




1993

## Rat Tryptase: Partial Amino Acid Sequence and Mast Cell Heterogeneity

Zehan Chen  
*Loyola University Chicago*

Follow this and additional works at: [https://ecommons.luc.edu/luc\\_diss](https://ecommons.luc.edu/luc_diss)

 Part of the [Biochemistry, Biophysics, and Structural Biology Commons](#)

---

### Recommended Citation

Chen, Zehan, "Rat Tryptase: Partial Amino Acid Sequence and Mast Cell Heterogeneity" (1993).  
*Dissertations*. 3118.  
[https://ecommons.luc.edu/luc\\_diss/3118](https://ecommons.luc.edu/luc_diss/3118)

This Dissertation is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Dissertations by an authorized administrator of Loyola eCommons. For more information, please contact [ecommons@luc.edu](mailto:ecommons@luc.edu).



This work is licensed under a [Creative Commons Attribution-NonCommercial-No Derivative Works 3.0 License](#).  
Copyright © 1993 Zehan Chen

LOYOLA UNIVERSITY MEDICAL CENTER

LIBRARY LOYOLA UNIVERSITY  
MEDICAL CENTER

RAT TRYPTASE:

PARTIAL AMINO ACID SEQUENCE AND MAST CELL HETEROGENEITY

A DISSERTATION SUBMITTED TO  
THE FACULTY OF THE GRADUATE SCHOOL  
IN CANDIDACY FOR THE DEGREE OF  
DOCTOR OF MOLECULAR AND CELLULAR BIOCHEMISTRY  
DEPARTMENT OF MOLECULAR AND CELLULAR BIOCHEMISTRY

BY

ZEHAN CHEN

MAYWOOD, ILLINOIS

MAY 1993

TABLE OF CONTENTS

ACKNOWLEDGEMENTS . . . . . iii  
VITA . . . . . iv  
LIST OF TABLES . . . . . vi  
LIST OF FIGURES . . . . . vii  
CONTENTS OF APPENDIX . . . . . viii

PART I  
PARTIAL AMINO ACID SEQUENCE OF RAT SKIN TRYPTASE

CHAPTER I  
INTRODUCTION . . . . . 2

CHAPTER II  
LITERATURE REVIEW . . . . . 4  
    1. Biological Function . . . . . 4  
    2. Chemical And Physical Properties . . . . . 7  
    3. Purpose Of The Project . . . . . 12

CHAPTER III  
MATERIALS AND METHODS . . . . . 14  
    A. Materials . . . . . 14  
    B. Methods . . . . . 15  
        1. Purification Of Rat Skin Tryptase . . . . . 15  
        2. SDS-PAGE . . . . . 18  
        3. Reversed-Phase HPLC . . . . . 20  
        4. Enzymatic Deglycosylation Of Tryptase . . . . . 21  
        5. Alkylation of Tryptase . . . . . 22  
        6. Enzymatic Cleavage Of Tryptase . . . . . 23  
        7. Separation And Purification Of Peptides . . . . . 24  
        8. Amino Acid Sequence Determination . . . . . 25

CHAPTER IV  
RESULTS . . . . . 26  
    1. Purification Of Rat Skin Tryptase . . . . . 26  
    2. Deglycosylation Of Tryptase . . . . . 35  
    3. Alkylation And Peptide Purification . . . . . 40  
    4. Peptide Sequences . . . . . 46

CHAPTER V  
DISCUSSION . . . . . 54  
    Partial Amino Acid Sequence Of Rat Skin Tryptase. . . . . 54

PART II  
RAT MAST CELL HETEROGENEITY

CHAPTER VI  
INTRODUCTION . . . . . 65

CHAPTER VII	
LITERATURE REVIEW . . . . .	68
1. Biological And Pathological Roles . . . . .	68
2. Mediators . . . . .	83
3. Mast Cell Heterogeneity . . . . .	88
4. Classification Of Mast Cells . . . . .	93
5. Purposes Of The Project . . . . .	103
CHAPTER VIII	
MATERIALS AND METHODS . . . . .	106
A. Materials . . . . .	106
B. Methods . . . . .	107
1. Western Blot . . . . .	107
2. Tissue Section Preparation . . . . .	112
3. Histochemical Stain . . . . .	113
4. Immunohistochemical Stain . . . . .	114
Single Stain . . . . .	115
Double Staining. . . . .	116
Simultaneous Double Labeling . . . . .	117
Sequential Double Labeling . . . . .	119
5. Photomicroscopy And Calculations . . . . .	120
CHAPTER IX	
RESULTS . . . . .	122
1. Specificity Of Goat Anti-Rat Tryptase Antibody . . . . .	122
2. Distribution Of Mast Cells And Tryptase Positive Cells . . . . .	125
3. Rat Tryptase Is Localized In Mast Cells .	126
4. Rat Tryptase Resides In The Safranin- Positive CTMC Cells . . . . .	133
5. Distribution Of Tryptase And Chymase I In Rat Mast Cells . . . . .	134
CHAPTER X	
DISCUSSION . . . . .	151
Rat Mast Cell Heterogeneity. . . . .	151
SUMMARY . . . . .	156
APPENDIX . . . . .	159
REFERENCES . . . . .	175

## ACKNOWLEDGEMENTS

The author is most grateful to Dr. William H. Simmons for the guidance and support throughout the work and to Allen Frankfater, Ph.D., Allen Samarel, M.D., Richard M. Schultz, Ph.D., and Donald F. Steiner, M.D., for taking time out of their busy schedule to serve in the committee.

The author would like to express his gratitude to Lawrence B. Schwartz, M.D., Ph.D., and Anne-Marie Irani, M.D., for their guidance in trypase localization and to Bassam Wakim, Ph.D., for the protein sequencing work.

The author would also like to thank Mary Druse Manteuffel, Ph.D., for her dedication and concern to his career at time of need.

This work is dedicated to his father Hanyang Chen, mother Xieren Cai, and Sister Yonghan Chen for their support and to his loving and patient wife Amy Chen who stood by him all the way.

## 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.

Mr. Chen has accepted a position as a postdoctoral fellow in the laboratory of Dr. Ruth Sager in the Division of Cancer

Genetics in Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts.

LIST OF TABLES

Table	Page
1. Purification Of Rat Skin Tryptase . . . . .	27
2. Peptide Sequence Homology . . . . .	57
3. Distribution Of Tryptase-Positive Mast Cells . . . .	132
4. Simultaneous Labeling Of Tryptase And Chymase I . .	144
5. Sequential Labeling Of Tryptase And Chymase I . . .	150



## LIST OF FIGURES

Figure	Page
1. SDS-PAGE Of Purified Trypsin . . . . .	29
2. Molecular Weight Of Trypsin By SDS-PAGE . . . . .	31
3. Reversed-Phase HPLC . . . . .	33
4. SDS-PAGE Of N-Glycanase Treated Trypsin . . . . .	36
5. Molecular Weight Change Following Deglycosylation .	38
6. Desalting Of Alkylated Trypsin On A Sephadex G-25- 80 Column . . . . .	42
7. Tryptic Digest Of Trypsin . . . . .	44
8. Gel Permeation Chromatography . . . . .	47
9. Separation By C4 Reversed-Phase Column . . . . .	49
10. Peptide Sequences . . . . .	52
11. Trypsin Sequence Alignment . . . . .	55
12. Specificity Of Goat IgG Anti-Rat Trypsin . . . . .	123
13. Sequential Staining With Alcian Blue And Anti- Trypsin Antibody . . . . .	127
14. Sequential Staining With Safranin And Anti-Trypsin Antibody . . . . .	135
15. Simultaneous Double Labeling With Anti-Trypsin And Anti-Chymase I Antibodies . . . . .	140
16. Sequential Double Labeling With Anti-Trypsin And Anti-Chymase I Antibodies . . . . .	146

## CONTENTS OF APPENDIX

Appendix	Page
A: ENZYME PURIFICATION SOLUTIONS . . . . .	160
B. SDS-PAGE SOLUTIONS . . . . .	163
C: ALKYLATION SOLUTIONS . . . . .	165
D: WESTERN BLOT SOLUTIONS . . . . .	167
E: TISSUE SECTION PREPARATION . . . . .	170
F: IMMUNOHISTOCHEMICAL STAINING . . . . .	172

PART I

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 future 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 evidence 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 hyperresponsiveness 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 bronchoconstrictor 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). Adrenocorticotrophic hormone (ACTH) is a pituitary

hormone which regulates the growth and function of the adrenal cortex. Human pituitary trypsin 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 trypsin.

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

A role for trypsin 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 trypsin (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 trypsin 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  $\alpha$ -tryptase and  $\beta$ -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  $\beta$ -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  $\alpha$ -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.  $\text{NaHPO}_4$ ,  $\text{NaH}_2\text{PO}_4$ ,  $\text{NaCl}$ , 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),  $\alpha$ -methyl-D-mannoside,  $\text{NaN}_3$ , 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 sulfete was obtained from Schwarz Mann Biotech. Div. of ICN Biomedicals, Inc., Camgridge, MA. ( $^3\text{H}$ )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%  $(\text{NH}_4)_2\text{SO}_4$  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%  $(\text{NH}_4)_2\text{SO}_4$  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  $\mu$ l, 2 ml of the dialysis buffer was added to each unit and concentrated until 50-100  $\mu$ l was left in each unit. This step was repeated once. Each unit was then rinsed twice with 100  $\mu$ l 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  $\mu$ l of the assay buffer (Appendix A), 10  $\mu$ l of the sample, and 50  $\mu$ l 10 mM BAEE. The reference cuvette contained 450  $\mu$ l of the assay buffer and 50  $\mu$ l 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  $\mu$ mole of substrate hydrolysed per minute. Specific activity is defined as unit activity per mg of protein and calculated according to the following formula:

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

ml)  
where  $\Delta A_{253}/\text{min}$  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  $\mu\text{g}/\text{ml}$  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),  $\beta$ -lactoglobulin (18,400), and lysozyme (14,300). The relative mobility (Rf) of a protein was determined according to the formula:  $Rf = \text{Distance of protein migration} / \text{Distance of tracking dye migration}$ . 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 guanidine-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 monitor 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  $\mu$ g) was denatured by boiling for 5 minutes in 20  $\mu$ l of 100 mM Tris-HCl, pH 8, containing 50 mM  $\beta$ -mercaptoethanol and 0.5% SDS. To the

denatured sample was added 5  $\mu$ l of 7.5% Nonidet P-40 followed by 5  $\mu$ l (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  $\mu$ l (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 occurred. 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  $\mu$ g) was dissolved in a solution containing 6 M guanidine hydrochloride by adding 0.7 g of the salt and 400  $\mu$ l 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  $\mu$ l solution of [<sup>3</sup>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  $\mu$ l of 6 N HCl solution (Appendix C).

The unincorporated 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  $\text{NH}_4\text{HCO}_3$  and 0.1 mM  $\text{CaCl}_2$ , pH 8. About 40  $\mu\text{g}$  of the alkylated tryptase was mixed with 0.4  $\mu\text{l}$  of the 1 mg/ml trypsin in 0.1 mM HCl, 10  $\mu\text{l}$  of 1 mM  $\text{CaCl}_2$ , and 90  $\mu\text{l}$  of 0.1 M  $\text{NH}_4\text{HCO}_3$ , 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  $\mu\text{l}$  of 50 mM Tris-HCl, pH 9, 1  $\mu\text{g}$  of Lys-C and 100  $\mu\text{g}$  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 guanidine-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 guanidine-HCl. The mobile phase contained 6 M guanidine-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  $\mu\text{g}$  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

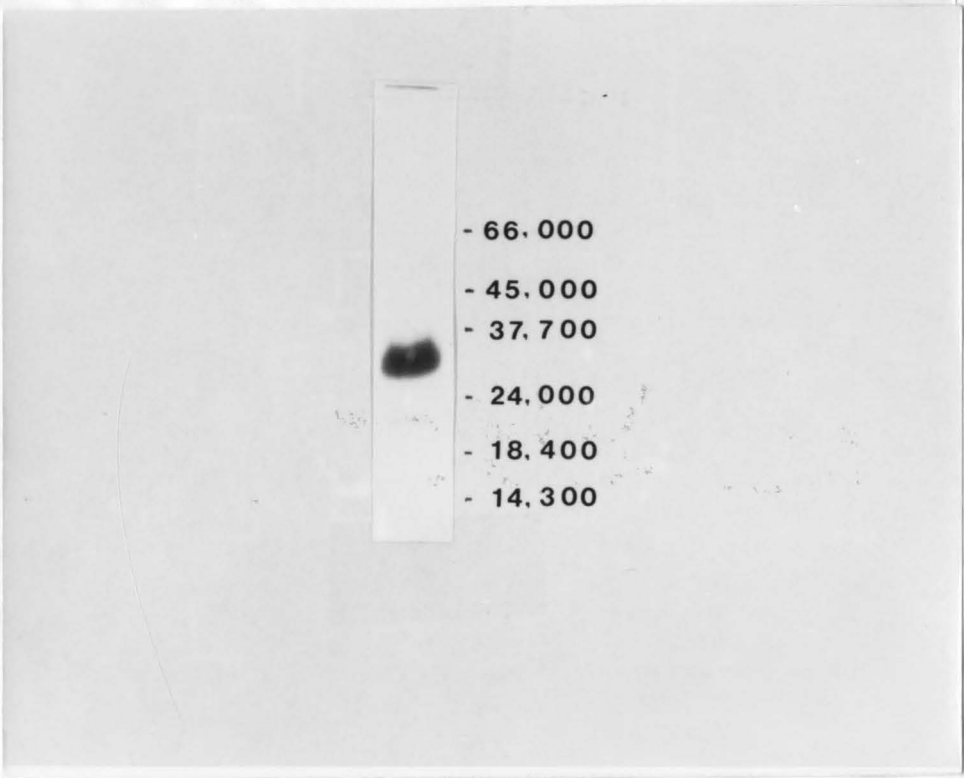
Fractions	Volume (ml)	Prot. Conc. (mg/ml)	Tot. Prot. (mg)	Act. (U/ml)	Sp. Act. (U/mg)	Tot. Act. (U)	Yield (U%)	Pur. Fact. (Fold)
Crude Extract	1530	2.23	3412	0.085	0.038	130.1	100	1
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 25-80%	95	9.81	932	0.216	0.022	20.5	16	0.6
PAB/Con A Columns	2.7	0.032	0.085	4.943	154	13.2	10	4053

Table 1. Purification Of Rat Skin Tryptase.

purified 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 consistent 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  $\mu$ g of purified tryptase. The procedure was used successfully a few times to obtain about 150  $\mu$ g of highly purified tryptase in one purification from a larger amount of skin. In one isolation, over 230  $\mu$ g 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  $31,000 \pm 1,000$  (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  $\mu\text{g}$ ) 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.



12.28  
ocular  
shows  
ords at  
ording  
of the



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.

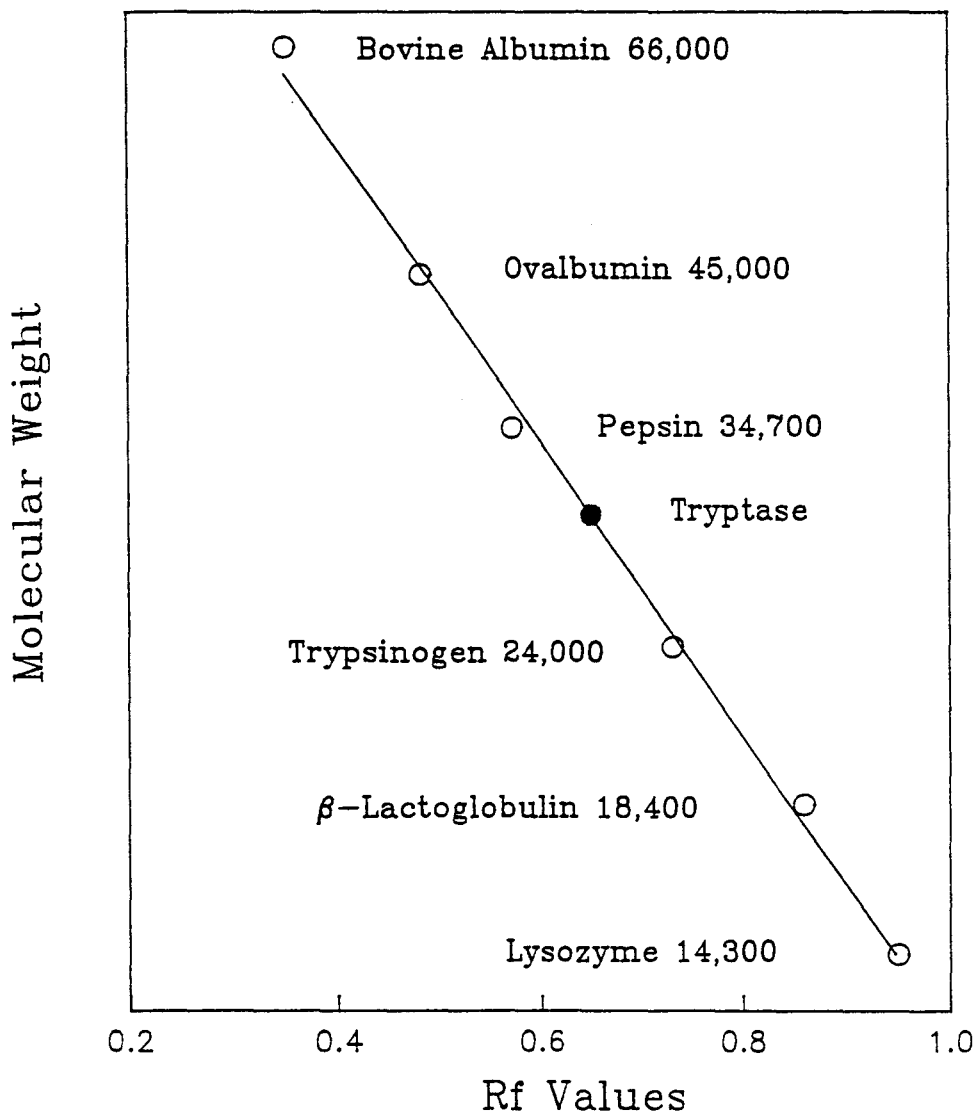
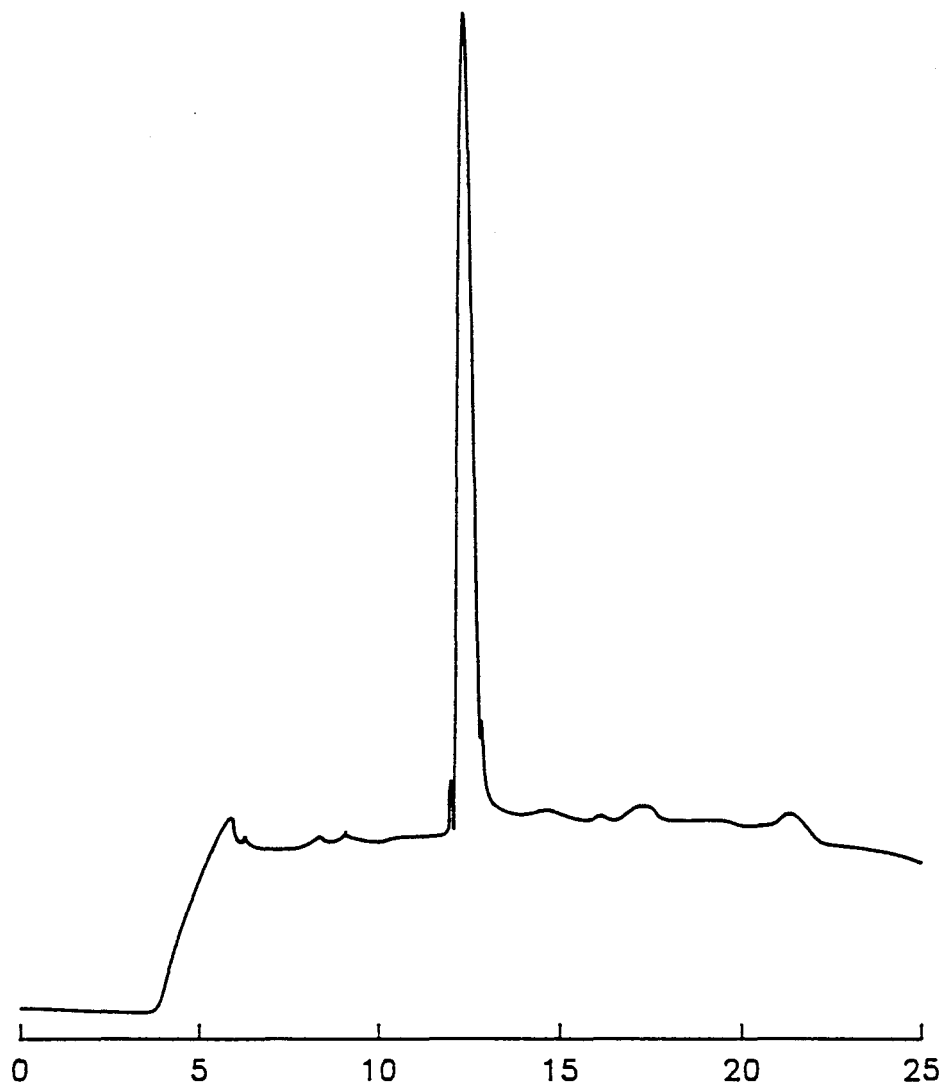


Figure 3. Reversed-Phase HPLC Of Purified Tryptase. Purified rat skin tryptase was subjected to reversed-phase HPLC chromatography using a C4 column. The separation conditions are shown below.

Column: Aquapore Butyl (C4)  
Mobile Phase: A: 0.1% TFA in H<sub>2</sub>O  
                  B: 0.08% TFA in CH<sub>3</sub>CN  
Gradient: 30-50% B, 20 minutes, linear  
Flow Rate: 0.75 ml/min  
Detection: UV, 206 nm  
Sample: 4 µg tryptase



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<sup>4</sup>-(N-acetyl- $\beta$ -glucosaminyl) asparagine amidase) (Plumer et al. 1984) catalyzes the hydrolysis of Asn-linked oligosaccharide at the  $\beta$ -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  $\mu\text{g}$ ). Lane 2 is the untreated tryptase (0.4  $\mu\text{g}$ ).

