost exclusively of the mucosal type. These distributions are similar to those previously reported (Enerback 1981, Gibson & Miller 1986, Huntley et al. 1990). Sequential staining with safranin and anti-tryptase antibody, in part, clarified the localization of tryptase. Safranin stains heparin-containing mast cells, and therefore is a marker of the mature connective tissue type of mast cell. Tryptase was present only in safranin-stained cells, but not in all connective tissue mast cells. This varied from tissue to tissue. The percentage of connective tissue mast cells staining for tryptase was 94% for lung, 20% for peritoneal cells, and 6% for skin. Thus, even though tryptase was purified from skin, the percentage of mast cells containing the enzyme in skin is markedly lower than in lung. The amount of tryptase in skin mast cells is also lower than that in lung

mast cells as indicated by weaker tryptase stain in the skin mast cells.

To confirm localization of tryptase to the connective tissue type mast cells, double labeling experiments were performed with anti-tryptase and anti-chymase I antibodies. Mast cell-specific chymases have been chosen to be markers that distinguish mast cells from other cell types, and mast cell subsets from one another (Karger 1990). In the rat, chymase I (also called rat mast cell protease I) is found only in connective tissue mast cells while chymase II (also celled rat mast cell protease II) is found only in mucosal mast cells (Miller et al. 1990). Using the simultaneous double labelling technique, all tryptase-stained mast cells in skin and peritoneal lavage cells also stained for chymase I. Only in lung was there a suggestion that tryptase could reside in mast cells lacking chymase I. In this case 4% of tryptase-stained mast cells did not develop detectable staining for chymase I by the simultaneous labeling technique. We view this as an upper limit, because variations in staining intensity from one cell to the next in tissue sections may lead to a more intense tryptase stain followed by a less intense stain for chymase I which, at the extreme, may not be detectable by this technique. Thus, the existence of a minor population of tryptase-positive, chymase I-negative cells in lung is questionable.

To more easily determine the distribution of tryptase

among the connective tissue mast cells, sequential rather than simultaneous double labelling was used. Staining with anti-tryptase antibodies was followed by staining with anti-chymase I antibodies such that tryptase-positive cells retained the stain for tryptase (even though they also contained chymase I) while those negative for tryptase stained for chymase I. The percentage of connective tissue mast cells that contained tryptase by this sequential chymase I technique compared with the simultaneous chymase I technique and the sequential safranin technique were similar: 84, 80 and 94 for lung; 7, 4 and 6 for skin; and 13, 15 and 20 for peritoneal cells. Thus, rat tryptase is present in a subset of the connective tissue type of mast cell.

Our results for tryptase in rat tissues more closely parallel previously made observations in mouse than in human. In human, all mast cells contain tryptase, whereas only a subset contain chymase (only one chymase in humans), carboxypeptidase and a cathepsin G-like protease (Irani & Schwartz 1990). In the mouse, cDNA molecules for at least 5 chymases, 2 tryptases and 1 carboxypeptidase have been identified (Reynolds et al. 1990a, 1990b). Northern blots with tissue extracts enriched for mucosal and connective tissue mast cells suggest that mouse tryptase (MMCP-6), like rat tryptase, is located in connective tissue mast cells, whereas chymases are distributed either in mucosal or connective tissue mast cell types or both.

The finding that only a subpopulation of connective tissue mast cells contain tryptase in the rat indicates a greater degree of heterogeneity than anticipated. One possible explanation for this finding is that tryptase expression is permissible only in connective tissue mast cells, but is regulated and inducible by environmental factors. An alternative explanation is that there are at least three distinct subsets of mast cells: tryptase-positive, chymase I-positive, chymase II-negative; tryptase-negative, chymase I-positive, chymase II-negative; and tryptase-negative, chymase I-negative, chymase II-positive. Further studies will be needed to clarify that to what extent mast cell heterogeniety is due either to lineage or to environment.

