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Neutral Proteases of Mast Cells

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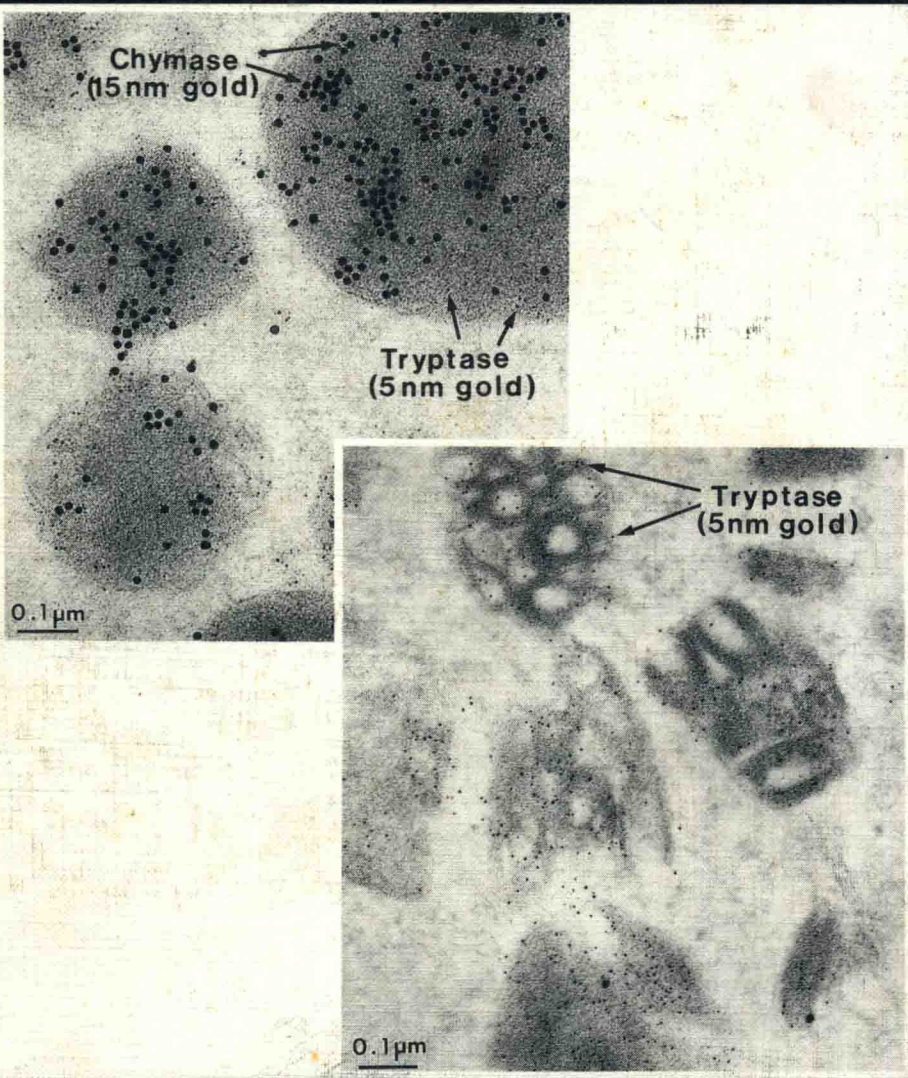

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Neutral Proteases of Mast Cells

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Preface

The major protein components of mast cells in mammals are proteolytic enzymes. These enzymes reside in secretory granules at acid pH, in forms that are catalytically active. With degranulation they invade the extracellular space, typically where the neutral pH environment is optimal for their catalytic activities. The multitude of mast cell proteases with chymotrypsin-like, trypsin-like and carboxypeptidase activities now described in mast cells was unforeseen in the early 1980s. Soon these will encompass at least 9 cDNA-derived protease sequences in murine mast cells, 4 in rat and 7 in human. Still, verification that each cDNA corresponds to a distinct gene is essential.