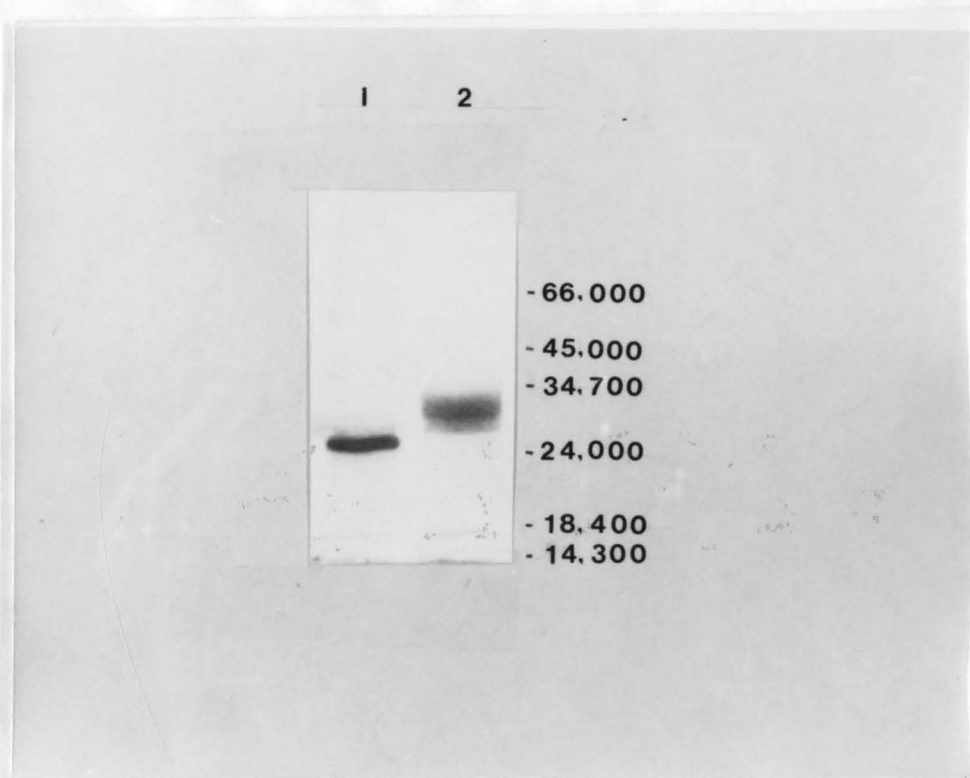
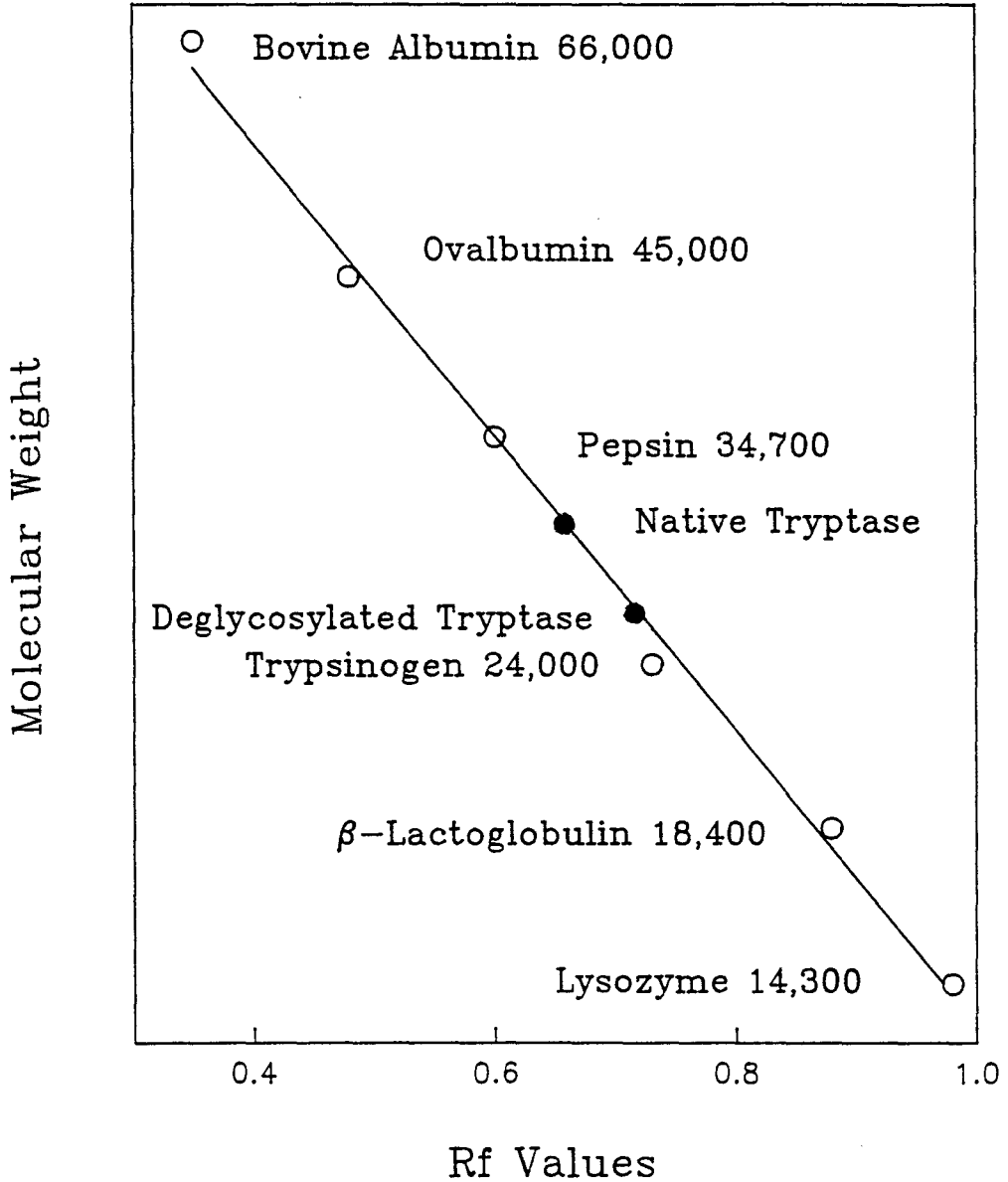


Figure 5. Molecular Weight Change Following Deglycosylation. Tryptase was enzymatically deglycosylated by incubation with N-Glycanase and subjected to SDS-PAGE as shown in Figure 4. The molecular weight standards are shown in the open circles. The molecular weights of the standards on a common logarithm 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.





tryptase is glycosylated through asparagine linkages. The fact that tryptase was purified with a concanavalin A-agarose column is consistent 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 (<sup>3</sup>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<sub>4</sub>HCO<sub>3</sub> 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  $\beta$ -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 trypsin in the same fraction numbers.

After alkylation, the protein was subjected to enzymatic fragmentation. The cleavage of trypsin 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.

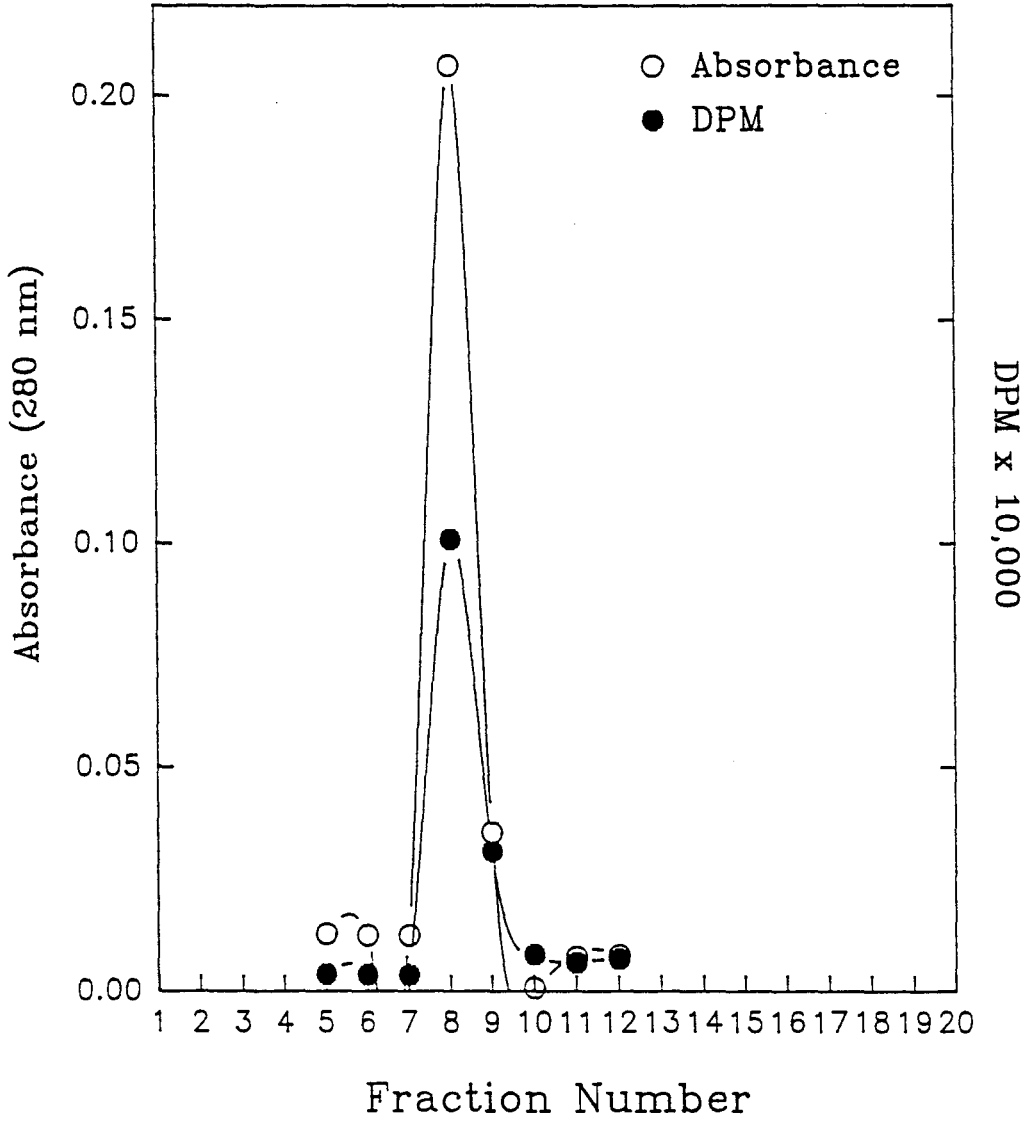
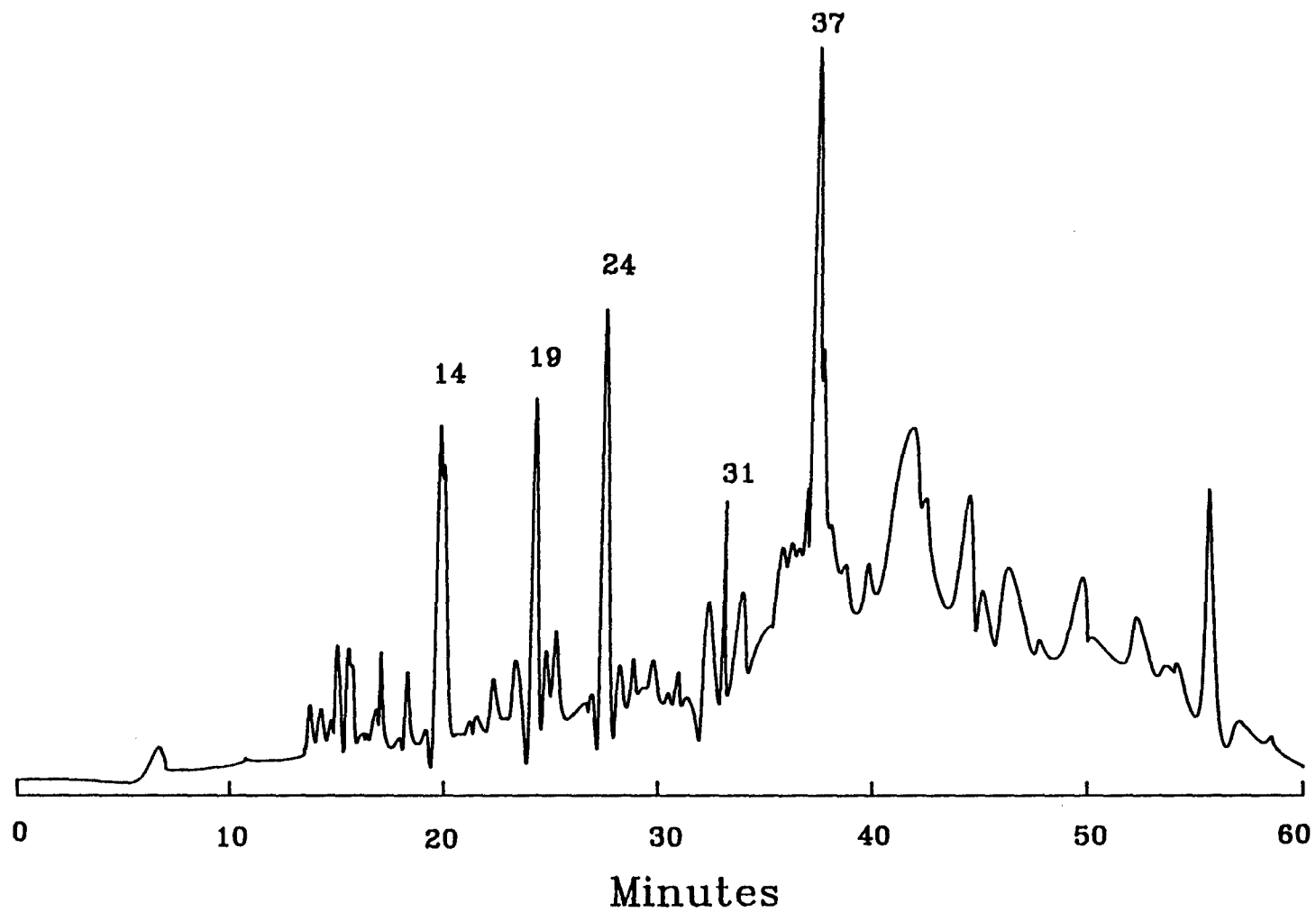


Figure 7. Tryptic Digest Of Trypsin. Rat skin trypsin fragments from a tryptic digest were separated by reversed-phase HPLC using a C18 column on a Varian Model 5500 system. All identifiable peaks were collected in separate plastic tube. Peak number 19, 24, 31, and 37 were sequenced. The conditions of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

Column: OD102 (C18)  
Mobile Phase: A: 0.1% TFA in H<sub>2</sub>O  
                  B: 0.08% TFA in CH<sub>3</sub>CN  
Gradient: 0-60% B, 60 minutes, linear  
Flow Rate: 0.5 ml/min  
Detection: UV, 206 nm  
Samples: 20 µg tryptic peptides



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 trypsin 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 trypsin 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 guanidine HCl  
10 mM potassium phosphate, pH 6  
Flow Rate: 0.5 ml/min  
Detection: UV, 226 nm  
Samples: Lys-C digest

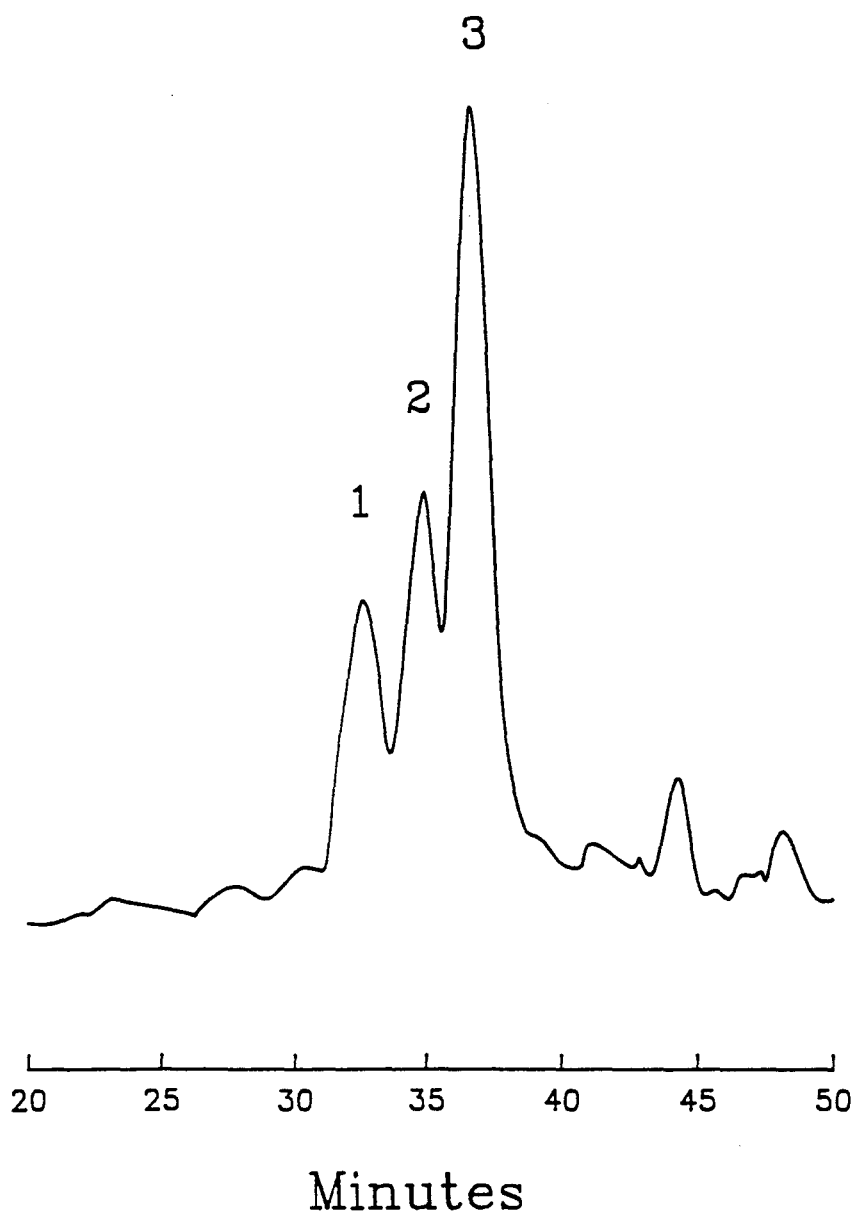


Figure 9. Separation By C4 Reversed-Phase Column. Peak number 1 from the gel permeation chromatography was subjected to reversed-phase HPLC using a C4 column. A single peak was identified. The separation conditions are shown below.

Column: BU300 (C4)

Mobile Phase: A: 0.1% TFA in H<sub>2</sub>O

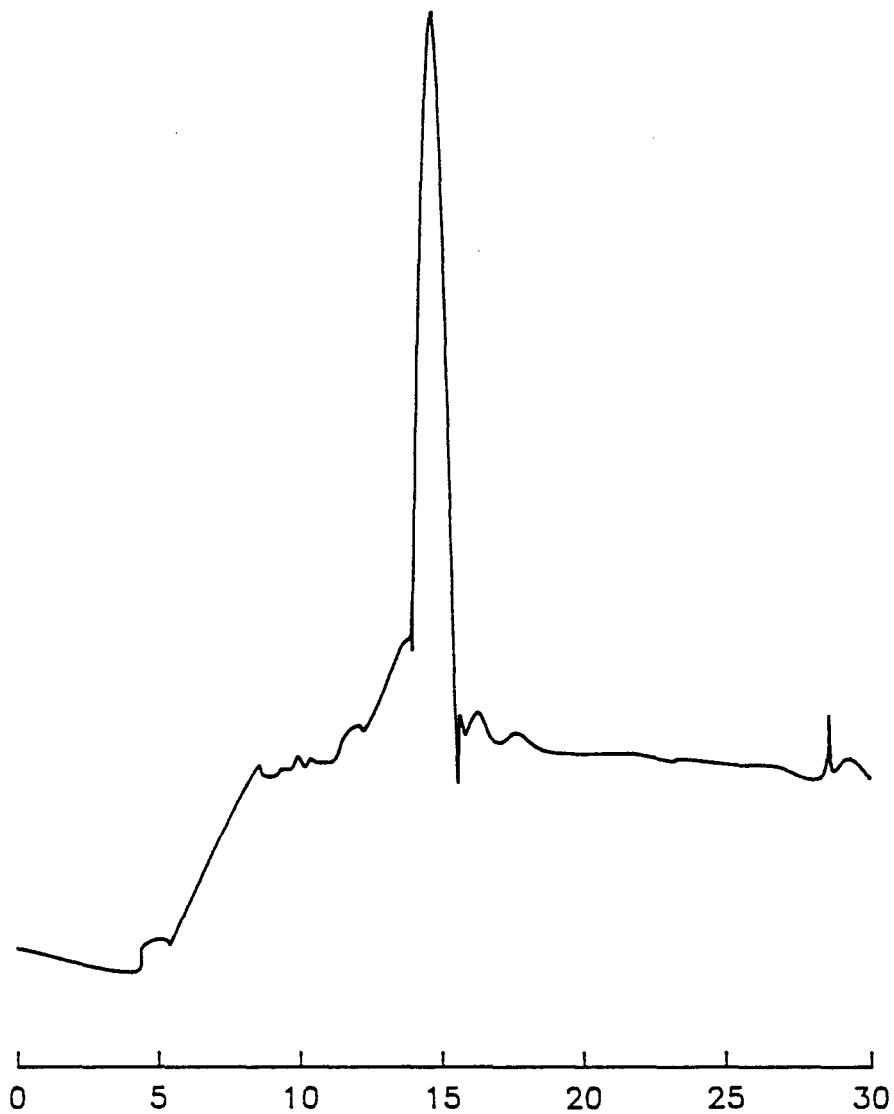
B: 0.08% TFA in CH<sub>3</sub>CN

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



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 trypsin 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 trypsin 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; HLT $\alpha$ , human lung tryptase  $\alpha$  (Miller et al. 89); HLT $\beta$ , human lung tryptase  $\beta$  (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    HFCGGSLINSQWVSAAHCYKSGIQVRLGEDNINVVEGN    EQFISASKSIVHPSYN  
 HLT $\alpha$     IVGGQEAPRSKWPWQVSLRVRDRYWMHFCGGSLIHPQWVLTAAHCLGPDVKDLATLRVN    SGTHTLYYQDQLLPVSRIMVHPQFY  
 HLT $\beta$     IVGGQEAPRSKWPWQVSLRVHGPYWMHFCGGSLIHPQWVLTAAHCVGPDVKDLAALRVQLREQHLYYQDQLLPVSRIIVHPQFY  
 HST1    IVGGQEAPRSKWPWQVSLRVHGPYWMHFCGGSLIHPQWVLTAAHCVGPDVKDLAALRVQLREQHLYYQDQLLPVSRIIVHPQFY  
 HST3    IVGGQEAPRSKWPWQVSLRVRDRYWMHFCGGSLIHPQWVLTAAHCVGPDVKDLAALRVQLREQHLYYQDQLLPVSRIVHPQFY  
 DMT    IVGGREAPGSKWPWQVSLRLKGQYWRHICGGSLIHPQWVLTAAHCVGPNVVCPEEIRWQLREQHLYYQDHLNPNRIVMHPNY  
 MMCT1    IVGGHEASESKWPWQVSLRFLNWIHFCGGSLIHPQWVLTAAHCVGPHIKSPQLFRVQLREQYLYYGDQLLSLNRIIVHPHY  
 MMCT2    IVGGQEAHGNKWPWQVSLRAndtYWMHFCGGSLIHPQWVLTAAHCVGPDVADPNKVRVQLRKQYLYYHDHLMTVSQIITHPDFY  
 RST    IVGGQEASGNKWPWQVSLRVxdtYWMHFCGGSLINPQ

♥                    \*                    111                    121                    131                    141                    151                    161  
 BT    SNTLNNDIMLIKLSAASLNSRVASISLPTSCA    SAGTQCLISGWGNTKSS    GTSYDPVLKCLDAPILSDSSCKSAYP  
 HLT $\alpha$     IIQTGADIALLELEEPVnisSRVHTVMLPPASETFPPGMPCWVTGWGDVDNDEPLPPPFPLKQVKVPIMENHICDAKYHLGAYT  
 HLT $\beta$     TAQIGADIALLELEEPVKVSSHVHTVTLPPASETFPPGMPCWVTGWGDVDNDERLPPPFPLKQVKVPIMENHICDAKYHLGAYT  
 HST1    TAQIGADIALLELEEPVnvsSHVHTVTLPPASETFPPGMPCWVTGWGDVDNDERLPPPFPLKQVKVPIMENHICDAKYHLGAYT  
 HST3    TAQIGADIALLELEEPVnvsSHVHTVTLPPASETFPPGMPCWVTGWGDVDNDERLPPPFPLKQVKVPIMENHICDAKYHLGAYT  
 DMT    TPENGADIALLELEDPVnvsAHVQPVTLPALQTFPTGTPCWVTGWGDVHSGTLPPLPPFPLKQVKVPIVENSMDVQYHLGLST  
 MMCT1    TAEGGADVALLELEGPVnvsTHIHPISLPPASETFPPGTSCWVTGWGDIDNDEPLPPPYPLKQVKVPIVENSCLDRKYHTGLYT  
 MMCT2    IVQDGADIALLLKLTNPVnisDYVHPVPLPPASETFPSGTLCWVTGWGNIDNGVNLPPPFPLKEVQVPIIENHLCDLKYHKLIT  
 RST                    STNPVxitSNVHTVSLPPASETF                    GLNT

171                    181                    ♦                    191♥                    \*                    211                    221                    231                    241  
 BT    GQ    ITSNMFCAGYLEGGKDSCQGDGGPVVC    SGK LQ    GIVSWGSGCAQKNKPGVYTKVCNYVSWIKQTIASN  
 HLT $\alpha$     GDDVRIIRDDMLCAG    NSQRDSCQGDGGPLVCKVngtWLQAGVVSWEDEGCAQPNRPGIYTRVTTYLWDWIHHYVPKKP  
 HLT $\beta$     GDDVRIVRDDMLCAG    NTRRDSCQGDGGPLVCKVngtWLQAGVVSWEDEGCAQPNRPGIYTRVTTYLWDWIHHYVPKKP  
 HST1    GDDVRIVRDDMLCAG    NTRRDSCQGDGGPLVCKVngtWLQAGVVSWEDEGCAQPNRPGIYTRVTTYLWDWIHHYVPKKP  
 HST3    GDDVRIVRDDMLCAG    NTRRDSCQGDGGPLVCKVngtWLQAGVVSWEDEGCAQPNRPGIYTRVTTYLWDWIHHYVPKKP  
 DMT    GDGVRIVREDMLCAG    NSKSDSCQGDGGPLVCRVRGVWLQAGVVSWEDEGCAQPNRPGIYTRVAYYLDWIHQYVPKEP  
 MMCT1    GDDFPIVHDGMLCAG    NTRRDSCQGDGGPLVCKVngtWLQAGVVSWEDEGCAQPNKPGIYTRVTTYLWDWIHRYVPEHS  
 MMCT2    GDNVHIVRDDMLCAG    NEGHSDSCQGDGGPLVCKVEDTTLQAGVVSWEDEGCAQPNRPGIYTRVTTYLWDWIHHYVPKDF  
 RST    GDNVHIVRVDMLCAG    NEGHSDCZGDSGGP    VEDTTLQAGVVSWEDEGCAQPN                    VTTYLD

	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
HLT $\alpha$	86	78	58	80	100	90	82
HLT $\beta$	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; HLT $\alpha$ , human lung tryptase  $\alpha$  (Miller et al. 89); HLT $\beta$ , human lung tryptase  $\beta$  (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- $\alpha$  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 cleft, for instance, the amino acid at position 23 among the tryptases varies significantly. HST-1 and HLT- $\beta$  have a hydrophobic proline residue at the position. HST-3 and HLT- $\alpha$  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 HLT $\alpha$  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- $\beta$ . 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 secretagogue 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). Bronchial 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 occurred 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 (Goldsehidt 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 (Mammaiorni 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 (Piotrowski & 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  $\beta$ -adrenergic receptors (Marquardt & 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 & Udem 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, Stehens 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 secretion 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. 1992).

