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Characterization and mast cell origin of a chymotrypsin-like proteinase isolated from intestines of mice infected with *Trichinella spiralis*

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SUMMARY

A proteinase was purified by cation exchange and affinity chromatography from the small intestines of mice infected with *Trichinella spiralis*. The enzyme was highly soluble and was chymotrypsin-like in its substrate specificities and susceptibility to inhibitors. It had a MW of 26,000, as determined by SDS–PAGE electrophoresis. Antibodies raised against the proteinase were affinity purified and their specificity confirmed by Western blot analysis. When used to localize the enzyme immunohistochemically, they reacted with granules of mast cells in the epithelium and lamina propria of the parasitized small intestine. The antibodies also bound to mast cell granules in a number of other sites, including tracheal epithelium, gastric mucosa, skin and tongue. Affinity-purified antibodies raised against rat mast cell proteinase II (RMCPII) cross-reacted with the mouse mast cell proteinase on Western blots.

INTRODUCTION

Mast cell heterogeneity in rat and man is associated with the presence, in the granules, of variant neutral proteinases (Woodbury, Gruzenski & Lagunoff, 1978; Gibson & Miller, 1986; Irani *et al.*, 1986). Immunoperoxidase studies with antibodies raised against human mast cell tryptase and chymotrypsin have revealed that 80–90% of the mast cells in skin and intestinal submucosa contain both tryptase and chymotrypsin, whereas the majority of mast cells in intestinal mucosa and lung lack chymotrypsin (Irani *et al.*, 1986). Similarly, in the rat, mast cell proteinase I (RMCPI) is found in connective tissue mast cells (CTMC) of rat lung, skin and muscle, whereas RMCPII is located in the mucosal mast cells (MMC) of gut and airways, and in bone marrow-derived mast cells grown in culture (Gibson & Miller, 1986; Haig *et al.*, 1982).

Antibodies against RMCPII have also been employed in immunoassays to quantify the concentrations of this enzyme in tissue (Woodbury & Neurath, 1978) and in body fluids (Miller *et al.*, 1983), thus providing a unique and highly selective marker for mast cell function *in vivo*. Similar results with a mucosal mast cell proteinase and a mast cell tryptase have also now been obtained in sheep and man, respectively (Huntley *et al.*, 1987; Wenzel *et al.*, 1986) and it would appear, therefore, that release of mast cell granule proteinases can be usefully monitored in several species.

Although, in mice, a thymus-dependent intra-epithelial mast cell population is generated in the gut during parasitic infection (Ruitenberg & Elgersma, 1976; Olsen & Levy, 1976), the relationship of these cells to mast cells elsewhere in the body is not known. Histochemical studies have shown that murine intestinal mast cells have fixation and glycosaminoglycan-staining properties that are similar to those of rat MMC (Crowle & Phillips, 1983) and, like rat MMC, the murine intestinal mast cells also contain serine esterases (Huntley *et al.*, 1985).

Biochemical studies revealed that a chymotrypsin-like enzyme (MW 25,000) was present in a murine mastocytoma cell line (Vensel, Komender & Bernard, 1971) and four neutral proteinases have been identified in cultured bone marrow-derived mast cells (BMMC) (Du Buske *et al.*, 1984). Since the latter have many of the histochemical features of MMC (Sredni *et al.*, 1983) and because, histochemically, murine MMC contain serine esterases (Huntley *et al.*, 1985) it seems likely that intestinal MMC may also be a good source of mast cell-derived proteinases.

Further phenotypic and functional characterization of murine mast cell subsets will probably be achieved best by analysis of

Abbreviations: DIPF, diisopropyl phosphofluoridate; EDTA, ethylenediamine tetracetic acid; 4HMP, 4-hydroxymercuribenzoate; NEM, *N*-ethylmaleimide; 1,10 Phe, 1,10 phenanthroline; PMS-F, phenyl methane sulphonyl fluoride; RMPCI, rat mast cell proteinase I; RMCPII, rat mast cell proteinase II; SBTI, soya bean trypsin inhibitor; Tos-Phe-CMK, 1-chloro-4-phenyl-3-tosylamido-L-butan-2-one; Tos-Lys-CMK, 7-amino-1-chloro-3-tosylamido-L-heptan-2-one.