Surprisingly, mast cells exclusively express many of these proteases. These enzymes are genetically and antigenically distinct from most proteases found in granules of myelomonocytic and lymphocytic cells. Accordingly, the names tryptase for the tetrameric enzymes with trypsin-like activities and chymase for those with chymotrypsin-like activities are preferable. The species should precede the name and the subtype within the species, by letter or number, should follow the name, e.g., rat chymase I and rat chymase II rather than rat mast cell protease I and II.

To call trypsin-like and chymotrypsin-like protease activities in other cell types tryptase and chymase, respectively, is inappropriate without sufficient justification. This should include sequence, antigenic and functional analyses that show such enzymes to be clearly derived from the mast cell protease families. Thus, the designation in this monograph of the trypsin-like enzyme in Molt 4 human lymphocytes as a tryptase is putative pending further analysis. Another putative designation concerns the cathepsin G-like protease found in human skin mast cells. This enzyme shows

immunologic cross-reactivity and apparent functional similarities with neutrophil cathepsin G and therefore is not called chymase. Also, the C-terminal exopeptidases in human, rat and mouse mast cells are distinct but related to the pancreatic carboxypeptidases and are called mast cell carboxypeptidases. To categorize them further, one should use letters according to whether their catalytic specificity and amino acid sequence is more similar to either pancreatic carboxypeptidase A or B.

The functional roles of mast cell neutral proteases in vivo, at this time, are an engima. Yet, their special and purposeful association with mast cells suggests their ultimate importance in inflammation, the repair or damage of tissue and immune defense. A practical consequence of their selective and abundant presence in mast cell secretory granules is the utilisation of such enzymes in body fluids as indicators of mast cell activation. Also, these proteases serve as sensitive and specific markers that distinguish mast cells from other cell types and different types of mast cells from one another. Thus, these enzymes factilitate a more precise diagnosis and evaluation of mast cell-dependent conditions.

I thank the authors who provided the manuscripts for this monograph and appreciate their many excellent and ongoing scientific contributions to the understanding of mast cell proteases.

Lawrence B. Schwartz

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Granule Chymases and the Characterization of Mast Cell Phenotype and Function in Rat and Mouse

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The presence of chymotrypsin-like esterases in rat mast cell granules was first demonstrated histochemically [1, 2] and subsequent biochemical analysis revealed that the granule esterases were, in fact, chymotrypsin-like neutral proteases [3]. These pioneering studies were carried out in the rat because of the abundance of serosal mast cells (SMC) which could be isolated from the peritoneal cavity and purified in relatively large numbers. When the granules were isolated from SMC, a chymotrypsin-like enzyme with a molecular weight (MW) of 29,000 was purified to homogeneity [4] and subsequently shown to be identical to a group-specific protease with chymotrypsin-like properties derived from muscle [5]. This is now known as rat mast cell protease I [6] or, for the purposes of this review, rat chymase I (RChy I).

Although rat SMC have long been used as the prototypes for studying connective tissue mast cell (CTMC) function, pharmacology, and biology, a second distinctive mast cell subset was identified by its unique fixation and histochemical properties [7]. This atypical or mucosal mast cell (MMC) population was predominantly located in rat mucosal tissues and, particularly, in the gastro-intestinal lamina propria [7]. Biochemical and immunohistochemical analysis indicated that intestinal MMC were the source of the variant and highly soluble neutral protease, rat mast cell protease II [8] or rat chymase II (RChy II). This was originally purified from intestinal mucosa and identified as group-specific protease which inactivated the apoproteins of pyridoxal enzymes [5].

[1] We thank Drs. D.A. Lammas and D. Haig for generously supplying BMMC. Dr. J. Irvine was supported by a grant from Fison's PLC.

Mast cells in the mouse also contain chymotrypsin-like enzymes [9, 10] and, based on fixation and histochemical properties, are currently subdivided into CTMC and MMC subsets [11]. In both mouse and rat, mast cell hyperplasia occurs in the gastro-intestinal mucosa as a consequence of parasitic nematode infection [12] and, in both species, in vitro growth of bone marrow cells in the presence of interleukin-3 (IL 3) promotes the differentiation of cells with the morphological appearance of MMC [13, 14].

