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Heterogeneity of murine bone marrow-derived mast cells: analysis of their proteinase content

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SUMMARY

The expression of granule proteinases by murine bone marrow-derived mast cells (BMMC) grown *in vitro* was compared with that of serosal mast cells (SMC) from the peritoneal cavity. The granules in a proportion of BMMC (0·4–13%) and in all SMC were labelled with fluorescent antibodies against rat mast cell protease I (RMCP I). The granules of 1–47% of BMMC and 100% of SMC were labelled with antibodies against a 30,000 molecular weight (MW) murine intestinal mast cell proteinase (MIMCP). Four antigens from BMMC, ranging in MW from 28,000 to 32,000 and a single 28,000 antigen from SMC were detected on Western blot using anti-MIMCP antibodies. Only the 28,000 MW antigens from BMMC and SMC were visualized in blots probed with anti-RMCP I. BMMC grown in the presence of conditioned medium from activated splenocytes or from the WEHI-3B myelomonocytic cell line contained 52–118 ng and 3–25 ng MIMCP/10^6 cells respectively, whereas SMC lacked detectable MIMCP. The selective labelling of the 28,000 MW antigens in BMMC and SMC with anti-RMCP I and the variable expression of this antigen in BMMC as detected by immunofluorescence indicates that BMMC are not a homogeneous population of cells.

INTRODUCTION

The development of techniques for culturing mast cells on fibroblast monolayers by Ginsburg and Lagunoff[1] and in suspension cultures from haemopoietic progenitors,[2-4] has provided substantial information on the biology of this cell type. Murine bone marrow-derived mast cells (BMMC) cultured *in vitro* have several features in common with mucosal mast cells (MMC) found mainly in the gastrointestinal mucosa. Both are T-cell-dependent populations;[5,6] they have similar histochemical staining properties,[7,8] and BMMC contain chondroitin sulphate E rather than the heparin present in serosal mast cells.[9] These similarities have led to BMMC being regarded as the *in vitro* analogues of MMC.[10]

Precise identification of mast cell phenotype is important because different subsets may be functionally distinct[11] and because there is increasing evidence of transdifferentiation between subpopulations.[12-14] However, identification of mast cell phenotypes by histochemical analysis of granule glycosaminoglycan (GAG) may be unreliable because the techniques lack specificity and because mast cell populations can contain several GAGs. For example, immature connective tissue mast cells of the rat resemble MMC but exhibit changes in GAG histochemistry as increasing quantitities of heparin are synthesized.[15] Similarly, biochemical analysis shows that rat serosal mast cells (SMC)[16] and mouse BMMC[17] contain both heparin and chondroitin sulphate E in varying proportions.

Rat BMMC synthesize and secret the neutral serine proteinase rat mast cell proteinase II (RMCP II) exclusively,[18] as do >99% of intestinal MMC.[19,20] By contrast, SMC of the peritoneal cavity contain the antigenically similar, but much less soluble, enzyme rat mast cell proteinase I (RMCP I).[21] Mast cell subsets in the rat are, therefore, readily distinguished by their content of neutral proteinases. In the mouse there is differential expression of a 30,000 molecular weight (MW) intestinal mast cell proteinase (MIMCP) in the gut and of a 28,000 MW putative serosal mast cell proteinase in connective tissues.[22] The distribution of these two proteinases in murine BMMC has not been described, nor is the relationship between murine BMMC and MMC defined in terms of their proteinase content. Immunohistochemical analysis of gastrointestinal MMC indicates that there is substantial proteinase heterogeneity.[22] Similarly, a family of serine proteinases ranging in MW between 28,000 and 31,000 is apparently differentially expressed in SMC and in immortalized BMMC,[23] suggesting that there is potentially a complex heterogeneity amongst murine mast cells both *in vivo* and *in vitro*.

In the present study we have analysed the granule proteinases of mouse BMMC grown under various culture conditions, and of SMC from the peritoneal cavity using paired immunofluorescence, immunoblotting, and ELISA, to characterize further these two mast cell populations.

