1986

Degradation of Human Anaphylatoxin C3a by Rat Peritoneal Mast Cells: A Role for the Secretory Granule Enzyme Chymase and Heparin Proteoglycan

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Degradation of Human Anaphylatoxin C3a by Rat Peritoneal Mast Cells: A Role for the Secretory Granule Enzyme Chymase and Heparin Proteoglycan

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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May, 1986

DEDICATIONS

There have been many people involved in the development of my personality, both socially and professionally. I would like to take this opportunity to thank some of these individuals by dedicating this work to them. I would like to dedicate this dissertation first and foremost to my father James E. Gervasoni, Sr. and my mother Jennie Mule' Gervasoni for without their complete devotion to the betterment of my soul I would have never achieved this goal. I would also like to extend this dedication to my loving sister Olga Marie Gervasoni whose enthusiasm and friendship is unparalleled.

I would also like to give special thanks to Dr. Daniel H. Conrad for his technical training early in my career. I would also like to extend my gratitude to Dr. Conrad for his faith in my ability to succeed in this program.

ACKNOWLEDGEMENTS

I would like to thank my mentor Dr. Shaun Ruddy for this excellent scientific guidance. I am honored to have had the opportunity to work with a scientist of Dr. Ruddy's caliber. I would like to thank my committee members for all of their assistance. I would also like to thank Dr. Lawrence B. Schwartz for all of his suggestions and helpful criticism on my work. I would like to thank Donald Purkall for his technical assistance and his introduction to me of a new ideology that is known in our laborotory as the "Purkonean Theory".

Finally, I would like to thank Barry Browder for the many discussions that we have had about my work and my well being over a few beers at the Village Cafe'.

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LIST OF ABBREVIATIONS

BS^3	bis(sulfosuccinimidyl)suberate
BSA	bovine serum albumin
BTEE	benzoyl-L-tyrosine ethyl ester
CMMC	cultured mouse mast cells
cpms	counts per minute
CTMC	connective tissue mast cell
$C3a_{des\ Arg}$	C3a minus the COOH-terminal arginine
DFP	diisopropylfluorophosphate
EDTA	ethylenediaminetetraacetic acid
^{125}I-C3a	radioiodinated C3a
MMC	mucosal mast cells
NP-40	nonidet
OVA	ovalbumin
PBS	phosphate buffered saline
RMC	rat peritoneal mast cells
RMCP I+II	rat mast cell protease
SBTI	soybean trypsin inhibitor
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel
TCA	trichloroacetic acid
TD	tyrode's buffer
TLCK	tosyl-L-lysyl chloromethyl ketone
TPCK	tosyl-L-phenylalanine chloromethyl ketone
Tyrode's$^{=}$	tyrode's buffer minus Ca^{++} and Mg^{++}

ABSTRACT

DEGRADATION OF HUMAN ANAPHYLATOXIN C3a BY RAT PERITONEAL MAST CELLS: A ROLE FOR THE SECRETORY GRANULE ENZYME CHYMASE AND HEPARIN PROTEOGLYCAN.

James Edmund Gervasoni, Jr, B.A.

Major Director: Shuan Ruddy, M.D.

Purified human C3a was iodinated (^{125}I-C3a) and used to study the interaction of labeled peptide with rat peritoneal mast cells (RMC). Cellular binding of ^{125}I-C3a occurred within 30 sec, followed by a rapid dissociation from the cell. Both the binding of ^{125}I-C3a and the rate of dissociation from the cell were temperature dependent. At 0^{o}C, the binding of ^{125}I-C3a was increased and the rate of dissociation reduced, as compared to 37^{o}C. Once ^{125}I-C3a was exposed to RMC, it lost the ability to rebind to a second batch of RMC. Analysis of the supernatants by trichloroacetic acid (TCA) precipitation and electrophoresis in sodium dodecyl sulfate polyacrylamide gels (SDS PAGE) revealed a decrease in the fraction of ^{125}I precipitable by TCA and the appearance of ^{125}I-C3a cleavage fragments. Pretreatment of RMC with enzyme inhibitors specific for chymotrypsin, but not trypsin, abrogated the degradation of ^{125}I-C3a. Treatment of RMC bearing ^{125}I-C3a with Bis (sulfosuccinimidyl) suberate (BS^3) covalently crosslinked the ^{125}I-C3a to chymase, the predominant enzyme found in the secretory granules. Antiserum directed against chymase precipitated ^{125}I-C3a from extracts of RMC treated with BS^3. Indirect immunofluorescence of RMC using the IgG fraction of goat

anti-rat chymase showed that chymase is present on the surface of unstimulated cells. Neither purified chymase nor heparin proteoglycan alone had any appreciable effect on ^{125}I-C3a, but together they resulted in prompt degradation of the ^{125}I-C3a. Immunoabsorption of RMC sonicates with specific antibody for chymase completely abrogated the ability of these sonicates to degrade ^{125}I-C3a. Intact RMC were pretreated with serine esterase inhibitors prior to ^{125}I-C3a and BS^3 exposure. The cells to which ^{125}I-C3a had been crosslinked to were solublized and analyzed by SDS PAGE and autoradiography. There were three bands visualized, a 35,000 dalton band which was defined as chymase, and two undefined 45,000 and 55,000 dalton bands. The results indicate that ^{125}I-C3a binds to RMC and is promptly degraded by chymase in the presence of heparin proteoglycan. In addition, this proteolysis of ^{125}I-C3a by chymase must be blocked in order to detect plasma membrane C3a binding components on RMC.

INTRODUCTION

The three systems that play an intricate role in the process of inflammation are the clotting, kinin, and complement systems. Upon activation each system generates primary and secondary products that have significant biological effects during the acute inflammatory response. The primary products continue on into the central pathways of their respective systems. The secondary products contain activation molecules that are involved in mediation of the inflammatory response. The complement system is made up of two distinct pathways, the classical and alternative pathways. The differences between the two pathways involves the generation of the C3 and C5 convertase enzymes. The C3 and C5 convertase enzymes are responsible for the cleavage of native C3 and C5, respectively. The fragments that are generated by the proteolytic cleavage of C3 and C5 are C3a and C3b and C5a and C5b, respectively. Both the C3b and C5b fragments are involved in the lytic function of the complement system. The smaller C3a and C5a fragments are termed "anaphylatoxins " and mediate other biological function involved in the acute inflammatory process. In the classical pathway, the complement components that are involved in generating C3 and C5 convertases are Clqrs, C2b, C4b, and C3b (1). In the corresponding alternative pathway, the molecules that are involved in the C3 and C5 convertase enzymes are Factor B, Factor D, C3b, and properdin (2). The C3a fragment generated by either the alterative or classical C3 convertase are chemically and biologically identical. This is also true for the C5a fragment generated by either pathway. Discovering the molecules responsible for anaphylatoxin activity took approximately sixty years.

Historical Background. In 1910 Friedberger observed that guinea pig serum exposed to immune precipitates acquired the ability to induce anaphylactic shock in animals injected with the treated serum. Friedberger called these hypothetical substances that caused this shock syndrome "anaphylatoxins" (3). In 1913 Bordet showed that anaphylatoxins could also be generated by treating serum with other agents such as polysaccharides (agar) (4). Agents such as immune complexes, agar, dextran, starch, and inulin were used to generate anaphylatoxin activity in sera from rats, rabbits, and guinea pigs. Novy and de Kruif in 1917 working with various types of activators of serum such as trypanosomes and agar showed that anaphylatoxin activity was thermolabile and could be generated in serum without addition of foreign proteins (5). It was concluded at this time that the toxic factor found in various animal sera might be derived from the complement system, which at this time was poorly understood. Friedberger proposed the "humoral anaphylatoxin theory" which stated that the shock-induced syndrome of activated serum was caused by humoral factors present in blood (complement) (3). The "humoral anaphylatoxin theory" was abandoned in the early 1950s when Hahn and Oberdorf showed that there was a correlation between anaphylactic shock syndrome and the liberation of histamine (6). The authors concluded that anaphylatoxin itself acts as a histamine liberator when injected into guinea pigs (8). Friedberger's hypothesis that complement may play a role in the generation of anaphylatoxins was not addressed until 1957 when Lepow showed a decrease in complement activity when serum was incubated with dextran, with a concomitant increase in anaphylatoxin activity (7). It was not until 1959 that Osler et al had

the first clear evidence that showed that complement was involved in anaphylatoxin generation (9). Osler et al, demonstrated that serum acquired the ability to induce smooth muscle contraction and enhance vasuclar permeability upon activation of the complement system. These investigators also showed that the hemolytic activity of serum decreased as the anaphylatoxin activity increased. Compounds such as chelators or hydrazine which prevent complement activation also showed to inhibit the production of spasmogeneic activity (9). Jensen in 1959 was the first investigator to assign the anaphylatoxin activity to a specific complement component. Jensen isolated guinea pig C5 from serum and digested the molecule with trypsin which generated anaphylatoxin activity (10). In addition, human C5 was also isolated and digested with trypsin. Two fragments C5b and C5a were identified and separated by gel filtration. The latter fragment showed anaphylatoxin activity (11). A new anaphylatoxin activity was reported by Dias da Silva et al (1967) in a reaction mixture that contained purified complement components C1 esterase (C1 esterase is activated C1s), C4, C2, and C3 but not C5. In the presence of Mg^{++} this reaction mixture resulted in the cleavage of C3. These investigators further demonstrated that a smaller fragment was generated from ^{125}I-C3 that showed anaphylatoxin activity (12). Cochrane and Muller-Eberhard showed that there were clearly two distinct anaphylatoxins that could be generated from the complement components C3 and C5 (11). They also showed that the C3a fragment is a polypeptide of 6,000 to 15,000 daltons and has all the anaphylatoxin activities as C5a. Shortly after the identification of the two anaphylatoxins C3a and C5a, Bokisch demonstrated a serum protein in humans that inhibited C3a

anaphylatoxin activity (13). In addition, Bokisch showed that if C3a was incubated with normal human serum for greater than 1 minute the C3a spasmogenic activity was completely abrogated (13). Furthermore, Bokisch and Muller-Eberhard isolated and identified the molecule responsible for the inactivation of C3a as a carboxypeptidase (14). Previous to their findings, anaphylatoxin generation in whole human serum could not be demonstrated. Failure to produce anaphylatoxin in human serum was a direct effect of this carboxypeptidase activity. The identification of this serum inactivator made it possible to isolate and characterize the C3a and C5a molecules in active form and compare them physiochemically. Although human C3a had previously been characterized by Cochrane (11), C5a had not. With the use of 1 M epsilon-aminocaproic acid, an inhibitor of the carboxypeptidase enzyme, Vallota and Muller-Eberhard (1972) were able to characterize C5a and compare it to C3a (15).

Chemical Nature of C3a. Vallota and Muller-Eberhard found that the electrophoretic mobility for C3a was + 2.1 x 10^{-5} cm^2 V^{-1} s^{-1} and for C5a it was +1.7 x 10^{-5} cm^2 V^{-1} S^{1}. In addition, the molecular weights of C3a and C5a were determined by two methods, gel filtration and gel electrophoresis. By gel filtration, the molecular weights of C3a and C5a were 8,700 and 17,500 daltons, respectively; by gel electrophoresis C3a was 7,200 and C5a was 16,500. Detailed chemical analysis of C3a was performed using human, rat, and porcine as a source of anaphylatoxins (16, 17, 18, 19). The amino acid sequence of the three animal types are very similar, although there are some noticeable differences. These differences do not greatly affect the functional activities of these three anaphylatoxins (20). The molecular weights of

these three C3a anaphylatoxin molecules are approximately 9,000 daltons according to gel filtration studies and direct calculations using the primary amino acid sequences (16, 17, 18, 19). The number of residues in human and porcine C3a are 77 amino acids, whereas in rat there are 78 amino acid residues. From the number of residues of C3a and from the amino acid sequence of C3 it can clearly be demonstrated that the C3a molecule comes from the α chain of native C3 and that the α chain is cleaved at position 77 or 78 by a serine hydrolase enzyme (C3 convertase) (21). All three C3a molecules from human, rat, and porcine have three disulfide bridges in the molecule. The isoelectric points of human and porcine C3a are 9.7 and 8.6, respectively. C3a is a highly cationic molecule made up of a single polypeptide chain lacking carbohydrate residues. It has been found that the inactive form of C3a is the $C3a_{des\ Arg}$ form. The $C3a_{des\ Arg}$ molecule is native C3a which has had its COOH-terminal arginine residue cleaved off (17). The $C3a_{des\ Arg}$ is nonspasmogenic and is the molecule produced following cleavage by the serum carboxypeptidase inactivator. It has been shown that the COOH-terminal octapeptide of C3a is 1 to 2% as active in all biological assays as compared to native C3a (22). Furthermore, as the length of the octapeptide is decreased going towards the arginine COOH-terminus there is a progressive decrease in activity. Below a tripeptide there is essentially no activity observed (23). From these studies it is clear that the COOH-terminal region of the C3a molecule is essential for biological activity. Structural studies to determine the tertiary conformation of human and a porcine C3a using circular dichoism (CD) have been performed (18,19). The CD spectra indicates that both human and

porcine C3a are 40 to 50% alpha helical in conformation. From the CD spectra it can be deduced that C3a has a tightly compact folding arrangement with the NH_2 and COOH-terminal regions in an alpha helical conformation with three small loops formed by the intrachain disulfide bonds (24). Recently, evidence to confirm this model has been demonstrated by Deisenhofer in Germany, who has purified, crystallized, and analyzed human C3a by x-ray crystallographic methods. C3a is folded into a conformation that resembles a drum stick, with the COOH-terminal end extending from a highly compacted region where the disulfide bridges are found. In addition, the NH_2-terminal region is also included in that compacted domain (25). Additional experiments were performed to determine the effect of various agents such as 6 M guanidinium chloride and mercaptoethanol on the conformation and spasmogenic activity of C3a (24). Both treatments show an alteration in conformation of the C3a molecule as measured by CD spectra, with less than 10% of the spasmogenic activity retained. The effect of these agents is reversible. After treatment of the C3a molecule by dialysis against buffer, full activity is restored. Irreversible inactivation of C3a occurs only after extensive exposure to 100°C. For example, C3a could be heated to 100°C for 30 minutes with more than 50% of the spasmogenic activity retained. Complete activity is lost after 3 hours at 100°C (24). The effect of pH on the CD spectra over the range from 1 to 9.6 indicate that significant conformational changes occur at the lower pH ranges between 1 and 3.7 (24).

Structural and Functional Aspects of C3a. The relationship between the structural and functional aspects of C3a have recently been

approached by using the synthetic peptides discussed earlier. The major thrust in structure-function studies is to elucidate the region of the C3a molecule that is responsible for its biological activity. As mentioned earlier, a synthetic octapeptide that has the identical amino acid sequence as native C3a at the COOH-terminus has the same biological actions except to a lesser degree (23). Experiments that compare native C3a to its analog the octapeptide C3a(70-77) indicate the latter is 40 to 50 fold less active on a mole basis in inducing contraction of guinea pig ileal and uterine smooth muscle. When C3a(70-77) is injected intracutaneously into humans there is a visible skin reaction caused by local edema and erythema that is maximal between 10^{-10} M to 10^{-9} M; using native C3a, a similar response is observed maximally over the range of 10^{-12} to 10^{-11} M. In addition, if C3a(70-77) or native C3a is applied to guinea pig ileal strips, the muscle contraction is abrogated towards the other factor. This cross tachyphylactic phenomena (i.e., desensitization of tissue by a substance) further indicates that the C3a(70-77) synthetic peptide is analogous to native C3a. The fact that C5a is not cross tachyphylactic with either native C3a or C3a(70-77) suggests that C5a and C3a mediate their cellular effects via different receptors. Hugli has devised a model based on studies using the synthetic peptides to explain the molecular interaction of C3a with its putative cellular receptor (23). The COOH-terminal sequence of native C3a is Ala-Ser-His-Leu-Gly-Leu-Ala-Arg-COOH. If one replaces both leucine residues with an alanine residue, C3a(70-77) activity is lost. Also if the arginine is removed, activity is lost and if the COOH group of arginine is replacead by glycine, the activity of the peptide is

lost. Based on these results, Hugli has proposed a model for the putative mast cell receptor. The putative receptor has a hydrophobic pocket for the two leucine residues, a negatively charged pocket for the arginine residue, and a positively charged pocket for the COOH-terminus.

The Tissue Effects of C3a. The tissue effects of C3a are well documented for smooth muscle preparations but not for vascular permeability. The most common test used to determine the presence of C3a is to measure the contraction of guinea pig ileal strips. Other tissue tested for smooth muscle contractility to C3a includes guinea pig ileum, uterus, trachea, lung, colon, atrium, rat ileum and rat uterus. The most reactive tissue for smooth muscle contraction is the guinea pig ileum, the least is lung strips (20). The smooth muscle contractions induced by C3a can be inhibited by antihistamines, except for the lung strips. These results suggest that C3a mediation of smooth muscle contraction is through liberation of histamine in all these tissues except the lung. It has been suggested that smooth muscle contraction of guinea pig lung is not inhibited by antihistamine because the substances responsible for contraction are prostaglandins (26).

In order to understand the effects of C3a in whole tissue in vivo, one must gain a better knowledge of the particular cells that this molecule interacts with. Furthermore, the interaction of C3a with a particular cell, on the molecular level, and how C3a effects that cell upon interaction is not well understood. C3a produces a variety of effects on cells including mediator release phenomena, aggregational responses, and suppression of the cellular immune responses. As a result, there is a growing list of cell types that C3a has been shown to interact with. The major cell target for C3a is the mast cell. The

effect of C3a on mast cells is well documented in early work of the 1950's. Many investigators have shown that C3a causes vasoamine release (27, 28, 29) and degranulation of mast cells (11,12). There has been no data to support the fact that $C3a_{des\ Arg}$ has any activity for vasoamine release or degranualtion of mast cells. Experiments to determine binding sites for C3a are few; one study found that ^{125}I-C3a binding to rat mast cells ranged from 5 to 10 million molecules per cell (29). These numbers are probably not correct since all cells do not have identical distribution of receptors on their surface. Ter Saan et al (30) has found that only 20% of the rat mast cells bind C3a by using indirect immunofluorescence techniques. In addition, if these investigators use inhibitors of mast cell degranulation such as ethylenediaminetetraacetic acid (EDTA) or disodium cromoglycate (DSCG) during the incubation period of C3a and RMC there was no fluorescence observed, indicating the degranulation of RMC increases the amount of C3a binding.

The other cell types to date that have been shown to have the capacity to bind C3a are eosinophils, neutrophils, basophils and monocytes (31). Becker et al are the only investigators to show a specific receptor site for C3a on guinea pig platelets (32). In addition, these investigators have shown that C3a interacts with guinea pig platelets and causes these cells to release serotonin (33). Recently, these investigators have shown that C5a causes serotonin release from guinea pig platelets and that the activation pathways for the anaphylatoxins are independent of each other (34). Other studies have been performed on neutrophils, macrophages, and lymphocytes to

observe the biological effect of C3a on these various cell types. Showell, et al has demonstrated that C3a can cause lysosomal enzyme release in a dose-dependent fashion (10-100 ug/ml) from human peripheral neutrophils (35). These investigators observed that both primary and secondary granules were released from neutrophils when exposed to C3a plus cytochalasin B. When C3a was exposed to neutrophils alone, there was limited lysosomal enzyme release. When the formyl peptide receptor antagonist, t-boc-(phe leu)$_2$-phe was added to neutrophils, this did not block the lysosomal enzyme release induced by C3a. These results suggest an independent receptor for C3a and the formyl peptides. In the macrophage system, Hartung, et al have shown that C3a induces the release of thromboxane A_2 from guinea pig peritoneal macrophages (36). These investigators also showed that the hexapeptide C3a(72-77) also caused thromoboxane release from guinea pig macrophages, and that both C3a and C3a(72-77) activation was blocked by a monoclonal anti-C3a antibody. $C3a_{des\ Arg}$ was completely ineffective in activating these macrophages. Finally, there have been studies done that show the effects of C3a on the cellular immune response. Charriaut, et al showed that C3a inhibits natural killer cell cytotoxicity in both mouse and human systems (37). Charriant, et al also found that the inhibitory effect was dose and time dependent, the inhibition was at the effector-cell level, and that $C3a_{des\ Arg}$ did not alter the inhibitory effect displayed by C3a. Morgan, et al have demonstrated the effect of C3a on suppression of both specific and polyclonal antibody responses in mouse and humans (38). In these experiments it was shown that the COOH-terminal arginine residue

was essential for suppressive active. In contrast, C3a had no effect on antigen or mitogen-induced B or T cell proliferative response. It was also found that the helper T cell was the target cell for this C3a suppressive activity. Human C3a and the synthetic octapeptide C3a(70-77) inhibited the generation of leukocyte inhibitory factor (LIF) activity by human mononuclear leukocytes and T lymphocytes cultured with mitogen (39). When lymphocytes were chromatographed onto a C3a(70-77)-Sepharose column the cells found in the effluent fractions lost their ability to generate LIF activity. When the lymphocytes from the effluent from C3a (70-77)-Sepharose columns were analyzed by using monoclonal antibodies to surface antigens, there was a selective depletion of the helper/inducer population of lymphocytes. It was concluded from these results that C3a may play an important role in the regulation of human T lymphocyte activity.

Similar studies that have been described above for C3a have also been performed with C5a anaphylatoxin. There have been specific receptors for C5a demonstrated on both human neutrophils and murine macrophages (40,41). Furthermore, C5a has been shown to cause lysosomal enzyme release from rabbit alveolar macrophages (42) and also has been implicated in regulating the immune response (43).