Our interest in mast cell granule chymases developed through a fruitful collaboration with Dr. Rick Woodbury in an attempt to characterize the mastocytosis associated with nematode infection in the rat [15]. We have, therefore, analysed the distribution of mast cell chymases in tissues and body fluids of the rat during intestinal nematodiasis and other immunological reactions in which mast cell activation was thought to occur. Using monoclonal and monospecific polyclonal antibodies against RChy I and II we have monitored mast cell activation both in vivo and in vitro. We have also purified and characterized a novel murine intestinal mast cell chymase now referred to as mouse chymase A (MChy A), and studied its distribution in normal and parasitized mice. The results shed new light on the in vivo biology of intestinal MMC in mouse and rat. They also raise questions about the validity of studies which apparently demonstrate transdifferentiation between MMC and CTMC in the mouse [16].

Rat Mast Cell Granule Chymases, Purification and Antigenic Properties

A variety of procedures for purification of RChy I have been described [6, 17]. We currently employ a modification of the method of Gibson and Miller [18] and RChy I is now purified from rat SMC by a combination of gel filtration and cation exchange chromatography on a Pharmacia FPLC Mono S column [19, and Newlands, unpublished]. The technique for the purification of RChy II from intestines of rats infected with *Nippostrongylus brasiliensis* is also based on cation exchange chromatography on Mono S [18, and Newlands, unpublished].

The physicochemical properties, amino-acid sequences and substrate and inhibitor profiles of RChy I and II are extensively described in the literature [6, 20] (table 1) and will not be discussed further. Instead, the antigenic relationships between RChy I and II and the development of specific immunoassays will be discussed in some detail.

Table 1. Properties and distribution of mast cell chymases in rat and mouse

	Rat		Mouse	
	RChy I	RChy II	MChy C	MChy A
MW (minigel)[a]	26,000	24,000	25,000	26,000–28,000
MW (large gel)[b]	29,188 ± 62	28,125 ± 72	28,625 ± 177[c]	30,063 ± 62
Solubility	low	high	low	high
Net charge	+18	+4	ND	+3
ELISA	Ab capture	Ab capture	–	Ab capture
limit of detection ng/ml	0.5	0.25		0.25
Distribution	connective tissues	gastro-intestinal tract	ND	gastro-intestinal tract
%[d]		94		99

ND = Not determined; Ab = antibody.

[a] Published MWs [11, 18, 30] derived from the Biorad Mini Protean (9×5 cm) SDS-PAGE system.

[b] MWs (n = 5) ± SE derived from larger format (16×14 cm) LKB vertical 12% slab gel SDS-PAGE system.

[c] MW derived from Western blot with an anti-RChy I antibody probe using the 16×14 cm format.

[d] Percent of total chymase measured in different tissues (fig. 2, table 2).

Woodbury et al. [8] immunized rabbits to produce antisera against RChy I and II which were apparently specific by gel diffusion. However, these antisera were not specific by Western blotting [18, 21] nor when used for tissue localization of RChy I and II by immunofluorescence [8]. Our approach was, therefore, to prepare monospecific rabbit $F(ab)_2$ antibodies to RChy I and II [18] and to raise murine monoclonal antibodies against RChy II. Rabbit $F(ab)_2$ anti-RChy I, prepared by cross-absorption and affinity purification on solid-phase RChy II and I, is highly specific by Western blotting (fig. 1), gel diffusion, and immunohistochemistry [18]. Rab $F(ab)_2$ anti-RChy II is equally specific by Western blot [18] although minimal cross-reactivity with RChy I was detected on dot blots [22]. A panel of three mouse monoclonal antibodies against RChy II have been raised in our laboratory [23], two of which, by Western and dot blotting, react uniquely with RChy II. Immunoprecipitation of RChy II and I, bound to the inhibitor [^{3}H]-di-isopropylfluorophosphate ([^{3}H]-DFP), with, respectively monoclonal anti-RChy II and Rab anti-RChy I has confirmed the specificity of each antibody [23].