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MATERIALS AND METHODS

Animals

Mice of the inbred NIH and SWR strains were bred and maintained at the Department of Zoology, University of Nottingham. All the mice were used at approximately 8–10 weeks of age and maintained under conventional conditions. Swiss white mice, randomly bred at Moredun Research Institute and maintained under conventional conditions were used, at 12–16 weeks old, for the isolation of SMC.

Preparation of T-cell-conditioned medium

Conditioned medium (CM) as a source of mast cell growth factor(s) was prepared by culturing SWR spleen cells (5×10^6/ml) for 40 hr at 37° in culture medium + 10 μg/ml concanavalin A (Sigma, Poole, Dorset, U.K.) in an atmosphere of 5% CO_2. The culture medium used both in the preparation of CM and bone marrow cultures was RPMI-1640 (Gibco, Paisley, Renfrewshire, U.K.) containing 23 mM sodium bicarbonate, 10 mM HEPES, $7{\cdot}5 \times 10^{-5}$ M monothioglycerol, 2 mM glutamine, 0·1 mM sodium pyruvate, 10% foetal calf serum (FCS), 100 IU/ml penicillin and 100 μg/ml streptomycin.

Preparation of WEHI-3B-conditioned medium

The mouse myelomonocytic tumour cell line WEHI-3B (E.C.A.C.C., Porton Down, Wilts, U.K.), which constitutively produces interleukin-3 (IL-3),[24] was cultured at between 3–9×10^5 cells/ml in RPMI-1640 + 10% FCS for 2–3 weeks at 37°/5% CO_2. The cell-free supernatant fluid was filtered (0·22 μm Falcon filter) before use in bone marrow cultures.

Bone marrow culture

Femoral bone marrow cells were obtained from NIH mice and cultured at 5×10^5 cells/ml in 10-ml aliquots in medium supplemented with 20% CM or WEHI-3B supernatant fluid in 25 cm^2 flasks (Gibco). The flasks were incubated upright at 37°/5% CO_2 for 21–28 days. Cultures were fed each week by replacing 50% of the culture medium supernatant fluid with fresh supplemented medium. Cells were then harvested and total viable counts made using fluorescein diacetate. Mast cell counts were made on cytocentrifuge preparations after fixing in Carnoy's fixative and staining with toluidine blue or after direct staining with Wright's or Leishman stain.

Mouse serosal mast cells

Peritoneal cells were recovered from Swiss white mice by lavage with Hanks' balanced salt solution contining 0·1% w/v gelatin and purified to 85%–90% mast cells by density gradient centrifugation over metrizamide (Sigma), as described by Hamaguchi *et al.*[25] For SDS–PAGE a pellet of purified mast cells was resuspended in reducing sample buffer and heated at 90° for 3 min.

Specific anti-proteinase antibodies

Antibodies, raised in rats and rabbits, against mouse intestinal mast cell proteinase[26] were affinity purified on MIMCP–Sepharose 4B and repeatedly cross-absorbed against RMCP I–Sepharose 4B.[19] Rabbit anti-RMCP I was cross-absorbed against MIMCP–Sepharose 4B, as described previously.[22]

Paired immunofluorescence

Cytocentrifuge preparations of BMMC were fixed in formaldehyde and proteinases detected by the method described previously.[22] Briefly, slides were incubated in 5% bovine serum albumin (BSA) (Sigma grade V) in phosphate-buffered saline (PBS) for 10 min before probing with optimally diluted, cross-absorbed, rabbit anti-RMCP I for 30 min. After washing, the slides were incubated in sheep anti-rabbit IgG-rhodamine (TRITC) conjugate (Cappel, Westchester, PA) for 30 min. The slides were washed again before incubation with rat anti-MIMCP for 30 min followed by further washes before addition of sheep anti-rat fluorescein (FITC) conjugate (The Binding Site Ltd, Birmingham, U.K.) for 30 min. All serum and conjugate dilutions were in 5% BSA/PBS. After final washing in PBS the smears were mounted with citifluor (Citifluor Ltd, London, U.K.) non-fluorescent mountant.