It is clear from the evidence presented above that the anaphylatoxins play an important role in the overall immunity of an organism, and that these molecules are involved in mediating a variety of cellular functions. These studies indicate the utmost importance of defining the specific biological role of C3a in the acute inflammatory

response. In order to accomplish this task, a better understanding of the molecular events involved in mediation of the function of C3a is imperative. These events involve the understanding of the specific C3a receptor and how these receptors operate on their respective cell types. The first step in gaining this knowledge is to establish, isolate and characterize a C3a receptor.

Mast Cell Heterogeniety.

Mast cells have long been recogized, with basophils, as being the major cell involved in immediate-type hypersensitivity reactions. The role of mast cells in inflammation and in host defence against parasitic infections gives credence for a central involvement of these cells in the overall immunity of an organism. The tissue location and the types of mediators released during activation of mast cells supports this notion. Mast cells are located around the venules and in the connective tissues of serosal, cutaneous, and mucosal surfaces where released mediators would have the most profound effect. The two types of mediators found in mast cells are preformed and newly-generated. The preformed mediators reside mostly in the secretory granules and include the biogenic amines (histamine and serotonin), proteoglycan (heparin or chondroitin sulfate), neutral proteases (chymase, carboxylpeptidase A, or tryptase), and the acid hydrolases (β-hexosaminidase, β-glucuronidase, β-galactosidase, and arylsulfatase). The newly-generated mediators are lipid-derived and are produced following activation of the mast cell. Among these lipid-derived mediators are the leukotrienes and prostaglandin D_2 (PGD_2), which are both derived from arachidonic acid, and platelet-activating factor (PAF).

There are several criteria used to characterized mast cells, namely, high affinity receptors for monomeric IgE, the ablility to produce histamine or serotonin, and the metachromatic staining of the cytoplasmic granules with basic dyes. Using these criteria it has become clear that there exists at least two different types of mast cells. The two types of mast cells under investigation are the connective tissue mast cell (CTMC) and the mucosal mast cell (MMC). The CTMC resides in skin and are isolated from serosal tissue of the lung and peritoneum, whereas C are found in the lamina propria and epithelium of the small intestines.

Connective Tissue Mast Cell. It is evident from morphologic, histologic, and ultrastructural studies that the rat CTMC found in the skin is different from the MMC located in the intestines (44). When CTMC are stained with alcian blue and safranin, both cationic dyes bind, indicating the presence of a highly sulfated glycosaminoglycan in the granules (45). Heparin proteoglycan is highly sulfated and is responsible for the type of staining pattern observed with CTMC. The concentration of heparin proteoglycan ranges between 10-20 µg per 10^6 cells with a molecular weight of 750,000 (46). CTMC is approximately 20 µm in diameter and contains amorphous graunles of uniform size (47). There are two types of biogenic amines found in the granules of CTMC and they are histamine and serotonin (48). The concentration of histamine in the cell is estamated to range between 10-30 µg per 10^6 cells. The amount of serotonin in these cells has not been determined. Both these mediators are involved in causing increased vasodilation and increased vascular permeability (49). The other type of mediators released by CTMC are the newly generated compounds such as PGD_2, SRSc (primarily leukotriene C (LTC)), and PAF. The function of PGD_2 is not clear,

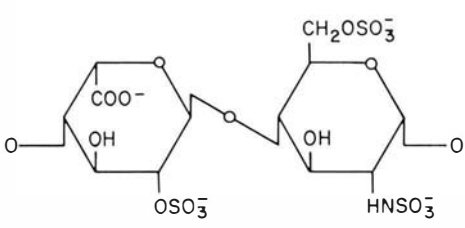
although it has been shown to cause bronchoconstriction of human (50) and canine airways (51) when administered by aerosol. There are several biological activities of LTC which include neutrophil chemotaxis and aggregation (52), bronchoconstriction (53), and a edema-erythema reaction following intracutaneous injections into humans (54). Finally, PAF has many biological activities such as platelet aggregation and secretion of platelet factor 4, serotonin and thromboxane B_2 release (55), smooth muscle contraction of guinea pig ileum (56), and edema-erythema reaction when injected intracutaneously into both human and animals (57).

RMC Heparin Proteoglycan. The major criterion for histological identification of mast cells is based on the ability of these cells to produce metachromasia following staining with basic dyes such as Alcian blue or Safranin (58). The mast cell component responsible for the production of metachromasia following addition of basic dyes is the highly sulfated proteoglycan found in the secretory granules (59,60,61). There are several types of proteoglycans found in different subsets of mast cells in various species. Heparin is the predominant proteoglycan found in human, mouse, and rat serosal mast cells, whereas chondroitin sulfate E is the major proteoglycan of mouse mucosal mast cells (59,60) (Fig. 1).

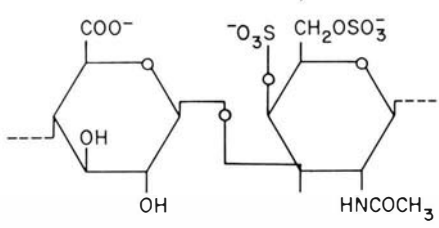
Chemical Composition of Heparin Proteoglycan. The heparin proteoglycan molecule is composed of many carbohydrate chains attached to a protein core. The type of carbohydrate chain is a glycosaminoglycan which is composed of a disacchaide unit with either glucuronic or iduronic acid linked to a glucosamine (62). Each disacchide unit has one carboxyl group and one to four sulfate groups. Both the carboxyl and

Figure 1. Repeating Disaccharide Subunits found in Heparin and Chondroitin Sulfate E.

Figure 1.



HEPARIN



CHONDROITIN SULFATE E

sulfate groups are reponsible for heparin proteoglycan having a net negative charge. The protein core of heparin proteoglycan is a polypeptide made up of alternating glycine and serine amino acids. The glycosaminoglycan is attached to glucuronic acid(GlcUA)-galactose(Gal)-Gal-xylose(Xyl) which in turn is O-linked to every second or third serine residue (63,64,65,66,67). The average molecular weight (MW) of the heparin proteoglycan molecule varies among different species. In the rat the MW of the proteoglycan is 750,000 and contains a protein core of 12,000-20,000 MW with glycosaminoglycan side chains of 50,000-100,000 MW (61,68). The human pulmonary mast cell proteoglycan has an average MW of 60,000 with glucosaminoglycan of 20,000 MW (69).

Biosynthesis of Heparin proteoglycan. The sequence of events leading to the biosynthesis of heparin proteoglycan involves several enzymatically catalyzed reactions. The initiation of the carbohydrate chain formation is facilitated by the serine-glycine sequence found in the protein core (70,71). The enzyme xylosyltransferase recognizes the serine-glycine sequence of the protein core and initiates the transfer of xylose from uridine diphosphate (UDP)-xylose to the hydroxyl group of serine (72). The elongation of the remaining carbohydrate sequence found in heparin involves the enzymes galactosyltransferase and glucuronosyltransferase yielding the sequence (GlcUA-N-acetyl glucosamine(GlcNAc))$_n$-GlcUA-Gal-Gal-Xyl-Serine (73,74,75). Two additional enzymes, N-acetylglucosaminyltransferase and glucuronosyltransferase are involved in formation of the repeating disaccharide unit (76). The elongation reaction occurs in the the Golgi apparatus or the endoplasmic reticulum by the addition of the

corresponding UDP-monosaccharide to the growing polysaccharide chain (77).

Once the polysaccharide chain is syntheized, there are five modifications of the disaccharide unit which produce a heparin type of glycosaminoglycan (77). The five step modification procedure has been studied in the mouse mastocytoma cell and has be shown to occur in less than 30 seconds. They are as follows: 1) the N-acetyl glucosamine residue is deacetylated to yield N-glucosamine, 2) N-glucosamine is sulfated, 3) particular glucuronic acid moieties are epimerized to iduronic acid, 4) the iduronic acid residue is sulfated at C-2, 5) and the N-sulfated glucosamine residue undergoes an additional sulfation at C-6 yielding the complete heparin glycosaminoglycan chain.

Function of Heparin Proteoglycan. The macromolecular size and negative charge of heparin proteoglycan makes this molecule well suited for being the matrix of the secretory granule in mast cells. In the secretory granule heparin proteoglycan acts as a cationic exchanger binding tightly with the neutral proteases chymase and carboxypeptidase A of rat serosal mast cells and tryptase of the human mast cell (78,79,80). Heparin proteoglycan has been shown to alter the activity of both chymase on particular protein substrates in the rat serosal mast cell and tryptase in the human mast cell (80,81,82). Both these findings suggest that heparin proteoglycan may regulate the activity of chymase and tryptase in the secretory granules and in the inflammatory milieu. The other component bound to heparin proteoglycan in the secretory granules is histamine via the carboxyl groups of both heparin and proteins at acidic pH (83). Unlike the neutral proteases, following degranulation of mast cells, histamine and the acid hydrolases are freely soluble at physiological pH.

Heparin proteoglycan has been shown to possess various activities that effect both cellular and humoral responses. The most well known activity of heparin is its anticoagulation effect on serum. Anticoagulation by heparin is mediated by its potentiation of antithrombin III inhibition of thrombin (84,85), coagulation factors XII (86), XIa (87), Xa (88), and IXa (89). Antitrombin III has be shown to bind heparin by interacting with specific carbohydrate moieties that are sulfated at C-3 of glucosamine residues (90). In addition, heparin also has been shown to affect the other two major serum protein systems, the complement and kinin systems. In the kinin system, heparin potentiates the inhibition of both plasmin (91) and kallikrein (92) and casues release of plasminogen activator from rat vascular endothelial cells (93). There have been several inhibitory effects of heparin described on both the classical and alterative complement pathways. These include C1s activation of C4 and C2 (94,95), C1q binding to immune complexes (96), activation of C3 by cobra-venom (97), C3bBb convertase assembly (98), and activation of the alterative pathway by zymozan (98). Heparin proteoglycan has been shown to enhance C1 inhibitor activity (98). Both the neutral proteases, chymase of the rat and tryptase of the human, have been shown to have enhanced proteolysis of the anaphylatoxin C3a in the presence of heparin (99). Some of the other effects of heparin include enhanced granulocyte elastase activity (100), release of lipoprotein lipase from human tissue *in vivo* (101) and phospholipase A_1 from rat liver plasma membranes (102), stimulation of endothelial cell migration (103), and inhibition of antibody-depentent killing of *Trypanosoma cruzi* by eosinophils (104).

Enzymes of Connective Tissue Mast Cells.

Neutral Proteases.

Chymase. There exists in the secretory granules of rat serosal mast cells (RMC) several types of enzymes which include the neutral proteases, oxidative enzymes, and the acid hydrolases. Chymase is one of two neutral proteases found in the secretory granules of RMC as assessed by histochemical techniques (105). Chymase is an endopeptidase with substrate specificity similar to α-chymotrypsin, both cleaving the peptide bond on the COOH side of aromatic amino acids (106). The involvement of both histidine and serine residues in the active site of chymase has been indicated by the inhibition of enzymatic activity by diisopropylfluorophosphate (DFP) and by L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) but not by N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK) (107,108,109,110).

Chymase has been shown to be associated noncovalently to heparin proteoglycan in the secretory granules of RMC (111). Under physiological conditions, chymase and heparin proteoglycan remain on the surface of RMC as an insoluble complex following IgE-mediated degranulation (111). The chymase-heparin proteoglycan complex can be dissociated with 1M NaCl. The insoluble chymase-heparin proteoglycan complex remains on the surface of RMC and doesn't diffuse away from the cell following RMC activation. This phenomenon has been implicated as relevant to the chronic nature of the host response involving mast cells (111).

There are several sources used for the purification of chymase, which include liver (112), cutaneous tissue (113), skeletal muscle

(112,114) and RMC. In RMC, chymase accounts for approximately 50% of the total protein found in the secretory granules and 25% of the total protein in the cell with a concentration of 24 pg per cell (111). Chymase is composed of a single polypeptide chain with a molecular weight range between 25,000 and 29,000 daltons (107,110). Chymase has a isoelectric point of 9.3 (110). The amino acid sequence of the N-terminal 35 residues reveal 100% homology between serosal and muscle chymase with 40% homology with α-chymotrypsin (114).

The enzymology of purified chymase has been studied in terms of synthetic and various protein substrates. The most efficient substrate for chymase found thus far is the synthetic peptide derivative of p-nitroanilide N-Suc-Phe-Leu-Phe-p-nitroanilide (107). The k_{cat}/K_m value for this synthetic peptide substrate was approximately 1×10^6 $M^{-1}s^{-1}$. There have been several protein substrates shown to be susceptible to proteolysis by chymase *in vitro*. These protein substrates are neurotensin (114), human plasma fibronectin (116), type IV but not type I collagen (117), and glucagon (115). The presence of heparin proteoglycan on chymase activity has been variable. In the presence of heparin proteoglycan chymase activity has been reduced with protein substrates such as ornithine amino transferase (118) and casein (107,110). Cleavage of the low molecular weight substrate N-benzoyl-L-tyrosine-ethyl ester (BTEE) by chymase is not affected when heparin proteoglycan is present (118).

Tryptase. Tryptase is an endopeptidase found in human pulmonary mast cells which cleaves peptide and ester bonds on the carboxyl side of basic amino acids. Tryptase can be measured by its ablility to hydrolyze

tosyl-L-arginine methyl ester (TAME) and certain synthetic peptide derivatives of p-nitroanilide (119,120). Tryptase has been isolated to homogeneity from human pulmonary mast cells. As is observed for chymase in the rat, tryptase has also been shown to bind very tightly to heparin proteoglycan in physiological buffer (121). The molecular composition is different from chymase, there being two subunits of 37,000 daltons and two subunits of 35,000 daltons making a 144,000 dalton tetramer. The concentration of tryptase in the mast cell is approximately 12 pg, accounting for about 23% of the total mast cell protein; this is comparable to that percentage of chymase found in RMC.

Purified tryptase generates C3b and C3a following incubation with C3 in vitro (99). The generation of the anaphylatoxin C3a has been confirmed both by bioassay and radioimmunoassay. In addition, if heparin is added to the incubation mixture the anaphylatoxin C3a is not detected on SDS PAGE. When purified C3a is incubated with tryptase in the presence of heparin, the C3a molecule was degraded, whereas in the absence of heparin tryptase had no effect (99). Additional activities of tryptase include the cleavage of high molecular weight kininogen which renders this molecule unable to generate kinin activity when incubated with purified human urinary kallikrein (122).

Carboxypeptidase A. The substrate specificity of mast cell carboxypeptidase A is similar to bovine carboxypeptidase A but the former has two to sixfold higher activity (123). This enzyme is a metalloexopeptidase that cleaves COOH-terminal aromatic amino acids. It is similar to chymase and tryptase of the human in terms of binding very tightly to heparin proteoglycan. Carboxypeptidase A has been purified

from RMC and analyzed by electrophoresis in sodium dodecyl sulfate (SDS) polyacrylamide gels which reveal one protein band of 35,000 daltons. The cellular content based on activity accounts for approximately 10 pg of protein per mast cell (124). Both carboxypeptidase and chymase together account for about 70% of the total protein found in the secretory granules. Biological substrates for carboxypeptidase A have not been defined.

Acid Hydrolases.

β-Hexosaminidase. The enzyme β-hexosaminidase is an exoglycosidase found in the secretory granules of mast cells (125). β-Hexosaminidase is measured by the catalytic cleavage of the synthetic substrate p-nitrophenyl-β-D-2-acetamido-2-deoxyglucopyranoside (126). This enzyme exists in two forms, the A isomer and the B isomer. The A isomer consists of two α and two β subunits ($\alpha_2\beta_2$) and the B isomer is composed of four β subunits (β_4) The α chains have a molecular weight of 25,000 and the β chain is 27,000 (127). The predominant form of the enzyme in rat mast cells is the A isomer, whereas in the human mast cell the B isomer prevails. The enzyme is soluble following degranulation of mast cells and serves as a good marker for mediator release of these cells.

β-Glucuronidase. This enzyme, like β-hexosaminidase, is an exoglycosidase found in the secretory granules of mast cells. β-Glucuronidase catalyzes the cleavage of β-glucuronate from the nonreducing termini of polysacchride chains. β-Glucuronidase is measured by cleavage of the synthetic substrate phenolphthalein-β-D-glucuronide (128). There are two isomeric forms of β-glucuronidase isolated from rat

liver, the lysosomal and the microsomal forms. Both of these forms are tetramers with a molecular weight of 75,000 that differ in their isoelectric points (129,130). Apparently, the only isomeric form found in rat serosal mast cells is the lysosomal form.

β-D-Galactosidase. This enzyme is also found in the secretory granules of mast cells. β-D-galactosidase is detected by the catalytic cleavage of p-nitrophenyl-β-D-galactopyranoside (131). The nature of its isomeric forms are not defined.

Arylsulfatases. This enzyme is found in the secretory granules of mast cells and catalyzes the hydrolysis of aromatic sulfate esters. There are two subtypes of arylsulfatase, A and B, which are both detected by their ability to cleave p-nitrocatechol sulfate (132). Arylsulfatase A has a molecular weight of 116,000 with an isoelectric point of 4.2. Arylsulfatase B has an isoelectric point of 6.4 with a molecular weightof 50,000 (133). Both arylsulfatase A and B are found in equal amounts in rat mast cells (134).

Mucosal Mast Cells. The discovery that MMC are a distinct and different cell type than the CTMC was made in 1966 by Enerback (44). Since then studies using MMC from isolated tissue have not been performed because of the difficulty in purification of these cells. Recently, there have been fully established cultured cells from both rat and mouse hematopoietic tissue that appear to be analogous to MMC. These cultured mast cells differ significantly from the CTMC. The most prominant difference is the fact that MMC can be cultured, but only in the presence of concanavalin A-spenocyte-conditioned medium, WEHI-3-conditioned medium, or

interleukin-3. MMC are morphologically smaller in diameter and have fewer and more heterogeneously sized metachromatically staining granules than CTMC. MMC also differ in their nuclear appearence, in that they have more indentations and lobes than do CTMC. When MMC are stained with basic dyes such as alcian blue and safranin, MMC only bind alcian blue. Cells that only bind alcian blue and not safranin, as do the MMC, is indicative of cells containing granules with a proteoglycan with less sulfated glucosaminoglycan (45). MMC do not contain heparin but rather have chondroitin sulfate (135). Chondroitin sulfates are glycosaminoglycan with disaccharide units composed of glucuronic acid linked to galactosamine (121) (Fig 1.). The protein core of chondroitin sulfates appears to be alternating serine and glycine residues, which is the same as heparin proteoglycan. The molecular weight of chondroitin sulfate is approximately 60,000 (47). The types of biogenic amines that MMC contain are histamine and serotonin. There is about 0.4-0.6 µg of histamine per 10^6 cells, whereas the amount of serotonin is unknown. The newly generated mediators produced following activation of MMC are LTC and PGD_2.

The types of enzymes found in the MMC are not as well defined as the CTMC enzymes. Only one enzyme has been defined in the MMC and that is the neutral protease rat mast cell protease II (RMCP II) (136,137). RMCP II is a serine esterase that is similar to chymase (RMCP I) found in CTMC. Like CTMC chymase, RMCP II can be measured by the cleavage of the synthetic substrate BTEE (138). The molecular weight of RMCP II is about 25,000 which is approximately 3,000-4,000 daltons less than RMCP I (139). The amino acid sequence of the N-terminal 51 amino acids show 33%

homology with chymotrypsin, trypsin, and elastase and 75% homology with skeletal muscle chymase (136). The activity of RMCP II, against p-nitroanilide derivatives is generally lower than RMCP I (140,141).

Mechanism of Mast Cell Activation. There are several agents that will cause the degranulation of mast cells. The most relevent to allergic diseases is the IgE dependent activation. Agents which induce nonimmunologic activation of mast cells include calcium ionophores (142), compound 48/80 (143), concanavalin A (144), hydrogen peroxide and mellitin found in bee venom (145), and the anaphlatoxins, C3a and C5a (20).

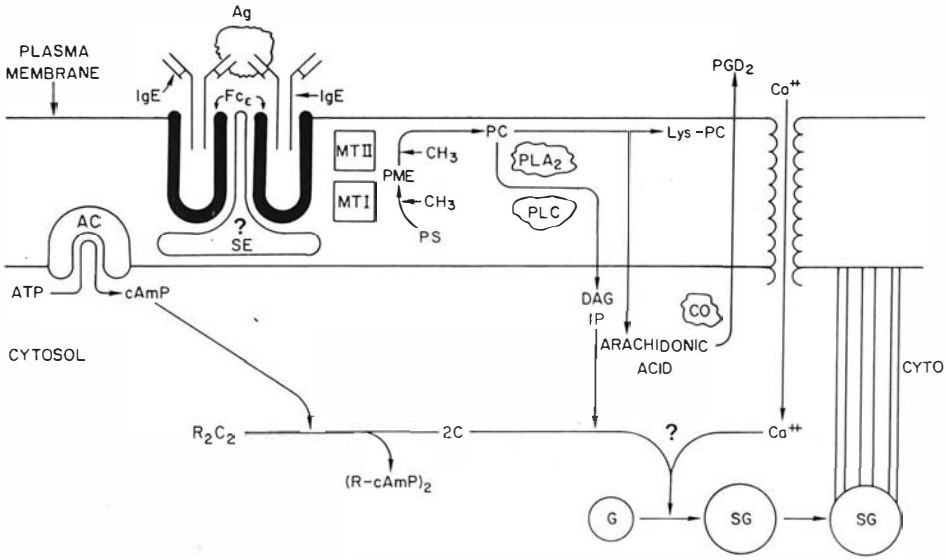
Following stimulation of human pulmonary mast cells with anti-IgE, there is a morphologic change that occurs with in the granules which involves both matrix breakdown and swelling as detected by electron microscopy (146). This type of cytoplasmic granular change seen in human mast cells is also observed in rat serosal mast cells following anti-IgE stimulation (146).