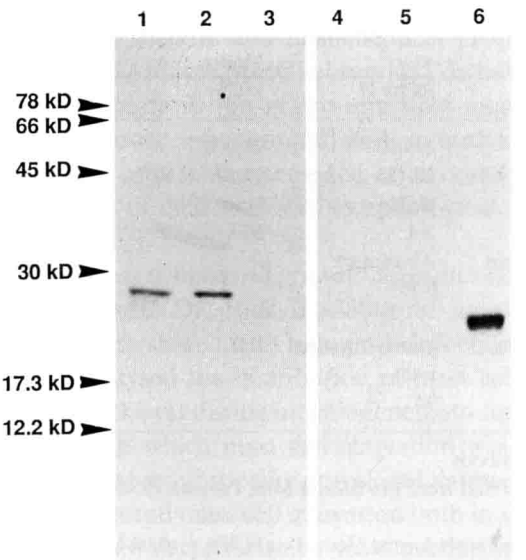

Fig. 1. Characterization of granule chymases in rat SMC by SDS-PAGE and Western blotting. Lanes 1–3 are probed with anti-RChy I and lanes 4–6 with anti-RChy II; lanes 1 and 4 purified RChy I (1 μg); lanes 2 and 5: SMC (95% pure; 5×10^4 cells extracted in SDS-2-mercaptoethanol) and, in lanes 3 and 6, 1 μg RChy II. All samples were run under reducing conditions using the Biorad Mini Protean electrophoresis apparatus (see text). Note that the purified SMC contain RChy I but lack RChy II. The polyclonal rabbit $F(ab)_2$ antibodies were affinity-purified and cross-absorbed as described in the text and show a high degree of specificity on Western blot. RChy I and II have apparent MWs of less than 27,000 on the minigel system (see text and table 1).

Rabbit [15, 24] or sheep antisera [25] have been used to quantitate RChy II in tissues by radial immunodiffusion, but this technique was not sufficiently sensitive to detect RChy II in blood of normal or parasitized rats [22]. Initially a competitive enzyme-linked immunosorbent assay (ELISA) was developed in which titration against RChy II standards (0–750 ng/ml) established a lower limit of detection of 150 ng RChy II/ml [26]. Because this assay was insufficiently sensitive to determine very low concentrations of RChy II and required relatively large quantities of pure RChy II with which to coat the plates, a modified antibody capture ELISA was developed. Initially, affinity-purified polyclonal antibodies were used [27], but plates are now coated with affinity-purified mouse monoclonal anti-RChy II (1 μg/ml coating buffer) and bound antigen is detected with