SUMMARY

Tryptase was purified from rat skin and found to be a glycoprotein with an apparent molecular weight of about 31,000 on SDS-PAGE gels. Incubation of the enzyme with N-glycanase converted the enzyme from a broad band to a sharp single band with a molecular weight of approximately 27,000. The amino acid sequences of the N-terminus, four tryptic peptides, and one lysylendopeptidase-C peptide were determined by the Edman procedure. These sequences accounted for 119 amino acids or about 45% of the total primary structure. The peptides have an overall 81-82% identity to human tryptases, 75% to dog tryptase, 77% to mouse tryptase-1, 93% to mouse tryptase-2, and 43% to bovine trypsin. Among the peptides sequenced are the active site region containing the catalytic serine residue and the substrate-binding aspartate. Two putative glycosylation sites have been identified. One of them is highly conserved among tryptases. The other only appears in mouse tryptase-2 but not the other tryptases. Four of the five tryptophan residues unique to tryptases but not to trypsin were found in the peptide sequences. The results revealed considerable structural differences between tryptase and trypsin as well as some significant structural variations among tryptases. This work has provided a solid support for future studies on the correlation of the functional characteristics of tryptases to their structural properties.

The cellular distribution of rat tryptase was examined in rat skin, lung, small intestine and peritoneal lavage cells by immunohistochemical techniques. Tryptase purified to apparent homogeneity from rat skin was used to generate goat polyclonal anti-rat tryptase antibody. Western blotting of extracts of skin, lung and peritoneal cells showed that the antibody was specific for rat tryptase. Tryptase containing cells were detected in lung, skin, and peritoneal lavage cells. Small intestinal mucosa, on the other hand, was found to have few, if any, tryptase-positive cells. Sequential staining with alcian blue and anti-tryptase antibody showed that tryptase was located only in mast cells. Sequential staining with safranin to identify the connective tissue type of mast cell, and anti-tryptase antibody showed that tryptase resides only in this mast cell type. However, only a subpopulation of the safranin-positive mast cells contained tryptase. In lung, 53% of the mast cells stained with safranin, and 94% of these were tryptase-positive. In skin, 80% of the mast cells stained with safranin, but only 6% of these were tryptase-positive. In peritoneal cells, more than 95% of the mast cells were safranin-positive, but onle 20% of these were tryptase-positive. In the bowel mucosa, where few safranin-positive cells exist, no tryptase-positive cells were detected. The simultaneous and sequential double-labeling with anti-tryptase and anti-chymase I antibodies were performed to confirm and extend the histochemical findings. The percentages of chymase

I-positive cells that were tryptase-positive by the simultaneous and sequential techniques, respectively, were 80 and 84 for lung, 4 and 7 for skin, and 15 and 13 for peritoneal cells. The data from our study suggest that the rat connective tissue type of mast cell is subdivided into two forms based on the presence or absence of tryptase. Rat mucosal mast cells are devoid of skin-derived tryptase, although it is possible that a tryptase could reside in mucosal mast cells that lacks immunological crossreactivity. These results contrast with those in humans, where tryptase is present in all mast cells. However, the results are similar to those in the mouse, where tryptase mRNA has been detected only in the connective tissue type.

APPENDIX

APPENDIX A: ENZYME PURIFICATION SOLUTIONS

Homogenization Buffer:

Dissolve 9.29 g of sodium phosphate monobasic, 43.9 g of sodium phosphate dibasic, 6.27 g of EDTA, and 438 g of NaCl in 15 l of Milli Q (MQ) water. The solution contains 25 mM of sodium phosphate, 1 mM EDTA, and 0.5 M of NaCl, pH 7.

Dialysis Buffer:

Dissolve 69 g of sodium phosphate monobasic, 8.3 g of EDTA, and 584.4 g of NaCl in MQ water; adjust to pH 6, and bring the volume to 20 l with MQ water. The solution contains 25 mM of sodium phosphate, 1 mM of EDTA, and 0.5 M of NaCl.

PAB Wash Buffer:

Dissolve 14.6 g of NaCl, and 240.4 g of urea in homogenization buffer to a final volume of 500 ml. The final solution contains 25 mM sodium phosphate, 1 mM EDTA, 1 M NaCl, and 8 M urea, pH 7.