Mast cells bear specific receptors for IgE on their surface within the plasma membrane. Crosslinking of cell-bound IgE by either multivalent antigen or anti-IgE antibody initiates the degranulation of mast cells (147,148). The biochemical events associated with IgE-dependent degranulation of mast cells are summarized in Figure 2. Following antigenic crosslinking of receptor-bound IgE, there appears to be a serine esterase activity required for the continuation of mast cell activation (149). This enzyme has not been isolated but is indicated for mast cells by inhibition of IgE-dependent degranulation with diisopropylfluorophosphate (DFP). DFP does not inhibit degranulation of

Figure 2. Schematic Model for IgE-Dependent Activation of RMC.

Ag, IgE specific antigen; Fc , IgE receptor; SE, serine esterase; MTI, methyltransferase I; MTII, methyltransferase II; PS, phosphatidylserine, PME, phosphatidyl-N-monomethylethanolamine; PC, phosphatidylcholine; PLA_2, phospholipase A_2; PLC, phosphalipase C; Lys-Pc, lysophosphatidylcholine; PGD_2, prostaglandin D_2; Cyto, cytoskeletal proteins; SG, swollen granules; G, granules; CO, cyclooxygenase; DAG, diacylglycerol; IP, phosphatidylinositol; AC, adenylate cyclase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; R_2C_2, inactive protein kinase; 2C, active protein kinase; $(R\text{-}cAMP)_2$, noncataylic protein kinase.

Figure 2.


Ag
PLASMA MEMBRANE
IgE
Fcε
IgE
MTII
MTI
CH3
PME
CH3
PS
PC
PLA2
PLC
Lys -PC
PGD2
Ca++
AC
?
SE
ATP
cAmP
DAG
IP
CO
ARACHIDONIC ACID
CYTOSOL
CYTO
R2C2
2C
(R-cAmP)2
?
Ca++
G
SG
SG

mast cell if added prior to immunologic stimulation. The process has to be initiated before DFP has any inhibitory effect. These results suggest that the active site for the putative protease is protected and that perturbation of the IgE receptor renders the enzyme susceptible to inhibition by DFP (121). Accompanying the esterase activity, following immunological stimulation of mast cells, is an early rise in the cAMP levels (150). Concentrations of theophylline appropriate for the inhibition of adenyl cyclase abrogate IgE-dependent mediator release, implicating the involvement of this enzyme in the degranulation process (151). The ultimate role of cAMP is to activate cAMP-dependent protein kinases which are involved in the phosphorylation essential proteins required for the secretory response (152,153). In addition to the transient rise in cAMP, the rate of phospholipid methylation increases following IgE-dependent activation of mast cells (150). Conversion of phosphatidylethanolamine to phosphatidylcholine by methylation appears to be catalyzed by two enzymes methyltransferase I and II (154). Two additional enzymes which may also be involved in mast cell activation are phospholipases A_2 and C (121). Phospholipase A_2 cleaves phospholipids at C-2 to generate arachidonic acid which is the precursor molecule used in the lipoxygenase and cyclooxygenase pathways (155). Phospholipase C cleaves phospholipids at C-3, generating phosphatidyl inositol and diacylglycerol which are involved in the activation of a calmodulin/calcium-dependent protein kinase and protein kinase C, respectively (121,156). Finally, there is an absolute requirement for calcium in the degranulation of mast cells (154). It appears that bridging of cell-bound IgE on mast cells by antigen induces not only a rapid Ca^{++} influx but also intracellular mobilization of free Ca^{++} (157).

MATERIALS AND METHODS

Buffers and Reagents. Phosphate buffered saline (PBS) is 0.15 M NaCl containing 0.05 M sodium phosphate, pH 7.4. TD/OVA is Tyrode's buffer (158) pH 6.8, containing 5 mM N-2-hydroxylethylpiperazine-N-2-ethane sulfonic acid (Hepes), 5 mM 2-(N-morpholino)-ethanesulfonic acid (Mes), and 0.1% ovalbumin (OVA). Bovine serum albumin, 5 mg/ml, was added to PBS to make PBS/BSA. The following reagents were purchased from the sources indicated: Hepes, Mes, and bovine serum albumin (BSA) (Calbiochem-Behring Corp., La Jolla, CA.); ovalbumin, Ficoll, trichloroacetic acid (TCA), porcine intestinal heparin, sucrose, benzoyl-L-tyrosine ethyl ester (BTEE), hippuryl-L-phenylalanine, N-tosyl-L-lysyl-chloromethyl ketone (TLCK), tosyl-L-phenylalanine chloromethyl ketone (TPCK), diisopropylfluorophosphate (DFP) (Sigma Chemical Co., St. Louis, MO.); soybean trypsin inhibitor (SBTI) and lactoperoxidase (Worthington Biochemical Co., Freehold, N.J).; Bis (sulfosuccinimidyl) suberate (BS^3) (Pierce Chemical Co., Rockford, Ill.); $Na^{125}I$ (Amersham Corp., Arlington Heights, Ill.); Sepharose CL-4B, Sephadex G-25, and phenyl-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.); Dowex 1-X2 chloride form 100-200 mesh, protein assay kit, acrylamide, bisacrylamide, ammonium persulfate, N,N,N',N'-tetramethyl ethylenediamine, sodium dodecyl sulfate (SDS), silver stain kit, bromophenol blue, molecular weight standards (Bio-Rad Laboratories, Richmond, CA.); TSK 3000 SW 7.5 mm x 600 mm column (Beckman Instruments, Inc., Irvine, CA.); Kodak XAR-2 x-ray film (Eastman Kodak Co., Rochester, N.Y.); 14" x 14" x-ray cassette (Manostat

Laboratory Instruments and Scientific Apparatus, N.Y., N.Y.); butyl phthalate and dinonyl phthalate (Fisher Scientific Co., Pittsburgh, PA.); fluorescein-conjugated IgG fraction rabbit anti-goat IgG (heavy chain specific) and IgG fraction goat anti-rat albumin (Cappel Laboratories, Cochranville, PA.). Staphylococcus aureus Cowan I strain was a gift from Dr. Gordon Archer. The organism was grown and formalin-fixed in our laboratory. Antiserum directed against chymase was produced in goats. The IgG fraction of the goat antiserum was prepared by sequential ammonium sulfate precipitation and DEAE-cellulose chromatography (159).

Purification of Rat Mast Cells. Lewis Strain rats (female retired breeder) were anesthetized with ether and sacrificed by cardiac puncture. Mast cells were obtained by lavaging the peritoneal cavity with TD/OVA buffer followed by sedimentation on 35% Ficoll (160). The number of RMC per rat and their purity and viability is shown in Table 1. The purity of mast cells, as assessed by staining with neutral red, was 90-97% and the viability, determined by trypan blue exclusion, was 85-95%. The yield of mast cells ranged between 0.9-2.0 x 10^6 per rat.

Cultured Cells. Cultured mouse mast cells (CMMC) and WEHI-3 monocyte/macrophage cell lines were donated by Dr. Lawrence Schwartz. CMMC were cultured at 10^6/ml in RPMI 1640 medium containing the following supplements: glutamine (2 mM final concentration), pyruvate (1 mM), nonessential amino acids (0.1 mM), penicillin (100 U/ml), streptomycin (100µg/ml), gentamicin (500 µg/ml), 2-mercaptoethanol (5 x 10^{-5} M), 10% FCS and 25% WEHI-3 conditioned medium. WEHI-3 cells were cultured at 5 x 10^5/ml in the same media as the CMMC. Culture flasks were incubated at 37°C in a humidified atmosphere of 5% CO_2, 95% air.

Table 1. Purification of Mast Cells from the Peritoneal Cavity

Number of cell (n=76)	$1.3 \times 10^6 \pm 4 \times 10^5$
Fraction staining with neutral red (n=76)[a]	93.5% ±4.0%
Fraction excluded by trypan blue (n=76)[b]	89.2% ±5.2%

[a]Purity was assessed by neutral red staining of cell preparation.

[b]Viability was determined by trypan blue exclusion.

C3a Purification. Human C3a was provided by Dr. Tony Hugli, having been purified by a modification of a method previously described (161). Briefly, human serum was activated with inulin in the presence of 2-mercaptomethyl-5-guanidinopentanoic acid. C3a was purified from this complement-activated serum by sequential chromatography on Sephadex G-200, carboxymethyl-Sephadex, Sephadex G-200, and DEAE-Sephadex. The C3a preparation was lyophilized, stored at -70^{o}C, and rehydrated with PBS. The C3a was homogeneous; SDS PAGE showed a single band at 9000 daltons. High performance liquid chromatography (HPLC) analysis on a TSK 3000 SW 7.5 mm x 600 mm size exclusion column, equilibrated in PBS, detected a single protein spike in the molecular weight range corresponding to 9000 daltons.

Iodination. Human C3a was iodinated with Na^{125}I using lactoperoxidase covalently bound to Sepharose CL-4B as previously described (162). Iodinated C3a was separated from free iodide by gel filtration over a 0.5 cm x 45 cm column of Sephadex G-25. The Sephadex G-25 had been presoaked in 0.1% ovalbumin to prevent nonspecific binding of iodinated C3a (^{125}I-C3a) to the G-25 matrix. In addition, Iodogen was also used to iodinate C3a. Iodogen (10 µg/test tube) was dissolved in chloroform and dispensed into silicated plastic test tubes. The chloroform was removed by volitilization with nitrogen. C3a was added to these Iodogenated test tubes (25-50 µg) with Na^{125}I and incubated for 20 min. The reaction was terminated by removing C3a from the test tube. The amount of free iodide was assessed by precipitation in a final concentration of 10% (w/v) TCA. The TCA precipitability ranged between 75-95%. Specific activities ranged between 0.5-5 µCi/µg.

Electrophoretic Analysis. Analysis in the presence of SDS was performed on either 10% (w/v) polyacrylamide or 5-17% (w/v) polyacrylamide linear gradient slab gels as previously described (163). Proteins were detected by silver staining. Molecular weight standards included myosin (200,000), β-galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

Binding of ^{125}I-C3a to RMC. All binding assays were performed in TD/OVA buffer. 5.0 x 10^6 RMC were incubated with B.0 µg of ^{125}I-C3a in a volume of 1 ml at 0^oC or 37^oC. At various times, a 100 µl portion was removed, layered over a mineral oil solution (dinonyl phthalate: butyl phthalate ratio 1:7), and centrifuged in a microcentrifuge (Beckman Microcentrifuge 12, Palo Alto, CA), at 8050 x g for 3 min. The entire centrifuge tube was counted for ^{125}I in order to obtain the total counts removed per aliquot. The cell pellet was cut off, and the number of ^{125}I counts bound to mast cells determined. Nonspecific intracellular trapping of ^{125}I-C3a in the pellet was less than 0.1% as determined by incubating ^{125}I-C3a with either buffer, rat basophilic leukemia cells or rat erythrocytes.

Analysis of ^{125}I-C3a Following Exposure to RMC. 2.0 x 10^6 RMC were incubated with 530 ng of ^{125}I-C3a in a volume of 200 µl TD/OVA at 37^oC or 0^oC. At various times, two 20 µl samples were removed. One sample was tested for solubility in TCA, and the second for electrophoretic mobility by SDS PAGE analysis. One of the samples was added to 1 ml PBS/BSA, centrifuged at 200 x g, and the supernatant

counted for ^{125}I to obtain initial cpms. An equal volume of 20% TCA was added to the supernatant, mixed, and centrifuged at 1600 x g for 10 min. One half of the supernatant was removed and counted for ^{125}I. The percent ^{125}I insoluble in TCA was calculated from the following formula:

$$\text{Net \% TCA-Insoluble } ^{125}\text{I-C3a} = \frac{\text{Initial cpms} - 2x\ (\text{TCA treated supernatant})}{\text{Initial cpms} - \text{Free iodide cpms}} \times 10^2$$

The free iodide term in the denominator represents the percent ^{125}I in the labeled C3a preparation that could not be precipitated by TCA. The second 20 µl sample was added to 100 µl of SDS PAGE sample buffer (0.062 M Tris, 2.3% (w/v) SDS, 10% (v/v) glycerol, pH 6.8) and boiled for 90 sec. Seventy-five microliters were loaded onto a 5-17% (w/v) polyacrylamide linear gradient slab gel and subjected to electrophoresis at 125 volts for 6 hours. The gel was stained, dried, and exposed to Kodak XAR-2 X-ray film in a 14" x 14" x-ray cassette. The exposure time was 1 day and 4 hours for the samples obtained at 37°C and 0°C, respectively. The x-ray film was developed by a Kodak RPX-omat processor.

Crosslinking of ^{125}I-C3a to RMC. 2.6×10^6 RMC were treated with 5×10^{-4} M SBTI in TD/OVA for 5 min at 37°C. Eighty nanograms of ^{125}I-C3a were then added to make a final volume of 500 µl, incubated 20 min at 0°C, and the tube centrifuged at 200 x g for 5 min. The cell pellet was resuspended in 1 ml TD/OVA/SBTI buffer containing a final concentration of 10 mM BS^3 (164) and incubated for 30 min at 0°C. The cells were washed and reexposed to 60 ng of fresh ^{125}I-C3a in 1 ml TD/OVA/SBTI. Following centrifugation and removal of the supernatant, an

additional 10 mM BS^3 was added and incubated for 30 min at 0^oC. The cell pellet was washed once with TD/OVA/SBTI buffer and twice with 0.01 M Mes, pH 6.0 containing 1.0 M NaCl (sonication buffer) and disrupted. A microtip attachment to a Cell Disruptor Model W-225R (Heat systems-Ultrasonics, Inc., Farmington, NY) was used at output power 2, 50% pulse cycle for 30 pulses. The RMC sonicate was then incubated with 100 µl of a S. aureus suspension (10% w/v) (165) for 15 min at 37^oC and centrifuged at 1600 x g for 10 min. One hundred µl portions of the S. aureus-treated RMC supernatant were incubated with anti-chymase serum for 1 hour at 37^oC at final dilutions of 1:2, 1:4, 1:8, and 1:20; controls with normal goat serum or sonication buffer were similarly incubated. Fifty nanograms of a S. aureus suspension were added, and incubated for 30 min at 37^oC. The reaction was stopped by centrifugation at 1600 x g for 10 min. The pellet was washed 3 times and the percent ^{125}I-C3a bound determined.

Isolation of Chymase and Heparin Proteoglycan. RMC that were greater than 95% pure were used in a modification of a previously described procedure (166). Between 6-9 x 10^6 RMC were sonicated in 0.01 M Mes, pH 6.0, containing 0.5 M NaCl, mixed with Dowex 1-X2 (2ml/5x10^6RMC) equilibrated in the sonication buffer, poured into a 1 cm x 10 cm column, and washed with 4 column volumes of 0.01 M Mes, pH 6.0, containing 0.6 M NaCl. The chymase was then eluted by increasing the NaCl concentration to 1.0 M. Chymase activity was assayed spectrophotometrically by cleavage of BTEE (one unit of enzyme cleaves one µmole of substrate/min at 22^oC) (166,167). Fractions containing chymase activity were pooled and concentrated by coating the dialysis bag

with sucrose. The concentrated chymase was dialyzed overnight against Tyrode's buffer and stored at -70°C and retained 95% of the initial activity after thawing. The specific activities ranged between 8.0-16.6 units/mg (protein was determined by the Bio-Rad protein assay kit using human γ-globulin as a standard). SDS PAGE analysis of purified chymase showed a single band of 29,000 daltons after electrophoresis under reducing conditions. Carboxypeptidase A (one unit hydrolyzes one μmole hippuryl-L-phenylalanine/min at 25°C, pH 7.5) (166,168) or heparin proteoglycan (metachromasia with Azure A (166)) could not be detected in the purified chymase preparation (169).

Heparin proteoglycan from the effluent fractions of the Dowex 1-X2 column were pooled and applied to a phenyl-Sepharose column equilibrated with 0.01 M Mes, pH 6.0, containing 3.0 M NaCl (166). The heparin proteoglycan eluted from the column in the effluent fractions, whereas carboxypeptidase A remained bound. Heparin proteoglycan was pooled, concentrated, dialyzed overnight against Tyrode's buffer, and stored at -70°C. The heparin proteoglycan had no detectable chymase or carboxypeptidase A activity and no detectable protein as assessed by the Bio-Rad protein assay.

Digestion of ^{125}I-C3a by Chymase. Fifty nanogram portions of ^{125}I-C3a were incubated with chymase alone (0.7 units), chymase plus heparin proteoglycan (60 μg), heparin proteoglycan alone, Tyrode's buffer, or RMC containing equivalent amounts of chymase and heparin proteoglycan in a volume of 680 μl at 37°C. At various times, 20 μl portions were removed and the ^{125}I-C3a soluble in 10% TCA determined.

Degradation of ^{125}I-C3a$_{des\ Arg}$ by Chymase as the Ratio of Chymase:C3a$_{des\ Arg}$ Varies. Various concentrations of chymase, ranging between 0.19 µg (2×10^{-5} units) to 100 µg (0.5 units), were incubated with 65 ng of ^{125}I-C3a$_{des\ Arg}$ in the presence of 25 µg of purified heparin proteoglycan (0.125 ml reaction volume). Following a 30 min incubation at 37°C 80 µl were removed and analyzed for percent TCA insoluble ^{125}I material.

Indirect Immunofluorescence of RMC. Forty thousand RMC were air dried onto microscope slides and incubated with 135 µg of the IgG fraction of goat anti-rat chymase for 30 min at room temperature. The slide was washed 3 times with P8S and incubated for an additional 30 min at room temperature with 400 µg of fluorescein-conjugated IgG fraction of rabbit anti-goat IgG. The slide was washed 3 times and mounted in polyvinyl alcohol. Normal goat serum (NGS) was incubated with RMC at a protein concentration corresponding to the primary antibody concentration. RMC showed no fluorescein staining following exposure to NGS and fluorescein-conjugated antibody.

Immunoabsorption of Chymase from RMC Sonicates using Chymase-Specific Antibody. Three million RMC were washed and sonicated in Tyrode's buffer, pH 6.8, containing 1 M NaCl. The RMC sonicates were treated twice with a S. aureus suspension (10% w/v) for 20 min at 37°Cand centrifuged at 1600 x g for 10 min. Chymase activity was measured and portions of the RMC sonicate corresponding to 0.1 units of chymase were incubated with 1 mg of either the IgG fractions of goat anti-rat chymase or goat anti-rat albumin or in buffer for 1 hr at 37°C. Fifty µl of a S. aureus suspension (10% w/v) was added, and

incubated for 20 min at 37°C and centrifuged at 1600 x g for 10 min. A portion of each supernatant was saved, and the exposure to antibody and S. aureus suspension repeated. The residual ability to digest C3a was then assessed by incubation with fifty nanograms of ^{125}I-C3a for 1 hr at 37°C and measured for percent ^{125}I insoluble in TCA.

Effect of SBTI on C3a-Induced Release of β-Hexosaminidase from RMC. Two hundred thousand RMC were pretreated in a volume of 0.3 ml with varying concentrations of SBTI (3.0×10^{-4}M-1.7×10^{-8}M) at 37°C for 5 min. Three micrograms of ^{125}I-C3a (0.3nM) were added and incubated for an additional 30 min at 37°C. A 90 µl portion was removed, dispensed in 1 ml of PBS/BSA solution, and analyzed for percent TCA insoluble ^{125}I material. An additional 200 µl portion was removed and put into 0.4 ml of ice cold Tyrode's buffer minus Ca^{++} and Mg^{++} (Tyrode's$^{=}$) and centrifuged. Both the cell pellet and supernatant were analyzed for β-hexosaminidase content. To serve as controls, both ^{125}I-C3a and the calcium ionophore A23187 were incubated with nontreated RMC.

β-Hexosaminidase Analysis. Both the cell pellets and supernatants were analyzed for β-hexosaminidase as described in Schwartz et al (170). Briefly, the RMC pellet was resuspened in Tyrode's solution adjusted to 1 M NaCl and sonicated. A 50 µl portion was removed and added to 0.45 ml of 0.2 M phosphate and 0.4 M citrate buffer, pH 4.5, containing 4mM p-nitrophenol-N-acetyl-β-D-glucosamide (β-hex-substrate buffer). The supernatant was also adjusted to 1 M NaCl and a 50 µl portion dispensed into 0.45 ml of β-hex-substrate buffer. Both portions from the pellet and the supernatant were incubated with the β-hex-substrate buffer at 37°C for 1 hr. The reaction was terminated by adding 1.5 ml of 0.2 M glycine, pH 10.7, and the optical density at 410 nanometers determined.