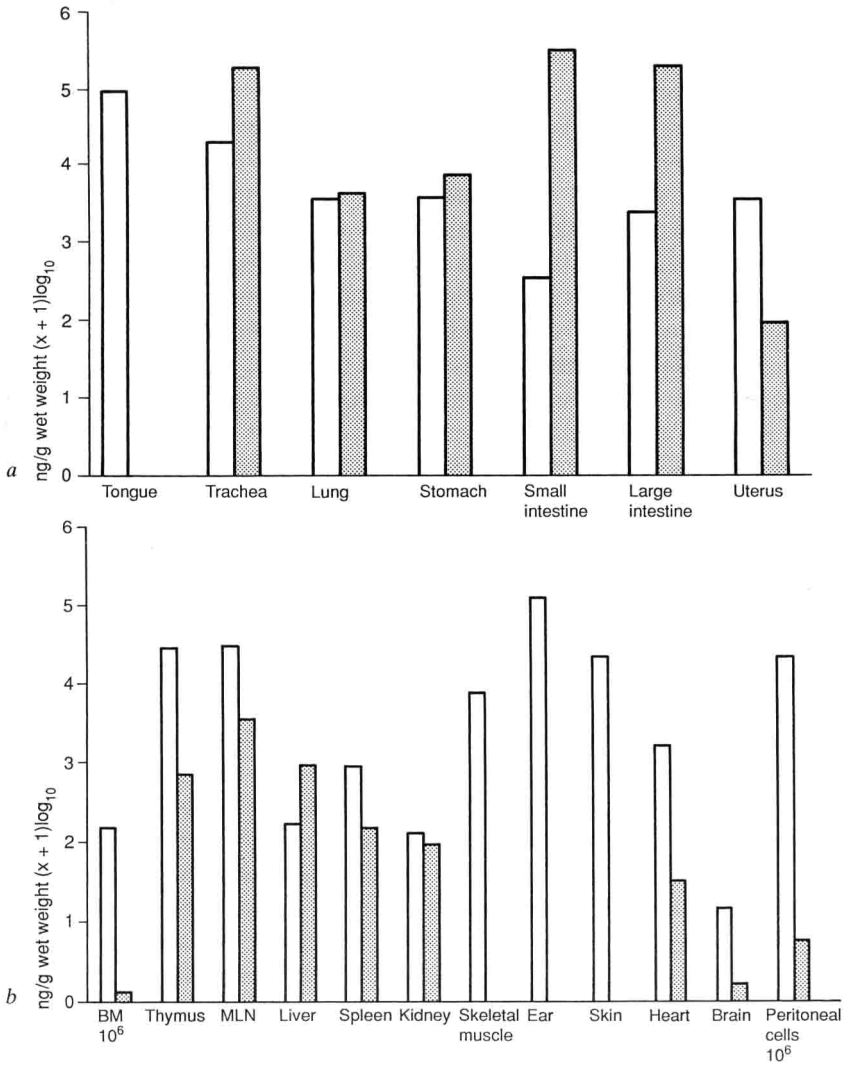

Fig. 2. The distribution of RChy I (open histograms) and RChy II (hatched histograms) in mucosal *(a)* and non-mucosal *(b)* tissues of the rats including bone marrow (BM) and peritoneal cells. Homogenates prepared from tissues or cells were assayed by ELISA for RChy I or II, and the results expressed as (x +1) $\log_{10}$ nanogrammes of RChy I or II per gramme wet weight of tissue, or per 10^6 BM or peritoneal cells. The small and large intestine are the principal sources or RChy II, although low concentrations of RChy II are present in non-mucosal sites including thymus, mesenteric lymph node (MLN), liver, spleen and kidney. Note the absence of RChy II in skeletal muscle, tongue, ear pinna and skin, which contain high concentrations of RChy I.

affinity-purified sheep IgG anti-RChy II labelled with peroxidase [23]. Both assays have similar sensitivities with linear colour development between 0.25 and 12 ng RChy II/ml [23]. Repeated tests have confirmed that this ELISA can detect low nanogram quantities of RChy II even in the presence of 30–40 μg RChy I/ml (fig. 2) [23].

Immunoassays for RChy I have also been developed. Sheep anti-RChy I serum is used for radial immunodiffusion with the agarose made up in 1.0 *M* NaCl and samples and standards are also prepared in 1 *M* NaCl to allow for the insolubility of RChy I [19]. For ELISA, with a sensitivity of 500 pg RChy I/ml, the plates are coated with affinity-purified sheep IgG anti-RChy I (1 μg/ml) and detection is with rabbit $F(ab)_2$ anti-RChy I followed by a sheep immunoglobulin anti-rabbit immunoglobulin-peroxidase conjugate. Samples and standards are prepared in 1 *M* NaCl and 4% bovine serum albumin is added to the diluent to minimize non-specific binding of RChy I to the microtitre plates. Like the ELISA for RChy II the RChy I ELISA is highly specific, measuring low nanogram concentrations of RChy I in the presence of 30–100 μg RChy II/ml (fig. 2)[23].

Mapping of Mast Cell Chymases in the Rat

Woodbury and Neurath [24], measured the concentrations of RChy II in the intestines of rats during early neonatal life up to 4 months of age and showed an age-related increase, reaching a plateau at about 6 weeks. We subsequently found that the mucosal mastocytosis in rats harbouring the intestinal nematode *N. brasiliensis* was associated with an increase in the concentration of RChy II from 0.3 mg in normal rats to 3.5 mg/g wet weight of intestinal tissue in immune animals [15]. These two experiments also demonstrated a positive correlation between MMC density and concentration of RChy II in the mucosa [15, 24].