SDS–PAGE

Discontinuous SDS–PAGE was with 0·75 mm thick 12% slab gels (Mini Protean II; Biorad, Richmond, CA) and samples were electrophoresed at a constant 200 volts for 1 hr. Samples were loaded at 40 ng/lane for the purified proteins and 5×10^4 cells per

Table 1. Murine bone marrow-derived mast cells: assessment of proteinase content by paired immunofluorescence and ELISA from four experiments

Exp.	Growth factor	Days of culture	% Mast cells	% Mast cells fluorescing	% FITC only	% Dual fluorescence	% TRITC only	MIMCP ELISA (ng/10^6 cells)
1	CM	26	92·5 ± 1·19	25·4 ± 2·62	24·0 ± 2·74	0·6 ± 0·2	0·8 ± 0·4	49·3 ± 1·69
2	CM	26	90·7 ± 0·3	61·7 ± 4·0*	46·5 ± 3·7*	13·1 ± 2·2‡	1·1 ± 0·6†	118·1 ± 20·9§
	WEHI	26	89·7 ± 1·3	18·8 ± 2·4	9·2 ± 1·9	4·0 ± 0·9	5·8 ± 0·9	25·2 ± 0·8
3	CM	28	77·0 ± 3·3	18·4 ± 1·2*	13·9 ± 1·2†	3·4 ± 0·6‡	1·1 ± 0·3	51·9 ± 7·7†
	WEHI	28	81·3 ± 1·9	1·7 ± 0·4	1·2 ± 0·3	0·4 ± 0·2	0	3·1 ± 0·5
4	CM	28	96·5 ± 0·8	29·0 ± 5·6	17·7 ± 3·1	11·3 ± 4·2	0	72·8 ± 12·9§
	CM/WEHI	21/7	94·0 ± 1·5	13·6 ± 0·9	11·5 ± 1·2	2·1 ± 0·2	0	28·4 ± 3·0

Data are mean ± SEM of four replicates from each culture protocol. Analysis by Student's two sample *t*-test.
Students two sample *t*-test: *$P < 0{\cdot}001$; †$P < 0{\cdot}01$; ‡$P < 0{\cdot}02$; §$P < 0{\cdot}05$.
CM, mitogen-stimulated T-cell conditioned medium; WEHI, WEHI-3B culture supernatant fluid.

lane for the cell extracts. Cell pellets and purified proteins were treated with reducing buffer and boiled for 3 min before they were loaded onto the gels.

Immunoblotting

Proteins from SDS–PAGE gels were transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) using a semi-dry transfer apparatus at 0·9 mA per cm^2 of gel for 1 hr at room temperature. After transfer, the nitrocellulose membranes were incubated in PBS, containing 0·1% v/v Tween 20, overnight at room temperature to block non-specific protein adsorption and then probed with affinity purified, cross-absorbed rabbit anti-RMCP I or unabsorbed rabbit anti-MIMCP optimally diluted with PBS/Tween 20 for 1·5 hr. The nitrocellulose membranes were washed 6 × 5 min in PBS/Tween 20 before incubation with sheep anti-rabbit horseradish peroxidase (HRPO) conjugate (Sera Lab, Crawley, Sussex, U.K.) optimally diluted with PBS/Tween 20. After further washing (6 × 5 min) peroxidase activity was revealed with diaminobenzidine/H_2O_2.

Enzyme-linked immunosorbent assay (ELISA)

Cell pellets were resuspended in 200 μl of 1·5 M NaCl in 20 mM Tris, pH 7·5, and rapidly freeze-thawed three times. The extracts were centrifuged at 8000 ***g*** for 5 min before assay of the supernatant fluids in an antibody-capture ELISA, described previously, for MIMCP.[27]

RESULTS

Mast cells (77–96%; Table 1) arising from mouse bone marrow cultured in the presence of conditioned medium (CM) or of supernatant fluid from the constitutive IL-3-producing cell line WEHI-3B (WEHI) were similar to the BMMC described previously.[3,9] Each had a single spherical or ovoid concentric nucleus and variable numbers of basophilic granules within the cytoplasm (Fig. 1). Some mast cells contained vacuoles, although the proportion of vacuolated cells and the extent of this vacuolation appeared greatest when BMMC growth was stimulated with WEHI supernatant fluid. The percentage of BMMC derived from the same batch of bone marrow was similar regardless of whether the cultures were stimulated with CM or WEHI (Table 1).