Binding of ^{125}I-C3a to RMC in the Presence of Heparin. Varying doses of RMC were pretreated with 10 mg/ml SBTI for 5 min at 0°C. These cells were treated with 50 units (0.3 mg) of heparin, 50 units of heparin in 0.3 M NaCl Tyrode's$^{=}$ buffer, or left untreated. Both heparin and NaCl were titrated separately and used at optimal concentrations. The cells were then incubated with 70 ng of ^{125}I-C3a for 30 min at 0°C. The cells were centrifuged and analyzed for percent ^{125}I-C3a bound to RMC. The viability of the cells in both Tyrode's$^{=}$ (0.15 M NaCl) and Tyrode's$^{=}$ containing 0.3 M NaCl were 86.0% and 88.0%, respectively.

Binding of ^{125}I-C3a to RMC in the Presence of Agents which Inhibit Degradation, Degranulation, and Binding to Heparin Proteoglycan. In all three analyses, the cells, prior to exposure to ^{125}I-C3a, are resuspended in Tyrode's$^{=}$ buffer containing 0.3 M NaCl, 1mM TPCK, 4 mM SBTI, and 1mM EDTA. For the dose-dependent binding study, increasing concentrations of ^{125}I-C3a were incubated with 5 x 10^5 RMC in a volume of 0.5 ml for 20 min at 0°C. A 0.4 ml portion was removed, layered onto oil, centrifuged, and the number of molecules per cell calculated. In the kinetic analysis of ^{125}I-C3a to RMC, 5.5 x 10^5 RMC were incubated with 65 ng of ^{125}I-C3a in a volume of 0.5 ml at 0°C. At various times, a 0.4 ml portion was removed and layered onto oil, centrifuged, and the cell pellet used to calculate the number of molecules per RMC. For the cold inhibition study, 6 x 10^5 RMC were incubated with 10 ng of ^{125}I-C3a in the presence of varying concentrations of C3a (ranging between 0.1 µg-20 µg) in a volume of 0.5

ml for 20 min at $0^{o}C$. A 0.4 ml portion was removed, layered onto oil, and analyzed for the number of molecules per RMC.

Percent TCA Insoluble ^{125}I Material and Binding of ^{125}I-C3a Following Exposure to CMMC. CMMC (1.2×10^{6}) were incubated with 18 ng of ^{125}I-C3a in a volume of 0.3 ml at $0^{o}C$, $25^{o}C$, and $37^{o}C$ in the presence or absence of 4mM SBTI. At various times, a 20 µl portion was removed, dispensed into 1 ml of PBS/BSA, and centrifuged. The supernatant was then analyzed for percent TCA insoluble ^{125}I material. To analyze the binding of ^{125}I-C3a to CMMC, 17.4×10^{6} CMMC were incubated with 1 µg Of ^{125}I-C3a in a volume of 1 ml at $0^{o}C$, $25^{o}C$, and $37^{o}C$. At various times, a 40 µl portion was removed, despenced into 0.4 ml of Tyrode's$^{=}$ buffer and layered onto oil. The number of ^{125}I-C3a molecules per cell were calculated from the cell pellet.

Crosslinking of Intact RMC with C3a. RMC were radioiodinated (^{125}I-RMC) using the lactoperoxidase method as described by Conrad et al (171). Briefly, 3.75×10^{6} RMC were incubated with 30 µg of lactoperoxidase and 200 µCi of $Na^{125}I$ in a volume of 0.2 ml. Hydrogen peroxide (0.1 mM) was added every min over a 3 min period. The reaction was terminated by diluting the cell suspension in 10 ml of Tyrode's$^{=}$ buffer and centrifuged. The cells were washed 5 times and resuspended to a volume of 0.2 ml in Tyrode's$^{=}$ containing 1mM DFP (Tyrode's$^{=}$/DFP) for 5 min at $0^{o}C$. In addition, 3.75×10^{6} noniodinated RMC were incubated separately with Tyrode's$^{=}$/DFP for 5 min at $0^{o}C$. Four micrograms of C3a and 4 µg of ^{125}I-C3a were added to the ^{125}I-RMC and RMC suspensions, respectively for 30 min at $0^{o}C$. Both cell suspensions were washed once and resuspended in 30 mM BS^{3} and incubated for an

additional 30 min at 0°C. The ^{125}I-RMC-C3a and RMC-^{125}I-C3a crosslinked material were both washed and resuspended in Tyrode's$^{=}$ containing the nonionic detergent Nonidet-40 (NP-40) and protease inhibitions (0.01 M benzamidine, 0.02 M iodoacetamide, 10µg/ml leupeptin, 400µg/ml SBTI, 13 mM phenanthroline, 30 U aprotinin, and 2mM DFP) then sonicated. Both cell lysates were centrifuged at 2000 x g for 20 min. The solubilzed portions were either analyzed directly by SDS PAGE or were incubated with anti-C3a antibodies and 10% Staph A suspension (as described in Crosslinking of ^{125}I-C3a to RMC section of Material and Methods) and then analyzed on SDS PAGE. In addition, the insoluble cell pellet was also analyzed on SDS PAGE. The gel was stained, dried and exposed to Kodak XAR-2 x-ray film in a 14 x 14 in. x-ray cassette for 24 hr. The x-ray film was processed and developed by a Kodak RPX-omat processor.

Crosslinking ^{125}I-C3a to Intact RMC in the Presence of Various Protease Inhibitors. Two million RMC were pretreated with either 4 mM SBTI, 0.04 mM SBTI, 1mM TPCK, 1mM TLCK, or 1 mM DFP for 10 min at 0°C. Two micrograms of ^{125}I-C3a were incubated with these cells for 20 min at 0°C. The cells were centrifuged and resuspended in Tyrode's$^{=}$ buffer containing 1 mM BS^3 and incubated for 30 min at 0°C. The cells were centrifuged and washed 5 times and resuspended to a volume of 0.2 ml with Tyrode's$^{=}$/NP-40/protease inhibition cocktail and sonicated. The cells were centrifuged at 2000 x g for 20 min and the supernatant analyzed by SDS PAGE and autoradiography.

RESULTS

Binding of ^{125}I-C3a to RMC. The number of C3a molecules bound to RMC at 37°C was examined as a function of time. Eight micrograms of ^{125}I-C3a was incubated with 5.0 x 10^6 RMC in a volume of 1 ml at 37°C. At various times, 100 µl aliquots were removed, layered over a mineral oil solution, and centrifuged. The supernatant was aspirated and the number of molecules bound per cell determined. For the experiment shown in Figure 3, the number of molecules offered per cell was 107 million and 10 million were bound at 30 sec. After 10 min, the number of ^{125}I-C3a molecules bound per cell had decreased to 2.5 million. Similar rapid binding and dissociation of ^{125}I-C3a by RMC was observed in 3 separate experiments using 2 different ^{125}I-C3a preparations. When the inactive form of C3a, $C3a_{des\ Arg}$, was used in binding studies with RMC, similar results were obtained (data not shown). The amount of degranulation of RMC following exposure to 107 million molecules of C3a per cell was $\leq$1% as assessed by β-hexosaminidase activity in the supernatant (170).

There is a temperature dependence of ^{125}I-C3a binding to RMC. At 30 sec, 17.0 x 10^6 ^{125}I-C3a molecules were bound per cell at 0°C, as compared to 8.9 x 10^6 molecules bound per cell at 37°C. By 60 min, of the ^{125}I-C3a molecules bound at 30 sec, 79.8% had dissociated at 37°C, whereas only 37.6% had dissociated at 0°C (Table 2). These data indicate that the kinetic equilibrium estabilished between ^{125}I-C3a and RMC does not follow the Henri-Michaelis-Menten model.

Figure 3. Dissociation of ^{125}I-C3a from RMC at 37^{o}C.

^{125}I-C3a (8 µg) was incubated with 5.0 x 10^{6} RMC in 1 ml of TD/OVA at 37^{o}C. At various times, a 100 µl aliquot was removed and centrifuged through mineral oil. The number of ^{125}I-C3a molecules bound per cell was calculated by using the specific activity of the ^{125}I-C3a preparation and a molecular weight of C3a of 9000.

Figure 3.

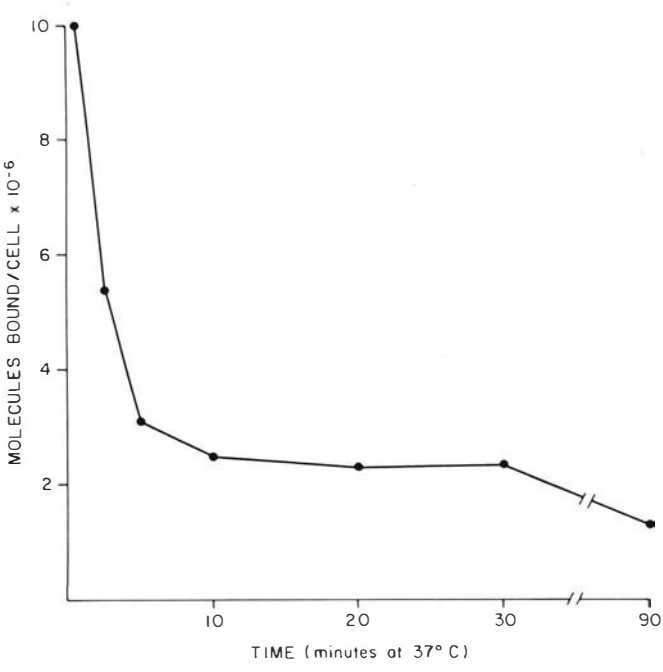

MOLECULES BOUND/CELL x 10-6
10
8
6
4
2
10
20
30
90
TIME (minutes at 37° C)

Table 2. Binding of ^{125}I-C3a to RMC at 37°C and 0°C.[a]

Time	Temperature Molecules bound per RMC x 10^{-6}	
Minutes	37°C	0°C
0.5	8.9	17.0
60.0	1.8	10.6

[a] 5.0×10^6 RMC were incubated with 8.0 µg of ^{125}I-C3a in a volume of 1 ml at 0°C or 37°C. A 100 µl portion was removed at 0.5 min and 60 min and the number of molecules bound per cell determined as described in Material and Methods.

Dose Dependent Binding of ^{125}I-C3a to RMC. Since the binding of ^{125}I-C3a to RMC is higher at 0°C than 37°C (Table 2) the dose response studies were performed at 0°C. When inceasing concentrations of ^{125}I-C3a are incubated with 5.0 x 10^5 RMC in 0.6 ml Tyrode's/OVA at 0°C for 5 min, saturation could not be obtained. The maximum number of ^{125}I-C3a molecules offered per cell was 530 million. At this concentration the number of molecules bound per cell was 97 million and still increasing as indicated in Figure 4. It was evident from the data of Table 2, Figure 3, and Figure 4 that the binding of ^{125}I-C3a to RMC does not reach equilibrium as would be expected by Henri-Michaelis-Menten model for a ligand and its specific receptor.

In order to investigate whether ^{125}I-C3a which had bound to and been released from RMC was capable of binding to fresh RMC the following experiment was performed. One million RMC were incubated with 280 ng of ^{125}I-C3a in a volume of 1 ml TD/OVA for 20 min at 37°C. Two aliquots were removed. One was layered over mineral oil and the binding of ^{125}I-C3a determined. The other aliquot was centrifuged at 200 x g for 5 min, the supernatant was removed, and a volume corresponding to 60 ng of ^{125}I-C3a (based on the cpms and original specific activity) was exposed to a fresh preparation of RMC for 20 min at 37°C. This procedure was repeated a total of four times. As shown in Fiqure 5, 3.2 million molecules were bound per cell during the first 20 min incubation. When the supernatant from the first incubation was re-exposed to a fresh batch of RMC the number of molecules bound per cell decreased to 1 million. After the fourth transfer, the number of

Figure 4. Dose Dependent Binding of ^{125}I-C3a to RMC at 0^{o}C.

RMC (5 x 10^5) were incubated with increasing concentrations of ^{125}I-C3a for 5 min at 0^{o}C in a volume of 0.6 ml. A portion was removed, layered over oil, and centrifuged. The pellet was analyzed for the number of ^{125}I-C3a molecules bound per RMC.

Figure 4.

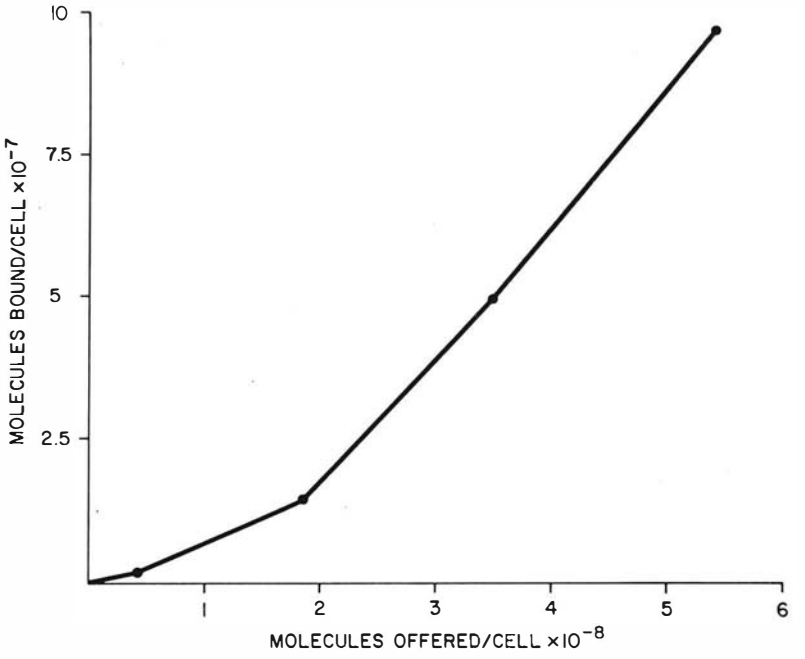
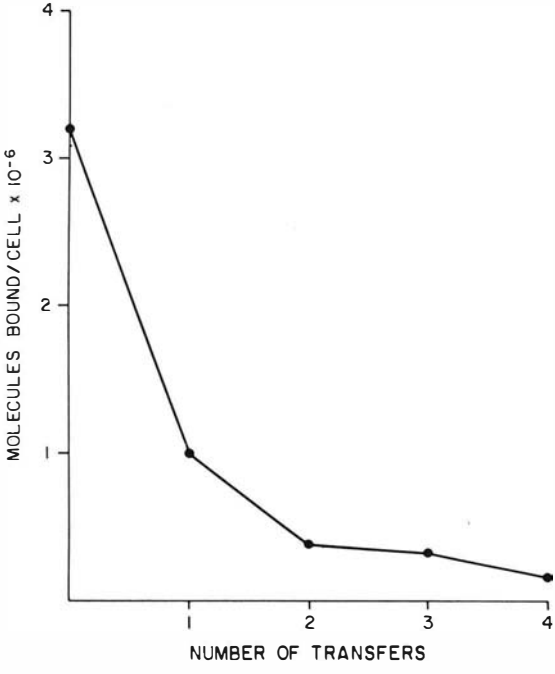

10
7.5
5
2.5
MOLECULES BOUND/CELL ×10⁻⁷
1
2
3
4
5
6
MOLECULES OFFERED/CELL ×10⁻⁸

Figure 5. Rebinding of ^{125}I-C3a Following Repeated Exposures to RMC.

^{125}I-C3a (280 ng) was incubated with 1.0 x 10^6 RMC in 1 ml of TD/OVA for 20 min at 37^{o}C. Two aliquots were removed. One aliquot was layered over mineral oil and centrifuged. The number of ^{125}I-C3a molecules bound per cell was calculated from the RMC pellet. The second aliquot was centrifuged and the supernatant was removed and reexposed to a new batch of RMC for 20 min at 37^{o}C. This procedure was repeated four times. Equal ^{125}I-C3a radioactive counts were transfered to each RMC mixture.

Figure 5.



molecules bound per cell was only 0.17 million. Thus, ^{125}I-C3a was altered upon exposure to RMC, such that it did not rebind to these cells.

Degradation of ^{125}I-C3a by RMC. To determine if ^{125}I-C3a was undergoing a conformational change or was being degraded, percent insolublility in TCA was measured and the size of the ^{125}I material in the supernatants following exposure of ^{125}I-C3a to RMC was analyzed by SDS PAGE. Figure 6 shows the % TCA precipitablity of ^{125}I-C3a at various times following incubation with RMC at 37^{o}C and 0^{o}C. By 30 sec, the % TCA precipitabilty decreased from the initial value of 100% to 30% and 63% at 37^{o}C and 0^{o}C respectively. A further decrease occurred during the ensuing 10 min after which the % TCA precipitabilty remained constant. SDS PAGE analysis of the supernatants is shown in Figure 7. Following incubation at 37^{o}C for 30 sec (left panel), there was a decrease in the amount of radioactivity detected on the autoradiograph due to counts lost in the buffer front and a shift towards a lower molecular weight species. With continued incubation, the detectable radioactivity further decreases. At 0^{o}C (right panel), the changes were less marked so that the ^{125}I-C3a shift to a lower molecular weight species occurs at 10 min and radioactivity persists throughout the incubation period. Thus the marked decrease in ^{125}I-C3a % TCA precipitabilty following exposure to RMC was accompanied by a decrease in molecular weight following SDS PAGE analysis.

Inhibition of ^{125}I-C3a Decrease in % TCA Precipitablity by Treatment of RMC with Various Protease Inhibitors. The temperature dependent degradation of ^{125}I-C3a suggested that this peptide is being cleaved by RMC proteases. The capacity of a variety of protease inhibitors to block this degradation was tested. Three hundred-thousand

Figure 6. Solubility in TCA of ^{125}I-C3a Following Exposure to RMC.

^{125}I-C3a (530 ng) was incubated with 2.0 x 10^6 RMC in 200 µl of TD/OVA at 37°C or 0°C. At various times, a portion was removed, centrifuged, and the supernatants analzyed for solubility in 10% TCA.

Figure 6.

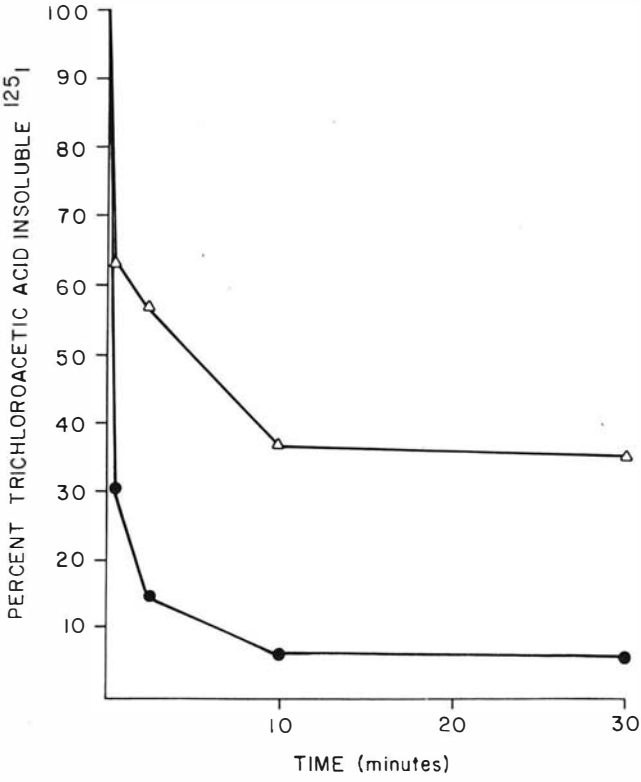
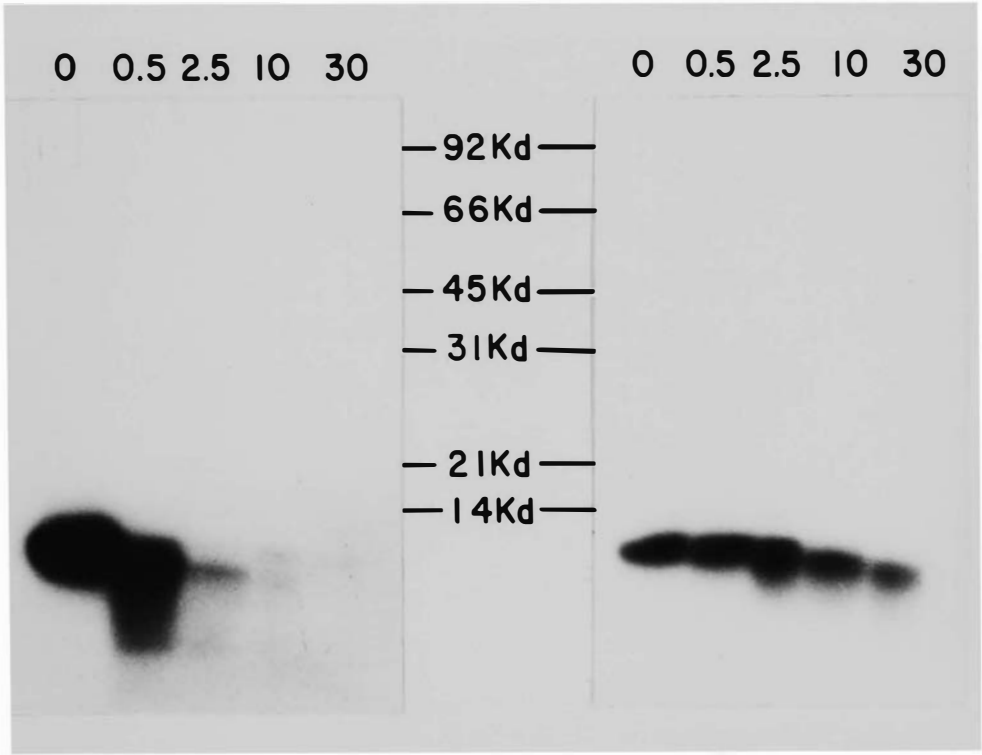

PERCENT TRICHLOROACETIC ACID INSOLUBLE ^{125}I
100
90
80
70
60
50
40
30
20
10
10
20
30
TIME (minutes)

Figure 7. SDS PAGE Analysis of ^{125}I-C3a Exposed to RMC at 37^{o}C and 0^{o}C.