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 (Metcalf 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 extent, 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	PGD <sub>2</sub>
5-HT	Thromboxane
Dopamine	LTB <sub>4</sub>
Heparin	LTC <sub>4</sub>
Chondroitin sulfate	LTD <sub>4</sub>
Tryptase	LTE <sub>4</sub>
Chymase	PAF
Cathepsin G	Cytokines
Carboxypeptidase	TNF- $\alpha$
VIP	IL-1, 2, 3, 4, 5, 6, 10
SLM	MIP-1 $\alpha$
	MIP-1 $\beta$
	MCAF
	TCA3
	GM-CSF
	TGF- $\beta$
	IF $\gamma$
	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  $\alpha$  (TNF- $\alpha$ ); 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 chondroitin (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- $\alpha$  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- $\beta$ , IF $\gamma$ , 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 IF $\gamma$  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 released from the lipids and metabolized to PGD<sub>2</sub> 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 phosphatidylcholine 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<sub>T</sub> (human mast cells containing tryptase only) and MC<sub>TC</sub> (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<sub>TC</sub> 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<sub>TC</sub> 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<sub>TC</sub> type is not T lymphocyte dependent.

Mast cells from different sources often respond differently to specific agents. Secretagogues 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 chondroitin 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). PGD<sub>2</sub> is produced predominantly by rat peritoneal mast cells but is consistently produced by both human skin and lung mast cells. LTC<sub>4</sub> and LTB<sub>4</sub> 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 LTD<sub>4</sub> and LTE<sub>4</sub> in human lung mast cells as opposed to LTC<sub>4</sub> 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 MC<sub>TC</sub> but not in MC<sub>T</sub> using immunoassay techniques (Meier et al. 1985). Carboxypeptidase was found at high concentration in MC<sub>TC</sub> type from human skin mast cells but in negligible amounts in MC<sub>T</sub>

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<sub>T</sub> or the MC<sub>TC</sub> (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<sub>T</sub> mast cells which contains only tryptase while the lattice or grating feature is characteristic of MC<sub>TC</sub> 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 chondroitin 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  $\pi$  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 isothiuronium 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 chondroitin sulfate does not bind safranin O well. Together with alcian blue, safranin 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 (Metcalf et al. 1979, Metcalf 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<sub>T</sub> type which contains tryptase only, and the MC<sub>TC</sub> 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<sub>TC</sub> cells contained both tryptase and chymase and that the MC<sub>T</sub> cell granules had only tryptase (Craig et al. 1988). Subsequent studies based on enzyme activity assay, mediator contents, and the response of the MC<sub>T</sub> and MC<sub>TC</sub> 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<sub>T</sub> type while the submucosal mast cells are MC<sub>TC</sub> 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<sub>T</sub> type was

detected from the analysis of gastrointestinal tissues of the patients while the MC<sub>TC</sub> type was virtually not affected (Irani et al. 1987a). In synovium of rheumatoid arthritis subjects, where heavy lymphocyte infiltration occurs, a mixture of MC<sub>T</sub> and MC<sub>TC</sub> types were found while usually only MC<sub>TC</sub> type is present in the normal synovium (Irani et al. 1987b). These observation indicate that activity of MC<sub>T</sub> 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<sub>T</sub> and MC<sub>TC</sub> 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 distinguished 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<sub>2</sub>O<sub>2</sub> (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  $\mu$ , while those for skin was 5  $\mu$ . Rat peritoneal cells were

collected from the lavage with PBS solutions (Appendex E) and subjected to cytocentrifugation. Each slide contained  $2 \times 10^4$  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 ( $4 \times 10^7$  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  $\mu$ l) 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  $\mu$ l of acetone twice. After air drying, the pellet was resuspended in 5  $\mu$ l of the SDS-PAGE sample buffer (Appendix B).

The crude extracts were assayed for trypsin and chymase activities. The trypsin assay reaction contained 850  $\mu$ l of the trypsin assay buffer (Appendix A), 50  $\mu$ l of the crude extract, and 100  $\mu$ l of 10 mM BAEE (Appendix A). The reaction was monitored at 253 nm for 5 minutes. The amount of trypsin was estimated using a specific activity of 160 units/mg protein in the formula presented in Part I. The chymase reaction contained 500  $\mu$ l of the chymase assay buffer (Appendix D), 33  $\mu$ l of the crude extract, and 467  $\mu$ l 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  $\mu$ l of the sample and 200  $\mu$ l of the reagents. Five BSA concentrations of 25, 100, 200, 300, and 500  $\mu$ g/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  $\mu\text{g}$  of the protein extracts or purposely over-loaded with 30  $\mu\text{g}$  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  $\mu\text{m}$ ), 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 PhastTransfer 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 peeled 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<sub>2</sub>O<sub>2</sub> 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  $\mu\text{m}$ ) and skin (5  $\mu\text{m}$ ) 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 abdomen 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  $2 \times 10^4$  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<sub>2</sub>O 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<sub>2</sub>O<sub>2</sub>/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  $\mu\text{g/ml}$  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<sub>2</sub>O<sub>2</sub> 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  $\mu\text{g/ml}$  was diluted into 1.6, 3.2, and 10  $\mu\text{g/ml}$  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<sub>2</sub>O<sub>2</sub> (Appendix F) as substrate for the color development. Cells stained red by safranin would turn dark brown after the DAB/H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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  $\mu\text{g}/\text{ml}$  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<sub>2</sub>O<sub>2</sub> 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  $\mu\text{g}/\text{ml}$  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 didydim filter and a TIFFEN 49 mm, 82 °C filter were used. For phase contrast, only the didydim 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  $\mu\text{m}$  was used as an estimate of the average mast cell diameter and 4  $\mu\text{m}$  was the tissue thickness for lung and bowel;

5  $\mu\text{m}$  was used for the thickness of skin.

## CHAPTER IX

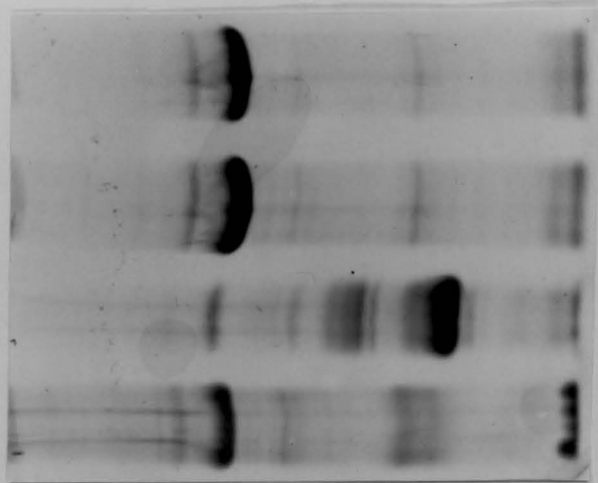
### RESULTS

#### 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  $\mu\text{g}$  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  $\mu\text{g}$  of the crude protein extract from rat peritoneal lavage cells. Lanes 4 and 5 contained 20  $\mu\text{g}$  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  $\mu\text{g}$  protein/lane), peritoneal cells (lanes 3 and 6, 20  $\mu\text{g}$  protein/lane) and lung (lane 4 and 5, 20  $\mu\text{g}$  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),  $\alpha$ 1-trypsin inhibitor (20,100), and  $\alpha$ -lactalbumin (14,400) (Pharmacia).

1 2 3 4 5 6 7 8



94,000 -  
67,000 -  
43,000 -  
30,000 -  
20,100 -  
14,400 -

...ain ... an ... tryptase  
ant ... No staining was observed using preimmune goat IgG  
a negative control. Tryptase-containing cells were most  
abundant in lung, where they were distributed in a

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 cytopsin 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

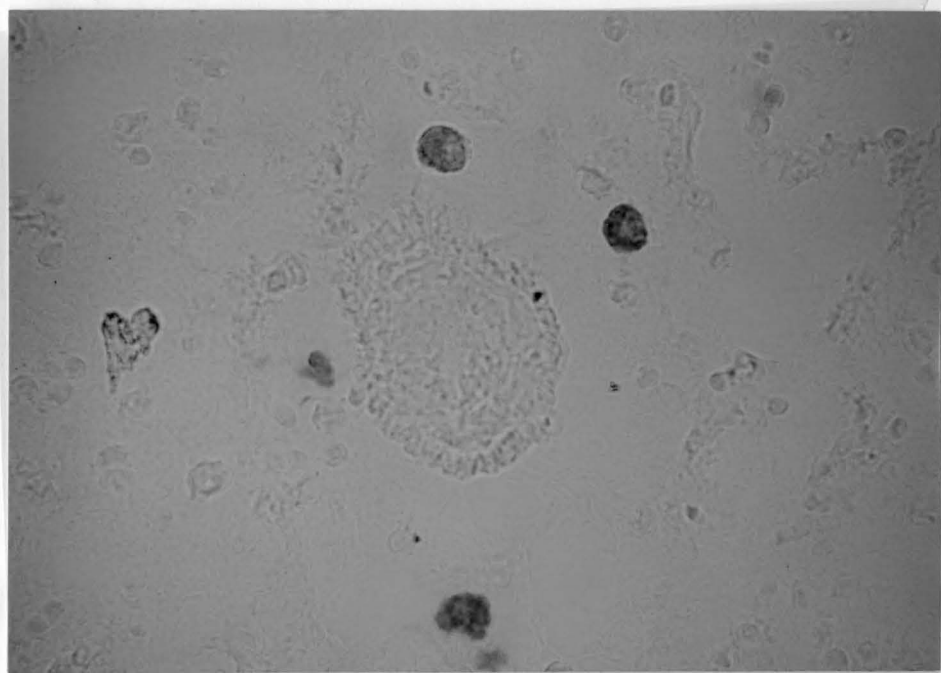
peribronchial location. Among peritoneal cells they accounted for only 20% of the cells. In skin, tryptase-positive cells were detected in the dermis and below the hypodermis, but at concentrations that appeared to be less than for mast cells detected histochemically. In bowel mucosa there were essentially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

### 3. Rat Tryptase Is Localized In Mast Cells

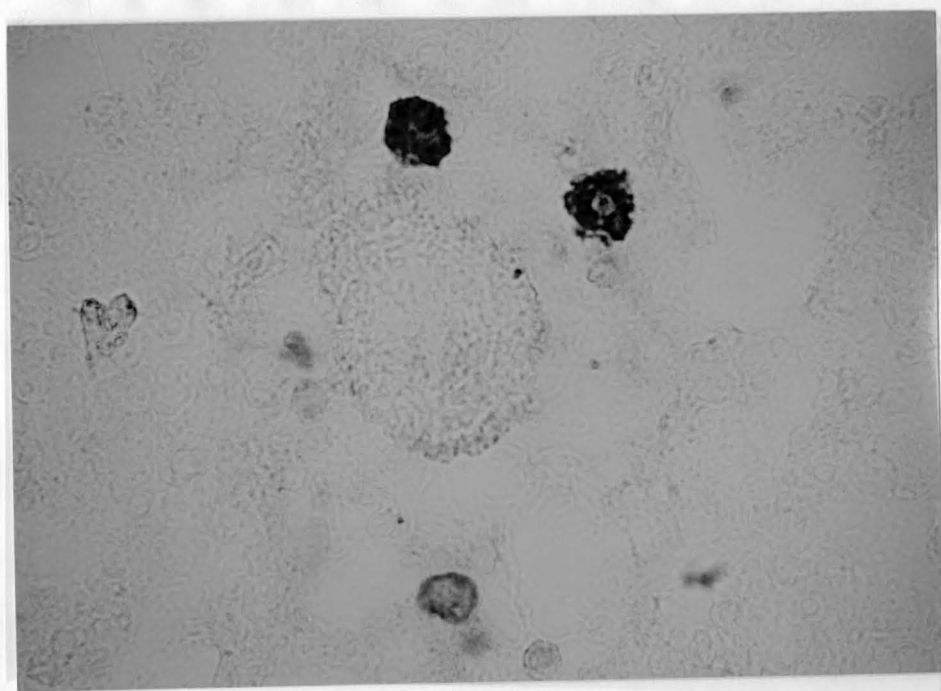
To determine whether tryptase-containing cells were mast cells and only mast cells, sequential staining with Alcian blue and anti-tryptase antibody was performed. For example, in a section of rat lung, among 4 Alcian blue-stained mast cells (Figure 13A), two stained positive for tryptase (Figure 13B). In these double labelling experiments, the concentration of mast cells in peribronchial tissue was about  $15 \times 10^3$  cells/mm<sup>3</sup>,  $63 \pm 8\%$  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  $7 \pm 4\%$  of the  $882 \pm 136$  mast cells/mm<sup>3</sup> in the dermis (Table 3). A cluster of 7 mast cells obtained from the



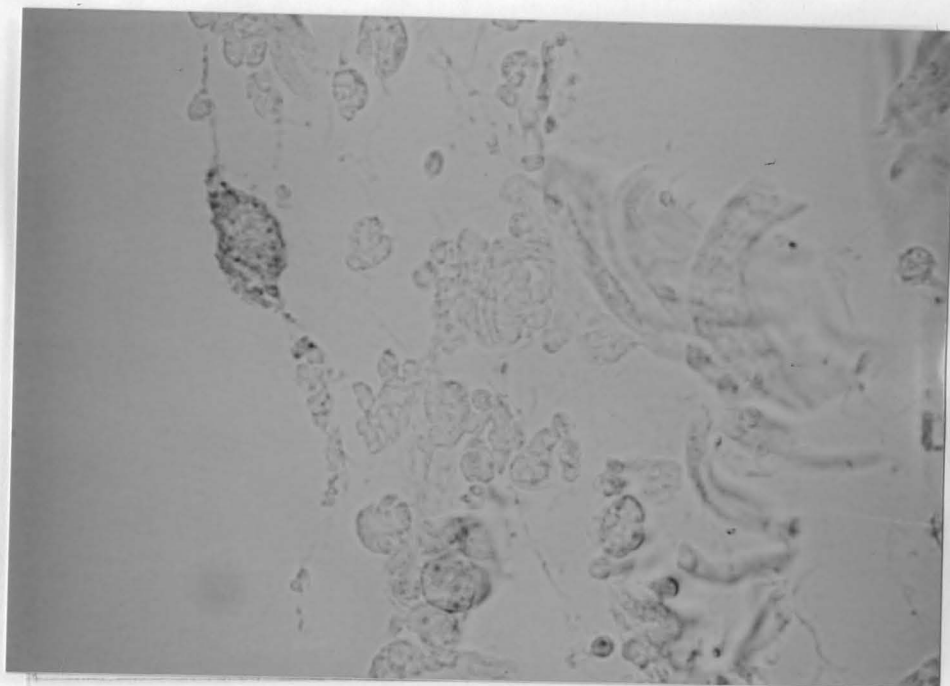
Figure 13. Sequential Staining With Alcian Blue And Anti-Tryptase Antibody. Tissue sections from lung (A, B), skin (C, D), a cytospin of peritoneal cells (E, F), and bowel (G, H) were stained first with alcian blue (A, C, E, G) and then with goat IgG anti-tryptase/oxidase (brown) (B, D, F, H). Identical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



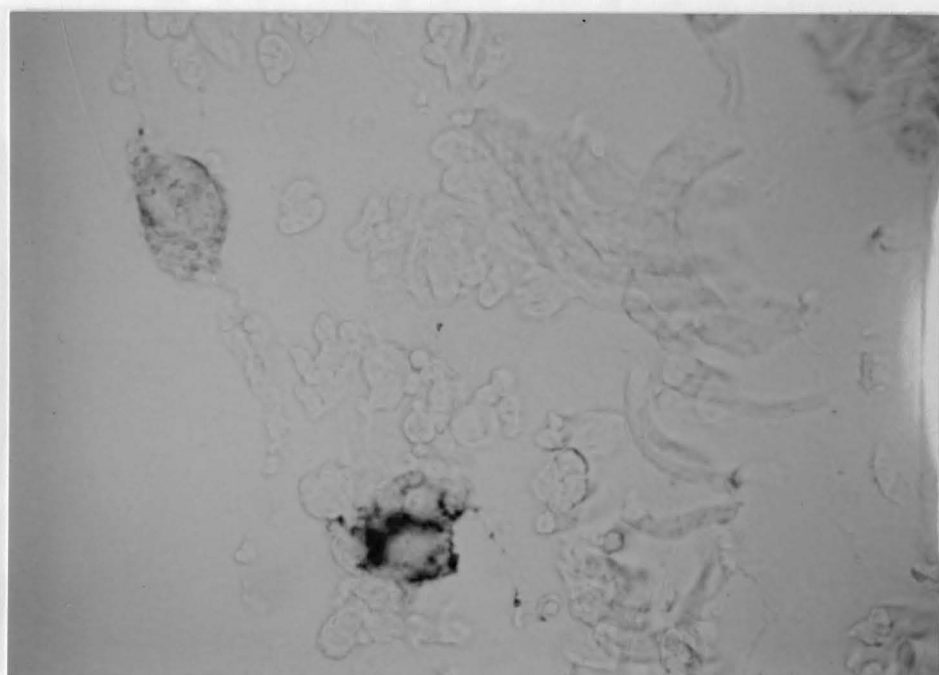
A



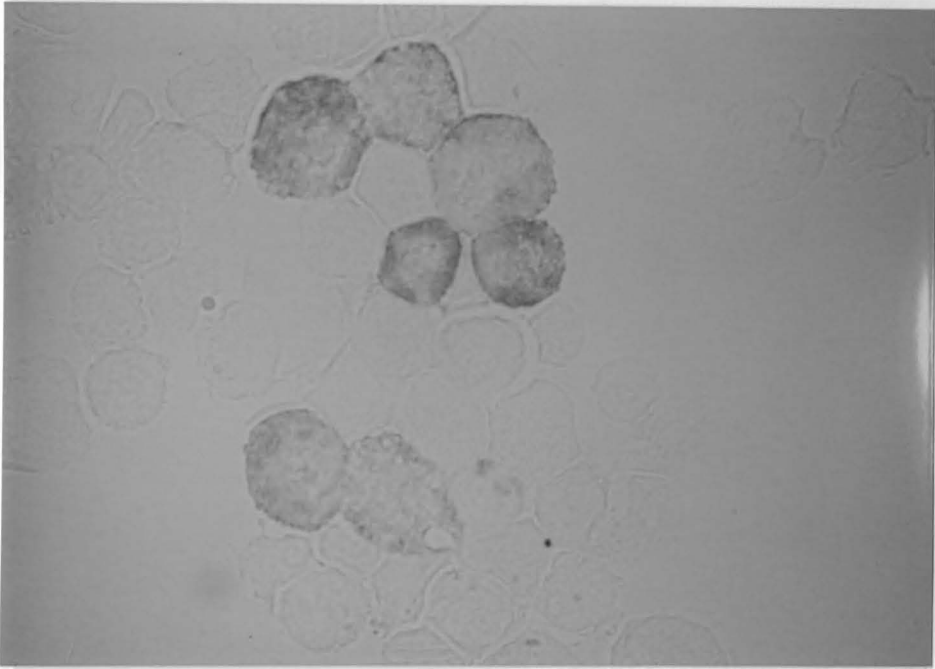
B



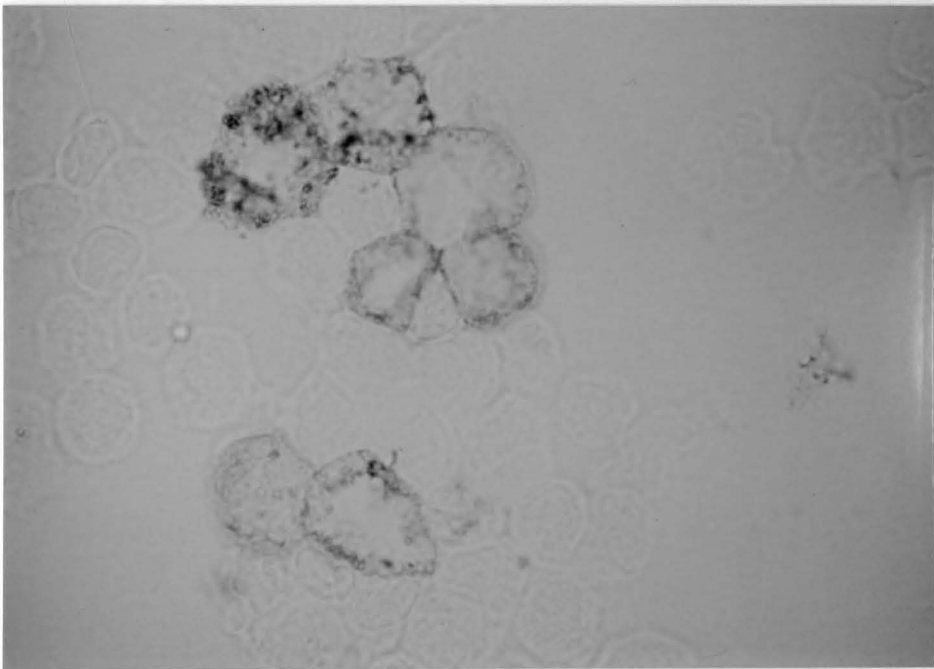
C



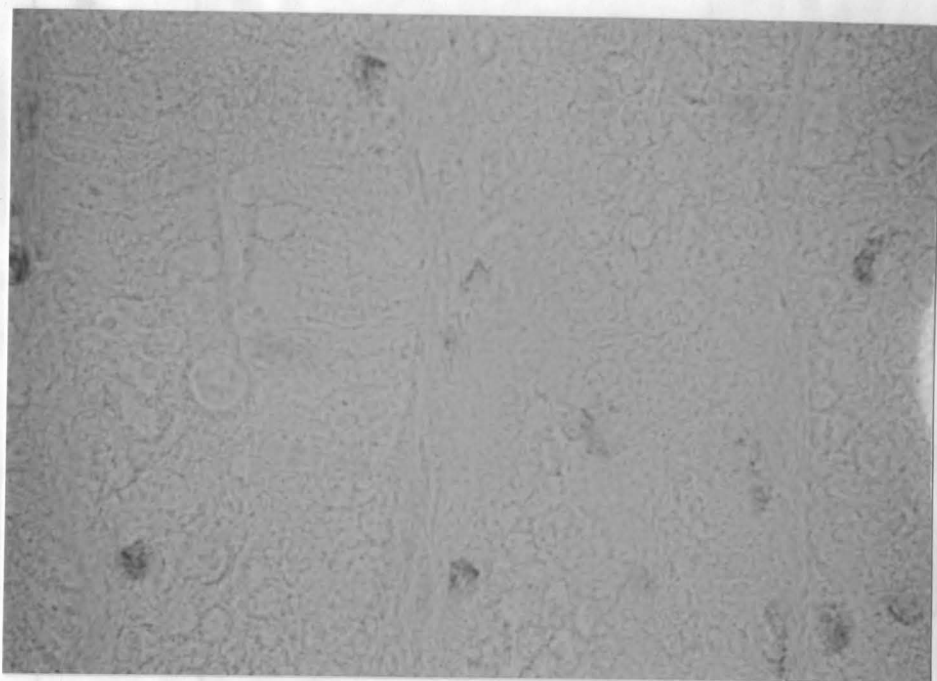
D



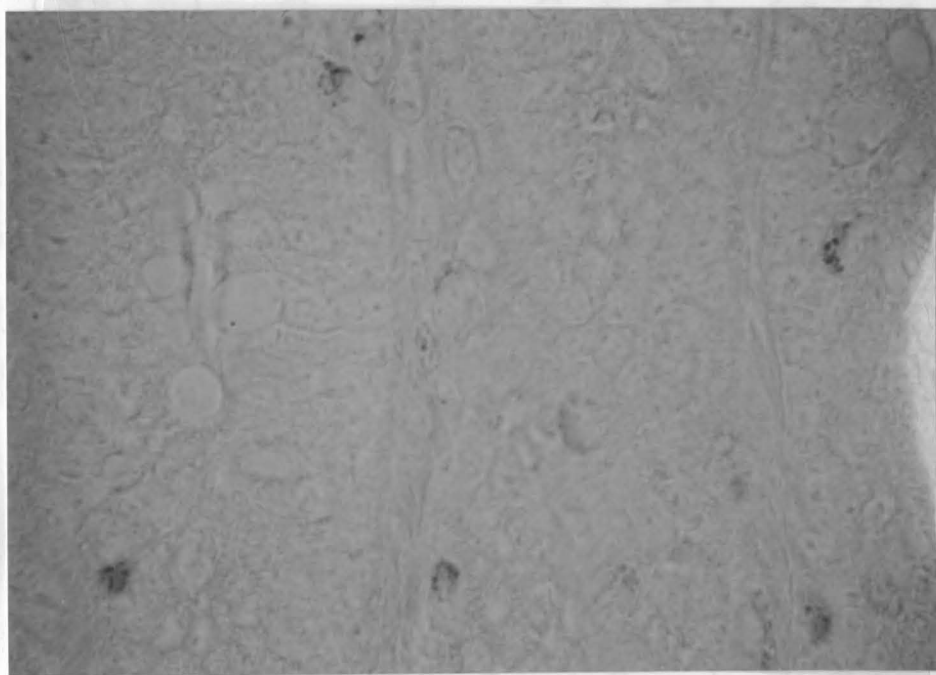
E



F



G



H

Cell Source	MC/mm <sup>3</sup> ± SD (n)	%Safranin <sup>-</sup> MC %Safranin <sup>+</sup> MC ± SD (n)	Tryptase <sup>+</sup> x 100 Alcian Blue <sup>+</sup> ± SD (n)	Tryptase <sup>+</sup> x 100 Safranin <sup>+</sup> ± 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<sup>+</sup> mast cells among 1600 Safranin<sup>+</sup>/Alcian Blue<sup>+</sup> mast cells. Areas of tissues examined were 2.69 mm<sup>2</sup> for peribronchus, 39.9 mm<sup>2</sup> for dermis, and 17.0 mm<sup>2</sup> for bowel mucosa. Sequential staining with alcian blue and safranin was used to determine %Safranin<sup>-</sup> and %Safranin<sup>+</sup> 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 ( $32,264 \pm 22,219$  cells/mm<sup>3</sup>) (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