PAB Equilibration Buffer:

Dissolve 3.45 g of sodium phosphate monobasic, 0.2 g of NaN_3, and 29.2 g of NaCl, adjust to pH 6, and bring the volume to 1 l in MQ water. The final solution contains 25 mM of sodium phosphate, 0.5 M of NaCl, and 0.02% (w/v) of NaN_3.

PAB Elution Buffer:

Dissolve 1566 mg of benzamidine in 100 ml of the PAB equilibration buffer. The final solution contains 25 mM sodium phosphate, 0.5 M NaCl, 0.02% NaN_3, and 0.1 M benzamidine, pH 6.

Con-A Activation Buffer:

Dissolve 6.8 g of sodium acetate, 10.2 g $MgCl_2$, 29.2 g of NaCl, 555 mg of $CaCl_2$, and 100 mg of NaN_3; adjust to pH 6, and bring to 500 ml in MQ water. The final solution contains 100 mM of sodium acetate, 100 mM of $MgCl_2$, 1 M of NaCl, 10 mM of $CaCl_2$, and 0.02% of NaN_3.

Con-A Elution Buffer:

Dissolve 10 g of α-methyl-D-mannoside in 100 ml of the PAB equilibration buffer. The final solution contains 25 mM of sodium phosphate, 0.5 M of NaCl, 0.02% of NaN_3, and 100 mg/ml of the mannoside, pH 6.

Con-A Wash Buffer:

Dissolve 987.5 mg of Tris-HCl, and 168 mg of EDTA in MQ water; adjust to pH 6, and bring the volume to 250 ml. The final solution contains 25 mM Tris-HCl, and 2 mM EDTA.

Enzyme Assay Buffer:

Dissolve 709.8 mg of sodium phosphate dibasic and 67.2 mg of EDTA in MQ water; adjust to pH 7.5, and bring the volume to 200 ml. The solution contains 25 mM sodium phosphate and 1 mM of EDTA.

10 mM BAEE Solution:

Dissolve 17.14 mg of BAEE in 5 ml of MQ water; stored at 4 °C.

APPENDIX B. SDS-PAGE SOLUTIONS

Sample Buffer:

The solution contains 2.5% of SDS (25 μl of 20% SDS stock), 10 mM of Tris and 1 mM EDTA (0.1 ml of 20 mM Tris, 2 mM EDTA), 0.5 mM DTT (15 μl of 10 mM/ml DTT), and 0.01% of Bromophenol Blue (2 μl of 1% Bromophenol Blue).

50% Ethanol, 10% Acetic Acid:

Mix 50 ml of 100% ethanol with 40 ml MQ water, then add 10 ml of acetic acid.

10% Ethanol, 5% Acetic Acid:

Mix 50 ml of 100% ethanol with 425 ml water, then add 25 ml acetic acid.

8.3% Glutaraldehyde:

Mix 40 ml of the 25% commercial glutaraldehyde with 80 ml of MQ water.

0.25% Silver Nitrate:

Desolve 250 mg of silver nitrate in 100 ml MQ water. Make

this solution fresh.

Developer:

12.5% Na_2CO_3 stock: Disolve 25 g of Na_2CO_3 in 200 ml of MQ water. Prior to the experiment, mix 50 ml of the 12.5% Na_2CO_3 stock with 200 ml of MQ water.

5% Acetic Acid (v/v):

Add 5 ml of acetic acid to 95 ml of MQ water.

10% Acetic Acid, 6% Glycerol (v/v):

Desolve 6 ml of glycerol in 84 ml of MQ water; add 10 ml of acetic acid.

APPENDIX C: ALKYLATION SOLUTIONS

Tris-HCl Buffer:

Dissolve 18.17 g of Tris-Base and 500 mg of EDTA in MQ water; adjust to pH 8.6 and bring to 100 ml. The solution contains 1.5 M Tris and 5 mg/ml EDTA.