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their granule proteinases. To this end, we have used the intestines of *T. spiralis*-infected mice, which are rich in MMC (Alizadeh & Wakelin, 1982), to isolate a putative mast cell proteinase. Immunohistochemistry, using affinity-purified antibodies, reveals that this chymotrypsin-like proteinase is of MMC origin and that it, or an antigenically very similar enzyme, is also present in CTMC.

MATERIALS AND METHODS

Animals

Male and female NIH mice reared at the University of Nottingham were used to provide parasitized tissue for enzyme preparation and histology. Swiss White mice raised at the Moredun Institute were the source of normal tissues for histology. All mice were fed and watered *ad libitum*. Antisera were raised in New Zealand white rabbits and Wistar rats.

Infection with Trichinella spiralis

Mice were each infected orally with 300 *T. spiralis* muscle larvae and killed 10 days later, at the peak of the mast cell response, as described elsewhere (Alizadeh & Wakelin, 1982), to provide source material for enzyme isolation and immune gut for histology.

Enzyme isolation

Small intestine from infected mice was excised, flushed with Hanks' balanced salt solution and stored at −20° until further processing. The tissue was homogenized in three volumes (3 ml per gram of tissue) of 20 mM Tris-HCl, pH 7·5, and centrifuged at 15,000 *g* for 1 hr. The supernatant was applied to CM-Fractogel (Merck, Darmstadt, FRG), a cation exchange resin, and equilibrated with 20 mM Tris-HCl, pH 7·5. Fractions were eluted with a linear 0–0·5 M NaCl gradient and those with enzyme activity were pooled and dialysed overnight against 20 mM Tris-HCl, pH 7·5. The dialysate was applied to a D-tryptophan methyl ester-agarose affinity column (Miles-Yeda, Israel). Fractions containing enzyme activity, eluted with 1·0 M NaCl, were pooled and subsequently diluted 10-fold with 20 mM phosphate buffer, pH 6·0. This material was then applied to a mono S cation exchange column (Pharmacia, Uppsala, Sweden) and the enzyme was eluted with a linear gradient of 0·075–0·150 mM NaCl in 20 mM phosphate buffer, pH 6·0.

Column chromatography

All ion exchange and affinity chromatography was carried out with the aid of fast protein liquid chromatography (FPLC) equipment (Pharmacia).

Enzyme assay

Enzyme activity was determined by the methods described by Knox, Gibson & Huntley (1986) using an I.L. Multistat III microcentrifugal analyser, except that the final reaction mixture consisted of buffer (150 μl), enzyme solution (5 μl), DMSO (50 μl) and substrate (5 μl). Stock enzyme solutions were diluted to give final concentrations in the reaction mixture of 1 μg bovine chymotrypsin A (Boehringer, Indianapolis, IN) or 1·15μg mouse intestinal proteinase. The following substrates were used, CBZ-L-Ala-NPE, CBZ-B-Ala-NPE, CBZ-L-Try-NPE, CBZ-L-Tyr-NPE, CBZ-L-Phe-NPE, B2-L-Tyr-4-Na, CBZ-L-Arg-NPE and CBZ-L-Lys-NPE (all from Sigma Chemical Co., Poole, Dorset).

Inhibitor studies

Enzyme activity in the presence of specific proteinase inhibitors was studied as described previously (Knox *et al.*, 1986). Residual activity against the substrate CBZ-L-Tyr-NPE was expressed as a percentage of the activity in the absence of inhibitors.

pH optimum

Enzyme activity using CBZ-L-Tyr-NPE as substrate was monitored over the range pH 5·0–10·2 using the assay technique described above.