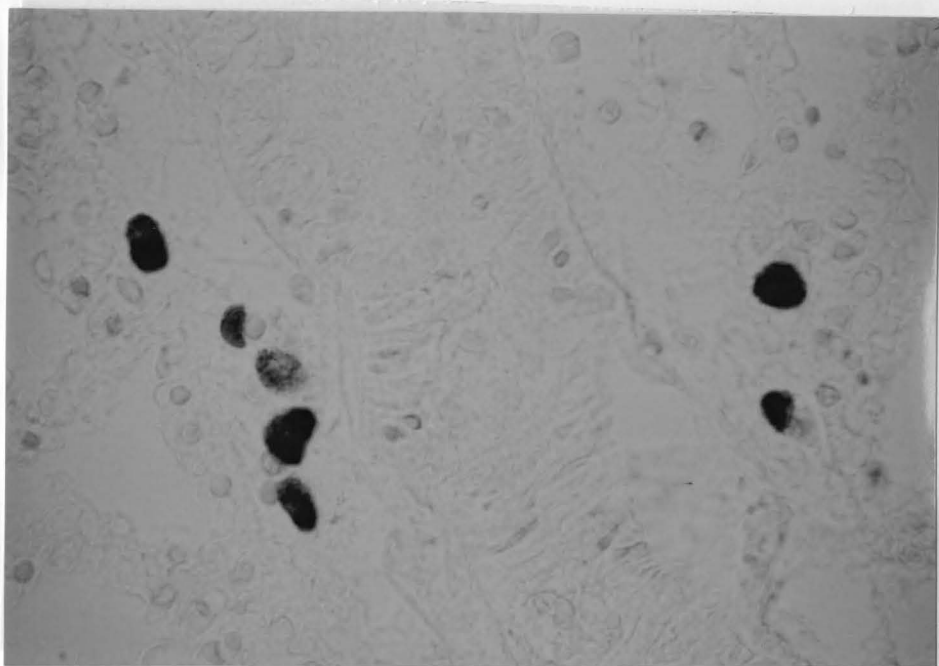
and then with anti-rat tryptase. The results revealed that all tryptase-positive cells were safranin-positive, but not all safranin-positive cells contained tryptase. For lung, Figure 14A shows 7 safranin-positive mast cells, six of which contained tryptase (Figure 14B). In rat lung, over 90% of the 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

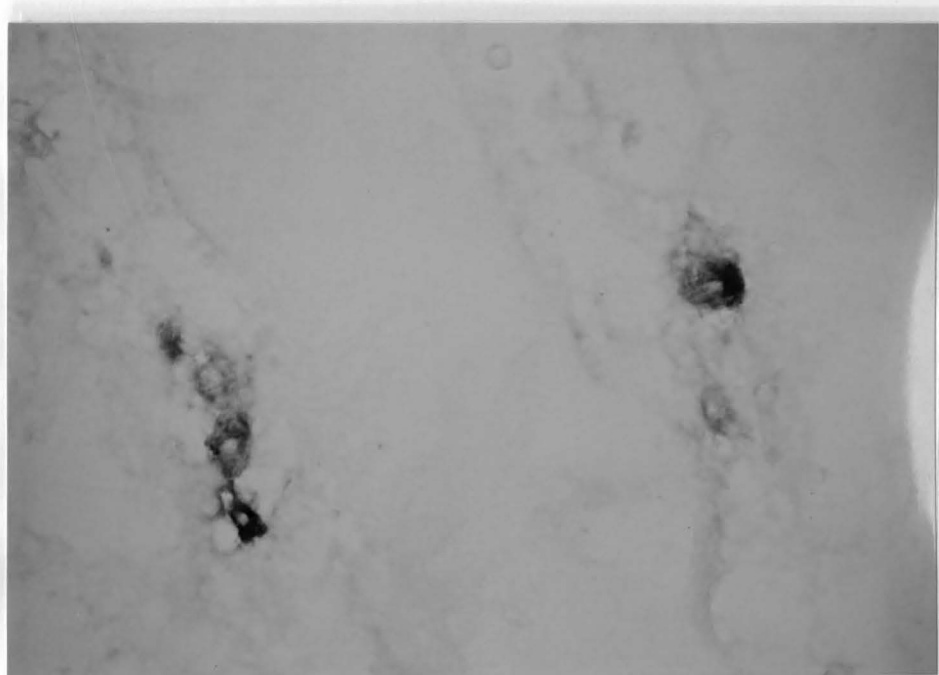
Simultaneous double-labeling experiments were performed to determine whether tryptase-positive cells are also chymase I-positive. Figure 15A shows a section of lung which had 6 blue tryptase-positive cells, all of which



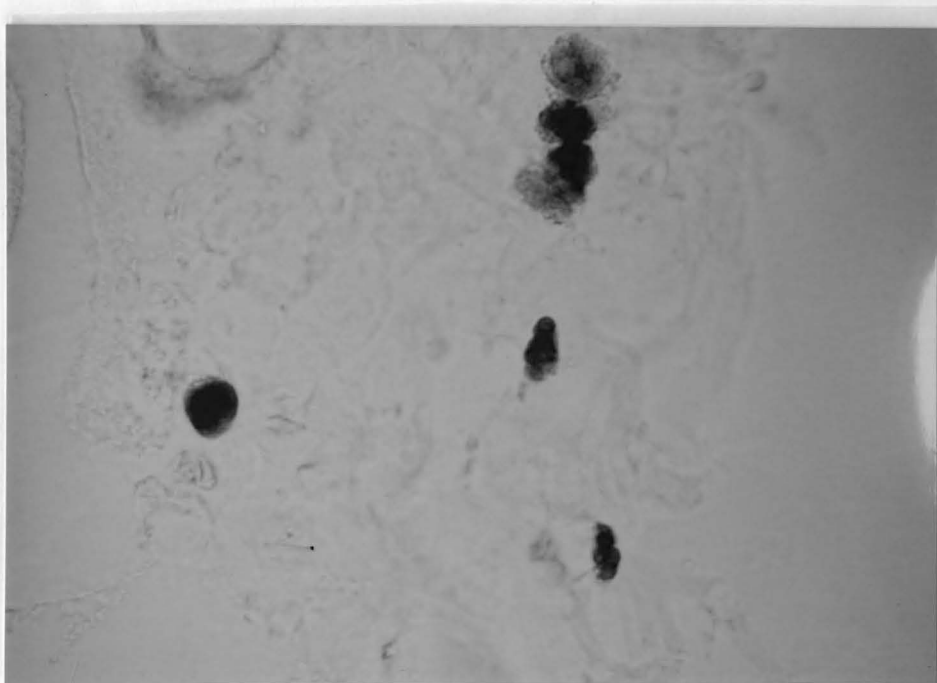
Figure 14. Sequential Staining With Safranin And Anti-Tryptase Antibody. Tissue sections from lung (A, B) and skin (C, D), and a cytospin of peritoneal cells (E, F) were stained first with safranin (red) (A, C, E) and then washed and stained with goat IgG anti-tryptase/oxidase (brown). Identical fields 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.



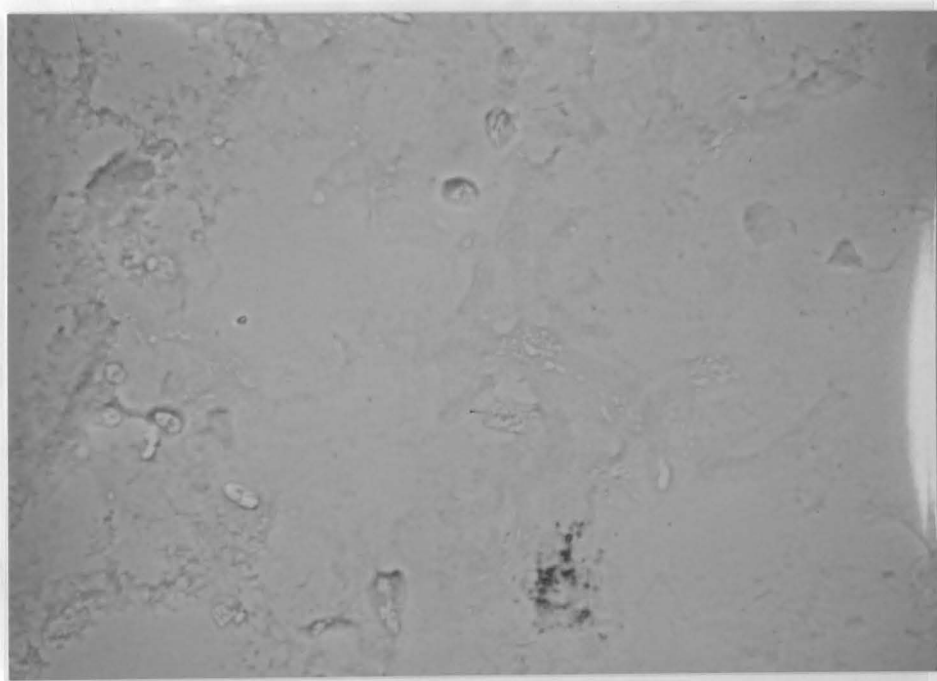
A



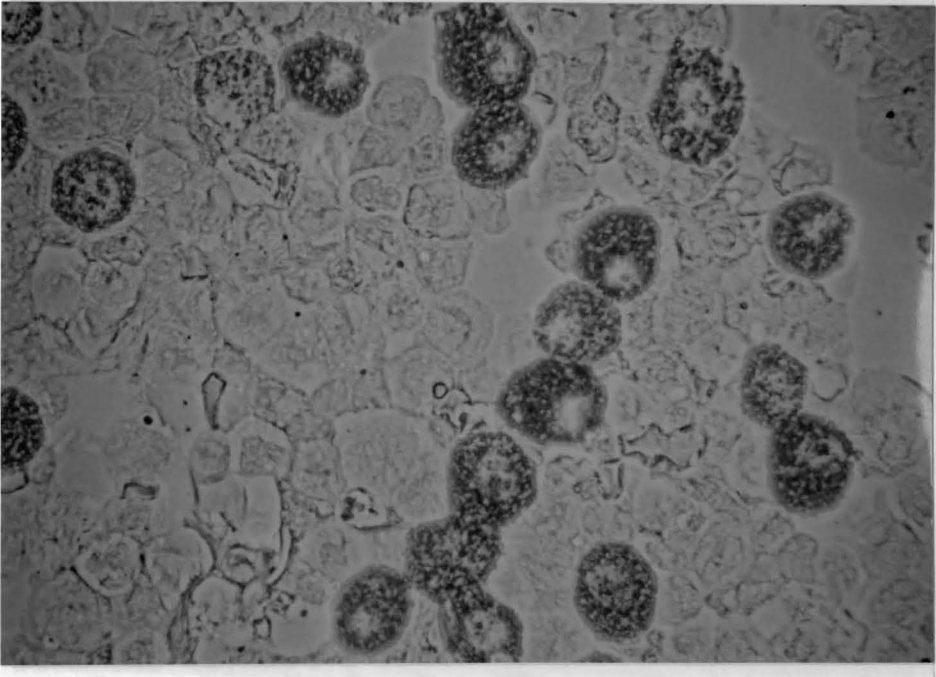
B



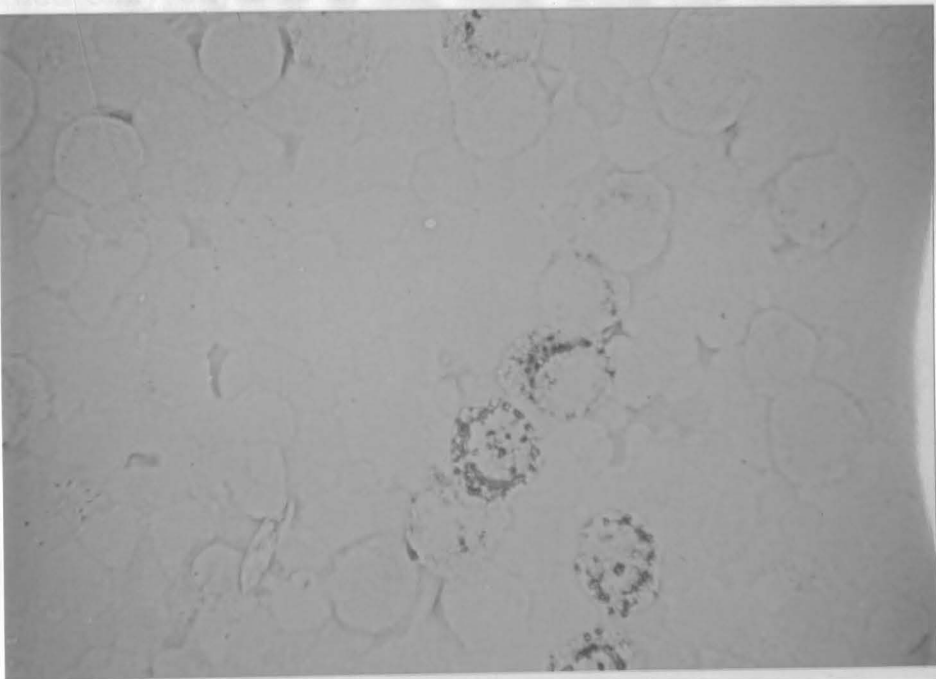
C



D



E

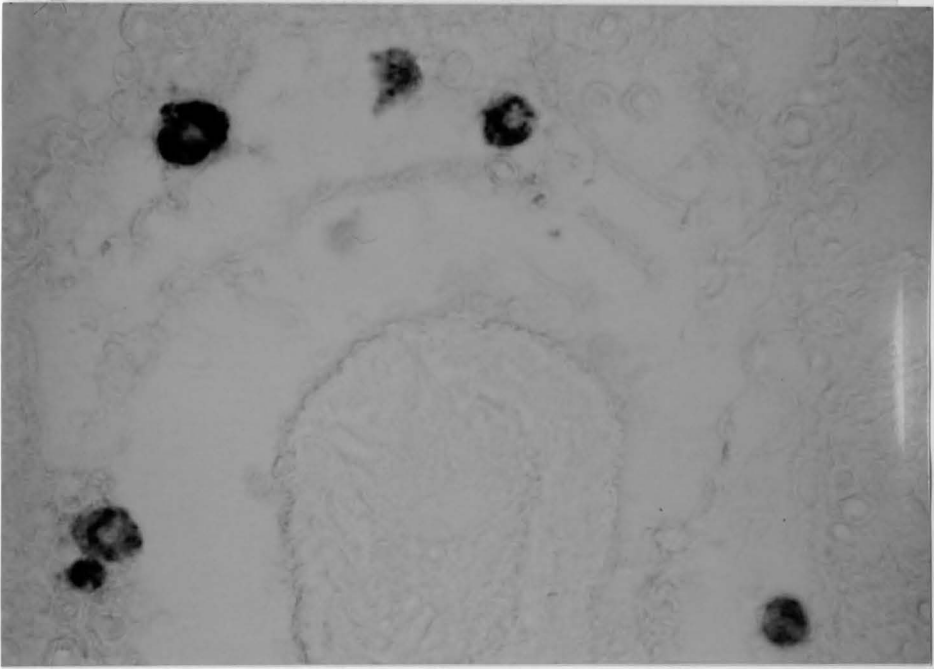


F

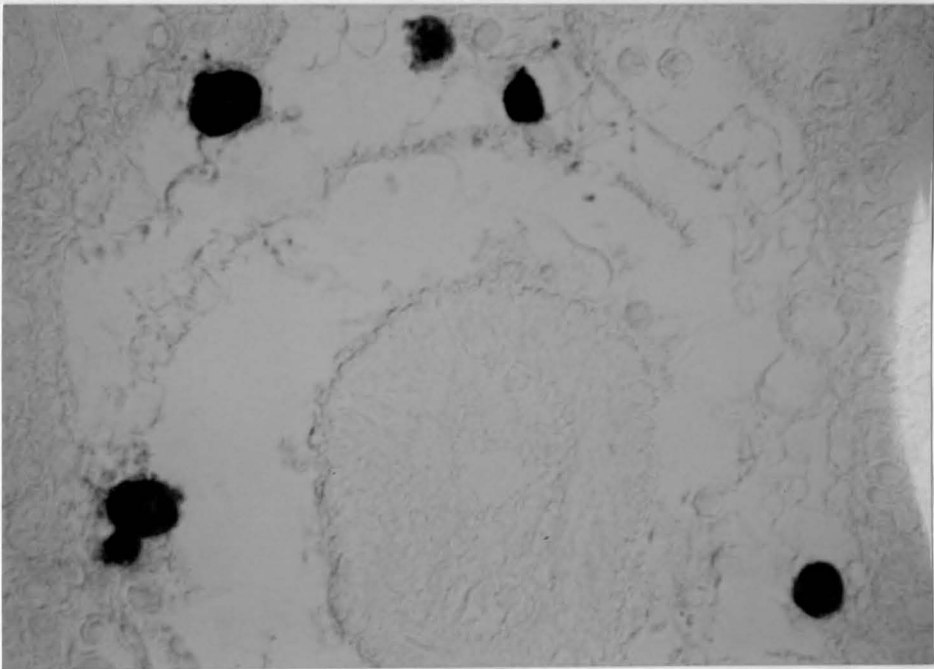
became dark brown (Figure 15B), indicating the co-localization of tryptase and chymase I in these cells. Of the lung mast cells which contained either tryptase, chymase I, or both, 77% contained both proteases, 4% contained only tryptase and 19% contained only chymase I (Table 4). Whether the small population 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

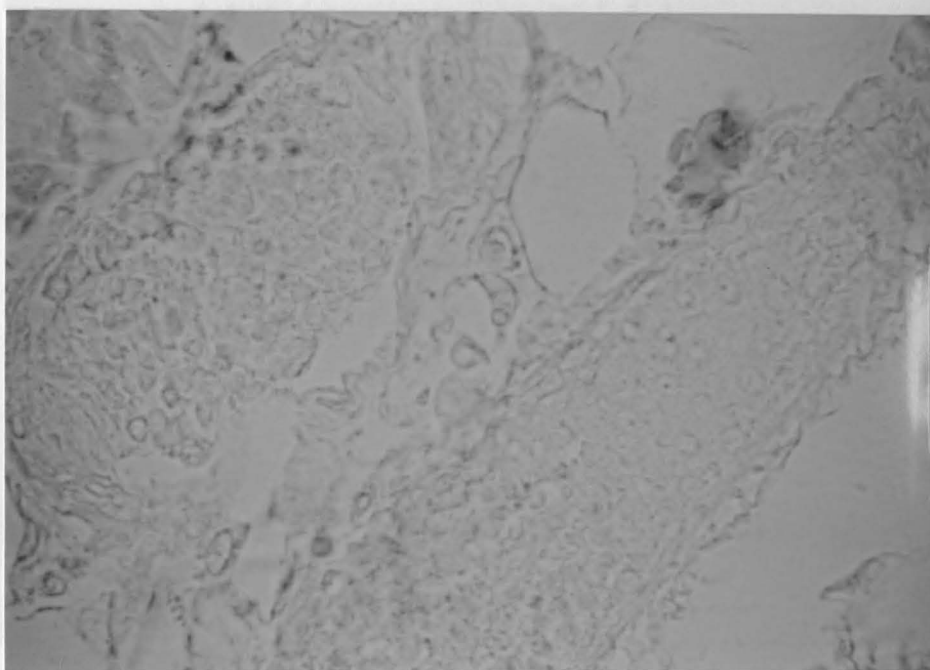
Figure 15. Simultaneous Double Labeling With Anti-Tryptase And Anti-Chymase I Antibodies. Sections of lung (A, B) and skin (C,D), and a cytopsin of peritoneal cells (E, F) (phase contrast) were incubated simultaneously with goat IgG anti-tryptase and rabbit IgG anti-chymase I antibodies. Sections were 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.