Iodo-[^{3}H]Acetic Acid Solution:

Dissolve 1 mCi of the iodo[2-^{3}H]acetic acid in 0.1 ml of MQ water to give a 0.1 mCi/ml solution. The specific activity was 175 mCi/mmol.

Cold Iodoacetic Acid Solution:

Dissolve 14.5 mg of iodoacetic acid in 108 μl of 0.5 N NaOH to 134 mg/ml.

6 N HCl Solution:

Add 500 μl of the concentrated HCl slowly to 500 μl of MQ water under the hood.

100 mM Ammonium Bicarbonate Buffer:

Dissolve 3.95 g of NH_4HCO_3 in 500 ml MQ water and adjust to pH 8.

9% Formic Acid:

Mix 51 ml of the 88% formic acid stock with 449 ml of MQ water.

APPENDIX D: WESTERN BLOT SOLUTIONS

Homogenization Buffer:

Dissolve 976 mg of MES and 14.5 g of NaCl in MQ water; adjust to pH 6 and bring to a final volume of 500 ml with water. The solution contains 10 mM MES and 0.5 M NaCl.

Chymase Assay Buffer:

Dissolve 960 mg of Tris-Base and 1.47 g of $CaCl_2$, adjust to pH 7.8 and bring to 500 ml with MQ water. The solution contains 80 mM Tris and 100 mM $CaCl_2$.

1 mM BTEE (N-Benzoyl-L-Tyrosine Ethyl Ester):

Dissolve 16.8 mg of BTEE in 25 ml of methanol and bring the volume to 50 ml with MQ water.

20% TCA:

Mix 0.8 ml of trichloroacetic acid with 3.2 ml of MQ water.

Staining Solution:

Dissolve 1 PhastGel Blue R tablet in 80 ml of MQ water; stir for 10-15 minutes; add 120 ml of methanol; stir

again for 2 minutes, and then filter the solution which contains 0.2% PhastGel Blue R.
Mix 10 ml of the 0.2% PhastGel Blue R with 90 ml of 20% acetic acid.

Destaining Solution (30% Methanol and 10% Acetic Acid):
Add 90 ml of methanol to 180 ml of MQ water; add 30 ml of acetic acid to the above.

Preservative Solution (5% Glycerol and 10% Acetic Acid):
Mix 5 ml of glycerol with 85 ml of MQ water; add 10 ml of acetic acid.

PhastTransfer Buffer:
Dissolve 1.5 g Tris-Base and 7.2 g glycine in 400 ml of MQ water; add 100 ml of methanol. The solution contains 25 mM Tris, 192 mM of glycine, and 20% methanol, pH 8.3.

10 X TBS:
Dissolve 6 g of Tris-Base and 43.5 g of NaCl; adjust to pH 7.5 and bring the volume to 500 ml with MQ water.

1 X TBS:
Make a 1:10 dilution from the 10 X TBS solution with MQ water. The final solution contains 10 mM of Tris and 50 mM NaCl, pH 7.5.

Blocking Buffer (3% BSA/TBS):

Dissolve 3 g of bovine serum albumin, fraction V in 100 ml of the 1 X TBS.

0.5% BSA/TBS:

Dissolve 0.5 g of BSA in 100 ml of the 1 X TBS.

Second Antibody Solution:

Dilute 15 μl of the peroxidase or alkaline phosphatase conjugated secondary antibody in 15 ml of the 0.5% BSA/TBS.

AEC/H_2O_2 Solution:

See appendix F.

APPENDIX E: TISSUE SECTION PREPARATION

Carnoy's Solution:

In a bottle, mix 60 ml of 100% ethanol, 30 ml chloroform, and 10 ml glacial acetic acid. Store the solution at 4 °C. The final solution contain s 60% ethanol, 30% chloroform, and 10% acetic acid.

PBS (Phosphate Balanced Saline):

Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g KH_2PO_4 in 800 ml of MQ water; adjust to pH 7.4 with HCl and add water to 1 liter.