Raising and affinity purification of specific antibodies

Rabbits and rats were immunized with proteinase (200 μg and 20 μg of protein, respectively), in Freund's complete adjuvant. Inoculations were intramuscular in rabbits and subcutaneous in the footpad of rats. Two subsequent injections of proteinase in Freund's incomplete adjuvant were given 4 and 7 weeks after the first. Rabbits and rats were bled at intervals after the final injection. Sera were tested by double diffusion and once they had reached a titre of > 1/32, 25 ml of blood were taken twice a month for 2 months from each rabbit, and rats were bled out under deep ether anaesthesia.

In order to affinity purify specific antibody, purified enzyme was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia) in accordance with the manufacturer's instructions.

Antisera were applied to proteinase–Sepharose 4B and specific antibody was eluted with 0·1 M acetic acid–0·5 M NaCl and neutralized with 1·0 M Tris.

SDS–PAGE

Discontinuous SDS–PAGE was carried out as described by Laemmli (1970) on 12% acrylamide slab gels, and electrophoresed at 20 mA per gel for 3 hr at 5°. A range of molecular weight standards was included in each run: cytochrome c (12,300), myoglobin (17,200), chymotrypsinogen A (25,700), ovalbumin (45,000), albumin (66,250) and ovotransferrin (76–78,000). Protein bands were visualized by the silver stain method of Morrissey (1981).

Immunoblotting

Proteins in SDS–PAGE gels were blotted onto nitrocellulose membranes using a semi-dry transfer apparatus (Kyhse-Andersen, 1984). The nitrocellulose was then washed 6 × 2 min in wash buffer and blanked in 50% horse serum for 1 hr at 37° to prevent non-specific adsorption of antibody. The blot was then probed with affinity-purified rabbit IgG anti-mouse proteinase or sheep $F(ab)_2$ anti-RMCPII (Gibson & Miller, 1986) at a concentration of 50 μg/ml, for 1 hr at room temperature. The nitrocellulose was again washed for 6 × 2 min in wash buffer before incubation with a sheep $F(ab)_2$ anti-rabbit F(ab)–horseradish peroxidase (HRPO) conjugate or rabbit $F(ab)_2$ anti-sheep F(ab)–HRPO at concentrations of 25 μg/ml.

All antibody and conjugate dilutions were made with 5% v/v horse serum in Western blot wash buffer which consisted of PBS (21), Tween 80 (10 ml), EDTA (0·75 g) and NaCl (40·8 g).

Histology and immunocytochemistry

Small intestine was excised, flushed through with fixative, and fixed by immersion in 4% paraformaldehyde in PBS for 4–6 hr