Conditions of incubation were the same as Fig. 3. Equal ^{125}I counts from each time interval were loaded onto a 5-17% (w/v) polyacylamide gradient slab gel. Autoradiography for the samples at 37^{o}C (left panel) and 0^{o}C (right panel) were for 1 day and 4 hours, respectively. The time, in minutes, of ^{125}I-C3a exposure to RMC is indicated above each lane. The 0 time represents ^{125}I-C3a exposed to TD/OVA alone. The apparent decrease of ^{125}I at 37^{o}C is due to loss of label into the anodal buffer.

Figure 7.



RMC were treated with inhibitors for 5 min at 37°C. Then 40 ng of ^{125}I-C3a was added in a total volume of 600 µl and incubated for 20 minat 37°C. Supernatants were analyzed for % TCA precipitable ^{125}I-C3a counts. As shown in Table 3, the most effective inhibitors were DFP, SBTI, and TPCK, which all inhibit serine esterases, the latter two with chymotrypsin-like specificities (172,173). The least effective inhibitor was TLCK, which inhibits serine esterases with trypsin-like specificity (45). These data indicate that the degradation of ^{125}I-C3a by RMC was abrogated by chymotrypsin but not trypsin-like protease inhibitors.

Effect of SBTI on the Binding of ^{125}I-C3a to RMC. It was established in Figures 6 & 7 and Table 2 that ^{125}I-C3a was being degraded by RMC and that this degradation could be abrogated if RMC were pretreated with chymotrypsin-specific inhibitors. In the following experiment, 2.0 x 10^5 RMC were pretreated with 4 mM SBTI for 5 min at 37°C. Increasing concentrations of ^{125}I-C3a were incubated with these pretreated RMC in a volume of 0.5 ml for 20 min at 37°C. A portion was removed, layered onto oil, centrifuged, and the number of molecules bound per RMC determined. As shown in Figure 8, up to 28 million molecules were offered per cell and 2 million ^{125}I-C3a molecules were bound with SBTI present. In contrast, when 28 million ^{125}I-C3a molecules were incubated with untreated RMC there were only 500,000 molecules bound per cell. The binding of ^{125}I-C3a to RMC is increased in the presence of SBTI.

RMC (2.0 x 10^5) were pretreated with 4 mM SBTI in a 0.5 ml volume for 5 min at 37°C. The cells were than transferred to ice for 15 min. Increasing concentrations of ^{125}I-C3a were incubated with these cells for 20 min at 0°C. As shown in Figure 9, even at concentrations of

Table 3. Inhibition of ^{125}I-C3a Degradation by RMC with Protease Inhibitors

Inhibitor	Concentration	Percent ^{125}I-insoluble in TCA	
		Expt. I[a]	Expt. II
^{125}I-C3a in buffer[b]	(--)	100	100
^{125}I-C3a plus RMC[c]	(--)	6.5	26.0
DFP	10^{-3}M	ND	65.0
SBTI	10^{-4}M	96.2	ND
TPCK	10^{-4}M	96.2	ND
TLCK	10^{-4}M	6.2	ND

[a]Expt. I and II represent the same experimental protocol (as described in results section) using different RMC and ^{125}I-C3a preparations.

[b]When ^{125}I-C3a is incubated with buffer alone for 25 min at 37°C.

[c]When RMC are incubated for 5 min at 37°C in buffer without protease inhibitors, followed by exposure to ^{125}I-C3a for 20 min at 37°C.

ND=Not Done

Figure 8. Effect of SBTI on the Binding of ^{125}I-C3a to RMC at 37^{o}C.

RMC (2.0×10^5) were pretreated with buffer or 4 mM SBTI for 5 min at 37^{o}C. Increasing concentrations of ^{125}I-C3a were incubated with these cells for 20 min at 37^{o}C in a volume of 0.5 ml. A portion was removed and analyzed for the number of ^{125}I-C3a molecules bound per cell.

Figure 8.

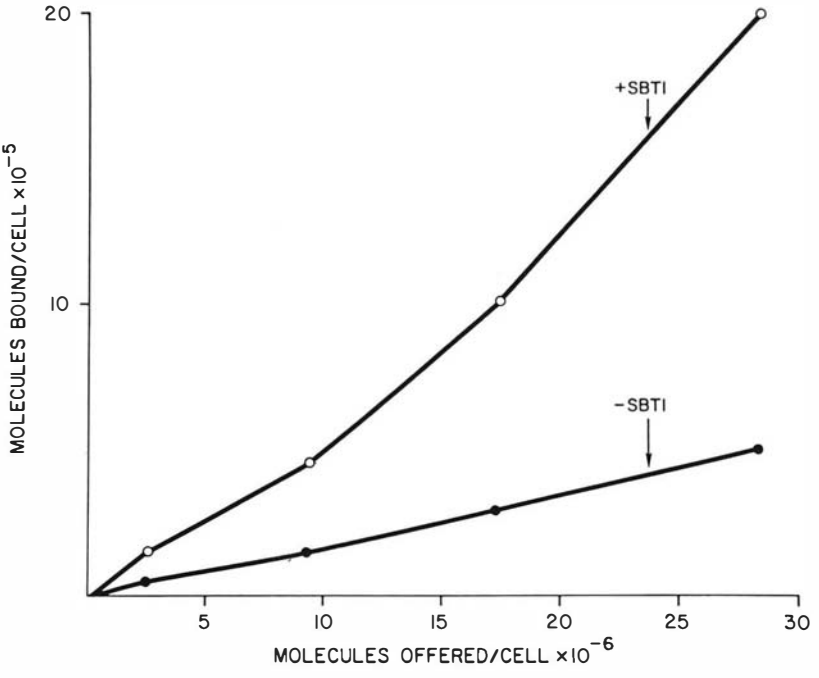
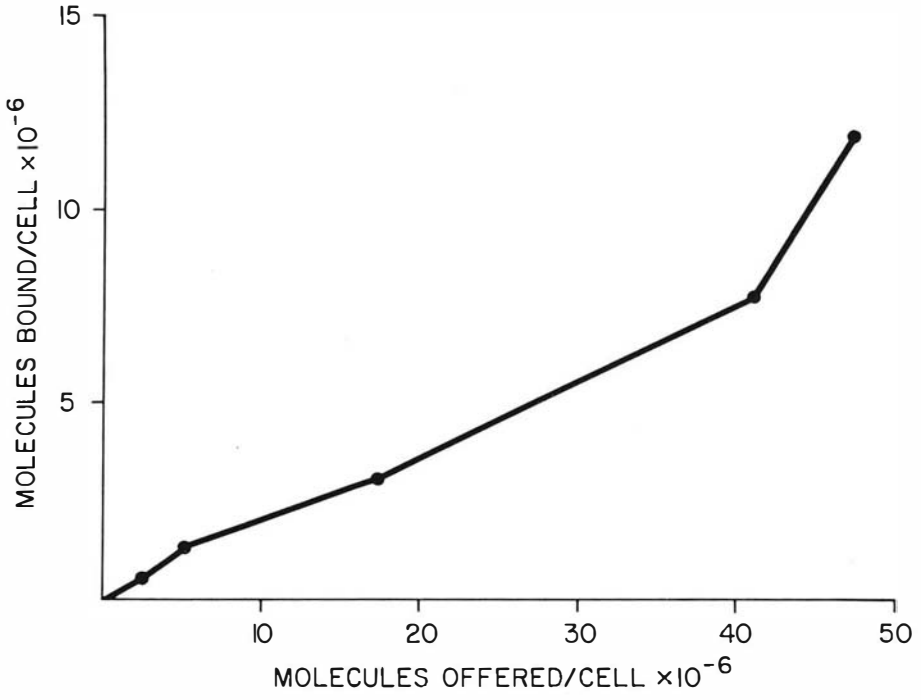

20
10
+SBTI
-SBTI
MOLECULES BOUND/CELL ×10⁻⁵
5
10
15
20
25
30
MOLECULES OFFERED/CELL ×10⁻⁶

Figure 9. Effect of SBTI on the binding of ^{125}I-C3a to RMC at 0^{o}C.

RMC (2 x 10^5) were pretreated with 4 mM SBTI for 5 min at 37^{o}C. The cells were than transferred to 0^{o}C for 15 min. Increasing concentrations of ^{125}I-C3a were added to these cells and incubated for 20 min at 0^{o}C in a volume 0.5 ml. A portion was removed and analyzed for the number of ^{125}I-C3a molecules bound per cell.

Figure 9.


15
10
5
MOLECULES BOUND/CELL ×10⁻⁶
10
20
30
40
50
MOLECULES OFFERED/CELL ×10⁻⁶

^{125}I-C3a of 48 million molecules offered per cell, saturation could not be obtained. ^{125}I-C3a was supplemented with noniodinated C3a and used in an experiment performed exactly as the above study. At concentrations of 210 million molecules offered per cell saturation could not be reached (data not shown).

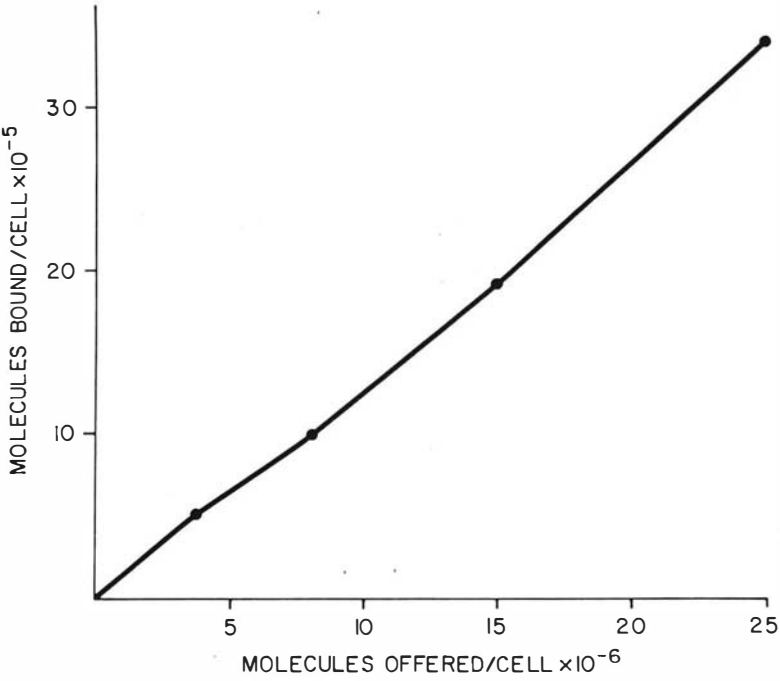
Effect of Protamine on the Binding of ^{125}I-C3a to RMC. C3a is a highly basic molecule capable of interacting electrostatically with negatively charged moieties on the surface of RMC. To test this hypothesis two very basic protiens, poly-L-lysine and protamine were used to pretreat RMC prior to ^{125}I-C3a exposure. Both poly-L-lysine and protamine were titrated to determine the optimal concentrations which would not cause degranulation of RMC. At all concentrations tested, poly-l-lysine caused degranulation. In contrast, protamine could be used at a concentration of 50 µg per million cells without causing degranulation or cell death as assessed by microscopic examination and trypan blue exclusion, repectively. To determine the effect of protamine on ^{125}I-C3a binding to RMC, 2.0×10^5 SBTI treated RMC were incubated with 10 µg of protamine for 15 min at 0^oC. Increasing concentrations of ^{125}I-C3a were added to these cells and incubated for 20 min at 0^oC. The cells were layered onto oil and the number of molecules per cell determined. As shown in Figure 10, RMC could not be saturated even when 25 million molecules of ^{125}I-C3a were offered per RMC. These data indicate that saturation is not observed in the presence of a cationic compound such as protamine.

Binding of ^{125}I-C3a to RMC in the Presence of Heparin. The large amount of binding and the unsaturable nature of the interaction between ^{125}I-C3a and RMC may be attributed to ^{125}I-C3a associating with surface bound heparin proteoglycan. Preliminary experiments using a

Figure 10. Effect of Protamine on the Binding of ^{125}I-C3a to RMC at $0^{o}C$.

RMC (2×10^{5}) were pretreated with 4 mM SBTI for 5 min at $0^{o}C$. These cells were incubated with 10 µg of protamine for 15 min at $0^{o}C$. Increasing concentrations of ^{125}I-C3a were incubated for an addition 20 min at $0^{o}C$ in a volume of 0.5 ml. A protion was removed and analyzed for the number of ^{125}I-C3a molecules bound per RMC.

Figure 10.


MOLECULES BOUND/CELL ×10⁻⁵
30
20
10
5
10
15
20
25
MOLECULES OFFERED/CELL ×10⁻⁶

column of agarose to which heparin had been covalently linked showed that passage of ^{125}I-C3a over such a column results in 26% binding. These molecules could be eluted from the heparin-agarose column by using 0.3 M NaCl (data not shown). The following experiment was performed to test if the negatively charged component of RMC to which ^{125}I-C3a was interacting with was heparin proteoglycan. Varying numbers of RMC which had been pretreated with SBTI were incubated with ^{125}I-C3a in the presence of Tyrode's buffer, Tyrode's buffer containing exogeneous commercial heparin, or Tyrode's buffer plus heparin containing 0.3 M NaCl for 20 min at 0^{o}C. Shown in Figure 11 is the percent ^{125}I-C3a bound to RMC under these three conditions. Untreated RMC bound up to 20% of the ^{125}I-C3a molecules offered. When heparin only or heparin plus 0.3 M NaCl were present in the reaction mixture there was 8% and 4% of the ^{125}I-C3a molecules offered, respectively. These data show a 60.0% and a 80.0% reduction in the percent ^{125}I-C3a bound for both heparin and heparin plus 0.3 M NaCl, respectively, as compared to untreated RMC. This indicates that the high amount of binding of ^{125}I-C3a to RMC could partially be explained by the electrostatic interaction of positively charged ^{125}I-C3a with the negatively charged heparin proteoglycan deposited on the surface of RMC.

Covalent Crosslinking of ^{125}I-C3a to Chymase. Chymase is the most abundant protease and protein component found in the secretory granules of RMC and has chymotrypsin-like specificity (174). In order to demonstrate the interaction of ^{125}I-C3a with chymase, a chemical crosslinking agent, BS^3, was added to RMC sonicates to bind ^{125}I-C3a covalently to chymase. The ^{125}I-C3a-chymase complex was then precipitated as described in Materials and Methods. Figure 12 shows the dose dependent immunoprecipitation of ^{125}I-C3a by antibody to chymase.

Figure 11. Binding of ^{125}I-C3a to RMC in the Presence of Heparin.

Varying numbers of RMC were pretreated with 4 mM SBTI for 5 min at 0^{o}C. These cells were than incubated with 70 ng of ^{125}I-C3a in Tyrode's buffer, Tyrode's buffer containing exogenous commercial heparin, or Tyrode's buffer plus exogenous commercial heparin containing 0.3 M NaCl in a volume of 0.6 ml for 30 min at 0^{o}C. A portion was removed and analyzed for percent ^{125}I-C3a molecules bound per cell.

Figure 11.

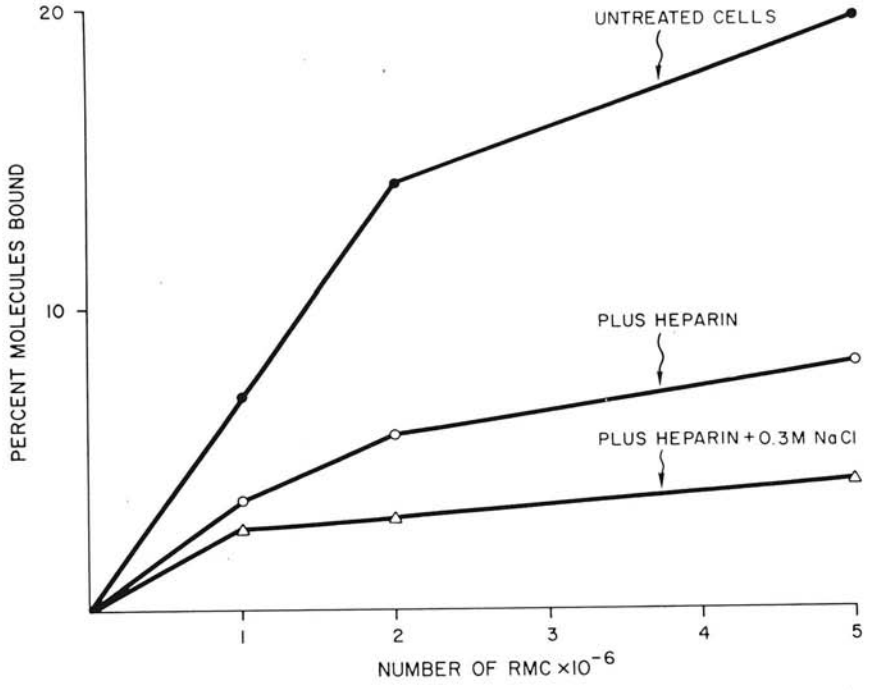
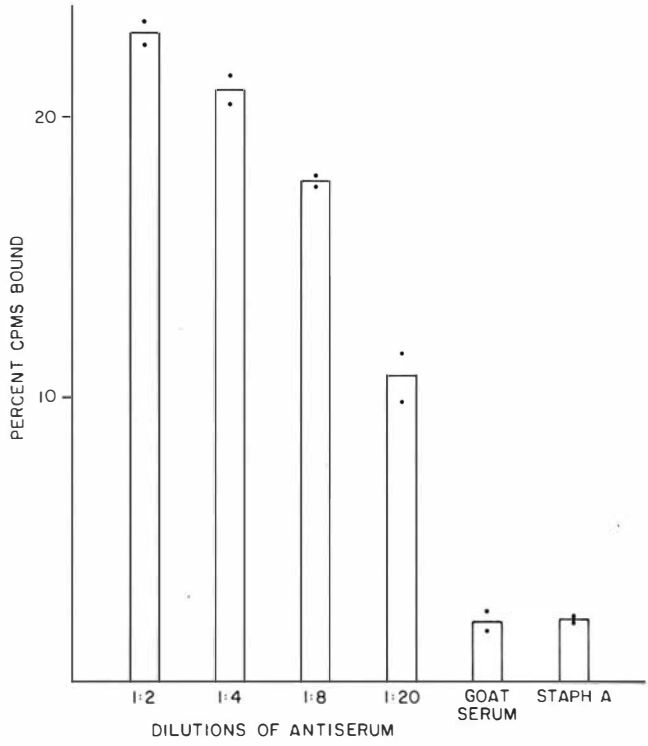

20
10
PERCENT MOLECULES BOUND
UNTREATED CELLS
PLUS HEPARIN
PLUS HEPARIN + 0.3M NaCl
1
2
3
4
5
NUMBER OF RMC ×10⁻⁶

Figure 12. Immunoprecipitation of ^{125}I-C3a Covalently Linked to RMC Chymase by Anti-Chymase Antibody.

RMC sonicates prepared as described in Materials and Methods were incubated first with dilutions of goat anti-chymase serum, goat serum, or buffer and then with a suspension of S. aureus. The ordinate shows the percent ^{125}I-C3a precipitated. Calculated from the formula, percent ^{125}I-C3a precipitated = cpms in precipitate after S. aureus treatment/cpms in supernatant before S. aureus treatment x 100.

Figure 12.


20
10
PERCENT CPMS BOUND
1:2
1:4
1:8
1:20
GOAT SERUM
STAPH A
DILUTIONS OF ANTISERUM

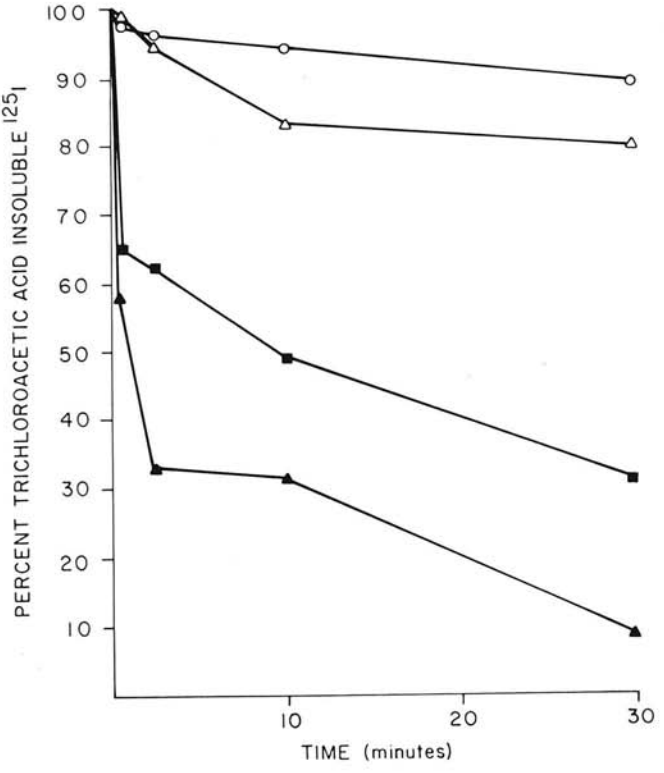
At the highest concentration of the antiserum, 23% of the initial ^{125}I counts wereprecipitated. At the lowest concentration, the percent ^{125}I counts precipitated decreased to 10.9%. This indicates that ^{125}I-C3a was covalently crosslinked to chymase following incubation with intact RMC and BS^3.