PBS/Heparin Buffer:

Dissolve 80 mg of gelatin in 80 ml of PBS by stirring on a hot plate; cool the solution to room temperature; add 0.8 ml of 1000 U/ml of heparin.

PBS/DNase Buffer:

Dissolve 10 mg of gelatin in 10 ml of PBS by stirring on a hot plate; cool the solution to room temperature; add 10 μl of DNase at 10 mg/ml.

H_2O_2/Methanol Solution:

Add 0.8 ml of 30% H_2O_2 to 40 ml of methanol. The solution which contains 0.6% of H_2O_2 in methanol was made prior to the reaction.

APPENDIX F: IMMUNOHISTOCHEMICAL STAINING

0.5% Alcian Blue Solution:

Dissolve 0.5 g of alcian blue 8 GX in 100 ml of MQ water by stirring; adjust to pH 1 with concentrated HCl; filter through a No. 4 filter from Millopore. The solution is stable for a few months at room temperature.

0.1% Safranin O Solution:

Add 1 ml of glacial acetic acid to 99 ml of MQ water; dissolve 0.1 g of safranin O in 100 ml of the 1% acetic acid solution. The solution is stable for a few months at room temperature.

PBS/BSA Solution:

Dissolve 1 g of bovine serum albumin fraction V and 10 mg of thimersol in 100 ml of BSA (appendix E); store the solution at 4 °C.

TTBS:

Dissolve 24 g of Tris-Base, 36 g of NaCl, 0.4 g thimersol, and 2 ml of Tween-20; adjust to pH 7.4; bring to 4 l with MQ water; store in 500 ml aliquot at 4 °C.

AEC/H_2O_2 Solution:

0.1 M acetic acid solution: Add 1.16 ml of glacial acetic acid in 100 ml of MQ water for solution A; dissolve 2.72 g of sodium acetate.$3H_2O$ in 100 ml of MQ water for solution B. Mix 14.8 ml of solution A with 35.2 ml of solution B and 50 ml of MQ water; adjust to pH 5.2 to render a 0.1 M acetic acid solution.

AEC solution: Dissolve 20 mg of 3-amino-9-ethylcarbazole in 5 ml of N-N-dimethylformimide; add 100 ml of the 0.1 M acetic acid solution; stir at room temperature for 5 minutes; filter the solution through a 0.45 μm membrane; store the AEC in 1 ml aliquot at -20 °C.

1% H_2O_2: Mix 29 ml MQ water with 1 ml of the 30% commercial H_2O_2 stock.

The AEC/H_2O_2 solution is prepared prior to the reaction by adding 10 μl of the 1% H_2O_2 to the 1 ml AEC.

DAB/H_2O_2 Solution:

DAB solution: Dissolve 3 g of Tris-Base in 500 ml MQ water; adjust to pH 7.6 with HCl to form a 0.05 M Tris buffer; dissolve 5 mg of diaminobenzidine in 10 ml of the 0.05 M Tris buffer; stir at room temperature for 5 minutes; filter through a 0.45 μm membrane.

1% H_2O_2: Mix 29 ml MQ water with 1 ml of the 30% commercial H_2O_2 stick.

Add 10 μl of the 1% H_2O_2 to 1 ml of the DAB solution. The DAB/H_2O_2 solution must be prepared fresh every time prior to the reaction.

Naphthol/Fast Blue Solution:

0.1 M Tris buffer: Dissolve 6.05 g of Tris-Base in 500 ml of MQ water; adjust to pH 8.2; store at 4 °C.

Prior to the reaction, dissolve 5 mg of naphthol AS-MX in 0.5 ml of N,N-dimethylformimide; add 24.5 ml of the 0.1 M Tris buffer warmed to 37 °C; add 6 mg of levamisole; dissolve 5-10 mg of Fast Blue RR in the above solution to 1 mg/ml; filter and use the solution within 20-30 minutes.

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The dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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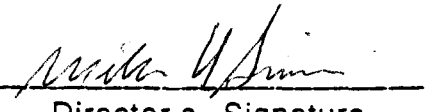
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