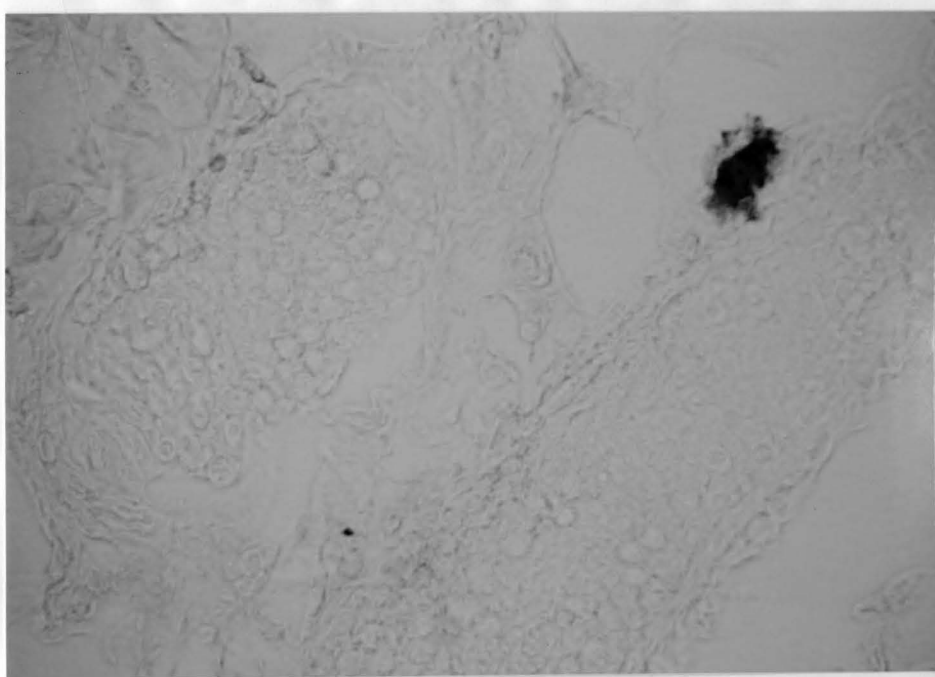
A



B

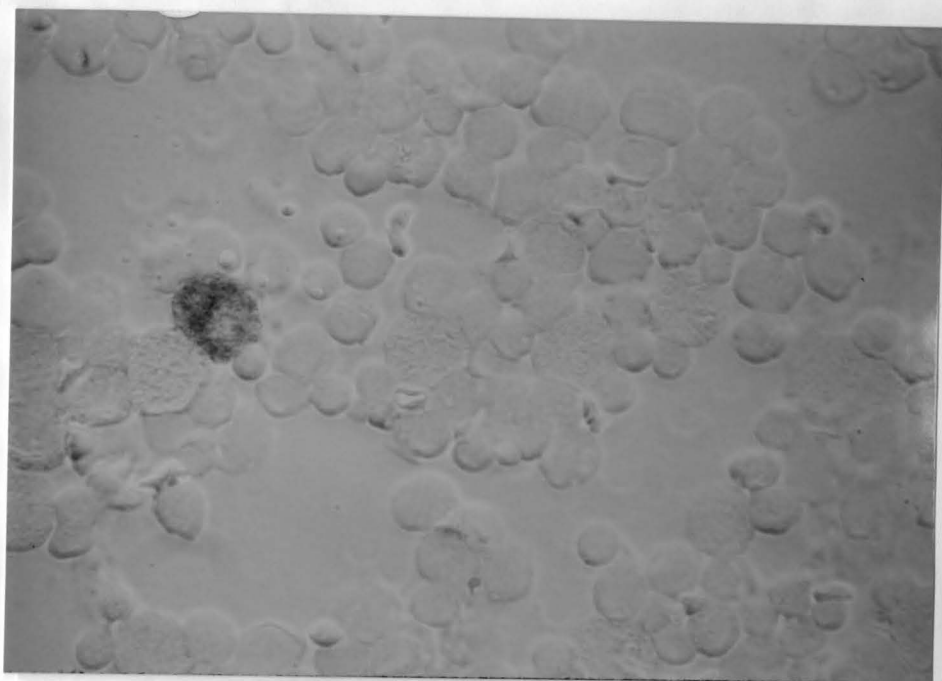


C

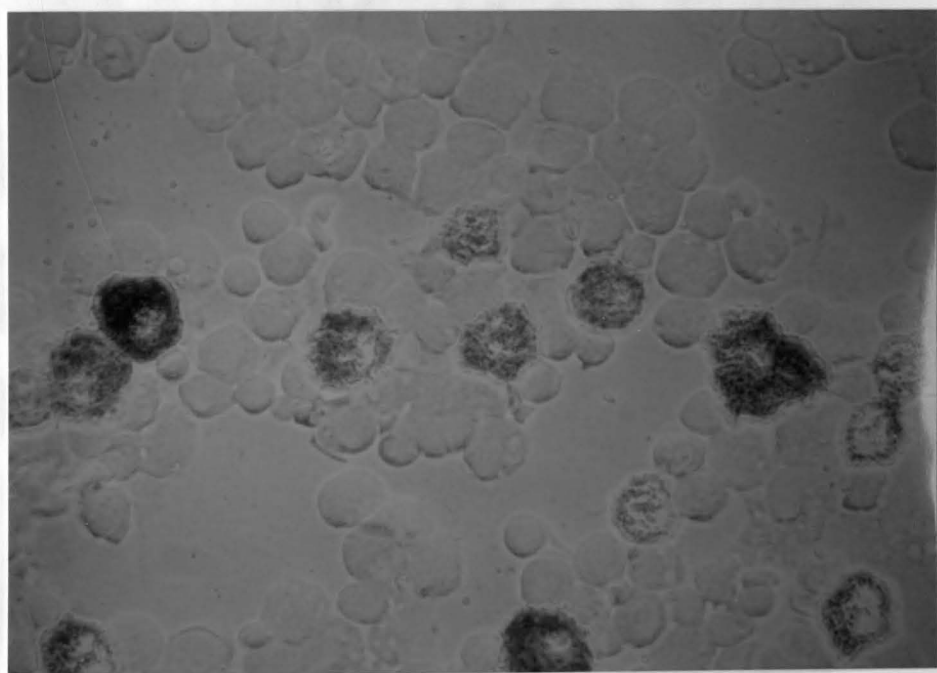


D





E



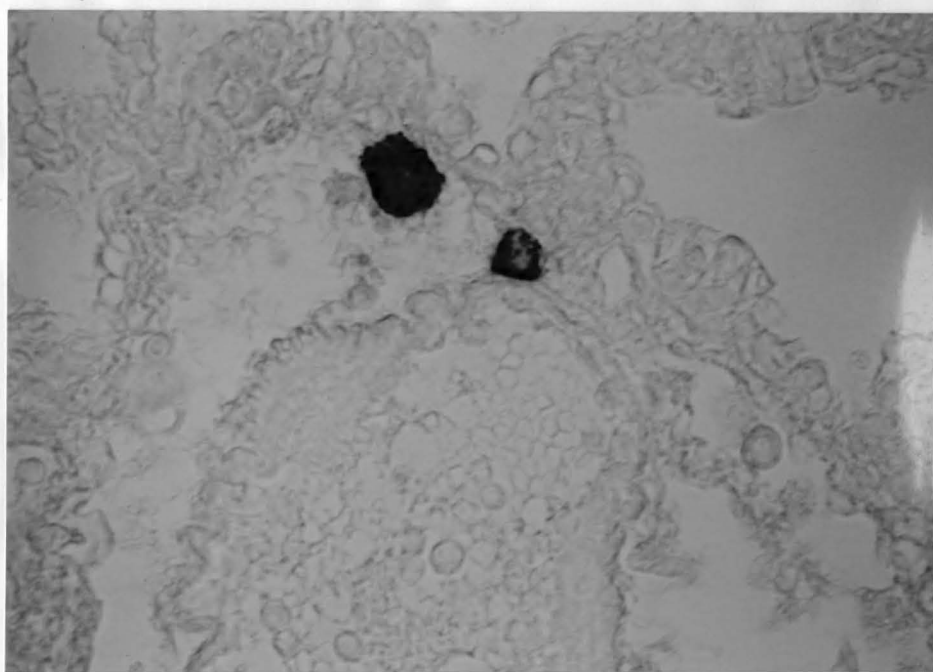
F

Cell Source	Cells* (n)	<u>Tryptase<sup>+</sup> x 100</u> Chymase I <sup>+</sup> ± SD	<u>Tryptase<sup>+</sup> x 100</u> Chymase I <sup>-</sup> ± SD	<u>Tryptase<sup>-</sup> x 100</u> Chymase I <sup>+</sup> ± 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

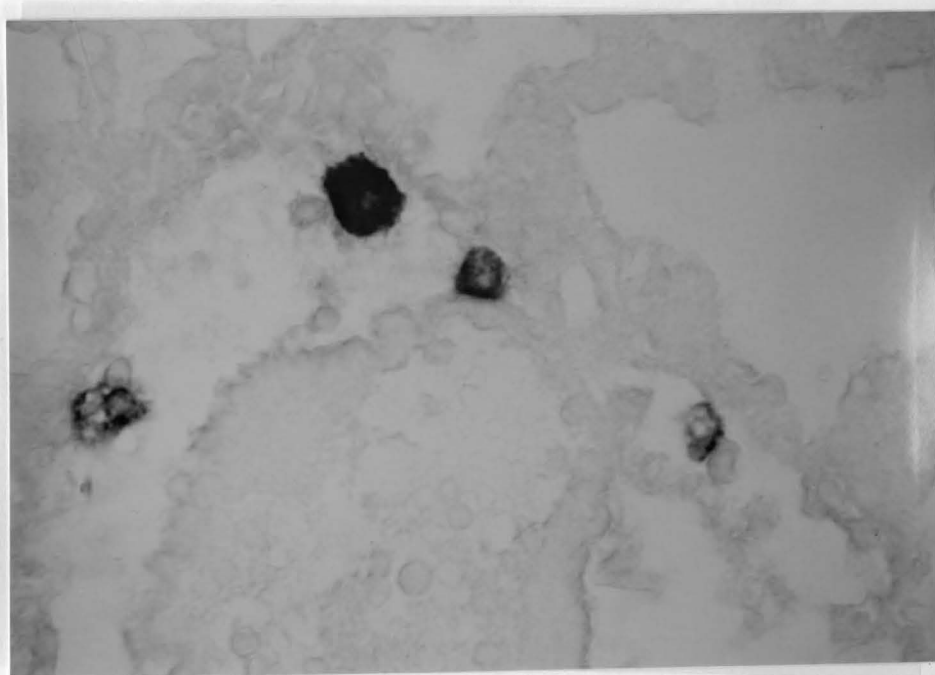
Table 4. Simultaneous Labeling Of Tryptase And Chymase I. This table shows the results of the simultaneous double labeling of mast cells with anti-tryptase and anti-chymase I antibodies. SD, standard deviation; n, number of tissues used in the experiment; \*, number of cells counted per section or slide.

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  $84 \pm 7\%$  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  $7 \pm 1\%$  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  $13 \pm 7\%$  of the chymase I-positive cells in peritoneal lavage fluid are also tryptase-positive (Table 5).

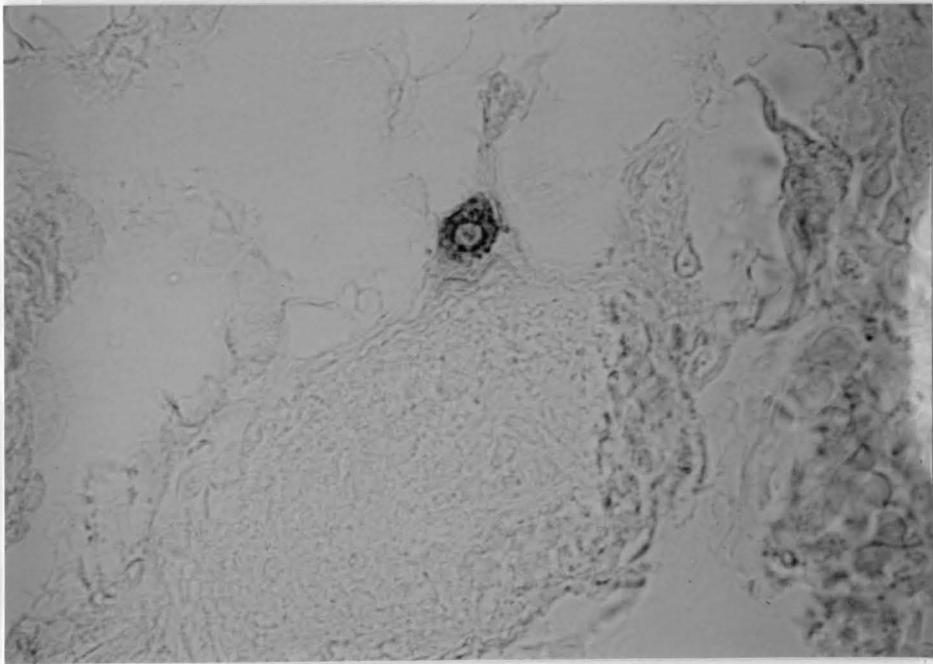
Figure 16. Sequential Double Labeling With Anti-Tryptase And Anti-Chymase I Antibodies. Sections of lung (A, B) and skin (C, D), and a cytospin of peritoneal cells (E, F) were incubated first with goat IgG anti-tryptase and developed with peroxidase-conjugated swine anti-goat IgG (brown) (A, C, E). Sections 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.



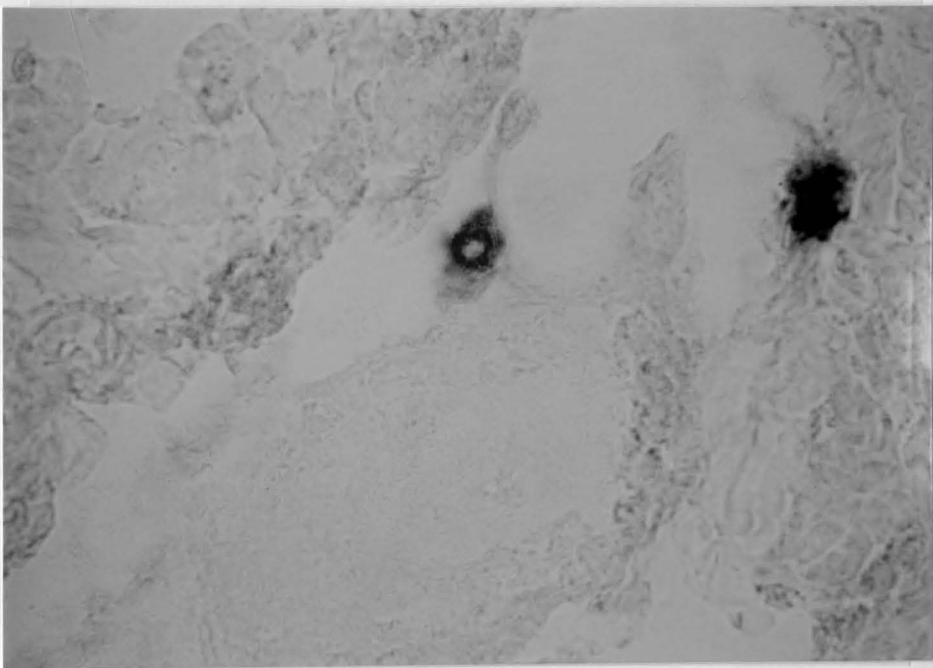
A



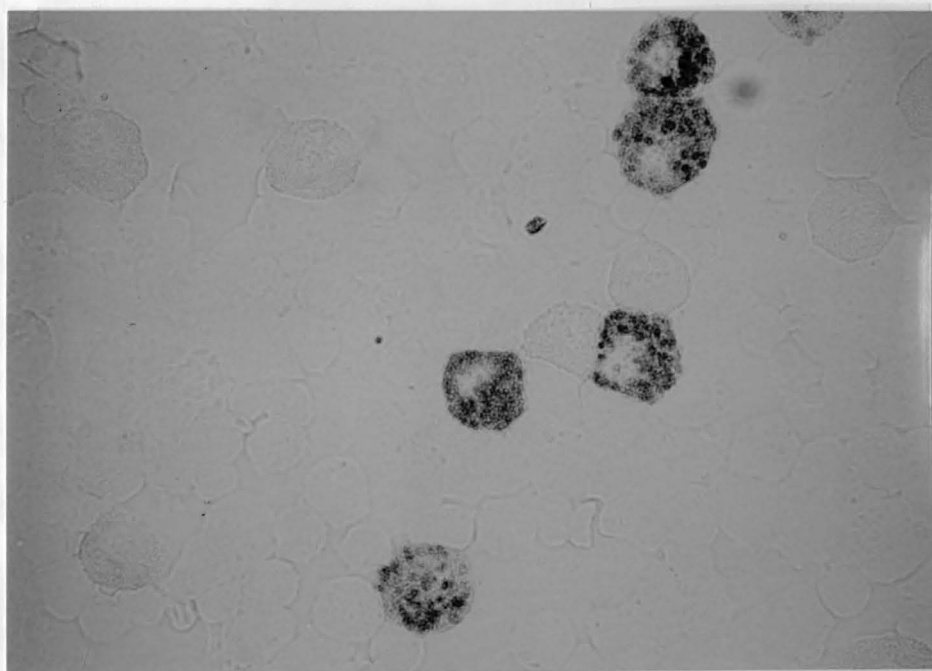
B



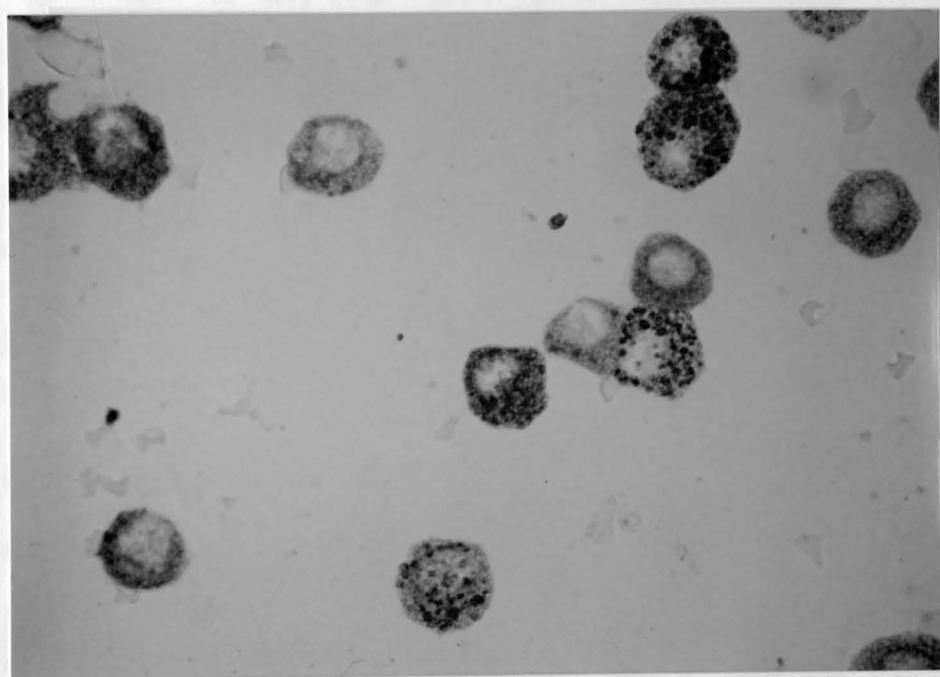
C



D



E



F

Cell Source	Cells* (n)	$\frac{\% \text{Tryptase}^+}{\% \text{Chymase I}^+} \pm \text{SD}$ **
Peribronchus	40-50 (4)	84/16 $\pm$ 7
Dermis	156-412 (4)	7/93 $\pm$ 9
Peritoneal	105-264 (12)	13/87 $\pm$ 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<sup>+</sup>/Tryptase<sup>+</sup> 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 called 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 heterogeneity 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 only 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  $\text{NaN}_3$ , and 29.2 g of  $\text{NaCl}$ , 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  $\text{NaCl}$ , and 0.02% (w/v) of  $\text{NaN}_3$ .

**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  $\text{NaCl}$ , 0.02%  $\text{NaN}_3$ , and 0.1 M benzamidine, pH 6.

**Con-A Activation Buffer:**

Dissolve 6.8 g of sodium acetate, 10.2 g  $\text{MgCl}_2$ , 29.2 g of  $\text{NaCl}$ , 555 mg of  $\text{CaCl}_2$ , and 100 mg of  $\text{NaN}_3$ ; adjust to pH 6, and bring to 500 ml in MQ water. The final solution contains 100 mM of sodium acetate, 100 mM of  $\text{MgCl}_2$ , 1 M of  $\text{NaCl}$ , 10 mM of  $\text{CaCl}_2$ , and 0.02% of  $\text{NaN}_3$ .

**Con-A Elution Buffer:**

Dissolve 10 g of  $\alpha$ -methyl-D-mannoside in 100 ml of the PAB equilibration buffer. The final solution contains 25 mM of sodium phosphate, 0.5 M of  $\text{NaCl}$ , 0.02% of  $\text{NaN}_3$ , 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  $\mu$ l 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  $\mu$ l of 10 mM/ml DTT), and 0.01% of Bromophenol Blue (2  $\mu$ l 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%  $\text{Na}_2\text{CO}_3$  stock: Dissolve 25 g of  $\text{Na}_2\text{CO}_3$  in 200 ml of MQ water. Prior to the experiment, mix 50 ml of the 12.5%  $\text{Na}_2\text{CO}_3$  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):

Dissolve 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- [<sup>3</sup>H]Acetic Acid Solution:

Dissolve 1 mCi of the iodo[2-<sup>3</sup>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  $\mu$ l of 0.5 N NaOH to 134 mg/ml.

### 6 N HCl Solution:

Add 500  $\mu$ l of the concentrated HCl slowly to 500  $\mu$ l of MQ water under the hood.

### 100 mM Ammonium Bicarbonate Buffer:

Dissolve 3.95 g of  $\text{NH}_4\text{HCO}_3$  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<sub>2</sub>, adjust to pH 7.8 and bring to 500 ml with MQ water. The solution contains 80 mM Tris and 100 mM CaCl<sub>2</sub>.

### 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  $\mu$ l of the peroxidase or alkaline phosphatase conjugated secondary antibody in 15 ml of the 0.5% BSA/TBS.

AEC/H<sub>2</sub>O<sub>2</sub> 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 contains 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<sub>2</sub>HPO<sub>4</sub> and 0.24 g KH<sub>2</sub>PO<sub>4</sub> 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<sub>2</sub>O<sub>2</sub>/Methanol Solution:

Add 0.8 ml of 30% H<sub>2</sub>O<sub>2</sub> to 40 ml of methanol. The solution which contains 0.6% of H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O 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<sub>2</sub>O<sub>2</sub>: Mix 29 ml MQ water with 1 ml of the 30% commercial H<sub>2</sub>O<sub>2</sub> stock.

The AEC/H<sub>2</sub>O<sub>2</sub> solution is prepared prior to the reaction by adding 10 μl of the 1% H<sub>2</sub>O<sub>2</sub> to the 1 ml AEC.

**DAB/H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub>: Mix 29 ml MQ water with 1 ml of the 30% commercial H<sub>2</sub>O<sub>2</sub> stick.

Add 10  $\mu$ l 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.



## REFERENCES

- Allansmith, MR, Bloch, KJ, Baird, RS, and Sinclair, K (1981) Ocular anaphylaxis: induction by local injection of antigen *Immunol.* 44:623-627
- Allansmith, MR, Baird, RS, and Ross, RN (1989) Morphologic evidence that compound 48/80-challenged rat eyelid mast cells differ in their state of maximal degranulation *Ophthalmic Res.* 21:206-215
- Allansmith, MR, Baird, RS, and Block, KJ (1985) Anaphylaxis of ocular adnexa induced by injection of anti-IgE antibody *Expl. Eye Res.* 40:797-804
- Alm, PE and Bloom, GD (1981) What-if any-is the role of adrenergic mechanisms in histamine release from mast cells? *Agents & Actions* 11:60-66
- Aloe, L (1988) The effect of nerve growth factor and its antibody on mast cells in vivo *J. Neuroimmunol.* 18:1-12
- Aloe, L and DeSimone, R (1989) NGF primed spleen cells injected in brain of developing rats differentiated into mast cells *Int. J. Dev. Neurosci.* 7:565-573
- Aloe, L (1977) Mast cells increase in tissues of neonatal rats injected with nerve growth factor *Brain Res.* 133:358-366
- Alter, SC, Metcalfe, DD, Bradford, TR, and Schwartz, LB (1987) Regulation of human mast cell tryptase. Effects of enzyme concentration, ionic strength and the structure and negative charge density of polysaccharide *Biochem. J.* 248:821-827
- Alving, K, Sundstrom, C, Matran, R, Panula, P, Hokfelt, T and Lundberg, JM (1991) Association between histamine-containing mast cells and sensory nerves in the skin and airways of controls and capsaicin-treated pigs *Cell Tissue Res.* 264:529-538
- Andre, F, Andre, C, and Cavagma, S (1985) Role of histamine in the cell turnover changes associated with

experimental gastric ulceration in mastomys  
Gastroenterol. 88:452-457

Arbaban, M, Graziano, FM, Jicinsky, J, Hadcock, J, Malbon, C, and Ruoho, AE (1989) Photoaffinity labeling of the guinea pig pulmonary mast cell beta-adrenergic receptor Am. J. Respir. Cell Mol. Biol. 1:351-359

Arizono, N, Matsuda, S, Hattori, T, Kojima, Y, Maeda, T, and Galli, SJ (1990) Anatomical variation in mast cell-nerve associations in the rat small intestine, heart, lung, and skin Lab. Invest. 62:626-634

Arzubiaga, C, Morrow, J, Roberts, LJ, and Biaggioni, I (1991) Neuropeptide Y, a putative cotransmitter in noradrenergic neurons, induces mast cell degradation but not prostaglandin D2 release J. Allergy Clin. Immunol. 87:88-93

Ashman, RI, Jarboc, DL, Contrad, DH (1991) The mast cell-committed progenitor: in vitro generation of committed progenitors from bone marrow J. Immunol. 146:211-216

Baggiolini, M, Horisberger, U, and Martin, U (1982) Phagocytosis of mast cell granules by mononuclear phagocyte, neutrophils and eosinophils during anaphylaxis Int. Arch. Allergy Appl. Immunol. 67:219-226

Barnes, PJ, Belvisi, MG, and Rogers, DF (1990) Modulation of neurogenic inflammation: novel approaches to inflammatory disease Trends in Pharmacol. Sci. 11:185-189

Barrett, KE (1991) Bidirectional communication between mast cells and nerves controls intestinal secretion Gastroenterol. 101:1134-1136

Barrett, KE, Szucs, EF, and Metcalfe, DD (1986) Mast cell heterogeneity in higher animals: a comparison of the properties of autologous lung and intestinal mast cells from nonhuman primates J. Immunol. 137:2001-2008

Beck, PL, Morris, GP, and Wallace, JL (1989) Reduction of ethanol-induced gastric damage by sodium cromoglycate and FPL-52694. Role of leukotrienes, prostaglandins, and mast cells in the protective mechanism Can. J. Physiol. Pharm. 67:287-293