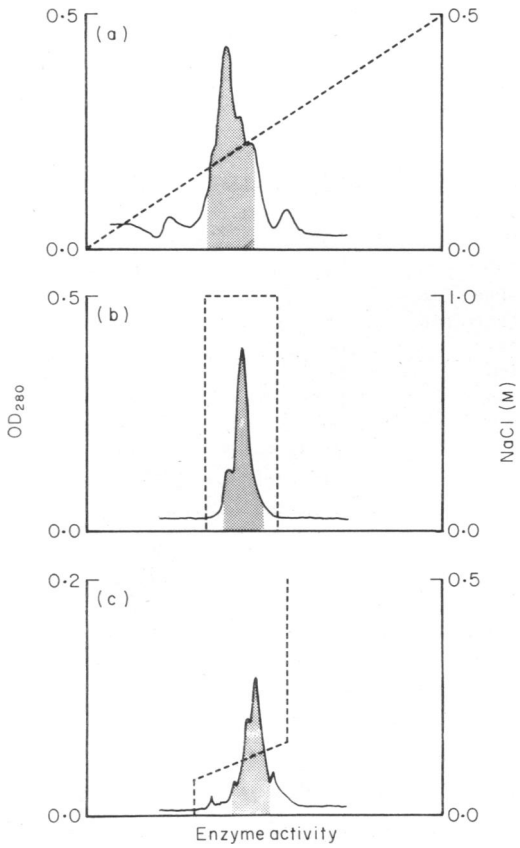

Figure 1. Purification of a serine proteinase from *T. spiralis*-immunized mouse intestine. (a) Elution of enzyme activity from CM-fractogel with a 0–0·5 M NaCl gradient (equilibration with 20 mM Tris-HCl, pH 7·5) after application of intestinal homogenate. (b) Fractions rich in proteinase activity from CM-fractogel and applied to D-tryptophan methyl ester-agarose in 20 mM Tris-HCl, pH 7·5, were eluted with 1·0 M NaCl. (c) Final purification of proteinase on a mono S cation exchange column equilibrated with 20 mM phosphate, pH 6·0. (– – –), NaCl gradient.

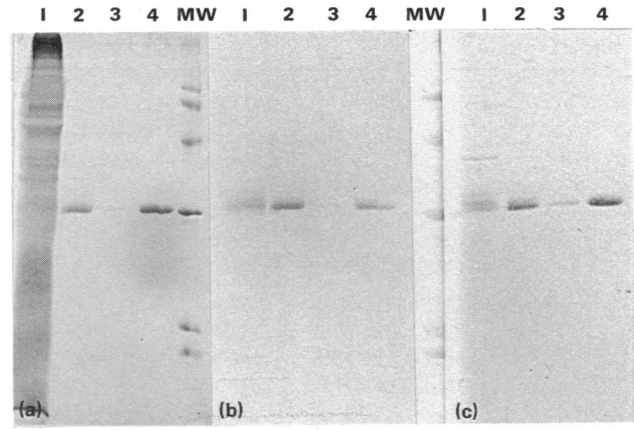

Figure 2. (a) SDS–PAGE showing the purification stages of proteinase from *T. spiralis*-infected mouse intestine. Individual lanes contain: (1), 25 μg of intestinal homogenate; (2), 10 μg of proteinase-rich fraction from CM fractogel; (3), 2 μg of active fraction from methyl-ester agarose; and (4), 5 μg of enzyme after final purification step on mono S 12% gel stained with silver nitrate. Molecular weight standards (cytochrome c, 12,300; myoglobin, 17,200; chymotrypsinogen A, 25,700; ovalbumin, 45,000; albumin, 66,250; transferrin, 76–78,000) are shown in lane marked MW. (b) Electroblot in which the fractions electrophoresed in (a) have been transferred to nitrocellulose and probed with affinity-purified rabbit anti-mouse intestinal proteinase. Lanes 1–4 as for (a). (c) Electroblot as in (b) but probed with affinity-purified sheep $F(ab)_2$ anti-rat RMCPII. Also shown are MW standards which have been electroblotted and stained with Coomassie Blue.

(Newlands, Huntley & Miller, 1984). The tissues were transferred to 70% ethanol overnight prior to processing to paraffin wax. Sections 5 μm thick were taken to water and treated with a periodic acid/sodium borohydride sequence to block endogenous peroxidase activity (Heyderman & Neville, 1977). The sections were then incubated in 10% bovine serum albumin in 100 mM Tris-HCl buffer, pH 7·5, for 1 hr prior to incubation with affinity-purified rabbit IgG or, for control purposes, normal rabbit IgG (optimally diluted with 10% BSA in Tris-HCl, pH 7·5) for a further hour. Sections were washed 3 × 5 min in 100 mM Tris-HCl, pH 7·5, before incubation with sheep $F(ab)_2$ anti-rabbit F(ab)–HRPO conjugate diluted to 25 μg/ml with 10% BSA in Tris-HCl, pH 7·5. In some experiments, immunostaining was with rat antiserum to mouse proteinase diluted 1/200 in 10% BSA in Tris-HCl, pH 7·5. For control purposes, normal rat serum at the same dilution was used. Staining was for 1 hr and, after washing, the slides were incubated with a sheep IgG anti-rat light chain–peroxidase conjugate (Sera Lab., Crawly Down, W. Sussex). Sections were washed again 3 × 5 min in Tris-HCl, pH 7·5, and peroxidase activity was revealed with 3-3 diaminobenzidine (Graham & Karnovsky, 1966).