Degradation of ^{125}I-C3a by Purified Chymase in the Presence of Heparin Proteoglycan. To show definitively that chymase is the enzyme directly responsible for the degradation of ^{125}I-C3a by RMC, purified chymase was assayed for proteolytic cleavage of the ^{125}I-C3a molecule. Both chymase and heparin proteoglycan were purified from RMC and assayed for activity just prior to each experiment as described in Materials and Methods. ^{125}I-C3a was incubated with 0.7 units of chymase alone, 0.7 units of chymase plus heparin proteoglycan (60 µg) or heparin proteoglycan alone (60 µg) at 37°C. ^{125}I-C3a was also incubated with RMC containing equivalent amounts of chymase and heparin proteoglycan. At various times, a portion was removed from the reaction mixture and the ^{125}I-C3a was analyzed for % TCA precipitablity. As shown in Figure 13, chymase or heparin proteoglycan alone had very little effect on the % TCA precipitablity of ^{125}I-C3a. In contrast, chymase and heparin proteoglycan together caused a decrease in the % TCA precipitability to 65.2% at 30 sec and 31.4% after 30 min. Chymase alone has very low C3a proteolytic activity and the addition of heparin proteoglycan greatly increases the degradation. Although the combination of chymase and heparin proteoglycan was not as effective as RMC containing equivalent amounts of these materials, overnight incubation at room temperature produced 5% TCA precipitabilty (data not shown). Similar results were obtained using ^{125}I-C3a$_{\text{des Arg}}$ (data not shown).

Figure 13. Degradation of ^{125}I-C3a by Purified Rat Chymase in the Presence or Absence of Rat Heparin Proteoglycan.

^{125}I-C3a (50 ng) was incubated with chymase alone (0.7 units)(○), chymase (0.7 units) plus heparin proteoglycan (60 µg) (■), heparin proteoglycan alone (60 µg) (△), or RMC containing equivalent amounts of chymase and heparin proteoglycan (▲) at 37°C. At various times, a portion of the reaction mixture was removed and analyzed for ^{125}I-C3a % TCA precipitablity.

Figure 13.


PERCENT TRICHLOROACETIC ACID INSOLUBLE ^{125}I
100
90
80
70
60
50
40
30
20
10
10
20
30
TIME (minutes)

Effect of Heparin Proteoglycan on the Degradation of ^{125}I-C3a$_{des\ Arg}$ by α-Chymotrypsin. The role of heparin proteoglycan in the synergism observed in the degradation of ^{125}I-C3a by chymase is not known. In an effort to define the role of heparin proteoglycan in this reaction, α-chymotrypsin was assessed for its capacity to degrade ^{125}I-C3a$_{des\ Arg}$ both in the presence and absence of purified rat heparin proteoglycan. ^{125}I-C3a was incubated with either α-chymotrypsin (0.5 units) or chymase (0.5 units) both in the presence and absence of 25μg of heparin proteoglycan in a volume of 0.5 ml at 37°C. In addition, 25 μg of heparin proteoglycan in buffer and intact RMC, containing equivalent amounts of chymase and heparin proteoglycan, were also incubated with ^{125}I-C3a$_{des\ Arg}$. At various times, a 60 μl portion was removed and dispensed into 1 ml of PBS/BSA, centrifuged, and the percent TCA ^{125}I insoluble ^{125}I material determined. As shown in Table 4, the percent TCA ^{125}I insoluble material found in the supernatant following ^{125}I-C3a$_{des\ Arg}$ exposure to α-chymotrypsin in the absence of heparin proteoglycan was 95% and 50% at 30 sec and 30 min, repectively. When heparin proteoglycan is present in the reaction mixture, the percent TCA ^{125}I insoluble material was 83% and 45% for 30 sec and 30 min, respectively. The overall activity of α-chymotrypsin in the presence, or absence of heparin proteoglycan in the degradation of ^{125}I-C3a$_{des\ Arg}$ appears to be very similar. In the presence of heparin the activity of α-chymotrypsin appears to be generally lower than chymase in the degradation of ^{125}I-C3a$_{des\ Arg}$.

Treatment of RMC Sonicates with Anti-Chymase Antibody. To determine whether chymase was the sole enzyme or one of several enzymes involved in the degradation of ^{125}I-C3a by RMC, immunoabsorption of RMC sonicates with antibody specific for chymase was performed. RMC sonicates in

Table 4. Effect of Heparin Proteoglycan on the Degradation of ^{125}I-C3a by Chymotrypsin

	Percent TCA ^{125}I insoluble Material	
Agent	30 sec	30 min
Tyrode's buffer	100	100
RMC	59	10
Chymase	97	60
Chymotrypsin	95	50
Heparin proteoglycan	100	75
Chymase plus Heparin proteoglycan	60	26
Chymotrypsin plus Heparin proteoglycan	83	45

Tyrode's buffer containing 1 M NaCl were absorbed one or two times with either goat anti-rat chymase or goat anti-rat albumin. Immune complexes were removed by addition of a S. aureus suspension. The supernatants were removed and incubated with ^{125}I-C3a for 1 hr at 37^{o}C and assayed for percent ^{125}I insoluble in TCA. The results in Table 5 show that following absorption of RMC sonicates with anti-albumin antibody there is no change in the ability of these sonicates to digest ^{125}I-C3a as compared to untreated sonicates. In contast, RMC sonicates treated with anti-chymase antibody lost their capacity to digest ^{125}I-C3a. There is a 68.0% and 100% inhibition of ^{125}I-C3a digestion by treated RMC sonicates following the first and second absorptions respectively. SDS PAGE analysis of a portion of the immunoabsorbed RMC sonicates revealed a loss of predominantly one protein at a molecular weight of 29,000. The results indicate that chymase is the major, if not the sole enzyme involved in the degradation of C3a by RMC.

Degradation of ^{125}I-C3a$_{des\ Arg}$ by Chymase as the Ratio of Chymase:C3a$_{des\ Arg}$ Varies. Varying amounts of chymase were incubated with a constant concentration of ^{125}I-C3a$_{des\ Arg}$ for 30 min at 37^{o}C. Since the degradation of ^{125}I-C3a by chymase was enhanced when heparin proteoglycan was present, 25 µg of purified rat heparin proteoglycan was added to the reaction mixture. As shown in Figure 14, there is a linear decrease as the ratio of chymase to ^{125}I-C3a$_{des\ Arg}$ decreases. The most efficient ratio of chymase to ^{125}I-C3a required for complete degradation within 30 min at 37^{o}C was 769. At the lowest ratio of chymase:^{125}I-C3a$_{des\ Arg}$ tested, 1.5, only 20% of the ^{125}I-C3a$_{des\ Arg}$ molecules were degraded as assessed by the percent TCA insoluble material found in the supernatant.

Table 5. Immunoabsorption of Chymase from RMC Sonicates.

	Percent ^{125}I insoluble in TCA	
	1st Absorption	2nd Absorption
RMC sonicate + buffer	61.8	58.8
RMC sonicate + anti-chymase	87.8(68)[a]	101.7(100)
RMC sonicate + anti-albumin	59.4(0)	60.0(2.9)

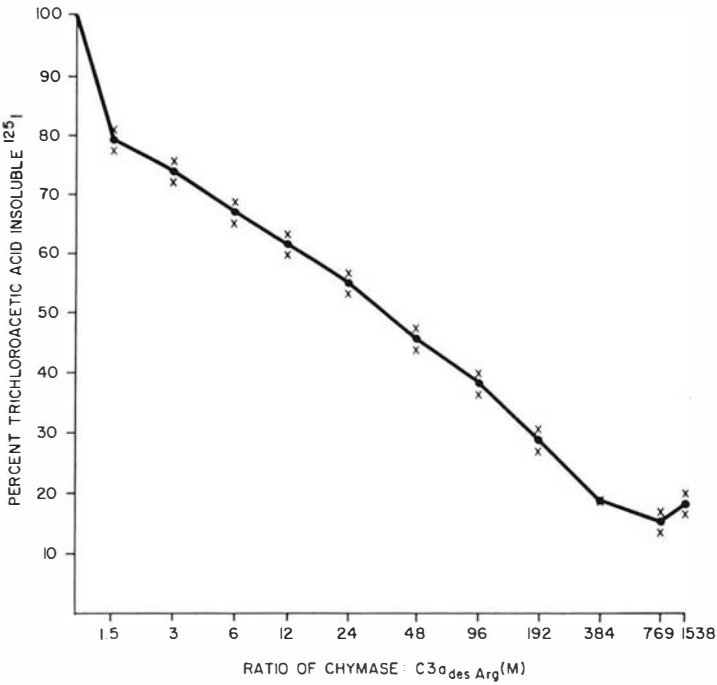
[a] Parentheses represent the percent inhibition of ^{125}I-C3a digestion.

Each value represents the mean from triplicate determinations which had a standard deviation of less than 1%.

Figure 14. Degradation of ^{125}I-C3a$_{des\ Arg}$ by Chymase as the Ratio of Chymase:^{125}I-C3a$_{des\ Arg}$ Varies.

^{125}I-C3a$_{des\ Arg}$ (65 ng) was incubated with various concentrations of purified RMC chymase (0.19 µg($0.2x10^{-4}$ units)-100µg(0.5 units)) in the presence of purified RMC heparin proteoglycan at 37°C for 30 min in a volume of 125 µl. An 80 µl portion was removed and analyzed for percent TCA insoluble ^{125}I material. The experiment was performed in duplicate. The closed circles represent the average value obtained from the duplicate values (X).

Figure 14.


100
90
80
70
60
50
40
30
20
10
PERCENT TRICHLOROACETIC ACID INSOLUBLE ^{125}I
1.5
3
6
12
24
48
96
192
384
769
1538
RATIO OF CHYMASE : $C3a_{des\ Arg}$(M)

The Role of Degranulation of RMC in the Degradation of ^{125}I-C3a. The inactive form of C3a, $C3a_{des\ Arg}$, does not have the capacity to degranulate RMC (20). Since $C3a_{des\ Arg}$ does not activate RMC there should not be any chymase exposed on the surface as a consequence of the incubation with $C3a_{des\ Arg}$. However, when $C3a_{des\ Arg}$ is exposed to RMC there is a similar rapid degradation as observed with C3a in Figure 6 (data not shown). Indirect immunofluorescence was performed using anti-chymase antibody followed by fluorescein-conjugated anti-goat IgG to determine if chymase is expressed on the surface of RMC without prior treatment. As shown in Figure 15, chymase is expressed on the surface of RMC purified from the peritoneal cavity following lavage and Ficoll sedimentation. In addition, chymase was also detected on the surface of RMC directly removed from the peritoneal cavity prior to Ficoll sedimentation (data not shown).

Effect of SBTI on C3a-Induced Release of β-Hexosaminidase from RMC. To test if the inhibition of ^{125}I-C3a degradation by RMC would enhance C3a-induced degranulation of RMC, a dose response experiment using SBTI was performed. Varying concentrations of SBTI were incubated with RMC prior to exposure to ^{125}I-C3a. ^{125}I-C3a was incubated with SBTI treated RMC for 30 min at 37^{o}C. Two portions were removed, one was analyzed for percent TCA insoluble ^{125}I material and the other assayed for β-hexosaminidase activity. As shown in Figure 16, the degradation of ^{125}I-C3a by RMC is inhibited by SBTI in a dose-dependent fashion as indicated by the increase in the percent TCA insoluble ^{125}I material. Maximum inhibition of ^{125}I-C3a degradation by RMC for SBTI is between 0.7 mM and 2.8 mM. The percent β-hexosaminidase release from RMC induced by ^{125}I-C3a in the presence of SBTI is not significantly different from that observed in the absence of SBTI.

Figure 15. Immunofluorescence of RMC using Goat IgG Anti-Rat Chymase Antibody.

Forty thousand RMC were air dried on a microscope slide and incubated with 135 µg of goat anti-rat chymase for 30 min and washed 3 times with PBS. The slides were then exposed to 400 µg of fluorescein-conjugated rabbit anti-goat IgG for 30 min and washed 3 times with PBS. All incubations were performed at room temperature. The purity of this RMC preparation was 97%. The viability of the RMC were 95% as assessed by trypan blue exclusion.

Figure 15.

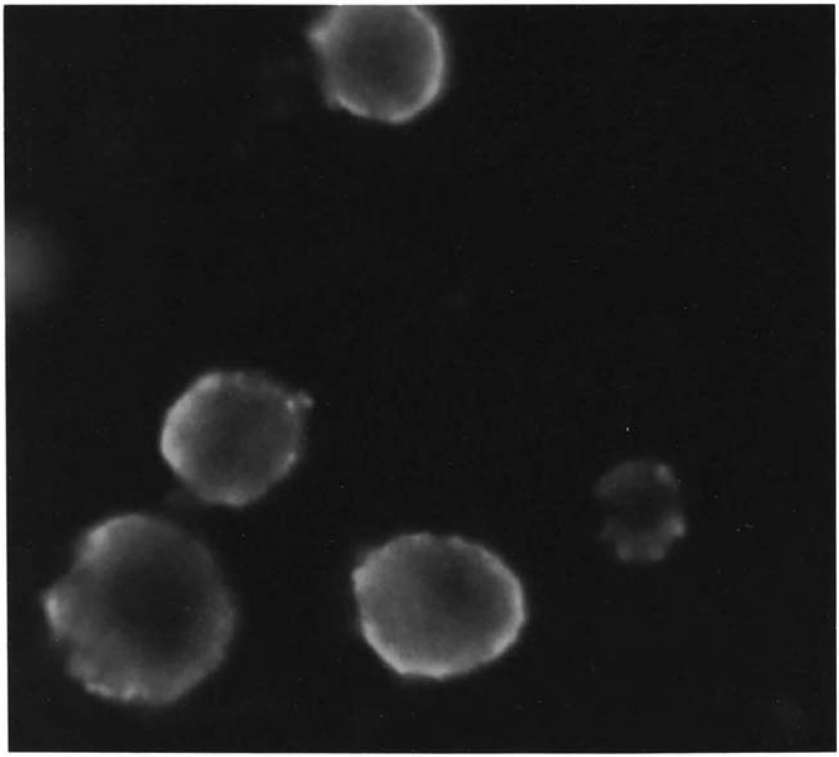
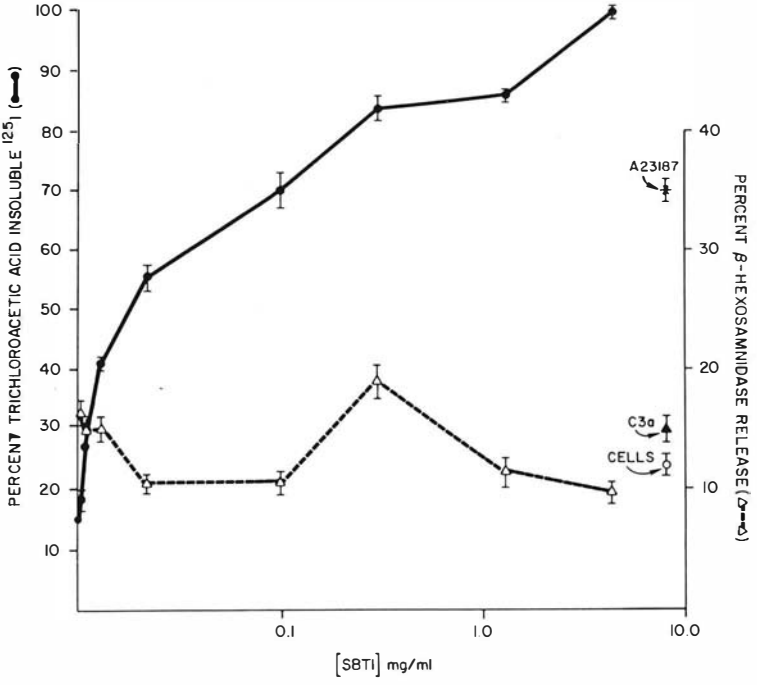


Figure 16. Effect of SBTI on C3a-Induced Release of β-Hexosaminidase from RMC.

2.0×10^5 RMC were pretreated with increasing concentrations of SBTI prior to incubation with 3 nM ^{125}I-C3a for 30 min at 37°C in a volume of 0.3 ml. A 90 µl portion was removed, centrifuged, and analyzed for % TCA insoluble ^{125}I material as described in Material and Methods. A second 0.2 ml portion was removed and despenced in 0.4 ml of ice cold Tyrode's$^{=}$ buffer and assayed for β-hexosaminidase. RMC incubated with A23187 alone, with C3a alone, and with buffer are shown. The experiment was performed in dupulicate. The error bars represent the range of the two values per test tube.

Figure 16.


PERCENT TRICHLOROACETIC ACID INSOLUBLE ^{125}I (●—●)
PERCENT β-HEXOSAMINIDASE RELEASE (△—△)
100
90
80
70
60
50
40
30
20
10
40
30
20
10
A23187
C3a
CELLS
0.1
1.0
10.0
[SBTI] mg/ml

Binding of ^{125}I-C3a to RMC in the Presence of Agents Which Inhibit Degradation, Degranulation, and Binding to Heparin Proteoglycan. The preliminary experiments all indicate that ^{125}I-C3a upon exposure to RMC is being degraded by the granular enzyme chymase and is interacting with heparin proteoglycan. The following set of binding studies was performed to test whether ^{125}I-C3a would show saturable binding to RMC in the presence of reagents that would block chymase activity, interactions with heparin proteoglycan, and C3a-induced degranulation. RMC were treated with Tyrode's$^{=}$ buffer containing 1 mM EDTA, 1mM TPCK, 4 mM SBTI, and 0.3 M NaCl. ^{125}I-C3a was incubated with these RMC at 0^{o}C. Three types of binding analyses were performed, dose-response, kinetic, and inhibition of binding by unlabelled C3a. As shown in Figures 17, when 3.6×10^{6} molecules are offered per RMC, saturation is not achieved even after a 60 min incubation. As shown in Figure 18, even when 590 million molecules of ^{125}I-C3a were offered per cell saturation was not observed. Finally, Figure 19 shows that 450 million molecules of unlabelled C3a per RMC could not block the binding of 1.2 million molecules of ^{125}I-C3a per cell. These data indicate that when ^{125}I-C3a is incubated with RMC under conditions which inhibit degradation, degranulation, and binding to heparin proteoglycan saturation was not observed.

Crosslinking of Intact RMC with ^{125}I-C3a. The cellular components on the surface of RMC to which ^{125}I-C3a could be covalently crosslinked to was determined. Two kinds of experiments were used to define the cellular components on RMC with which C3a interacts: 1) ^{125}I-C3a was incubated with and covalently crosslinked to RMC, 2) unlabelled C3a was incubated with and crosslinked to labelled RMC. RMC were vectorially iodinated by the lactoperoxidase method (4.0 cpm/cell). Both ^{125}I-RMC and RMC were

Figure 17. Time Course of ^{125}I-C3a Binding to RMC in the Presence of Agents Which Inhibit Degradation, Degranulation, and Binding to Heparin Proteoglycan.

RMC (5.5×10^5) were pretreated with Tyrode's buffer containing 1 mM TPCK, 4 mM SBTI, 1 mM EDTA, and 0.3 M NaCl for 5 min at 0^oC. ^{125}I-C3a (30 ng) was incubated with these cells at 0^oC in a volume of 0.5 ml. At various times, a portion was removed and analyzed for the number of ^{125}I-C3a molecules bound per RMC.

Figure 17.

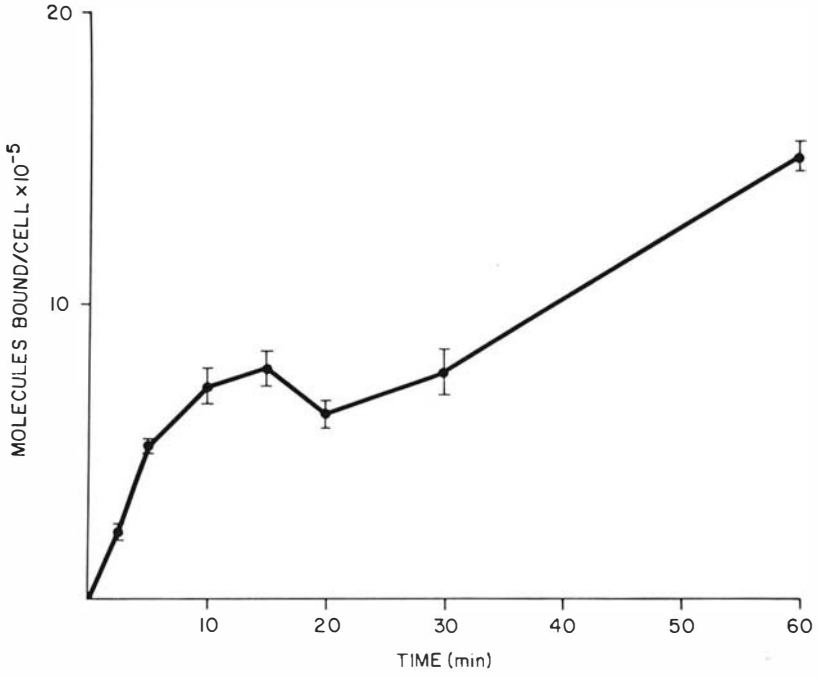


Figure 18. Dose Dependent Binding of ^{125}I-C3a to RMC in the Presence of Agents Which Inhibit Degradation, Degranulation, and Binding to Heparin Proteoglycan.