Befus, AD and Bienenstock, J (1979) Immunologically mediated intestinal mastocytosis in *Nippostrongylus brasiliensis*-infected rats Immunol. 38:95-101

Befus, AD, Dyck, N, Goodacre, R, and Bienenstock, J (1987) Mast cells from the human intestinal lamina propria: isolation, histochemical subtypes, and functional characterization J. Immunol. 138:2604-2610

Benditt, EP and Arase, M (1959) An enzyme in mast cells with properties like chymotrypsin J. Exp. Med. 110:451-460

Bernstein, JA and Lawrence, KD (1990) The mast cell: a comprehensive update review Allergy Proc. 11:209-223

Bienenstock, J, MacQueen, KG, Sestini, P, Marshall, JS, Stead, RH, and Perdue, MH (1991) Mast cell/nerve interactions in vitro and in vivo Am. Rev. Respir. Dis. 143:55-58

Bienenstock, J, Perdue, M, Blennerhassett, M, Stead, R, Kakuta, N, Sestini, P, Vancheri, C, and Marshall, J (1988) Inflammatory cells and the epithelium. Mast cell/nerve interactions in the lung in vitro and in vivo Am. Rev. Respir. Dis. 138:S31-34

Bieninstock, J (1988) An update on mast cell heterogeneity J. Allergy Clin. Immunol. 81:763-769

Blankenship, DT, Brankamp, RG, Mantey, GD, and Cardin, AD (1990) Amino acid sequence of ghillanten: anticoagulation-antimetastatic principle of the south america leech, Haementeria ghilliani Biochem. Biophys. Res. Commun. 166:1384-1389

Bo, L, Olsson, T, Nyland, H, Kruger, PG, Taule, A, and Mork, S (1991) Mast cell in brains during experimental allergic encephalomyelitis in Lewis rats J. Neurol. Sci. 104:135-142

Bolana, LM, Espinosa, J, and Eleno, N (1987) Adrenergic activity on rat pleural and peritoneal mast cells. Loss of beta-receptors during the purification Gen. Pharmacol. 18:141-148

Bosso, JV, Stevenson, DD, Schwartz, LB (1989) Tryptasemia with aspirin-induced respiratory reaction J. Allergy Clin. Immunol. 83:184

Botstein, GR, Sherer, GK, and LeRoy, EC (1982) Fibroblast selection in scleroderma: an alternative model of fibrosis Arthritis Rheum. 25:189-195

Braganza, VJ and Simmons, WH (1991) Tryptase from rat skin: purification and properties Biochem. 30:4997-5007

- Brain, SD and Williams, TJ (1988) Substance P regulates the vasodilator activity of CGRP *Nature* 335:73-75
- Bridges, AJ, Malone, DG, Jicinsky, J, Chen, M, Ory, P, Engber, W, and Graziano, FM (1991) Human synovial mast cell involvement in rheumatoid arthritis and osteoarthritis *Arthritis Rheumatism* 34:116-1124
- Briggaman, RA, Schechter, NM, Fraki, J, Lazarus, GS (1984) Degradation of the epidermal-dermal junction by proteolytic enzyme from human skin and human polymorphonuclear leukocytes *J. Exp. Med.* 160:1027-1042
- Brown, MA, Pierce, JH, Watson, CJ, Falco, J, Ihle, JN, Paul, WE (1987) B cell stimulatory factor-1/interleukin-4 mRNA is expressed by normal and transformed mast cells *Cell* 50:809-818
- Bueno, L, Fargeas, MJ, Theodorou, V, and Fioramonti, J (1991) Involvement of 5-hydroxytryptamine in the intestinal motor disturbances by mast cell degranulation in rats *Eur. J. Pharmacol.* 192:263-269
- Burd, PR, Rogers, HW, Gordon, JR, Martin, CA, Jayaraman, S, Wilson, SD, Dvorak, AM, Galli, SJ, and Dorf, ME (1989) IL-3-dependent and -independent mast cells stimulated with IgE and antigen express multiple cytokines *J. Exp. Med.* 170:245-257
- Butterfield, JH, Weiler, DA, Hunt, LW, Wynn, SR, and Roche, PC (1990) Purification of tryptase from human mast cell line *J. Leukocyte Biol.* 47:409-419
- Canfield, SP and Curwain, BP (1983) Inhibition of gastric acid secretion in the conscious dog by the mast cell stabilizing agent, FPL-52694 *Br. J. Pharmacol.* 80:27-32
- Casale, TB, Bowman, S, and Kaliner, M (1984) Induction of human cutaneous mast cell degranulation by opiates and endogenous opioid peptides: evidence for opiate and nonopiate receptor participation *J. Allergy Clin. Immunol.* 73:775-781
- Casale, TB (1983) The role of the autonomic nervous system in allergic disease *Ann. Allergy* 51:423-459
- Caughey, GH (1989) Roles of mast cell tryptase and chymase in airway function *Am. J. Physiol.* 257:L39-46
- Caughey, GH, Viro, NF, Ramachandran, J, Lazarus, SC, Borson, DB, and Nadel, JA (1987) Dog mastocytoma tryptase: affinity purification, characterization, and

amino-terminal sequence Arch. Biochem. Biophys. 258:555-563

Caughey, GH, Leidig, F, Viro, NF, and Nadel, JA (1988) Substance P and vasoactive intestinal peptide degradation by mast cell tryptase and chymase J. Pharm. Exp. Therap. 244:133-137

Caulfield, JP, Hein, A, and Helfgott, SM (1988) Intraarticular injection of arthritogenic factor causes mast cell degranulation, inflammation, fat necrosis, and synovial hyperplasia Lab. Invest. 59:82-95

Chen, Z, Wakin, BT, and Simmons, WH (1990) A tryptase-like enzyme from rat skin: partial amino acid sequence FASEB J. 4:A2159 (Abstract)

Chin, JR, Murphly, G, and Werb, Z (1985) Stromelysin, a connective tissue-degrading metalloendopeptidase secreted by stimulated rabbit synovial fibroblasts in parallel with collagenase J. Biol. Chem. 260:12307-12376

Chiu, H and Lagunoff, D (1972) Histochemical comparison of vertebrate mast cells Histochem. J. 4:135-144

Cho, CH and Ogle, CW (1979) Cholinergic-mediated gastric mast cell degranulation with subsequent histamine H1- and H2- receptor activation if stress ulceration in rats Eur. J. Pharmacol. 55:23-33

Christian, EP, Udem, BJ, and Weinreich, D (1989) Endogenous histamine excited neurons in the guinea pig superior cervical ganglion in vitro J. Physiol. 409:297-312

Church, MK, Benyon, RC, Rees, PH, Lowman, MA, Campbell, AM, Robinson, C, and Holgate, ST (1989) Functional heterogeneity of human mast cells. In: Galli SJ and Austen, KF eds. Mast cell and basophil differentiation and function in health and disease New York: Raven Press pp116-170

Church, MK, el-Lati, S, Okayama, Y (1991) Biological properties of human skin mast cells Clin. Exp. Allergy 21:1-9

Church, MK, Pao, GJK, and Holgate, ST (1982) Characterization of histamine secretion from mechanically dispersed human lung mast cells: effect of anti-IgE, calcium ionophore A23187, compound 48/80, and basic polypeptides J. Immunol. 129:2116-2121

Claman, HN (1987) New hope for neurofibromatosis? The mast cell connection JAM 258:823

Claman, HN (1989) Mast cells, endothelial cells, and fibroblasts JAM 262:1206-1209

Cleggs, LS, Church, MK, Holgate, ST (1985) Histamine secretion from human skin slices induced by anti-IgE and artificial secretagogues and the effects of sodium cromoglycate and salbutamol Clin. Allergy 15:-321-328

Cocchiara, R, Albeggian, G, Di Trapani, G, Azzolina, A, Lampiasi, N, Cervello, G, and Geraci, D (1988) Dispersal of rat uterine mast cells and their functional response to an embryo-derived histamine releasing factor: a possible model for embryo implantation J. Reprod. Immunol. 14:191-201

Combs, JW, Lagunoff, D, and Benditt, EP (1965) Differentiation and proliferation of embryonic mast cells of the rat J. Cell. Biol. 25:577-592

Craig, SS, Sehechter, NM, and Schwartz, LB (1988) Ultrastructural analysis of human T and TC mast cells identified by immunoelectron microscopy Lab. Invest. 58:682-691

Craig, SS, Schechter, NM, and Schwartz, LB (1989) Ultrastructural analysis of maturing human T and TC mast cells in situ Lab. Invest. 60:147-157

Craig, SS and Schwartz, LB (1989) Tryptase and chymase, markers of distinct types of human mast cells Immunol. Res. 8:130-148

Crisp, AJ, Chapman, CM, Kirkham, SE, Schiller, AL, Krane, SM (1984) Articular mastocytosis in rheumatoid arthritis Arthritis Rheum. 27:845-851

Cromlish, JA, Seidah, NG, Marcinkiewicz, M, Hamelin, J, Johnson DA, and Chretien, M (1987) Human pituitary tryptase: molecular forms, NH<sub>2</sub>-terminal sequence, immunocytochemical localization and specificity with prohormone and fluorogenic substrates J. Biol. Chem. 262:1363-1373

Crowle, PK and Starkey, JR (1989) Mast cells and tumor-associated angiogenesis In: mast cell and basophil differentiation and function in health and disease Galli, SJ and Austen, KF eds. Raven Press, New York pp307-315

Csaba, G, Acs, T, Horvath, C, and Mold, K (1961) Genesis

and function of mast cell: mast cell and plasmacyte reaction to induced, homologous and heterogous tumors Br. J. Can. 15:327-335

Cutz, E, Chan, W, and Track, NS (1978) Release of vasoactive intestinal peptide in mast cells by histamine liberators Nature 275:661-662

Dabbous, MK, Haney, L, Nicolson, GL, Eckley, D, and Woolley, DE (1991) Mast cell modulation of tumor cell proliferation in rat mammary adenocarcinoma 13762 NF Br. J. Cancer 63:873-878

Dabbous, MK, Woolley, DE, Haney, J, Carter, LM, and Nicolson, GL (1986) Host mediated effectors of tumor invasion: role of mast cells in matrix degradation Clin. Exp. Metast. 4:141

Davidson, S, Mansour, A, Gallily, R, Smolarski, M, Rofolovitch, M, Ginsburg, H (1983) mast cell differentiation depends on T cells and granule synthesis on fibroblasts Immunol. 48:439-452

Dayer, JM, Beutler, B, and Cerami, A (1985) Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E2 production by human synovial cells and dermal fibroblasts J. Exp. Med. 162:2163-2168

Dayton, ET, Caulfield, JP, Hein, A, Austen, KF, and Stevens, RL (1989) Regulation of the growth rate of mouse fibroblasts by IL-3-activated mouse bone marrow-derived mast cells J. Immunol. 142:4307-4313

De Young, MB, Nemeth, EF, and Scarpa, A (1987) Measurement of the internal pH of mast cell granules using microvolumetric fluorescence and isotopic techniques Arch. Biochem. Biophys. 254:222-233

Debold, AJ (1985) Atrial natriuretic factor: a hormone produced by the heart Sci. 230:767-770

Devereux, J, Haerberli, P, and Smithes, O (1984) A comprehensive set of sequence analysis program for the VAX Nuc. Acids Res. 12:387-395

Dimlich, RWW, Keller, JT, Strauss, TA, and Fritts, MJ (1991) Linear arrays of homogeneous mast cells in the dura mater of rat J. Neurocytol. 20:485-503

Dropp, JJ (1976) Mast cells in mammalian brain Acta. Anat. 94:1-24

Drudy, L, Sheppard, BL, and Bonnar, J (1991) The mast cell and histamine concentration of the human post-menopausal uterus Eur. J. Obst. Gynecol. Reprod. Biol. 42:39-42

Druvefors, P and Norrby, K (1988) Molecular aspects of mast-cell-mediated mitogenesis in fibroblasts and mesothelial cells in situ Virchows Arch. [B] 55:187-192

Dvorak, AM, Dvorak, HF, Galli, SJ (1983) Ultrastructural criteria for identification of mast cells and basophils in humans, guinea pigs, and mice Am. Rev. Respir. Dis. 128:S49-52

Dvorak, AM, Dvorak, HF, Peters, SP, Shulman, ES, MacGlashan, DWJr, Pyne, K, Harvy, VS, Galli, SJ (1983) Lipid bodies: cytoplasmic organelles important to arachidonate metabolism in macrophages and basophils J. Immunol. 131:2965-2976

Dvorak, AM (1989) Human mast cells. Advances in anatomy embryology and cell biology Beck, F, et al. eds. Vol. 114, Springer-Verlag

Ebertz, JM, Hirshman, CA, Kettlekamp, MS, Uno, H, and Hainfin, JM (1987) Substance P-induced histamine release in human cutaneous mast cells J. Invest. Dermatol. 88:682-685

Ehrlich, P (1878) Beitrage zur theorie und praxis der histologischen farbung Doctoral Thesis, University of Leipzig, Germany

Eliakim, R, Gilead, L, Ligumsky, M, Okon, E, Rachmilewitz, D, and Razin, E (1986) Histamine and chondroitin sulfate E proteoglycan released by cultured human colonic mucosa-indication for possible presence of E mast cells Proc. Natl. Acad. Sci. USA 83:461-464

Enerback, L (1966) Mast cells in rat gastrointestinal mucosa I. Effects of fixation Acta Pathol. Microbiol. Scand. 66:289-302

Enerback, L (1981) The gut mucosal mast cell Monogr. Allergy 17:222-232

Erjavec, F, Lembeck, F, Florjane-Irman, T, Skofitsch, G, Donneres, J, Saria, A, and Holzg, P (1981) Release of histamine by substance P Naunyn Schmiedebergs Arch. Pharmacol. 317:67-70

Everitt, MT and Neurath, H (1980) Rat peritoneal mast



cell carboxypeptidase: localization, purification and enzymatic properties FEBS Lett. 110:292-296

Fantozzi, R, Moroni, F, Blandina, P, Mannaioni, PF, and Bani-Sacchi, T (1978) Release of histamine from rat mast cells by acetylcholine Nature 273:473-474

Farnoush, A and McKenzie, IC (1983) Sequential histological changes and mast cell response in skin during chemically-induced carcinogenesis J. Pathol. 12:300-306

Ferrante, F, Ricci, A, Felici, L, Cavallotti, C, and Amenta, F (1990) Suggestive evidence for a functional association between mast cells and sympathetic nerves in meningeal membranes Acta Histochem. Cytochem. 23:637-646

Fewtrell, CM, Foreman, JC, Jordan, CC, Oehme, P, Renner, H, and Stewart, JM (1982) The effect of substance P on histamine and 5-hydroxytryptamine in the rat J. Physiol 330:393-411

Finn, CA (1986) Implantation, menstruation, and inflammation Biol. Rev. 61:313-328

Fiorucci, L, Erba, F, and Ascoli, F (1992) Bovine tryptase: purification and characterization Biol. Chem. 373:483-490

Flint, KC, Leung, KBP, Pearce, FL, Hudspith, BN, Brostoff, J, Johnson, NMCI (1985) Human mast cells recovered by bronchoalveolar lavage: their morphology, histamine release and the effect of sodium cromoglycate Clin. Sci 68:427-432

Flynn, EA, Schwartz, JL, and Shklar, G (1991) Sequential mast cell infiltration and degranulation during experimental carcinogenesis J. Cancer Res. Clin. Oncol. 117:115-122

Fogelman, AM, Van Lenten, BJ, Warden, C, Haberland, ME, and Edwards, PA (1988) Macrophage lipoprotein receptors J. Cell Sci. 9:135-149

Foreman, JC (1987) Substance P and calcitonin gene-related peptide: effects on mast cells and human skin Int. Arch. Allergy Appl. Immunol. 82:366-371

Fort-Hutchinson, AW, Bray, MA, Doig, MV Shipley, ME, and Smith, JH (1980) Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes Nature 286:264-265

Fox, B, Bull, TB, and Guz, A (1981) Mast cells in the human alveolar wall: an electronmicroscopic study J. Clin. Pathol. 34:1333-1342

Fox, CC, Dvorak, AM, Peleas, SP, Kagey-Sobotka, A, and Lichtenstein, LM (1985) Isolation and characterization of human intestinal mucosal mast cells J. Immunol. 135:483

Franco-Browder, S, Masson, GMC, and Corcoran, AC (1959) Induction of acute gastric lesions by histamine liberators in rats J. Allergy 30:1-11

Fraser, RA and Simpson, JG (1983) Role of mast cells in experimental tumor angiogenesis In: development of the vascular system Ciba Found. Symp. 100:120-131

Frater-Schroder, M, Risan, W, Hallmann, R, Gautshi, P, and Bohlen, P (1987) Tumor necrosis factor type  $\alpha$ , a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo Pro. Natl. Acad. Sci. USA 84:5277-5281

Furitsu, T, Saito, H, Dvorak, AM, Schwartz, LB, Irani, AM, Burdick, JF, Ishizaka, K, and Ishizaka, T (1989) Development of human mast cells in vitro Proc. Natl. Acad. Sci. USA 86:10039-10043

Galli, SJ (1990) New insights into "The riddle of the mast cells": microenvironmental regulation of mast cell development and phenotypic heterogeneity Lab. Invest. 62:5-33

Galli, SJ, Bose, R, and Szabo, S (1985) Mast cell-dependent augmentation of ethanol-induced acute gastric damage Dig. Dis. Sci. 30:375 (Abstr)

Gibson, S, Mackeller, A, Newland, GFJ, and Miller, HRP (1987) Phenotypic expression of mast cell granule proteinases. Distribution of mast cell proteinases I and II in the rat digestive system. Immunol. 62:621-627

Gilead, L, Livni, N, Eliakim, R (1987) Human gastric mucosal mast cells are chondroitin sulfate E-containing mast cells immunol. 62:23-28

Gleich, GJ (1986) Mast cell heterogeneity: biological and clinical significance In: Befus AD, eds., mast cell differentiation and heterogeneity Raven Press, N.Y. pp347-366

Glennier, GC and Cohen, LA (1960) Histochemical demonstration of species-specific trypsin-like enzyme in

mast cells Nature 185:846-847

Glodschmidt, RC, Hough, LB, Glick, SD, and Padawer, J (1984) Mast cells in rat thalamus: nuclear localization, ex difference and left-right asymmetry Brain Res. 323:209-217

Godfrey, HP, Ilardi, C, Engber, W, Graziano, FM (1984) Quantitation of human synovial mast cells in rheumatoid arthritis and other rheumatic diseases Arthritis Rheum. 27:852-856

Goetzl, EJ, Chernov-Rogan, T, Cooke, MP, Renold, F, and Payan, DG (1985) Endogenous somatostatin-like peptides of rat basophilic leukemia cells J. Immunol. 135:2707-2711

Goldstein JL, Ho, YK, Basu, SK, and Brown, KS (1979) Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition Proc. Natl. Acad. Sci. USA 76:333-337

Goldstein, SM, Kaempfer, CE, Kealey, JT, and Wintroub, BY (1989) Human mast cell carboxypeptidase: purification and characterization J. Clin. Invest. 83:1630-1636

Goldstein, SM, Kaempfer, CE, Proud, D, Schwartz, LB, Irani, AM, and Wiontroub, BY (1987) Detection and partial characterization of human mast cell carboxypeptidase J. Immunol. 139:2724-2729

Goossens, J, Van Reempts, J, and VanWauve, JP (1987) Cytoprotective effects of disodium cromoglycate on rat stomach mucosa Br. J. Pharmacol. 91:165-169

Gordon, JR and Galli, SJ (1991) Release of both preformed and newly synthesized tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )/cachectin by mouse mast cells stimulated via the Fc $\epsilon$ 6RI. A mechanism for the sustained action of mast cell-derived TNF- $\alpha$  during IgE-dependent biological responses J. Exp. Med. 174:103-107

Gordon, JR, Burd, PR, and Galli, SJ (1990:) Mast cells as a source of multifunctional cytokines Immunol. Today 11:458-464

Goto, T, Befus, D, Low, R, and Bienenstock, J (1984) Mast cell heterogeneity and hyperplasia in bleomycin-induced pulmonary fibrosis of rats Am. Rev. Respir. Dis. 130:797-802

Green, KR, Fowler, J, MacGlashan, D, and Weinreich, D

(1988) IgE-challenged human lung mast cells excite vagal sensory neurons in vitro J. Appl. Physiol. 64:2249-2253

Greenberg, G and Burnstock, G (1983) A novel cell-to-cell interaction between mast cells and other cell types Expl. Cell. Res. 147:1-13

Greer, J (1990) Comparative modeling methods: application to the family of the mammalian serine proteases Prot. Struct. Funct. Genet. 7:317-334

Griffen, M, Weiss, JW, Leitch, AG, McFadden, Jr, ER, Corey, EJ, Austen, KF, and Drazen, JM (1983) Effects of leukotriene D on the airway in asthma N. Eng. J. Med. 308:436-439

Griffin, DE and Mendoza, QP (1986) Identification of the inflammatory cells present in the central nervous system of normal and mast cell-deficient mice during Sinbis virus encephalitis Cell. Immunol. 97:454-459

Gruber, BL (1991) Immunoglobulin E, mast cells, endogenous antigens, and arthritis Rheum. Dis. Clin. North. Am. 17:333-342

Gruber, BL, Marchese, MJ, Suzuki, K, Schwartz, LB, Okada, Y, Nagase, H, and Ramamurthy, NS (1989) Synovial procollagenase activation by human mast cell tryptase dependence upon matrix metalloproteinase 3 activation J. Clin. Invest. 84:1657-1662

Gruber, BL, Schwartz, LB, Ramamurthy, NS, Irani, AM, and Marchese, MJ (1988) Activation of latent rheumatoid synovial collagenase by human mast cell tryptase J. Immunol. 140:3936-3942

Gryle, A, Sanders, PM, and Gardner, DL (1971) The mast cell in early rat adjuvant arthritis Ann. Rheum. Dis. 30:24-29

Gushchin, IS, Prozorovshy, NS, Yamshchikova, OA, and Kiselev, AV (1991) Mast cell regulatory effect on lymphoid cell proliferation Agents & Actions 33:185-188

Guth, PH and Kozbur, X (1969) Microcirculatory and mast cell changes in restraint stress Am. J. Dig. Dis. 14:113-117

Guyton JR, Kemp, KF, Black, BL, and Bocan, TMA (1990) Extracellular lipid deposition in atherosclerosis Eur. Heart J. 11:20-28

- Haig, DM, McKee, TA, Jarrett, EEE, Woodbury, R, Miller HRP (1982) Generation of mucosal mast cells is stimulated in vitro by factors derive from T cells of helminth-infected rats Nature 300:188-190
- Hamaguchi, Y, Kanakura, Y, Fujita, J, Takeda, S, Nakano, T, Tarui, S, Honjo, T, and Kitamura, Y (1987) Interleukine 4 as an essential factor for in vitro clonal growth of murine connective tissue-type mast cells J. Exp. Med. 165:268-273
- Hartveit, F (1981) Mast cells and metachromasia in human breast cancer: their occurrence, significance and consequences: a preliminary report J. Path. 134:7
- Hartveit, FS and Sandstad, E (1982) Stromal metachromasia: marker for area of infiltrating tumor growth? Histopathol. 6:423-428
- Hartveit, F, Thoresen, S, Tangen, M, and Maartmann-Moe, H (1984) Mast cell changes and tumor dissemination in human breast carcinoma Invasion & Metastasis 4:146
- Harvima, IT, Harvima, RJ, Penttila, IM, Eloranta, TO, Horsmanheimo, M, and Fraki, JE (1989) Effect of human mast cell tryptase on human plasma proenzymes Int. Arch. Allergy Immunol. 90:104-108
- Harvima, IT, Schechter, NM, Harvima, RJ, and Fraki, JE (1988) Human skin tryptase: purification, partial characterization and comparison with human lung tryptase Biochem. Biophys. Acta 957:71-80
- Hashimoto, K, Yamanishi, Y, Maeyens, E, Dabbous, MK, and Kanzaki, T (1973) Collagenolytic activities of squamous cell carcinoma of the skin Cancer Res. 33:2790-2801
- Hawkins, RA, Claman, HN, Clark, RAF (1985) Increased dermal mast cell populations in progressive systemic sclerosis: a link in chronic fibrosis? Ann. Intern. Med. 102:182-186
- Hayaishi, O (1991) Molecular mechanisms of sleep-wake regulation: roles of prostaglandins D2 and E2 FASEB J 5:2575-2581
- Heavey, DJ, Ernst, PB, Stevens, RL, Befus, AD, Bienenstock, J, and Austen, KF (1988) Generation of leukotrienes C4, leukotriene B4, and prostaglandin D2 by immunologically activated rat intestinal mucosa mast cells J. Immunol. 140:1953-1957