The distribution of RChy I and II in the gastro-intestinal tract was compared using radial immunodiffusion. RChy I was abundant in tongue, oesophagus, stomach and rectum but was not detected in the small intestine [19]. Because the lower limit of detection by radial immunodiffusion was 3–5 μg of RChy I or II/ml, we re-examined the distribution of RChy I and II throughout the body in normal adult rats by ELISA. The results show that RChy II is undetectable in tongue, ear pinna, skeletal muscle and skin even though several of these organs contain 30–40 μg RChy I/g wet weight of tissue (fig. 2). However traces of RChy II (11–70 ng/g tissue) were present

in heart, uterus, kidney and spleen. RChy I was found in varying concentrations in all tissues examined, although, relative to RChy II, concentrations of RChy I were very low in the small intestine (fig. 2) and this is in agreement with the results from our previous study [19].

There is, therefore, a hierarchy of expression of RChy I and II in different tissues. RChy I is abundant in muscle and connective tissues, including skin; sites where RChy II is undetectable. By contrast, RChy II is abundant in mucosal tissues and, interestingly, is relatively abundant in lung, larynx/trachea, mesenteric lymph node and thymus (fig. 2).

Mouse Mast Cell Chymases, Purification and Antigenic Properties

By comparison with RChy I and II, chymases in murine mast cells have received less attention, probably because of the comparatively low protease content and paucity of murine peritoneal mast cells. Vensel et al. [9] described the purification of a 25,000 MW chymase from a murine mastocytoma cell line. The complexity of murine mast cell chymases was, however, highlighted by the analysis of cultured murine bone marrow-derived mast cells (BMMC) in which four neutral proteases of 27,000, 29,000, 30,000 and 31,000 MW, which bound [^{3}H]-DFP, were identified by sodium dodecyl sulphate-polyacrylamide electrophoresis and subsequent autoradiography [28].

Because of our interest in the role of MMC during parasitic infection we attempted to purify mast cell chymases from the intestines of mice infected with the nematode *Trichinella spiralis* [29]. Advantage was taken of the observation that mouse mast cells were detected in tissue sections with anti-RChy II [29] and that this same antibody identified a series of bands of 25,000–28,000 MW on Western blot [29]. Purification of a soluble chymase was achieved by homogenizing immune intestines (day 10 of infection with *T. spiralis*) in 20 m*M* Tris buffer, pH 7.5, followed by ion exchange, hydroxyl apatite and affinity chromatography, with a final purification step on a Pharmacia Mono S high-resolution cation exchange column [29]. These techniques have been simplified and modified so that MChy A, is purified more rapidly and using fewer steps by ion exchange chromatography [11]. Although MChy A was originally reported to have a MW of 26,000 [29], recent measurements using the larger format SDS-PAGE rather than the minigel system used previously [29] indicate an average MW of 30,063 ± 62.5 (table 1).

Biochemical analysis confirmed the chymotrypsin-like properties of MChy A, which was inhibited by DFP and Tos-Phe-CMK and which catabolized the substrates CBZ-L-Try-NPE and CBZ-L-Tyr-NPE with a pH optimum of 7.4 [29]. Determination of the amino-acid composition and sequence of MChy A has confirmed its relationship to RChy I and II with which it shares 74.3 and 74.1% sequence homology, respectively [30]. However, MChy A has a low net charge of +3 when compared with a net charge of +18 for RChy I at physiological pH [30] and this may explain its high solubility, which is comparable to that of RChy II [22].