RMC (5×10^5) were pretreated with Tyrode's$^{=}$ buffer containing 1 mM TPCK, 4 mM SBTI, 1 mM EDTA, and 0.3 M NaCl for 5 min at 0^{o}C. Increasing concentrations of ^{125}I-C3a were incubated with these cells for 20 min at 0^{o}C in a volume of 0.5 ml. A portion was removed and analyzed for the number of ^{125}I-C3a molecules bound per RMC.

Figure 18.

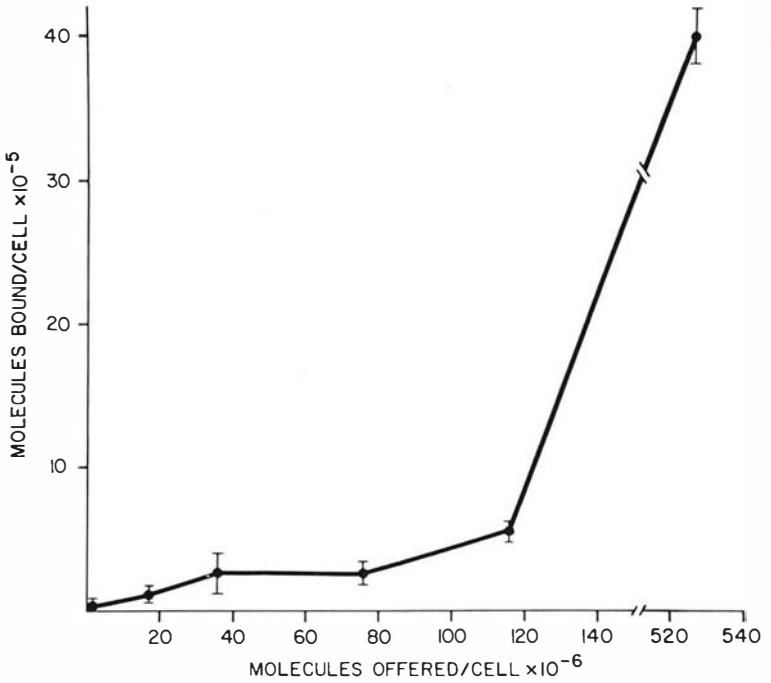
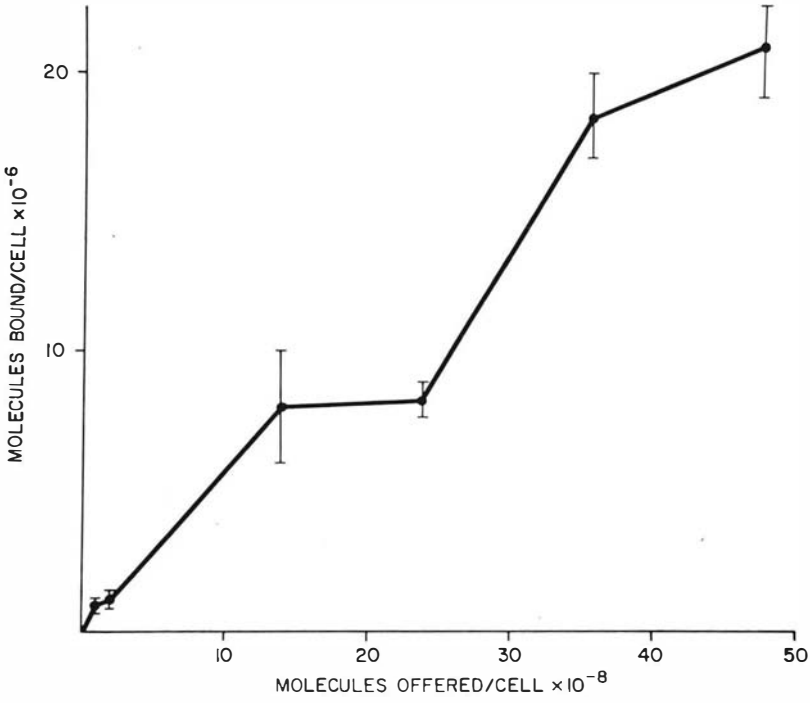

40
30
20
10
MOLECULES BOUND/CELL ×10⁻⁵
20
40
60
80
100
120
140
520
540
MOLECULES OFFERED/CELL ×10⁻⁶

Figure 19. Binding of ^{125}I-C3a to RMC in the Presence of Excess C3a.

RMC (6×10^5) were pretreated with Tyrode's$^{=}$ buffer containing 1 mM TPCK, 4 mM SBTI, 1 mM EDTA, and 0.3 M NaCl for 5 min at 0^{o}C. ^{125}I-C3a (11.5 ng) was incubated with these cells in the presence of excess C3a ranging between 100 ng and 20 µg for 20 min at 0^{o}C in a volume of 0.5 ml. A portion was removed and analyzed for the number of ^{125}I-C3a molecules bound per RMC.

Figure 19.


20
10
MOLECULES BOUND/CELL ×10⁻⁶
10
20
30
40
50
MOLECULES OFFERED/CELL ×10⁻⁸

pretreated with 1 mM DFP for 5 min at $0^{o}C$. C3a and ^{125}I-C3a were incubated with ^{125}I-RMC and RMC, respectively for 30 min at $0^{o}C$. These cells were centrifuged and resuspended in Tyrode's$^{=}$ buffer containing 30 mM BS^{3} and incubated for 30 min at $0^{o}C$. Both cell suspensions were centrifuged, washed 5 times, resuspended in Tyrode's$^{=}$ buffer/NP-40, and sonicated. These lysates were centrifuged and both the supernatants and pellet were analyzed directly by SDS PAGE or immunoprecipitated with anti-C3a antibody and Staph A and then analyzed by SDS PAGE followed by autoradiography. The results obtained by both methods were identical. The intensity of the autoradiography with the immunoprecipitated material was very low, therefore is not shown. Figure 20 shows the autoradiography of an SDS PAGE analysis. Shown in lanes 1 and 3 are the insoluble pellet and soluble supernatant from the ^{125}I-C3a-RMC crosslinked material, respectively. There are three distinct bands visualized at 35,000, 45,000, and 55,000 molecular weight, as determined by the standard curve generated by radioiodinated standards shown in Figure 21 and Table 6. When ^{125}I-C3a-RMC lysates are treated with anti-chymase antibody and precipitated with Staph A, the only band detected is the 35,000 molecular weight species (data not shown). Lanes 2 and 4 are the insoluble pellet and soluble supernatant from the C3a-^{125}I-RMC crosslinked material, repectively. None of the bands correspond to the ^{125}I-C3a-RMC bands visualized in lanes 1 and 3, with the possible exception of the 35,000 molecular weight band. These data indicate that crosslinking ^{125}I-C3a to RMC in the presence of DFP enables the detection of 3 bands of 35,000, 45,000, and 55,000 daltons following SDS PAGE analysis. In addition, the only band that is similar following SDS PAGE analysis of unlabelled C3a crosslinked to labelled RMC is the 35,000 dalton band.

Figure 20. Crosslinking ^{125}I-C3a to Intact RMC and C3a to ^{125}I-RMC

RMC (3.75×10^6) or radiolabelled RMC (3.75×10^6) were pretreated with 1 mM DFP for 5 min at 0^oC. Both RMC and ^{125}I-RMC were incubated with 4 µg of ^{125}I-C3a and C3a, respectively, for 30 min at 0^oc in a volume of 0.2 ml. The cells were washed and resuspended in Tyrode's$^=$ buffer containing 30 mM BS^3 and incubated for 30 min at 0^oC. The cells were centrifuged, washed 3 times, and resuspended in Tyrode's$^=$ buffer containing 1% NP-40 and sonicated. The cell lysate was centrifuged and both the insoluble pellet and soluble fraction was analyzed by SDS PAGE and autoradiography. Lanes 1 and 2 are the insoluble pellet from ^{125}I-C3a-RMC and C3a-^{125}I-RMC crosslinked material, respectively. Lanes 3 and 4 are the soluble fraction from ^{125}I-C3a-RMC and C3a-^{125}I-RMC crosslinked material, respectively.

Figure 20.

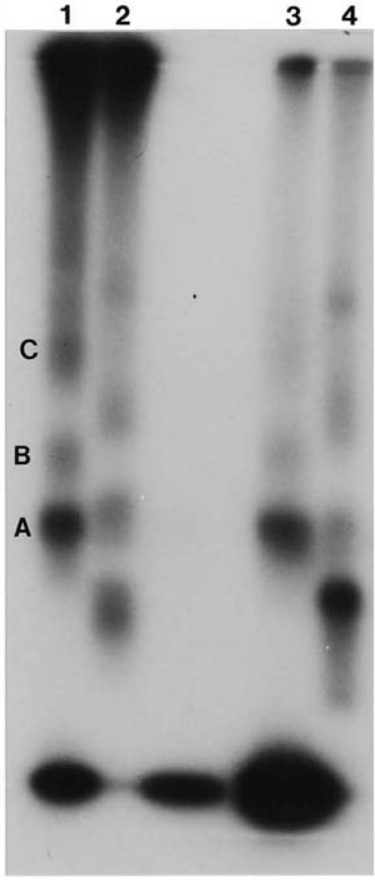


Figure 21. Standard Curve for the Determination of Molecular Weights.

The standard curve for the determination of molecular weights was generated by measuring the distance (cm) from the cathode each protein of known molecular weight migrated. This value was divided by the distance the tracking dye (bromphenol blue) migrated and is represented on the ordinate (Rf). On the abcissa are the log values of β-galactosidase (116,250) (■), phosphorylase B (92,500) (▲), BSA (66,200) (□), OVA (45,000) (○), carbonic anhydrase (31,000) (△), SBTI (21,500) (●).

Figure 21.

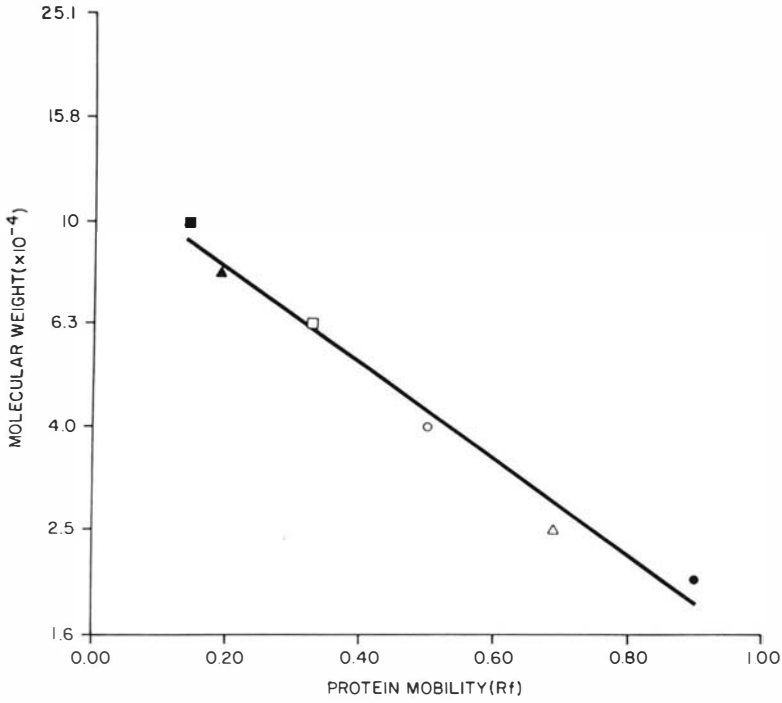

25.1
15.8
10
6.3
4.0
2.5
1.6
MOLECULAR WEIGHT(x10^{-4})
0.00
0.20
0.40
0.60
0.80
1.00
PROTEIN MOBILITY(Rf)

Table 6. Approximate Molecular Weights of Labelled Proteins Observed in Figure 22.

		Rf	MW
Unknown	A	0.6	35,000
Unknown	B	0.47	45,000
Unknown	C	0.38	55,000

Crosslinking ^{125}I-C3a to Intact RMC in the Presence of Various Protease Inhibitors. RMC were either incubated with 4 mM SBTI, 0.04 mM SBTI, 1 mM TPCK, 1 mM TPCK, 1 mM DFP, or buffer prior to exposure to ^{125}I-C3a. As shown in Figure 22, when RMC were pretreated with 4 mM SBTI (lane 2), 1mM TPCK (lane 5), and 1 mM DFP (lane 6) there were three bands of 35,000, 45,000, and 55,000 daltons visualized. These three bands of 35,000, 45,000, and 55,000 daltons were also visualized in Figure 20. When RMC are pretreated with 0.04 mM SBTI (lane 3) or 1 mM TLCK (lane 4) prior to exposure to ^{125}I-C3a there were no bands visualized. When RMC were not incubated with protease inhibitors prior to exposure to ^{125}I-C3a there were no bands visualized (lane 1). There are no bands visualized when the inhibitors 1 mM TPCK (lane 7) or 1 mM TLCK (lane 8) are added to the RMC at the same time as the ^{125}I-C3a. These data indicate that the 3 35,000, 45,000, and 55,000 dalton bands are only detected on SDS PAGE following preincubation of RMC with chymotrypsin-like inhibitors prior to exposure to ^{125}I-C3a.

Percent TCA Insoluble ^{125}I-C3a Material and Binding of ^{125}I-C3a Following Exposure to CMMC. ^{125}I-C3a was tested for its ability to bind to CMMC at 0^{o}C, 25^{o}C, and 37^{o}C. CMMC (5.0×10^{6}) were incubated with 1 µg of ^{125}I-C3a at 0^{o}C, 25^{o}C, and 37^{o}C. At various times, a portion was removed and analyzed for the number of ^{125}I-C3a molecules bound per CMMC. As shown in Figure 23, the highest binding of ^{125}I-C3a to CMMC is at 37^{o}C with approximately 29,000 molecules bound per cell after 30 min which appears to be similar at 60 min. At 25^{o}C, there are 12,000 and 22,000 ^{125}I-C3a molecules per cell at 30 min and 60 min, respectively. At 0^{o}C, the binding is the lowest with 9,000 molecules of ^{125}I-C3a bound per CMMC at 30 min which also appears to remain unchanged at 60 min. In a separate experiment the

binding of ^{125}I-C3a to CMMC was assessed at 30^{o}C (data not shown). The maximum number of ^{125}I-C3a molecules bound per cell were about 25,000. At all temperatures tested, with possibly the exception of 25^{o}C, it appears equilibrium was obtained.

To determine if C3a was being degraded by CMMC as assessed by percent TCA ^{125}I insoluble material, ^{125}I-C3a was incubated with CMMC at 0^{o}C, 25^{o}C, and 37^{o}C in the presence or absence of SBTI. As shown in Figure 24, when the cells were exposed to ^{125}I-C3a at 37^{o}C for 60 min there was a reduction in the percent TCA ^{125}I insoluble material by about 40.0%. When SBTI is present with CMMC at 37^{o}C the degradation of ^{125}I-C3a is inhibited. At 25^{o}C, there was a 10% reduction in the percent TCA ^{125}I insoluble material that could be inhibited with SBTI. The degradation of ^{125}I-C3a by CMMC was completely inhibited by performing the experiment at 0^{o}C.

DISCUSSION

When radioactive ^{125}I-C3a is incubated with RMC for 30 sec at 37^{o}C, there were as many as 10 million molecules bound per RMC (Fig. 3). These numbers are in agreement with those of a previous report for RMC (15), but considerably higher than those for other cells using ^{125}I-C3a or ^{125}I-C5a. Guinea pig platelets were found to have 4.8 x 10^{3} C3a binding sites per cell with a K_d of 6.6 x 10^{-12}M (32). Human neutrophils or murine macrophages had approximately 2.0 x 10^{5} and 1-3 x 10^{5} binding sites for C5a respectively (175,41). It is evident from Table 2 that the binding of ^{125}I-C3a to RMC is temperature dependent. There were 17 million ^{125}I-C3a molecules bound per RMC at

Figure 22. Crosslinking of ^{125}I-C3a to Intact RMC in the Presence of Various Protease Inhibitors.

RMC (2.0×10^6) were incubated with Tyrode's$^{=}$ buffer (lane 1), 4 mM SBTI (lane 2), 0.04 mM SBTI (lane 3), 1 mM TLCK (lane 4), 1 mM TPCK (lane 5), or 1 mM DFP (lane 6) for 10 min at 0^oC. ^{125}I-C3a (2 µg) were incubated with these cells for 20 min at 0^oC. Both 1 mM TPCK (lanes 7) and 1 mM TLCK (lane 8) were added to RMC at the same time as the ^{125}I-C3a and incubated for 20 min at 0^oC. All the cells were washed and resuspended in Tyrode's$^{=}$ buffer containing 10 mM BS^3 and incubated for 30 min at 0^oC. The cells were washed 3 times and resuspended in Tyrode's/ 1% NP-40 and sonicated. The cells were centrifuged and the supernatants analyzed by SDS PAGE and autoradiography.

Figure 22.

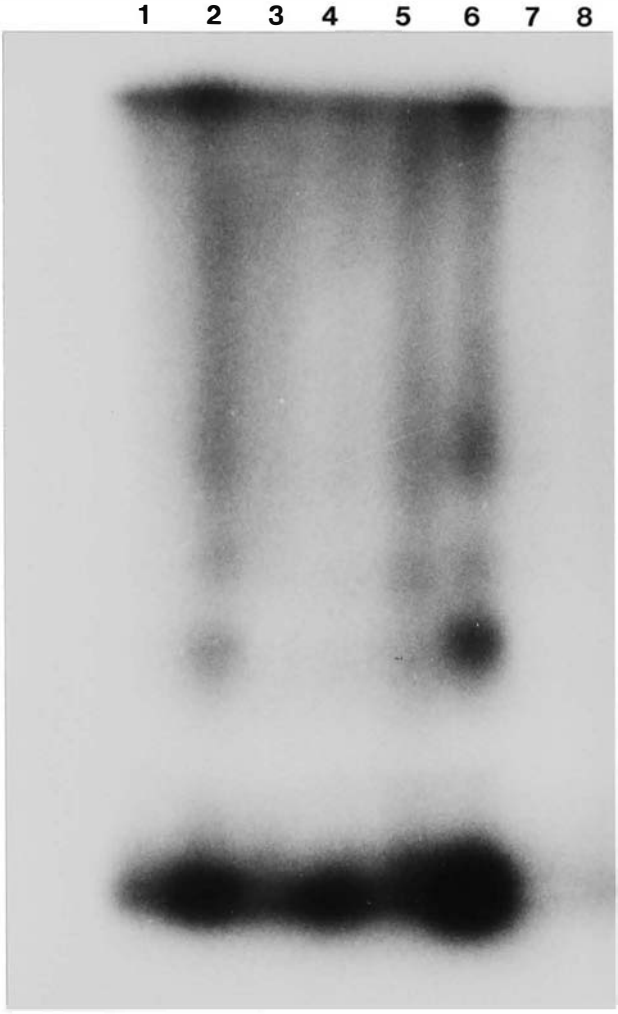


Figure 23. Kinetic Equilibrium Analysis of ^{125}I-C3a Binding to CMMC.

CMMC (17.4×10^6) were incubated with 1 µg of ^{125}I-C3a at 0^oC, 25^oC, or 37^oC in a volume of 1 ml. At various times, a 40 µl portion was removed, dispenced into 0.4 ml of Tyrode's= buffer, layered onto a mineral oil solution, and centrifuged. The CMMC pellet was used to calculate the number of ^{125}I-C3a molecules bound per cell.

Figure 23.