Heine, H and Forster, FJ (1975) Histophysiology of mast cells in skin and other organs Arch. Dermatol. Res. 253:225-228

Hinson, JP, Vinson, GP, Kapas, S, and Teja, R (1991) The relationship between adrenal vascular events and steroid secretion: the role of mast cells and endothelia J. Steroid Biochem. Mol. Biol. 40:381-389

Hinson, JP, Vinson, GP, Pudney, J, and Whitehouse, BJ (1988) Adrenal mast cells modulate vascular and secretory responses in the intact adrenal gland of the rat J. Edocrinol. 121:253-260

Hoff, JF, O'Neil, J, Pepin, JM, and Cole, TB (1990) Macrophage uptake of cholesterol-containing particles derived from LDL and isolated from atherosclerotic lesions Eur. Heart J. 11:105-115

Houlgatte, R, Mallat, M, Brachet, P, and Prochiantz, A (1989) Secretion of nerve growth factor in cultures of glial cells and neurons derived from different regions of the mouse brain J. Neurosci. Res. 24:143-153

Huff, RF and Justus, DE (1988) Mast cell differentiation in culture of T cell- depleted mesenteric lymph node cells from Nippostrongylus brasiliensis-infected mice Int. Arch. Allergy Appl. Immunol. 85:137-144

Huntley, JF, George, AM, Newlands, FJ, Irvine, J, and Miller HRP (1990) Mapping of the rat mast cell granule proteinases RMCPI and II by enzyme-linked immunosorbent assay and paired immunofluorescence APMIS 98:933-944

Ibrahim, MZM (1974) The mast cells of the mammalian central nervous system part I. Morphology, distribution and histochemistry J. Neurol. Sci. 21:431-478

Inoue, F, Hasegawa, H, Nishimura, M, Yanagisawa, M, and Ichiyama, A (1985) Distribution of 5-hydroxytryptamine (5HT) in tissue of a mutant mouse deficient in mast cell (W/W<sup>v</sup>). Demonstration of the contribution of mast cells to the 5HT content in various organs Agents & Actions 16:395-301

Ionov, ID (1989) Mast cell-basophil system in tumor growth Neurofibromatosis 2:204

Irani, AMA and Schwartz, LB (1989) Mast cell heterogeneity Clin. Exp. Allergy 19:143-455

Irani, AMA, Craig, SS, DeBois, G, Elson, CO, Schechter,

NM, and Schwartz, LB (1987) Deficiency of the tryptase-positive, chymase-negative mast cell type in gastrointestinal mucosa of patients with defective T lymphocyte function J. Immunol. 138:4381-4386

Irani, AMA, Schechter, NM, Craig, SS, DeBlois, G, and Schwartz, LB (1986) Two types of human mast cells that has distinct neutral protease compositions Proc. Natl. Acad. Sci. USA 83:4461-4468

Irani, AMA and Schwartz, LB (1990) Neutral proteases as indicators of human mast cell heterogeneity Monogr Allergy 27:146-462

Irani, AMA, Bradford, TR, Kepley, CL, Schechter, NM, and Schwartz, LB (1989) Detection of MC<sub>T</sub> and MC<sub>TC</sub> types of human mast cells by immunohistochemistry using new monoclonal anti-tryptase and anti-chymase antibodies J. Histochem. Cytochem. 37:1509-1515

Irani, AMA, Butrus, SI, Tabbara, KF, and Schwartz, LB (1990) Human conjunctival mast cells: distribution of MC<sub>T</sub> and MC<sub>TC</sub> in vernal conjunctivitis and giant papillary conjunctivitis J. Allergy Clin. Immunol. 86:34-40

Iriarte, LM, Mateu, J, Cruz, G, and Escudero, J (1988) Chorea: a new manifestation of mastocytosis J. Neurol. Neurosurg. Psych. 51:1457-1463

Isaacson, P (1976) Mast cells in benign sheath tumors J. Pathol. 119:193-196

Ito, A and Nagase, H (1988) Evidence that human rheumatoid synovial matrix metalloproteinase 3 is an endogenous activator of procollagenase Arch. Biochem. Biophys. 267:211-216

Ito, A, Ihara, H, and Mori, Y (1980) Partial purification and characterization of a novel neutral proteinase from human uterine cervix Biochem. J. 185:443-450

Itoh, N, Obata, K, Yanaihara, N, and Okamoto, H (1983) Human preprovasoactive intestinal polypeptide contains a novel PHI-27-like peptide PHM-27 Nature 304:547-549

Izumo, S, Liningto, C, Wekerle, H, and Meyemann, R (1985) Morphologic study on experimental allergic neuritis mediated by T cell line specific for bovine P2 protein in Lewis rats Lab. Invest. 53:209-218

Janiszewski, J, Bienenstock, J, and Blennerhassett, MG

(1990) Activation of rat peritoneal mast cells in coculture with sympathetic neurons alters neuronal physiology Brain Behav. Immunol. 4:139-150

Jarjour, NN, Calhoun, WJ, Schwartz, LB (1991) Elevated bronchoalveolar lavage fluid histamine levels in allergic asthmatics are associated with increased airway obstruction Ann. Rev. Respir. Dis. 144:83

Jenkinson, DM, Thompson, GE, Kenny, JDR, and Pearson, JM (1970) Histochemical studies on mast cells in cattle skin Histochem. 2:419-424

Johnson, D and Krenger, W (1992) Interaction of mast cells with the nervous systems-recent advanced Neurochem. Res. 17:939-951

Johnson, MD, Kamso-Pratt, J, Federspiel, CF, and Whetsell, WOJr (1989) Mast cell and lymphoreticular infiltrates in neurofibromas. Comparison with nerve sheath tumors Arch. Pathol. Lab. Med. 113:1263-1270

Johnson, DA and Barton, GJ (1992) mast cell tryptases: Examination of unusual characteristics by multiple sequence alignment and molecular modeling Prot. Sci. 1:370-377

Jones, RE, Duvall, D, and Guillette, LJ, Jr. (1980) Rat ovarian mast cells: distribution and cyclic changes Anat. Rec. 197:489-493

Jontell, M, Hansson HA, and Nygren, H (1986) Mast cells in oral lichen planus J. Oral Pathol. 15:273-275

Kai Kai, MA and Keen, P (1985) Localization of 5-hydroxytryptamine to neurons and endoneurial mast cells in rat sensory ganglia J. Neurocytol. 14:63-78

Kamino, A, Taguchi, T, Shiraishi, M, Shimata, K, Takebayashi, S, Cleveland, JC, and Kummerow, FA (1979) Mast cells in human aortal Arterial Wall 5:125-136

Karger, B (1990) Neutral proteases of mast cells Monogr. Allergy P1

Kazimierczak, W, Adamas, B, and Malinski, CZ (1980) Failure of acetylcholine to release histamine from rat mast cells Agents & Actions 10:1-3

Kerdel, FA and Soter NA (1989) The mast cell in mastocytosis and pediatric dermatologic disease Adv. Dermatol. 4:159-182



- Kessler, DA, Langer, RS, Pless, NA, and Folkman, J (1976) Mast cells and tumor angiogenesis Int. J. Cancer 18:16-27
- Kido, H, Fukusen, N, and Katunuma, N (1985) Chymotrypsin- and trypsin-type serine proteases in rat mast cells: Properties and functions Arch. Biochem. Biophys. 239:436-443
- Kido, H, Yokogoshi, Y, and Katunuma, N (1988) Kunitz-type protease inhibitor found in rat mast cells J. Biol. Chem. 263:18104-18107
- Kiernan, JA (1990) Interactions between mast cells and nerves. Neurogenic inflammation. Trends In Pharmacol. Sci. 11:316
- Kjellen, L and Lindahl, U (1991) Proteoglycan: structures and interactions Ann. Rev. Biochem. 60:443-475
- Kokkonen, JO and Kovanen, PT (1985) Low density lipoprotein degradation by rat mast cells. Demonstration of extracellular proteolysis caused by mast cell granules J. Biol. Chem. 260:14756-14763
- Kokkonen, JO and Kovanen, PT (1987a) Low-density-lipoprotein binding by mast-cell granules. Demonstration of binding of apolipoprotein B to heparin proteoglycan of exocytosed granules Biochem. J. 241:583-589
- Kokkonen, JO and Kovanen, PT (1987b) Stimulation of mast cells leads to cholesterol accumulation in macrophages in vitro by a mast cell granule-mediated uptake of low density lipoprotein Proc. Natl. Acad. Sci. USA 84:2287-2291
- Kokkonen, JO, Vartiainen, M, and Kovanen, PT (1986) Low density lipoprotein degradation by secretory granules of rat mast cells Sequential degradation of apolipoprotein B by granule chymase and carboxypeptidase A J. Biol. Chem. 261:16067-16072
- Kokkonen, JO and Kovanen PT (1989) Proteolytic enzymes of mast cell granules degrade low density lipoproteins and promote their granule-mediated uptake by macrophages in vitro J. Biol. Chem. 264:10749-10755
- Kovanen PT (1990) Atheroma formation: defective control in the intimal round-trip of cholesterol Eur. Heart J. 11:238-246
- Kovanen, PT and Kokkonen, JO (1991) Modification of low

density lipoproteins by secretory granules of rat serosal mast cells J. Biol. Chem. 266:4460-4436

Kovanen, PT (1991) Mast cell granule-mediated uptake of low density lipoproteins by macrophages: a novel carrier mechanism leading to the formation of foam cells Annals of Med. 23:551-559

Krenger, W, Yasui, DH, Al-Sabbagh, A, Sriram, S, and Johnson D (1991) Mast cell activation in murine EAE J. neuroimmunol. (Suppl 1):45

Krishna, A and Terranova, PF (1991) Compartmentalized mast cell degranulations in the ovarian hilum, fat pad, bursa and blood vessel regions of the cyclic hamster: relationship to ovarian histamine and blood flow Act Anat. 141:18-25

Krishna, A and Terranova, PF (1985) Alterations in mast cell degranulation and ovarian histamine in the proestrous hamster Biol. Reprod. 32:1211-1217

Lagunoff, D, Richard, A, and Marquart, C. (1991) Rat mast cell tryptase Arch Biochem. Biophys. 291:52-58

Lal, SM, Brooks, CS, Luger, AM, Davenport, JG, Weber, RD, Loy, TS, and Haibach, HH (1988) Systemic mastocytosis associated with membranous nephropathy, and peripheral neuropathy Am. J. Kidney Dis. 12:538-543

Lambracht-Hall, M, Konstantinidon, AD, and Theoharides, TC (1990) Serotonin release from brain mast cells in vitro Neurosci. 39:199-207

Lawrence, ID, Warner, JA, Cohan, VL, Hubbard, WC, Kagey-Sobotka, A, and Lichtenstein, LM (1987) Purification and characterization of human skin mast cells: evidence for human mast cell heterogeneity J. Immunol. 139:6062

Lazarus, LH, Perrin, MH, and Brown, MR (1977) Mast cell binding of neurotensin, iodination of neurotensin and characterization of the interaction of neurotensin with mast cell receptor sites J. Biol. Chem. 252:7174-7179

Leibovich, SJ, Polverini, PJ, Shepard, HM, Wiseman, DM, Shively, V, Nuseir, N (1987) Macrophage-mediated angiogenesis mediated by tumor necrosis factor-alpha Nature 329:630-632

Levi-Schaffer, F and Shalit, M (1989) Differential release of histamine and prostaglandin D2 in rat peritoneal mast cells activated with peptides Int. Arch.

Allergy Appl. Immunol. 90:352-357

Levi-Schaffer, F, Austen, KF, Gravallesse, PM, and Stevens, RL (1986) Coculture of interleukin 3-dependent mouse mast cells with fibroblasts results in phenotypic change of the mast cells Proc. Natl. Acad. Sci. USA 83:6485-6488

Lewis, RA, Soter, NA, Damond, PT, Austen, KF, Oates, JA, Roberts, LJ (1982) Prostaglandin D2 generation after activation of rat and human mast cells with anti-IgE J. Immunol. 129:1627-1631

Lindahl, U, Pertoft, H, and Seljelid, R (1979) Uptake and degradation of mast-cell granules by mouse peritoneal macrophages Biochem. J. 182:189-193

Linder, S, Barkhem, T, Norberg, A, Persson, H, Schalling, M, Hokfelt, T, and Magnusson, G (1987) Structure and expression of the gene encoding the vasoactive intestinal peptide precursor Proc. Natl. Acad. Sci. USA 84:605-609

Lindholm, D, Heumann, R, Meyer, M, and Thoenen, H (1987) Interleukin-1 regulates synthesis of nerve growth factor in non-neural cells of rat sciatic nerve Nature 330:658-659

Lindstedt, KA, Kokkonen, JO, Kovanen, PT (1992) Soluble heparin proteoglycans released from stimulated mast cells induce uptake of low density lipoprotein by macrophages via scavenger receptor-mediated phagocytosis J. Lipid Res. 33:65-75

Liotta, LA, Tryggvasson, K, Garbisa, S, Hart, I, Foltz, CM, and Shofic, S (1980) Metastatic potential correlates with enzymatic degradation of basement membrane collagen Nature 284:64-68

Lowman, MA, Benyon, RC, and Church, MK (1985) Characterization of neuropeptide-induced histamine release from human dispersed skin mast cells Br. J. Pharmacol. 95:121-130

Lowman, MA, Rees, PH, Benyon, RC, and Church, MK (1988) Human mast cells dispersed from skin, lung, adenoids, tonsils, and colon in response to IgE-dependent and nonimmunologic stimuli J. Allergy Clin. Immunol. 81:590-597

Lundberg, JM, Martling, CR, and Saria, A (1983) Substance P and capsaicin-induced contraction of human bronchi Acta Physiol. Scand. 119:49-53

Lundberg, JM, Fahrenberg, J, Hokdelt, T Martling, CR, Larsson, O, Tatemoto, K, and Anggard, A (1984) Co-existence of peptide HI (PHI) and VIP in nerve regulating blood flow and bronchial smooth muscle tone in various animals, including man Peptides 5:593-606

MacDermot, J, Kelsey, CR, Waddell, KA, Richmond, R, Knight, RK, Cole, PI, Dollery, CT, Landon, DN, and Blair, IA Synthesis of leukotriene B<sub>4</sub>, and prostanoids by human alveolar macrophages: analysis by gas chromatography/mass spectrometry Prostaglandins 27:163-179

Maier, M, Spragg, J, and Schwartz LB (1983) Inactivation of human high molecular weight kininogen by human mast cell tryptase J. Immunol. 130:2352-2356

Malone, DG and Metcalfe, DD (1988) Demonstration and characterization of a transient arthritis in rats following sensitization of synovial mast cells with antigen-specific IgE and parenteral challenge with specific antigen Arthritis Rheum. 31:1063-1067

Mannaioni, PF, Moroni, F, Fantozzi, R, and Masini, E (1975) Studies on the <sup>14</sup>C-histamine release induced by noradrenaline in mouse neoplastic mast cells Agents & Actions 5:417-423

Marom, ZM (1991) The role of mast cells in bronchial asthma: mechanisms and possible therapeutic implications Mount Sinai J. Med. 58:472-482

Marshall, JS, Bienenstock, J, Perdue, MH, Stanisz, AM, Stead, RH, and Ernst, PB (1989) Novel cellular interactions and networks involving the intestinal immune system and its microenvironment APMIS 97:383-394

Masini, E, Fantozzi, R, Conti, A, Blandina, P, Brunelleschi, S, and Mannaioni, PF (1985) Mast cell heterogeneity in response to cholinergic stimulation Int. Arch. Allergy Appl. Immunol. 77:184-185

Masini, E, Fantozzi, R, Blandina, P, Brunelleschi, S, and Mannaioni, PF (1983) Muscarinic cholinergic receptor binding in rat mast cells Agents & Actions 13:327-332

Massey, WA, Guo, CB, Dvorak, AM, Hubbard, WC, Bhagavan, BS, Cohan, BL, Warner, JA, Kagey-Sobotka, A, and Lichtenstein, LM (1991) Human uterine mast cells. Isolation, purification, characterization, ultrastructure, and pharmacology J. Immunol. 147:1621-1627

Matsson, P, Enander, I, Andersson, AS, Nystrand, J, Schwartz, LB, Watkins, J (1991) Evaluation of mast cell activation (tryptase) in two patients suffering from drug-induced hypotensoid reactions Agents & Actions 33:218-220

Matsuzaki, Y, Hamasaki, Y, and Said, SI (1980) Vasoactive intestinal peptide: a possible transmitter of nonadrenergic relaxation of guinea pig airways Sci. 210:1252-1253

Mayrhofer, G and Bazin, H (1981) Nature of the thymus dependency of mucosal mast cells III. Mucosal mast cells in nude mice and nude rats, in B rats and in a child with the Di George syndrome Int. Arch. Allergy Appl. Immunol. 64:320-331

Mayrhofer, G (1979a) The nature of the thymus dependency of mucosal mast cells I. An adaptive secondary response to challenge with *Nippostrongylus brasiliensis* Cell. Immunol. 47:304-311

Mayrhofer, G (1979b) The nature of the thymus dependency of mucosal mast cells II. The effect of thymectomy and of depleting recirculating lymphocytes on the response to *Nippostrongylus brasilliensis* Cell. Immunol. 47:312-322

McGill, HCJr (1968) Fatty streaks in the coronary arteries and aorta Lab. Invest. 18:560-564

Meier, HL, Heck, LW, Schulman, ES, MacGlashan, DWJr (1985) purified human mast cells and basophils release human elastase and cathepsin G by an IgE mediated mechanism Int. Arch. Allergy Appl. Immunol. 77:179-183

Metcalfe, DD, Soter, NA, Wasserman, SI, and Austen, KF (1980) Identification of sulfated mucopolysaccharides including heparin in the lesional skin of a patient with mastocytosis J. Invest. Dermatol. 74:210-215

Metcalfe, DD, Lewis, RA, Silbert, JE, Rosenberg, RD, Wasserman, SI, and Austen, KF (1979) Isolation and characterization of heparin from human lung J. Clin. Invest. 64:1537-1543

Metcalfe, DD, Kaliner, M, Donlon, MA (1981) The mast cell In: CRC Critical Reviews in Immunology Ed. 3, Boca Raton, CRC Press, p23

Metcalfe, DD (1991) The liver, spleen, and lymph nodes in mastocytosis J. Invest. Dermatol. 96;45S-46S

- Mican, JM and Metcalfe, DD (1990) Arthritis and mast cell activation J. Allergy Clin. Immunol. 86:677-683
- Michels, NA (1963) The mast cells Ann. N.Y. Acad. Sci. 103:232
- Mihm, MCJr, Solter, NA, and Dvorak, HF (1976) The structure of normal skin and the morphology of atopic eczema J. Invest. Dermatol. 67:305-312
- Miller, JS, Moxley, G, and Schwartz, LB (1990) Cloning and characterization of a second complementary DNA for human tryptase J. Clin. Invest. 86:864-870
- Miller, HRP, Huntley, JF, Newlands, GF, and Irvine, J (1990) Granule chymases and the characterization of mast cell phenotype in rat and mouse Monogr. Allergy 27:1-30
- Miller, HRP and Walshaw, R (1972) Immune reactions in mucous membranes IV. Histochemistry of intestinal mast cells during helminth expulsion in the rat Am. J. Path. 69:195-208
- Miller, JS, Westin, EH, and Schwartz, LB (1989) Cloning and characterization of complementary DNA for human tryptase J. Clin. Invest. 84:1188-1195
- Mokhtarian, F and Griffin, DE (1984) The role of mast cells in virus-induced inflammation in the murine central nervous system Cell. Immunol. 86:491-500
- Mori, H, Kawada, K, Shang, F, Uesugi, Y, Sakamoto, O, and Koda, A (1991) Bleomycin-induced pulmonary fibrosis in genetically mast cell-deficient WBB6F,-W/W<sup>m</sup> mice and mechanism of the suppressive effect of tranilast, an antiallergic drug inhibiting mediator release from mast cells, on fibrosis Int. Arch. Allergy Appl. Immunol. 95:195-201
- Muller, S and Weihe, E (1991) Interrelation of peptidergic innervation with mast cells and ED01-positive cells in rat thymus Brain Behav. Immunol. 5:55-72
- Murquardt, DL and Wasserman, SI (1982) Characterization of the rat mast cell beta-adrenergic receptor in resting and stimulated cells by radioligand binding J. Immunol. 1129:2122-2127
- Newson, B, Dahlstrom, A, Enerback, L, and Ahlman, H (1983) Suggestive evidence for a direct innervation of mucosal mast cells Neurosci. 10:565-570

Nicolson, GL (1984) Generation of phenotypic diversity and progression in metastatic tumor cells Cancer Metastasis Res. 3:25-42

Nishioka, K, Kobayashi, Y, and Katayana, I (1987) Mast cell numbers in diffuse scleroderma Arch. Dermatol. 123:205-208

Norrby, K, Jakobson, A, and Sorbo, J (1988) Mast cell secretion and angiogenesis, a quantitative study in rats and mice Virchows Arch. B, 57;251-256

Norrby, K and Enstrom, S (1984) Cellular and extracellular changes following mast cell secretion in a vascular rat mesentery Cell Tiss. Res. 2235:339-345

Norrby, K, Jakobson, A, and Sorbo, J (1986) Mast cell mediated angiogenesis: a novel experimental model using the rat mesentery Virchows Arch. B, 52:195-206

Oelz, O, Oelsz, R, Knapp, HR, Sweetman, BJ, and Oates, JA (1977) Biosynthesis of prostaglandin D2. I. Formation of prostaglandin D2 by human platelets Prostaglandins 13:225-234

Ogle, CW and Lau, HK (1980) Stomach mast cell involvement in gastric ulceration induced by polymyxin B in rat IRCS Med. Sci. 8:64

Okada, Y, Nagase, H, and Harris, ED (1986) A metalloproteinase from human rheumatoid synovial fibroblasts that digest connective tissue matrix components J. Biol. Chem. 261:14245-14255

Okada, Y, Konomi, H, Yada, T, Kimata, K, and Nagase, H (1989) Degradation of type IX collagen by matrix metalloprotease 3 (stromelysin) from human rheumatoid synovial cells REBS Lett. 244:473-476

Olsson, Y (1968) Mast cells in the nervous system Int. Rev. Cytol. 24:27-70

Orr, EL (1988) Presence and distribution of nervous system-associated mast cells that may modulate experimental allergic encephalomyelitis Ann. N.Y. Acad. Sci. 723-726

Osman, IAR, Garrett, JR, and Smith, RE (1989) Enzyme histochemical discrimination between tryptase and chymase in mast cells of human gut J. Histochem. Cytochem. 37:415-421

Otsuka, H, Denburg, J, and Dolovich, J (1985) Heterogeneity of metachromatic cells in the human cells in the human nose: significance of mucosal mast cells J. Allergy Clin. Immunol. 76:695-702

Palmer, JBD, Cuss, FMC, Mulderry, PK, Ghatel, MA, Springall, DR, Cadieux, A, Bloom, SR, Polak, JM, and Barnes, PJ (1987) Calcitonin gene-related peptide is localized to human airway nerves and potently constricts human airway smooth muscle Br. J. Pharmacol. 91:95-101

Palmer, JBD, Cuss, FMC, and Barnes, PJ (1986) VIP and PHM and their role in nonadrenergic inhibitory response in isolated human airways J. Appl. Physiol. 61:1322-1328

Parwaresch, MR, Hornym, HP, and Lennart, K (1985) Tissue mast cells in health and disease Path. Res. Pract. 179:439

Pearce, FL (1968) Functional differences between mast cells from various locations. In: Befus AD, eds., Mast cell differentiation and heterogeneity Raven Press, N.Y. pp215-222

Pearce, FL (1989) Mast cells; function, differentiation and activation Curr. Opin. Immunol. 1:630-636

Pearse, AGE (1968) Histochemistry: Theoretical and applied 3rd. Ed. vol.1, Little, Brown, Boston

Peters, SP, MacGlashan, DWJr, Schulman, ES, Schleimer, RP, Hayes, EC, Rokach, J, Adkinson, NFJr, and Lichtenstein, LM (1984) Arachidonic acid metabolism in purified human lung mast cells J. Immunol. 132:1972-1979

Piotrowski, W and Foreman, JC (1985) On the actions of substance P, somatostatin, and vasoactive intestinal peptide on rat peritoneal mast cells and in human skin Naunyn Schmiedebergs Arch. Pharmacol. 331:364-368

Pollak, OJ (1957) Mast cells in the circulatory system of man Circulation 16:1084-1089

Powell, HC, Braheny, SL, Myers, RR, Rodriguez, M, and Lampert, PW (1983) Early changes in experimental allergic neuritis Lab. Invest. 48:332-338