Table 1. Enzyme activity recovered during stages of purification of mouse intestinal proteinase

Stage	Total protein (mg)	Total activity (n Kats)	Specific activity (n Kats/mg)	Yield (%)
Homogenate	125	9452	75·6	100
Fractogel	53·5	6019	112·6	64
Affinity	3·8	1413	371·8	15
Mono S	0·26	617	2378·0	7

n Kat(nanoKatal) = the activity hydrolysing 1 nmol substrate per second.

Adjacent sections were stained with 0·5% toluidine blue in 0·5 N HCl (Enerback, 1966) or with naphthol As-D chloroacetate as described elsewhere (Huntley *et al.*, 1985) to demonstrate mast cells.

RESULTS

Purification of mouse intestinal proteinase

The elution profile obtained from the CM-fractogel cation exchanger and the location of activity against the substrate CBZ-L-Tyr-NPE show that the enzyme activity was in a broad series of peaks eluting between 0·23 M and 0·38 M NaCl (Fig. 1a).

Table 2. Substrate specificity of mouse intestinal proteinase compared with that of chymotrypsin

Specificity	Substrate	Mouse proteinase (n Kat/mg) pH 7·4	Chymotrypsin (n Kat/mg) pH 7·8
Elastase	CBZ-L-Ala-NPE	85·1	412
	CBZ-B-Ala-NPE	6·0	NA
Chymotrypsin	CBZ-L-Try-NPE	491	2436
	CBZ-L-Tyr-NPE	2282	3620
	CBZ-L-Phe-NPE	40·6	59
	BZ-L-Tyr-4-NA	16·1	52
Trypsin	CBZ-L-Arg-NPE	NA	NA
	CBZ-L-Lys-NPE	NA	NA

NA, no activity.

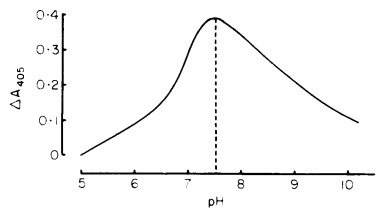

Figure 3. Graph illustrating the pH optimum for activity of mouse intestinal proteinase against the substrate CBZ-L-Tyr-NPE.

Following dialysis to remove salt, and application to the D-tryptophan-methyl ester-agarose affinity column, the major activity was associated with the peak eluted with 1·0 M NaCl (Fig. 1b). The active material was diluted 1/10 with 20 mM phosphate buffer, pH 6·0, prior to final separation on a mono S cation exchange column in which the enzyme activity was associated with three peaks. The third peak was the largest and eluted between 0·075 M and 0·150 M NaCl in 20 mM phosphate buffer, pH 6·0 (Fig. 1c). On SDS–PAGE this peak resolved as a single band with a MW of 26,000 (Fig. 2a). The stages of the purification procedure and the recovery of enzyme are recorded in Table 1.

Specificity of intestinal protease against low molecular weight substrates

The activity of the isolated intestinal proteinase against a variety of low molecular weight synthetic substrates is shown in Table 2. CBZ-L-Tyr-NPE and CBZ-L-Try-NPE were the substrates hydrolysed most rapidly by the proteinase with specificities similar to those of chymotrypsin but at slower rates. No activity was detected against the trypsin substrates CBZ-L-Arg-NPE or CBZ-L-Lys-NPE, but there was some slow hydrolysis of the synthetic elastase substrates CBZ-L-Ala-NPE and CBZ-B-Ala-NPE.