The structural homology between MChy A and RChy II is reflected by the antigenic similarities of the two chymases demonstrated by Western blot [29]. Antibodies against MChy A were raised by immunizing both rabbits and rats [11]. However, it was not possible to render these antibodies monospecific and they cross-reacted with a single polypeptide in mouse SMC [11]. The latter was shown to be RChy I-like, when, after cross-absorption on MChy A-Sepharose, rabbit $F(ab)_2$ anti-RChy I proved to be monospecific for this antigen in mouse SMC [11]. This reagent detected a 28,625 MW antigen from 90–95% pure SMC by Western blotting (table 1), which was assumed to be a murine SMC chymase, putatively referred to as mouse chymase C (MChy C). Definitive identification of this antigen as MChy C and its relationship to RChy I awaits analysis of the purified protein. The antibody did not react with MChy A by Western blot, dot blot or double diffusion [11].

Despite their continued cross-reaction with MChy C on Western blot, polyclonal antibodies against MChy A did not react with MChy C when tested against extracts of 90–95% pure SMC by double diffusion (fig. 3). They did, however, form a single line of precipitation against a crude extract of intestine from mice infected 10 days previously with *T. spiralis* (fig. 3) or against purified MChy A. Similarly, sheep and rabbit anti-RChy I formed a precipitin arc against extracts of mouse SMC (fig. 3) but not against intestinal homogenates from *Trichinella*-infected mice (fig. 3) or pure MChy A. Complete specificity was also demonstrable when rabbit anti-MChy A was used to quantify MChy A by ELISA [31]. The technique is similar to that described for RChy II and the sensitivity of detection is 500 pg MChy A/ml with linear development of colour between 0.5 and 12 ng MChy A/ml. This ELISA does not detect MChy C even when 85–90% pure mouse SMC (10^6/ml) are extracted in 1*M* NaCl [31].

The proteases of higher MW identified both by their antigenic similarity to MChy A (fig. 4) and by their capacity to bind [^{3}H]-DFP (fig. 5)

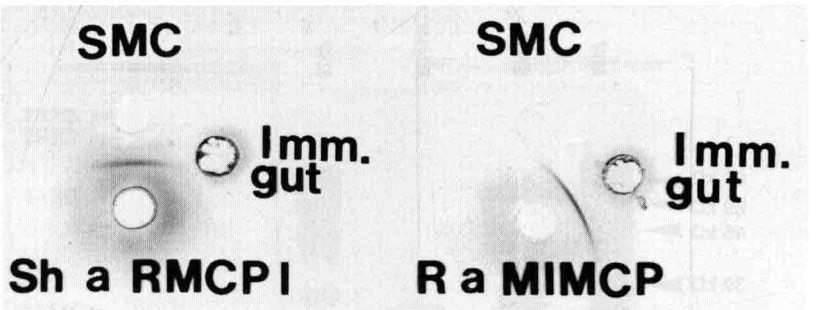

Fig. 3. Comparison of the antigenicity of chymases from mouse SMC and from small intestines (Imm. gut) of mice infected 10 days previously with the nematode *T. spiralis*. Precipitin arcs have developed between extracts of SMC and sheep anti-RMCPI (RChy I) and between homogenates of immune gut and rabbit anti-MIMCP (MChy A). Intestinal homogenates apparently lack RChy I-like antigens and SMC lack MChy A.

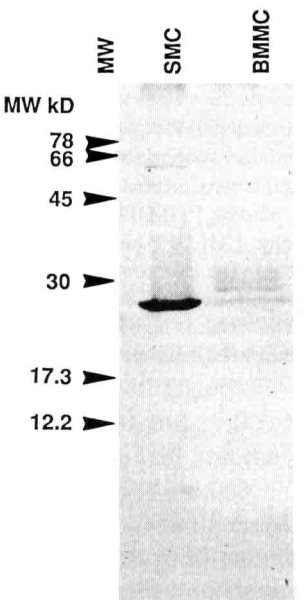

Fig. 4. Demonstration of four distinct antigens in > 95% murine BMMC (lane 2) by Western blotting revealed with rabbit anti-MChy A. Mouse SMC (4×10^5 cells; 90–95% pure) are blotted in lane 1. The major antigen in SMC and the lowest MW antigen in BMMC are apparently of identical MW. Conditions for electrophoresis and blotting are as described in figure 1.