Proctor, GB, Chan, KM, Garrett, JR, and Smith, RE (1991) Proteinase activities in bovine atrium and the possible role of mast cell tryptase in the processing of atrial natriuretic factor (ANF) Comp. Biochem. Physiol. B:Comp. Biochem. 99:839-844



- Prouchlev, A, Youroukova, Z, and Kiprova, D (1966) A study of changes in the number of mast cells in the human arterial wall during the stages of development of atherosclerosis *J. Atheroscler Res.* 6:342-351
- Proud, D, Siekierski, ES, and Bailey, GS (1988) Identification of human lung mast cell kininogenase as tryptase and relevance of tryptase kininogenase activity *Biochem. Pharmacol.* 37:1473-1480
- Purcell, WM and Hanahoe, THP (1991) A novel source of mast cells: the human placenta *Agents & Actions* 33:8-12
- Rakusan, K and Campbell, SE (1991) Spatial relationship between cardiac mast cells and coronary capillaries in neonatal rats with cardiomegaly *Can. J. Physiol. Pharmacol.* 69:1750-1753
- Rakusan, K, Sarkar, K, Turek, Z, and Wicker, P (1990) Mast cells in the rat heart during normal growth and in cardiac hypertrophy *Cir. Res.* 66:511-516
- Razin, E, Ihle, JN, Seldin, D, Mencia-Huerta, JM, Katz, HR, Leblanc, PA, Hein, A, Caulfield, JP, Austen, KF, and Stevens, RL (1984) Interleukin 3: a differentiation and growth factor for the mouse mast cell that contains chondroitin sulfate E proteoglycan *J. Immunol.* 132:1479-1486
- Reccardi, VM (1989) Genetic alterations and growth factors in the pathogenesis of von Recklinghausen's neurofibromatosis *Neurofibromatosis* 2:292-298
- Reichardtson PD, Davies, MJ, and Born GV (1989) Influence of plaque configuration and stress distribution on fissuring of coronary atherosclerotic plaques *Lancet* II:941-944
- Reilly, CF, Tewksbury, DA, Schechter, NM, and Travis, J (1982) Rapid conversion of angiotensin I to angiotensin II by neutrophil and mast cell proteinases *J. Biol. Chem.* 257:8619-8622
- Reynolds, DS, Stevens, RL, Lane, WS, Carr, MH, Austen, KF, and Serafin, WE (1990) Different mouse mast cell populations express various combinations of at least six distinct mast cell serine proteases *Proc. Natl. Acad. Sci. USA* 87:3230-3232
- Reynolds, DS, Stevens, RL, Gurley, DS, Lane, WS, and Austen, KF (1989) Isolation and molecular cloning of mast cell carboxypeptidase A: a novel member of the

carboxypeptidase gene family J. Biol. Chem. 264:20094-20099

Reynolds, DS, Gurley, DS, Austen, KF, and Serafin, WE (1991) Cloning of the cDNA and gene of mouse mast cell protease-6 J. Biol. Chem. 266:3847-3853

Reynolds, DS, Gurley, DS, Stevens, RL, Sugarbaker, DJ, Austen, KF, and Serafin WE (1989) Cloning of cDNAs that encode human mast cell carboxypeptidase A and rat pancreatic carboxypeptidases Proc. Natl. Acad. Sci. USA 86:9480-9484

Riccardi, VM (1987) Mast cell stabilization to decrease neurofibroma growth. Preliminary experience with ketotifen Arch. Dermatol. 123:1011-1016

Riccardi, VM (1981) Cutaneous manifestation of neurofibromatosis: cellular interaction, pigmentation, and mast cells Birth Defects 17:129-145

Riccardi, VM and Eichner, RE (1986) Neurofibromatosis: phenotype, natural history, and pathogenesis Johns Hopkins Press, Baltimore

Robinson, C (1988) Mast cells and newly -generated lipid mediators. In: Holgate, ST eds., immunology and medicine: mast cells, mediators and diseases, Kluwer Academic Publishers, London, p149

Robinson, C, Benyon, C, Holgate, ST, Church, MK (1989) The IgE-and calcium-dependent release of eicosanoids and histamine from human purified cutaneous mast cells J. Invest. Dermatol. 93:397-404

Roche, WR (1985) mast cells and tumors: the specific enhancement of tumor proliferation in vitro Am. J. Pathol. 119:57-64

Roger, MP, Bloomingdale, K, Murawski, BJ, Soter, NA, Reich, P, and Austen, KF (1986) Mixed organic brain syndrome as a manifestation of systemic mastocytosis Psychosomatic Med. 48:437-447

Rothe, MJ, Nowak, M, and Kerdel, FA (1990) The mast cell in health and disease J. Am. Acad. Derm. 23:615-624

Ruokonen, H (1992) Mast cells in oral erythema multiform Acta Derm. Venereol. 72:92-94

Ruoslahti, E and Yamaguchi, Y (1991) Proteoglycans as modulators of growth factor activities Cell 64:867-869

Ruoss, SJ, Hartmann, T, and Caughey, GH (1991) Mast cell tryptase is a mitogen for cultured fibroblasts J. Clin. Invest. 88:493-499

Ryan, JJ and Huff, TF (1990) Schwann cell-conditioned medium supports development of mast cell-committed progenitors FASEB J: A1006

Schayer, RW (1963) Histidine decarboxylase in mast cells Ann. N.Y. Acad. Sci. 103:164

Schechter, NM, Irani, AMA, Sprows, JL, Abernethy, J, Wintroub, B, Schwartz, LB (1990) Identification of a cathepsin G-like proteinase in the MC<sub>TC</sub> type of human mast cell J. Immunol. 245:2652-2661

Schmidt, G, Owman, CH, and Sjoberg, NO (1988) cellular localization of ovarian histamine, its cyclic variations and histaminergic effects on ovulation in the rat ovary perfused in vitro J. Reprod. Fert. 82:409-417

Schmutzler, W, Delmich, K, Eichelberg, D, Gluck, S, Greven, T, Jurgensen, H, Riesener, KP, Risse, G, and Pult, P (1985) The human adenoidal mast cells. susceptibility to different secretagogues and secretion inhibitors Int. Arch. Allergy Appl. Immunol. 77:177-178

Schubert, M and Hamberman, D (1956) Metachromasia; chemical theory and histochemical use J. Histochem. 4:159-189

Schulman, ES, MacGlashan, DWJr, Peters, SP, Schleimer, RP, Newball, HH, and Lichtenstein, LM (1982) Human lung mast cells: purification and characterization J. Immunol. 129:2662

Schwartz, LB (1985) Monoclonal antibodies against human mast cell tryptase demonstrate shared antigenic sites on subunits of tryptase and selective localization of the enzyme to mast cells J. Immunol. 134:526-531

Schwartz, LB (1987) Mediators of human mast cells and human mast cell subsets Ann. Allergy 58:226-235

Schwartz, LB (1990a) Tryptase from human mast cells: biochemistry, biology, and clinical utility In: Neutral proteases of mast cells. Monogr Allergy, Basel Karger, vol.27, pp90-113

Schwartz, LB (1990b) Tryptase, a mediator of human mast cells J. Allergy Clin. Immunol. 86:594-598

Schwartz, LB (1991) Mast cells and their role in urticaria J. Am. Acad. Dermatol. 25:190-203

Schwartz, LB and Bradford, TR (1986) Regulation of tryptase from human lung mast cells by heparin: stabilization of the active tetramer J. Biol. Chem. 261:7372-7379

Schwartz, LB and Huff, TF (1991) Mast cells. In: The Lung, Crystal, RG et al. eds., Raven Press, Ltd., N.Y. pp601-616

Schwartz, LB, Bradford, TR, Littman, BH, and Wintroub, BY (1985) The fibrinogenolytic activity of purified tryptase from human lung mast cells J. Immunol. 135:2762-2767

Schwartz, LB, Kawahara, MS, Hugli, TE, Vik, D, Fearon, DT, and Austen, KF (1983) Generation of C3a anaphylatoxin from human C3a by human mast cell tryptase J. Immunol. 130:1891-1895

Schwartz, LB, Lewis, RA, and Austen, KF (1981a) Tryptase from human pulmonary mast cells Purification and characterization J. Biol. Chem. 256:11939-11943

Schwartz, LB, Lewis, RA, Seldin, D, and Austen, KF (1981b) Acid hydrolases and tryptase from secretory granules of dispersed human lung mast cells J. Immunol. 126:1290-1294

Schwartz, LB, Metcalfe, DD, Miller, JS, Earl, H, and Sullivan, T (1987) Tryptase levels as an indicator of mast cell activation in systemic anaphylaxis and mastocytosis N. Eng. J. Med. 316:1622-1626

Schwartz, LB, Maier, M, and Spratgg, J (1986) Interaction of human low molecular weight kininogen with human mast cell tryptase In: Adv. In Exp. Med. Biol, Greenbaum, LM, and Margolius HS eds, Plenum Press, Y.N., pp105-111

Schwartz, LB, Riedel, C, Caulfield, JP, Wasserman, SI, and Austen, KF (1981c) Cell association of complexes of chymase, heparin proteoglycan and protein after degranulation by rat mast cells J. Immunol. 126:2071-2078

Schwartz, LB, Riedel, C, Schratz, JJ, and Austen, KF (1982) Localization of carboxypeptidase A to the macromolecular heparin proteoglycan-protein complex in secretory granules of rat serosal mast cells J. Immunol. 128:1128-1133

Schwartz, LB, Yunginger, JW, Miller, J, Brokhari, R, and Dull, D (1989) Time course of appearance and disappearance of human mast cell tryptase in the circulation after anaphylaxis J. Clin. Invest. 83:1551-1555

Scott, JE (1972) Histochemistry of alcian blue. II. The structure of alcian blue 8GX Histochem. 30:215-234

Sedzwick, JB, Calhoun, WJ, Gleich, GJ, Kita, H, Abrams, JS, Schwartz, LB, Volovitz, B, Ben-Yaakov, M, and Busse, WW (1991) Immediate and late airway response of allergic rhinitis patients to segmental antigen challenge. Characterization of eosinophil and mast cell mediators Am. Rev. Respir. Dis. 144:1274-1281

Seeldrayers, PA, Levin, L, and Johnson, D (1989) Astrocytes support mast cell viability in vitro J. Neuroimmunol. 36:239-243

Segal, DM, Taurog, JD, and Metzger, H (1977) Dimeric immunoglobulin E serves as a unit for mast cell degranulation Proc. Natl. Acad. Sci. USA 74:2993-2997

Sekizawa, K, Caughey, GH, Lazarus, SC, Gold, WM, and Nadel, JA (1989) Mast cell tryptase causes airway smooth muscle hyperresponsiveness in dog J. Clin. Invest. 83:175-179

Serafin, WE, and Austin, KF (1987) Mediators of immediate hypersensitivity reactions N. Eng. J. Med. 317:30-34

Shanahan, F, Lee, TD, Bienenstock, J, and Befus, AD (1984) The influence of endorphins on peritoneal and mucosal mast cell secretion J. Allergy Clin. Immunol. 74:499-504

Sheu, HM, Yu, HS, Chang, CH (1991) Mast cell degranulation and elastolysis in the early stage of striae distensae J. Cut. Path. 18:410-416

Shibata, H, Mio, M, and Tasaka, K (1985) Analysis of the mechanism of histamine-release induced by substance P Biochem. Biophys. Acta 846:1-7

Shinohara, H, Nakatani, T, Morisawa, S, Matsuda, T, and Narwe, Y (1987) Mast cell in the ovarian bursa of the golden hamster Biol. Reprod. 36:445-450

Skofitsch, G, Savitt, JM, and Jacobowitz, DM (1985) Suggestive evidence for a functional unit between mast cells and substance P fibers in the rat diaphragm and

mesentery Histochemistry 82:5-8

Shotton, D.M. and Watson, H.C. (1970) Three-dimensional structure of Tosyl-elastase, Nature 225:811-816

Smith, TJ and Johnson, DA (1991) Human lung tryptase activity is stabilized by heparin Red. Proc. 43:1760 (Abstr)

Smith, TJ, Hougland, MW, and Johnson DA (1984) Human lung tryptase J. Biol. Chem. 259:11046-11051

Smith EB (1974) The relationship between plasma and tissue lipids in human atherosclerosis Adv. Lipid Res. 12:1-49

Smith, CJ, Smith, JC, and Finn, MC (1987) The possible role of mast cells (allergy) in the production of keloid and hypertrophic scarring J. Burn Care Rehabil. 8:126-131

Sommerhoff, CP, Caughey, GH, Finkbeiner, WE, Lazarus, SC, Basbaum, CB, Nadel, JA (1989) Mast cell chymase. A potent secretagogue for airway gland serous cells J. Immunol. 142:2450

Starkey, JR, Crowle, PK, and Taubenberger, S (1988) Mast cell deficient W/W<sup>v</sup> mice exhibit a decreased rate of tumor angiogenesis Int. J. Cancer 42:48-52

Sary, HC (1987) Macrophages, macrophage foam cells, and eccentric intimal thickening in the coronary arteries of young children Atherosclerosis 64:91-108

Sary, HC (1990) The sequence of cell and matrix changes in atherosclerotic lesions of coronary arteries in the first forty years of life Eur. Heart J. 11:3-19

Stead, RH, Tomioka, M, Quinoez, G, Simon, GT, Felten, SY, and Bienenstock, J (1987) Intestinal mucosal mast cells in normal and nematode-infected rat intestines are in intimate contact with peptidergic nerves Proc. Natl. Acad. Sci. 84:2975-2979

Stead, RH, Dixon, MF, Bramwell, MH, Riddell, RH, and Bienenstock, J (1989) mast cells are closely apposed to nerves in the human gastrointestinal mucosa Gastroenterol. 97:575-585

Stehbens, WE (1987) Localization of atherosclerotic lesions in relation to hemodynamics. In: Olsson, AG, Ed.

Atherosclerosis biology and clinical science Edinburgh:  
Churchill Livingstone, pp175-182

Steinberg, D, Parthasarathy, S, Carew, TE, Khoo, JC, and Witztum JL (1989) Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity N. Eng. J. Med. 320:945-924

Steinbusch, HWM and Mulder, AH (1984) Immunohistochemical localization of histamine in neurons and mast cells in the rat brain In: Bjoerklund et al. eds, Handbook of chemical neuroanatomy vol.3 pp126-140

Stevens, RL, Fox, CC, Lichtestein, LM, Austen, KF (1988) Identification of chondroitin sulfate E proteoglycans and heparin proteoglycans in the secretory granules of human lung mast cells Proc. Natl. Acad. Sci. USA 85:2284-2287

Stevens, RL, Lee, TDG, Seldin, DC, Austen, KF, Befus, AD, and Bienenstock, J (1986) Intestinal mucosal mast cells from rats infected with *Nippostrongylus brasiliensis* contain protease-resistant chondroitin sulfate di-B proteoglycans J. Immunol. 137:291-295

Sydbom, A (1982) Histamine release from isolated rat mast cells by neurotensin and other peptides Agents & Actions 12:91-93

Tainsh, KR, Lau, HY, Liu, WL, and Pearce, FL (1991) The human skin mast cell: a comparison with the human lung cell and a novel mast cell type, the uterine mast cell Agents & Actions 33:16-19

Takeuchi, K, Ishihara, Y, Kunimi, H, and Okabe, S (1984) Effect of FPL-52694, a new mast cell stabilizer, on gastric secretion and various acute gastric lesions in rats Agents & Actions 14:637-642

Takeuchi, K, Ohtsuki, H, and Okabe, S (1986) Pathogenesis of compound 48/80 induced gastric lesions in rats Dig. Dis. Sci. 31:392-400

Tam, EK and Caughey, GH (1990) Degradation of airway neuropeptides by human lung tryptase Am. J. Respir. Cell Mol. Biol. 3:27-32

Theoharides, TC (1990) mast cells: the immune gate to the brain Life Sci. 46:617-647

Theoharides, TC and Douglas, WW (1978) Somatostatin induces histamine secretion from rat peritoneal mast cells Endocrinol. 102:1143-1145

Thomas, PS, Schreck, RE, and Lazarus, SC (1992) Tobacco smoke releases preformed mediators from canine mast cells and modulates prostaglandin production Am. J. Physiol. 263:L67-72

Thompson, HL, Burbelo, PD, Segui-Real, B, Yamada, Y, and Metcalfe, DD (1989) Laminin promotes mast cell attachment J. Immunol. 143:2323-2327

Thompson, HL, Schulman, ES, and Metcalfe, DD (1988) Identification of chondroitin sulfate E in human lung mast cells J. Immunol. 140:2708-2713

Tsai, M, Shih, LS, Newlands, GF, Takeishi, T, Langley, KE, Zsebo, KM, Miller, HR, Geissler, EN, and Galli, SJ (1991) The rat C-kit ligand, stem cell factor, induces the development of connective tissue-type and mucosal mast cells in vivo. Analysis by anatomical distribution, histochemistry, and protease phenotype J. Exp. Med. 174:125-131

Valent, P, Sillaber, C, and Betklheim P (1991) The growth and differentiation of mast cells Prog. Growth Fact. Res. 3:27-41

Van der linden, PWG, Hack, CE, Poortman, J, Vivie-Kipp, YC, Struyvenberg, A, and van der Zwan, JK (1992) Insect-sting challenge in 138 patients: relation between clinical severity of anaphylaxis and mast cell activation J. Allergy Clin. Immunol. 90:110-118

Van den Boek, MF, van den Berg, WB, and van de Putte, LBA (1988) The role of mast cells in antigen induced arthritis in mice J. Rheumatol. 15:544-551

Van den Hooff, A and Tigchelaar-Guttar, W (1983) Stromal changes in experimental carcinogenesis in the skin. I. Ultrastructural aspects of collagen degradation Proc. of the K.N.A. van W, Series C: Biol. Med. Sci. 86:179-190

Vanderslice, P, Ballinger, SM, Tam, EK, Goldstein, SM, Craik, CS, and Caughey, GH (1990) Human mast cell tryptase: multiple cDNAs and genes reveal a multigene serine protease family Proc. Natl. Acad. Sci. USA 87:3811-3815

Vanderslice, P, Craik, CS, Nadel, JA, and Caughey, GH (1989) Molecular cloning of dog mast cell tryptase and a related protease: structural evidence of a unique mode of serine protease activation Biochem. 28:4148-4155

Vassimon, CS and Rothschild, AM (1990) Compound 48/80-



induced secretion of histamine from rat peritoneal mast cells depends on a tryptase controlled step also leading to chymase activity *Agents & Actions* 30:150-152

Vater, CA, Nagase, H, and Harris, ED (1983) Purification of an endogenous activator of procollagenase from rabbit synovial fibroblast culture medium *J. Biol. Chem.* 258:9374-9382

Villena, F, Montoya, GA, Roa, J, Jofre, A, and Goset, C (1986) Mast cells and synaptic transmission in sympathetic ganglia *Cell Mol. Biol.* 32:253-256

Wada, H, Inagaki, N, Yamatodani, A, and Watanabe, T (1991) Is the histaminergic neuron system a regulatory center for whole brain activity? *TINS* 14:45-48

Wagner, MMF, Edwards, RE, Moncrieff, CB, and Wagner, JC (1984) Mast cells and inhalation of asbestos in rats *Thorax* 39:539-544

Walden, SM, Proud, D, Bascom, R, Lidtenstein, LM, Kagey-Sobotka, A, Adkinson, NFJr, and Naclerio, RM (1988) Experimentally induced nasal allergic responses *J. Allergy Clin. Immunol.* 81:940-949

Wallace, JL, Morris, GP, Krausse, EJ, and Greaves, SE (1982) Reduction of ethanol-induced gastric mucosal damage by cytoprotective agents: a correlated morphological and physiological study *Can. J physiol. Pharm.* 60:1686-1699

Wallengren, J and Hakanson, R (1987) Effect of substance P, neurokinin A, and calcitonin gene-related peptide in human skin and their involvement in sensory nerve-mediated responses *Eur. J. Pharmacol.* 143:267-273

Walls, AF, Brain, SD, Desai, A, Jose, PJ, Hawkings, E, Church, MK, and Williams, TJ (1992) Human mast cell tryptase attenuates the vasodilator activity of calcitonin gene-related peptide *Biochem. Pharm.* 43:1243-1248

Weihe, E, Mueller, S, Fink, T, and Zentel, HJ (1989) Tachykinins, calcitonin gene-related peptide and neuropeptide Y in nerves of the mammalian thymus: interactions with mast cells in autonomic and sensory neuroimmunomodulation? *Neurosci. Lett.* 100:77-82

Weinreich, D and Udem, BJ (1987) Immunological regulation of synaptic transmission in isolated guinea pig autonomic ganglia *J. Clin. Invest.* 79:1529-1532

- Weitzman, G, Galli, SJ, Dvorak, AM and Hammel, I (1985) Cloned mouse mast cells and normal mouse peritoneal mast cells. Determination of serotonin content and ability to synthesize serotonin in vitro Int. Arch. Allergy Appl. Immunol. 77:189-191
- Wenzel, SE, Fowler, AA, Schwartz, LB (1988) Activation of pulmonary mast cells by bronchoalveolar allergen challenge. In: vivo release of histamine and tryptase in atopic subjects with and without asthma Am. Rev. Respir. Dis. 137:1002
- Williams, JM and Feltern, DL (1981) Sympathetic innervation of murine thymus and spleen: a comparative histofluorescence study Anat. Rec. 199:531-542
- Wintroub, BY, Schechter, NB, Lazarus, GS, Kaempfer, CE, Schwartz, LB (1984) Angiotensin I conversion by human and rat chymotryptic protenases J. Invest. Dermatol. 83:336-339
- Wintroub, BY, Mihm, MCJr, Goetzl, EF, Soter, NA, and Austen KF (1978) Morphologic and functional evidence for release of mast cell products in bullous pemphigoid N. Eng. J. Med. 298:417-421
- Woodbury, RG, Miller, HR, Huntley, JF, Newlands, GF, Palliser, AC, and Wakelin D (1984) Mucosal mast cells are functionally active during spontaneous expulsion of intestinal nematode infections in rat Nature 312:450-452
- Woodbury, RG and Miller, HRP (1982) Quantitative analysis of mucosal mast cell protease in the intestine of Nippostrongylus-infected rats Immunol. 46:487-495
- Wooley, D (1984) Collagenolytic mechanisms in tumor cell invasion Cancer Metastasis Rev. 3:61-372
- Yamanishi, Y, Dabbous, MK, Hashimoto, K (1972) Effects of collagenolytic activity in basal cell epithelioma of the skin on reconstituted collagen and physical properties and kinetics of the crude enzyme Cancer Res. 32:2551-2560
- Yawasaki, Y, Shimamura, O, Kizu, A, Nakagawa, M, and Ijichi, H (1982) IgE-mediated <sup>14</sup>C-serotonin release from rat mast cells modulated by morphins and endorphins Life Sci. 31:471-478
- Yoshimura, A and Mori, W (1991) Renal tissue mast cells in liver disease Intern. Urol. Nephrol. 23:511-515

Young, JDE, Liu, CC, Butler, G, Cohn, ZA, and Galli, SJ (1987) Identification, purification, and characterization of a mast cell-associated cytolytic factor related to tumor necrosis factor Proc. Natl. Acad. Sci. USA 84:9175-9179

Yunginger, JW, Nelson, DR, Squillace, DL, Jones, RT, Holley, KE, Hyma, BA, Biedrzycki, L, Sweeney, KG, Sturner, WQ, Schwartz, LB (1991) Laboratory investigation of deaths due to anaphylaxis J. Forensic Sci. 36:857-865

Yurt, RW, Leid, RW, Austen, KF, and Silbert, JE (1977) Native heparin from rat peritoneal mast cells J. Biol. Chem. 252:518-521

Zachrisson, BY (1968) Mast cell of the human gingiva IV. Experimental gingivitis J. Periodont. Res. 4:46-55

DISSERTATION APPROVAL SHEET

The dissertation submitted by Zehan Chen has been read and approved by the following committee:

William H. Simmons, Ph.D., Director  
Associate Professor, Department of Molecular and Cellular  
Biochemistry, Loyola University of Chicago.

Allen Frankfater, Ph.D.  
Associate Professor, Department of Molecular and Cellular  
Biochemistry, Loyola University of Chicago.

Allen Samarel, M.D.  
Professor of Medicine and Physiology, Director of the Cardiovascular  
Basic Research Laboratories, Loyola University of Chicago.

Richard M. Schultz, Ph.D.  
Professor and Chairman, Department of Molecular and Cellular  
Biochemistry, Loyola University of Chicago.

Donald F. Steiner, M.D.  
Senior Investigator and Pritzker Professor of Biochemistry and  
Molecular Biology and Medicine, Howard Hughes Medical Institute,  
Department of Biochemistry and Molecular Biology, University of  
Chicago.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

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

2/15/93

Date



Director's Signature