Table 3. Effect of proteinase inhibitors on activity of mouse intestinal proteinase

Inhibitor	Concentration (mM)	% activity: Mouse proteinase	% activity: Chymotrypsin
DIPF	1·0	0	0
PMS-F	1·0	0	0
Tos-Phe-CMK	1·0	1·5	5
Tos-Lys-CMK	1·0	100	100
NEM	1·0	100	100
4HMB	1·0	92	90
1,10 Phe	1·0	90	98
EDTA	2·0	100	100
SBTI	0·1 mg/ml	85	43
Ovomucoid	0·1 mg/ml	100	40

Effect of pH on intestinal proteinase activity

Using CBZ-L-Tyr-NPE as a substrate, the enzyme, when monitored over the range pH 5·0–10·2, showed maximal activity at pH 7·4 (Fig. 3) and none at pH 5·0. Only 25% of maximum activity was detected at pH 10·2.

Inhibitors of intestinal proteinase

The results of the inhibition studies are summarized in Table 3. The activity of mouse proteinase, like that of chymotrypsin, was totally inhibited by DIPF and PMS-F. Again, like chymotrypsin, it was markedly inhibited by Tos-Phe-CMK. No substantial inhibition of either enzyme was noted after incubation with Tos-Lys-CMK, NEM, 4HMB, or the chelating agents 1,10 phenanthroline or EDTA. The naturally occurring chymotrypsin inhibitor SBTI had only a slight inhibitory effect on mouse proteinase and chick ovomucoid caused no detectable reduction in activity.

Immunoblotting of intestinal proteinase

When the intestinal proteinase fractions were transferred from SDS–PAGE gel to nitrocellulose and probed with affinity-purified rabbit anti-mouse proteinase antibodies, single bands were detected in each of the lanes containing intestinal homogenate or active fractions from the CM-fractogel, D-Try-ME and mono S columns in lanes 1, 2, 3 and 4, respectively (Fig. 2b).

When a parallel blot was probed with sheep $F(ab)_2$ anti-RMCPII, four bands were detected in the crude gut homogenate, two in the CM-fractogel proteinase fractions and single bands in both the D-Try-ME and Mono S proteinase fractions (Fig. 2c).

Immunohistochemical localization of intestinal proteinase in mast cell granules

Immunoperoxidase staining of paraformaldehyde-fixed tissues with rat or rabbit antibodies raised against mouse proteinase demonstrated the presence of immunoreactive antigen uniquely in the granules of mast cells in both mucosal and connective tissue sites. No staining was detected when normal rabbit IgG or