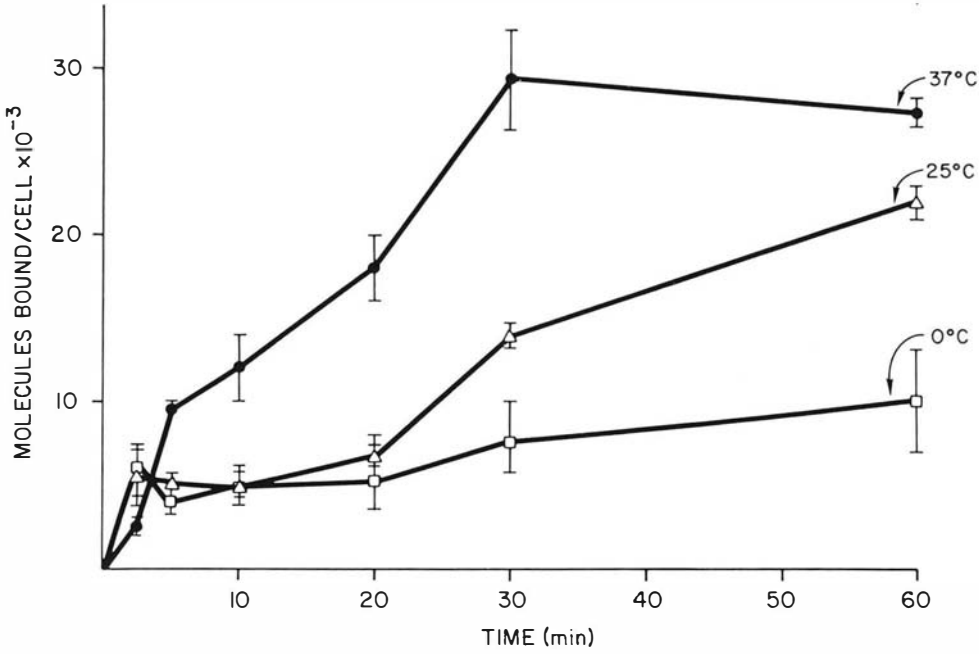
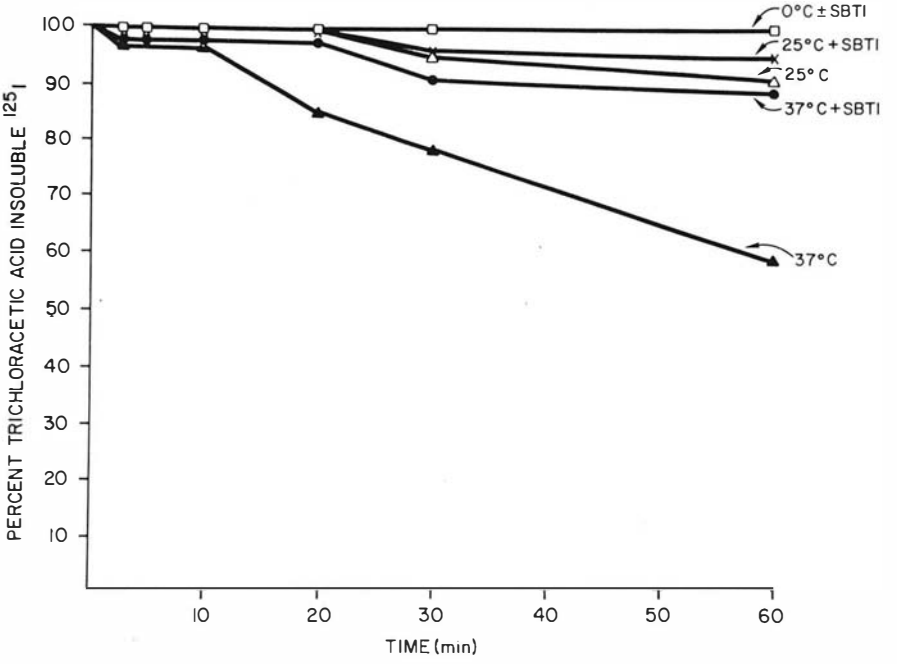

MOLECULES BOUND/CELL x10⁻³
30
20
10
37°C
25°C
0°C
10
20
30
40
50
60
TIME (min)

Figure 24. Percent TCA Insoluble ^{125}I-C3a Material Following Exposure to CMMC.

CMMC (1.2×10^6) were pretreated with Tyrode's buffer or Tyrode's$^{=}$ buffer containing 4mM SBTI at 0^oC, 25^oC or 37^oC for 5 min. ^{125}I-C3a (18 ng) was incubated with cells at 0^oC plus or minus SBTI, 25^oC plus or minus SBTI, and 37^oC plus or minus SBTI in a volume of 0.3 ml. At various times, a 20 µl portion was removed and despenced into 1 ml of PBS/BSA and centrifuged. The supernatants were than analyzed for percent insoluble ^{125}I material.

Figure 24.


0°C ± SBTI
25°C + SBTI
25° C
37°C + SBTI
37°C
PERCENT TRICHLORACETIC ACID INSOLUBLE ^{125}I
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
TIME (min)

30 sec, as compared to 8.9 million at 37°C. Figure 4 shows that the binding of ^{125}I-C3a to RMC at 0°C is not saturable. An equlibrium was not estabalished between ^{125}I-C3a and RMC even when increasing the number of ^{125}I-C3a molecules to 530 million offered per RMC

Additional binding studies were therefore performed in order to investigate the reasons for the high amount of binding and unsaturable nature of the C3a-RMC interaction. In an effort to obtain conditions in which binding of C3a to RMC was saturable and the number of molecules bound per cell more realistic, a series binding studies were performed in which the experimental conditions were varied. When 25 million molecules of ^{125}I-C3a were offered per cell in the absence of SBTI there was 450,000 molecules bound per cell; in the presence of SBTI this increased to 1.7 million. At 0°C, in the presence of SBTI (Fig. 9), the number of ^{125}I-C3a molecules bound per RMC is even higher at 4.5 million. In the presence of a highly basic protein, such as protamine, saturation could not be achieved. There was a reduction in the number of ^{125}I-C3a molecules bound per cell, from 4.5 million to 3 million in the presence of protamine (Figs. 9&10). This inhibition by the highly positively charged protamine suggests an interaction of C3a with a negatively charged component on the surface of RMC. The very large amount of ^{125}I-C3a bound to RMC shown in Figures 3,4,8,9, and 10 may reflect the interaction of this positively charged polypeptide with heparin proteoglycan present on the surface of the RMC. Passage of ^{125}I-C3a over a column of agarose to which heparin has been covalently linked and equilibrated in PBS, results in the binding of up to 26% of the ^{125}I-C3a molecules. These molecules can be eluted from the

heparin-agarose column using 0.3 M NaCl (data not shown). Addition of commercial intestinal mucosal heparin to the incubation of RMC and ^{125}I-C3a, decreases the number of molecules bound per cell in a dose-dependent fashion. The number of molecules bound per cell further decreases if the incubation is performed in buffer containing 0.3 M NaCl in the presence of commercial heparin, as compared to the effect of either alone (Fig. 11). The results of Figures 8, 11, and Table 2 all indicated that the binding studies could best be performed in the presence of 1 mM TPCK, 4 mM SBTI, 1 mM EDTA, and 0.3 M NaCl at 0^{o}C. As shown in Figures 17, 18, and 19, saturation was still not be obtained. The inability to show saturation of ^{125}I-C3a binding to RMC in either a dose-dependent, kinetic, or C3a inhibition study indicates that there are large amounts of non-specific binding of C3a, possibly to chymase and heparin proteoglycan on the surface of RMC. These compounds on the surface of RMC make it impossible to determine either the number of C3a receptors per cell or their affinity using classic methods for studing ligand-receptor interactions.

Following the initial binding of ^{125}I-C3a by RMC, there is a rapid dissociation of the label from the cells (Fig. 3). This labeled material is in an altered form that is no longer capable of binding to fresh RMC (Fig. 5). Analysis of the released material demonstrates lack of precipitability by TCA (Fig. 6) and smaller peptides by SDS PAGE (Fig. 7), presumably the products of enzymatic cleavage. Approximately 50% of ^{125}I is dissociated within 30 sec and the remainder appears more slowly over the next 30 min. One explanation for this phenomenon may be the selective cleavage between two ^{125}I labeled tyrosine residues which are located 43 amino acid residues apart (16). When mast cells degranulate, much of the released heparin proteoglycan and chymase remain as an

insoluble complex in an extracellular position at the cell surface within lacunae (111). Conceivably, C3a is ionically bound to heparin proteoglycan within these lacunae and enzymatic cleavage occurs releasing one fragment rapidly, while the remaining portion is bound to the heparin proteoglycan. The gradual decrease in the percent ^{125}I-C3a insoluble in TCA after the 30 sec incubation time may be due to the release of this remaining fragment into the fluid phase.

Cleavage of ^{125}I-C3a by RMC was abrogated with inhibitors that block the activity of chymotrypsin-like enzymes (Table 3). The inhibition by DFP and TPCK indicate that both serine and histidine residues are in the active site (174). Chymase, being the most abundant protease in rat serosal mast cells and also bearing chymotrypsin-like specificity (174) was the most likely enzyme involved. The immunoprecipitation of ^{125}I-C3a by antibody to chymase following covalent crosslinking is direct evidence for the interaction of these two molecules (Fig. 12). Although the precise mode of interaction is unknown, the ^{125}I-C3a and chymase molecules must be in close proximity so as to allow covalently linkage of adjacent amino groups (epsilon amino group of lysine or terminal alpha amino groups) of these two proteins (164). Since chymase cleaves C3a, it is likely that some of the ^{125}I-C3a molecule are bound to the active site on chymase and that the peptide is crosslinked to the enzyme adjacent to its active site.

The effect of immunoabsorption of chymase from RMC sonicates on the degradation of ^{125}I-C3a is shown in Table 5. The degradation of ^{125}I-C3a by RMC sonicates is completely abrogated by preabsorption with anti-chymase antibody, whereas anti-albumin antibody had no effect. It appears that chymase is the major enzyme responsible for the digestion of C3a by RMC.

In order for C3a to bind to chymase and be degraded by it, the enzyme must be accessible for such an interaction. The concentration of C3a used in these experiments (8.8×10^{-7}M) induced no detectable degranulation of RMC ($\leq$1% above background as assessed by release of β-hexosaminidase activity). Johnson et al. found that concentrations of C3a less than 9×10^{-6}M did not induce histamine release from purified RMC. When increasing concentrations of C3a ranging up to 10^{-5}M were incubated with RMC, the maximum total histamine released was 25-30% (29). In a similar study, Glovsky et al., using purified human leukocytes, showed that C3a concentrations of 10^{-6}M induced 30% release of total histamine (31). Since no degranulation was detectable in our experiments, it seems likely that chymase-heparin proteoglycan complexes on the surface of the RMC are responsible for the destruction of C3a. The observation that chymase can be visualized by immunofluoroescence microscopy on the surface of unstimulated RMC (Fig. 15) and that these cells can digest $C3a_{des\ Arg}$, an inactive from of C3a that does not induce degranulation, supports this notion.

The contribution of heparin proteoglycan to the degradation of ^{125}I-C3a by purified chymase is demonstrated in Figure 13. Neither chymase nor heparin proteoglycan alone had the capacity to cleave ^{125}I-C3a to any appreciable extent, but together 68.6% was cleaved in 30 min. The enhancing effect of heparin proteoglycan on chymase activity is in contrast to other reports. Diminished chymase proteolytic activity in the presence of heparin proteoglycan was found with protein substrates such as ornithine amino transferase apoenzyme (81) and casein (23,110). Chymase activity against the ester substrate BTEE, in the presence of heparin proteoglycan, has been variably affected (23,110).

An analogous effect on the degradation of C3a by chymase has also been shown to occur with tryptase, the major neutral protease of human mast cells (99). Tryptase purified from human pulmonary mast cells cleaves the third component of complement into five fragments. One of these fragments, with a molecular weight of 9000, was shown to be authentic C3a that expressed antigenic determinents on radioimmunoassay and anaphylatoxic activity on bioassay. When heparin together with tryptase was incubated with C3 this C3a-like fragment was not detectable by SDS PAGE analysis. When purified C3a was incubated with tryptase in the presence of heparin, the C3a molecule was rapidly degraded, whereas in the absence of heparin tryptase had no appreciable effect (99). These observations precisely parallel those for chymase in the present study.

The rate of ^{125}I-C3a degradation (Fig. 13) by RMC was faster than that catalyzed by identical amounts of chymase and heparin proteoglycan. Differences between the results with purified materials and intact RMC may reflect the higher local concentrations of chymase and heparin proteoglycan in the lacunae, promoting more efficient interaction with ^{125}I-C3a. In addition, carboxypeptidase A, the second most abundant protease that resides in rat mast cell secretory granules and is bound to heparin proteoglycan, cleaves aromatic amino acid residues from the carboxy-terminal end of peptides. Carboxypeptidase A may act in concert with chymase to release ^{125}I-tyrosine residues from ^{125}I-C3a.

The role of heparin proteoglycan in the enhancement of chymase proteolysis of ^{125}I-C3a is not defined. In order to better understand the requirement for heparin proteoglycan, chymotrypsin was assessed for its capacity to degrade ^{125}I-C3a in the presence and absence of heparin proteoglycan. As shown in Table 4, when chymotrypsin is incubated with

^{125}I-C3a in the presence of heparin proteoglycan there appears to be a slight augmentation of about 20% in the ability of this enzyme to degrade ^{125}I-C3a, as compared to chymotrypsin alone at 30 sec. At 60 min the augmentation is not as obvious. When ^{125}I-C3a was incubated with chymase alone, there was 97% and 60% TCA insoluble ^{125}I material at 30 sec and 60 min, respectively. When chymase was incubated with ^{125}I-C3a in the presence of heparin proteoglycan there was 60% and 26% TCA insoluble ^{125}I material at 30 and 60 min, respectively. These data suggests that heparin proteoglycan may function by making C3a more susceptible to proteolysis by chymase or chymotrypsin. Whether the heparin proteoglycan conformationally changes the C3a molecule so that the peptide cleavage site is more accessible to the enzyme needs to be proven experimentally. The alternative, that heparin stabilizes chymase activity, as it does for human tryptase, also requires investigation.

The rate at which chymase degrades for C3a is greater than chymotrypsin (Table 4). Chymase differs structurally from chymotrypsin in the number of disulfide bridges. The chymase molecule has one less disulfide bond than does chymotrypsin. It is thought that this structural difference may be relevant to the unusual ability of chymase to cleave substrates with proline residues 3 amino acids away from the cleavage site on the NH_3-terminal side (121). Such a proline residue exists in the primary structure of C3a at amino acid number 31 and may account for the more rapid rate of C3a cleavage by chymase.

The activity of purified chymase on the proteolysis of ^{125}I-C3a in the presence of heparin proteoglycan (Fig. 14) appears to be lower than would have been initially predicted by the rate of degradation observed in Figures 6 and 7. As the ratio of chymase to ^{125}I-C3a$_{des\ Arg}$

decreases the amount of proteolysis decreases in a linear fashion. There are several possibilities for the reduced activity. There may be a low percentage contaminant that is reponsible for the degradation of C3a. A second possibility is that the experimental conditions were not optimized for maximum chymase activity. The data that argue against the former explanation are 2 fold. The reaction is abrogated by inhibitors specific for chymotrypsin-like enzymes. In addition, when this chymase preparation is analyzed on SDS PAGE there is only one protein band detected at 29,000 molecular weight following silver staining. Chymase is a neutral protease and is optimally active at a pH range between 7.4 and 7.8. This experiment was performed at pH 6.8. The activity of chymase at pH 6.8 does not appear to be affected when using the synthetic substrate BTEE, the activity of chymase at pH 6.8 on substrates such as complex proteins such as C3a is not known. It is conceivable that the optimal pH for proteolysis of C3a is closer to pH 7.4-7.8 than to pH 6.8.

The C3a concentrations required to cause greater than 15% release of total histamine from RMC are approximately 100 µg/ml which is 100 fold more than C5a-induced histamine release (personal communication with T. Ishizaka). The reason for the lower activity of C3a in RMC activation as compared to C5a is not known. One possiblility is that C3a is being degraded by RMC chymase and that the increased concentrations of C3a required for RMC activation is directly affected by this phenomena. The experiment shown in Figure 16 illustrates the effect of pretreating RMC with various concentrations of SBTI prior to ^{125}I-C3a exposure on both the degradation of ^{125}I-C3a and β-hexosaminidase release. There is a dose-dependent inhibition of ^{125}I-C3a proteolysis by RMC using SBTI. There appears to be little correlation between the concentrations of SBTI used to inhibit proteolysis of C3a by RMC to the amount of

β-hexosaminidase released. The concentrations of SBTI between 0.1 (.015 mM) and 1.6 (0.24 mM) µg/ml appear to have very slight enhancement (20%) on the percent β-hexosaminidase release following stimulation with C3a as compared to C3a incubated with untreated RMC (15%) and spontaneous RMC release (12%). It has been shown that pretreatment of RMC with inhibitors that block serine esterase activity will completely abrogate IgE-mediated release of histamine from these cells (154). In addition, it has been recently shown by Kido et al (177) that specific inhibition of chymase, using both $F(ab')_2$ fragments of anti-chymase antibody and chymostatin, completely blocked histamine release induced by rat anti-IgE. Both $F(ab')_2$ fragments of anti-chymase antibody or chymostatin had no affect on histamine release as induced by compound 48/80 (154). It is evident from these experiments that chymase plays an essential role in the degranulation process of RMC. It is conceivable that there is a concentration of SBTI that inhibits the proteolysis of C3a but not the chymase activity required for RMC degranulation and that this concentration may be between 0.015 mM and 0.24 mM.

With the use of BS^3, the binding components for ^{125}I-C3a on RMC could be analyzed by electrophoresis on SDS polyacrylamide slab gels. Figure 20 shows the autoradiography of an SDS PAGE analysis following the crosslinking of ^{125}I-C3a to RMC (lanes 1 and 3) and C3a to radioiodinated RMC (lanes 2 and 4). There are three distinct bands visualized in both the insoluble pellet (lane 1) and the soluble fraction (lane 3) following treatment of RMC with ^{125}I-C3a. The molecular weights of these 3 components, as ascertained by the molecular weight standards shown in Figure 21 and Table 6, are 35,000, 45,000, and 55,000. The 35,000 molecular weight species is ^{125}I-C3a crosslinked to chymase as determined by immunoprecipition with anti-chymase antibody

(data not shown). The molecular weight of chymase is between 26,000 and 29,000. The difference between the molecular weight of C3a (9,000) and 35,000 is 26,000, making this band (35,000) in the correct molecular weight range for chymase. The other bands have not been defined. The molecular weight differences between the 3 bands are 10,000. It is conceivable that the 2 bands at 45,000 and 55,000 molecular weight could be chymase crosslinked to two and three ^{125}I-C3a molecules, respectively. There are two arguments that suggest that this is not the case; 1) when the solublized RMC fractions are immunoprecipitated with anti-chymase antibody and Staph A there is only one band at 35,000 molecular weight detected on SDS PAGE and autoradiography, 2) these two bands at 45,000 and 55,000 molecular weight are not detected when C3a is crosslinked to ^{125}I-RMC (lanes 2 and 4), but the 35,000 molecular weight band is. Another possiblility is that the bands at 45,000 and 55,000 molecular weight represent two components of the C3a receptor. The band at the bottom of the gel is free ^{125}I-C3a, as assessed by the electrophoretic pattern observed with ^{125}I-C3a alone. The intense radioactivity at the top of the gel may be ^{125}I-C3a crosslinked to heparin proteoglycan or to chymase which in turn is crosslinked to heparin proteoglycan. The fact that this intense radioactivity is not found in the soluble fractions (lanes 3 and 4) where heparin proteoglycan would not be found, supports this notion. The only band that is similar between ^{125}I-C3a-RMC and C3a-^{125}I-RMC is the 35,000 molecular weight species. The other two bands at 45,000 and 55,000 molecular weight are not found in the C3a-^{125}I-RMC (lanes 2 and 4). The 45,000 and 55,000 molecular weight species are not labeled with ^{125}I because they are either inaccessible to the ^{125}I or they lack tyrosine residues exposed on the surface of the plasma membrane.

The effect of various inhibitors and incubation conditions on the ability of BS^3 to crosslink ^{125}I-C3a to RMC was tested. As shown in Figure 22, there is an absolute requirement for the pretreatment of RMC with a chymotrypsin-specific inhibitor prior to ^{125}I-C3a exposure for the detection of the 35,000, 45,000, and 55,000 molecular weight species.

Recently, a second type of mast cell (mucosal mast cell) has been idenifed that is distinctively different in their proteoglycan and neutral protease. It has been shown that the mucosal mast cell is equivalent to the cultured mouse mast cell (CMMC). Preliminary experiments using the CMMC indicate that these cells may be useful in determining the number of C3a receptors of the surface of mast cells. As shown in Figure 23, when ^{125}I-C3a is incubated with CMMC at 0^oC, 25^oC and 37^o there appears to be an equilibrium established within 30 min at all of the temperatures tested, with the possible exception of 25^oC. The number of C3a binding sites per CMMC (30,000) in Figure 23 is much lower than that observed for RMC (Figures 3,4,8,9,and 10). In addition, the binding curves for RMC and CMMC are entirely different. As shown in Figure 3, when RMC are incubated with ^{125}I-C3a at 37^oC, there was a high amount of binding followed by a rapid dissociation of label from these cells. In contrast, when CMMC are incubated with ^{125}I-C3a at 37^oC, there are fewer molecules bound per cell with a steady increase during the ensuing 30 min. One explanation for this difference observed in C3a binding is that CMMC does not degrade C3a as rapidly as does RMC, as illustrated in Figure 24. The type of proteoglycan found in CMMC is not as sulfated as RMC heparin, therefore it may not bind as much C3a.

C3a has been shown to possess several biological activities in various animal species both in vitro and in vivo. It causes smooth muscle contractions, induces increased vascular permeablity (12,13), and triggers mast cells and basophils to degranulate and release histamine (12,13,31,176). Other inflammatory processess can be mediated by C3a such as thromboxane release from macrophages (36), lysosomal enzyme secretion from neutrophils (35) and serotonin release from platelets (33). The control of these biological activities is mediated systemically by serum carboxypeptidase N, which cleaves the carboxyl terminal arginine changing C3a to $C3a_{des\ Arg}$, rendering it biologically inactive (20). The inactivation of human C3a by carboxypeptidase N is similar in human, guinea pig, rat, and rabbit sera (20). The data from the present study suggests a regulatory role for chymase in response to C3a stimulation of mast cells. During an acute inflammatory episode, C3 is converted to C3b and C3a (20). This newly generated C3a then acts on mast cells causing degranulation and histamine release (29,20,23). Following degranulation of mast cells, C3a is rapidly degraded by released chymase in the presence of heparin proteoglycan. Once C3a interacts with mast cells it is inactivated and can not degranulate other mast cells in the area. C3a that has not interacted with mast cells may be inactivated by the serum carboxypeptidase N. While the carboxypeptidase N may control systemic levels of C3a, the effect of chymase and heparin proteoglycan may be important in the microenvironment of the inflammatory lesion.

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