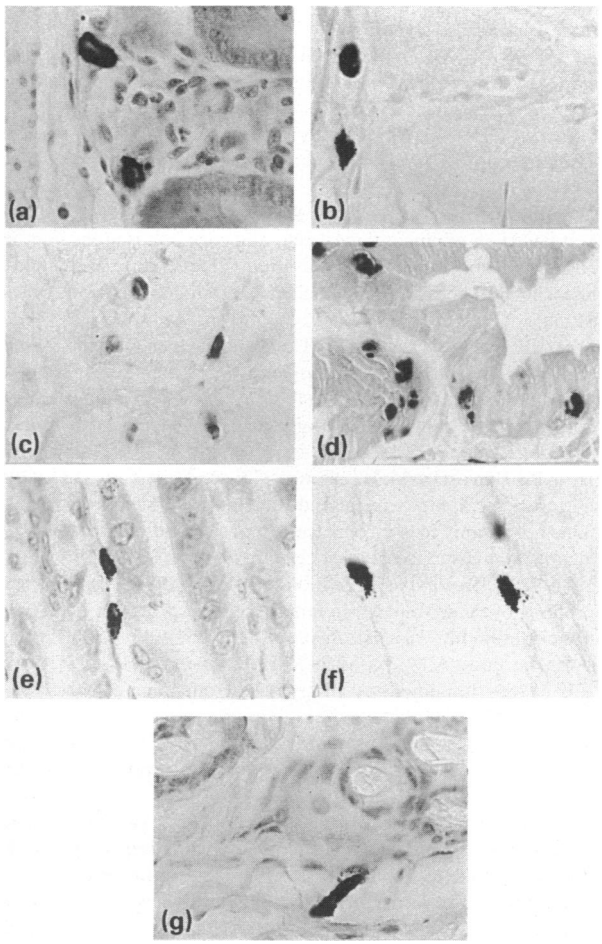

Figure 4. Photomicrographs of murine tissues fixed in 4% paraformaldehyde and stained immunohistochemically to demonstrate the localization of intestinal proteinase or histochemically to detect mast cells. (a) Immunoperoxidase reaction localizing proteinase in two mast cells in the basal mucosa of normal mouse intestine. (b) Toluidine blue, pH 0·5, of a section adjacent to (a). (c) Intra-epithelial mast cells in *T. spiralis*-immune gut stained with rat anti-mouse proteinase antiserum (1/200) and peroxidase-labelled anti-rat immunoglobulin. (d) Section adjacent to (c) stained with Naphthol As-D chloroacetate. Note the intense reaction of intraepithelial mast cell granules. (e) Gastric mast cells from normal mouse stained as in (a). (f) Adjacent section to (e) stained with toluidine blue, pH 0·5. (g) Section of skin from a normal mouse stained as in (a). Magnification × 360.

normal rat serum were substituted for proteinase-specific antibodies. The few immunoreactive mast cells detected in the mucosa of normal small intestine were confined to a region adjacent to the muscularis mucosae (Fig. 4a, b). In gut from *T. spiralis*-immune mice, >95% of the peroxidase-labelled cells were intra-epithelial (Fig. 4c). Cells of similar morphology and tissue location stained very intensely with Naphthol As-D chloroacetate (Fig. 4d) as has been reported previously for intra-epithelial MMC in parasitized mice (Huntley *et al.*, 1985). Immunoperoxidase-positive and comparable toluidine blue-staining mast cells were detected in the mucosa of stomach (Fig. 4e and f) and trachea and skin (Fig. 4g).

DISCUSSION

A serine proteinase from intestines of *T. spiralis*-infected mice has been isolated and purified to homogeneity. The isolated enzyme was similar in its substrate specificity, chromatographic properties, molecular weight and solubility to the rat MMC-derived proteinase RMCPII. Furthermore, it was strongly cross-reactive with polyclonal, affinity-purified, sheep anti-RMCPII antibody on Western blot analysis. When affinity-purified rabbit anti-mouse proteinase was used for immunoperoxidase studies it reacted with the granules of both intra-epithelial and connective tissue mast cells; so too did sera from rats immunized with proteinase. These results suggest that murine intra-epithelial mast cells are the source of a soluble RMCPII-like enzyme and that it, or an antigenically very similar enzyme, is present in connective tissue mast cells.

Mouse mast cells derived in culture from bone marrow stem cells contain four neutral proteases ranging in molecular weights from 27,000 to 31,000 (Du Buske *et al.*, 1984). By contrast, rat mast cells contain only two major chymotrypsin-like enzymes in their granules, RMCPI and RMCPII (Woodbury & Neurath, 1980). The present results indicate that homogenates from *T. spiralis*-infected mouse intestine contain several enzymes that cross-react with anti-RMCPII. Clearly, therefore, the relationship of this gut-derived enzyme to those in parasitized intestine, in cultured BMMC, and in connective tissue mast cells requires further investigation.

The relationship between intra-epithelial granule-containing cells and mast cells elsewhere in the tissues of mice has yet to be clearly defined (reviewed in Miller, 1980). Differentiation between mast cells and granulated intra-epithelial lymphocytes is currently based on the fixation and staining properties of the granule glycosaminoglycans (Ruitenberg & Elgersma, 1976; Crowle & Phillips, 1983), on the IgE-binding properties of these cells (Guy-Grand *et al.*, 1984) and on their content of serine esterases (Huntley *et al.*, 1985). The isolation from intra-epithelial mast cells of a 26,000 MW serine proteinase will, we believe, provide further, more definitive, biochemical characterization of this cell type. Once the amino acid sequence of this enzyme has been obtained and when a panel of monoclonal antibodies has been raised against it and against other similar mast cell-derived enzymes, it may finally prove possible to define mast cell subsets and their functions more definitively in the mouse. These studies are currently in progress.

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