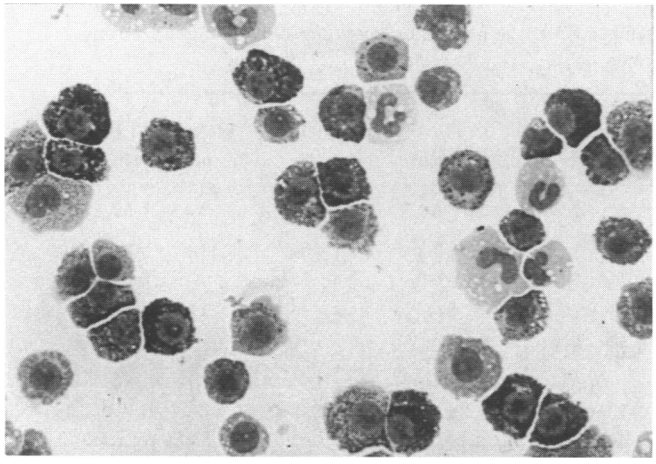

Figure 1. Photomicrograph of Leishman-stained mouse bone marrow-derived mast cells, grown in splenocyte conditioned medium, showing variable expression of basophilic granules in the cytoplasm (×225).

In four separate experiments, BMMC, grown either in CM or WEHI were analysed for their content of proteinases by ELISA and by paired immunofluorescence (Fig. 2, Table 1). The latter technique revealed four types of BMMC: mast cells uniquely fluorescing with anti-MIMCP (FITC), or with anti-RMCP I (TRITC); dual fluorescent mast cells detected with anti-MIMCP and anti-RMCPI (FITC+TRITC); and unlabelled mast cells. The pattern of fluorescence was similar to that described previously for intestinal mast cells,[28] with sometimes diffuse cytoplasmic staining for anti-MIMCP and a more distinct granular location for anti-RMCP I (Fig. 2). The numbers of fluorescing cells of each phenotype, and estimates of the MIMCP content of BMMC are summarized in Table 1.

The proportion of fluorescing cells from different cultures was extremely variable, ranging from 18·4% to 61·7% in CM-derived cells and between 1·7% and 18·8% for WEHI-cultured BMMC. From the same batches of bone marrow (Exps 2 and 3) cultures stimulated with CM had significantly greater ($P < 0·001$) proportions of fluorescent cells than those stimulated with WEHI (Table 1). There were also more cells which fluoresced for the presence of MIMCP alone ($P < 0·01$; Table 1) and more dual fluorescing cells ($P < 0·02$; Table 1) in CM-stimulated cultures, but in Exp. 2 there were more cells ($P < 0·01$; Table 1) which fluoresced for the presence of the RMCP I-like enzyme alone in WEHI-grown cells. Cells labelled uniquely with TRITC were relatively scarce in both cultures when compared to single FITC-labelled or dual labelled cells.

When cultures were stimulated with CM for 3 weeks followed by 1 week with WEHI (Exp. 4), the intensity of fluorescence of BMMC labelled with FITC was reduced when compared with BMMC continuously cultured in CM, although there was no decrease in the proportion of cells detected by immunofluorescence.

When measured by ELISA, concentrations of MIMCP/10^6 BMMC were significantly greater in cultures stimulated with CM ($P < 0·05$ for Day 26 and $P < 0·01$ for Day 28) (Table 1) than in cultures from the same batches of bone marrow grown exclusively on WEHI supernatant fluid. Cultures grown for 21 days on CM followed by 7 days on WEHI which had less intense fluorescence also had significantly lower concentrations of MIMCP ($P < 0·05$) when compared with cultures from the same batch of marrow grown exclusively on CM for 28 days (Table 1).

Serosal mast cells isolated from the peritoneal cavity had typical mast cell morphology with the cytoplasm densely packed with blue staining granules in Leishman-stained cytospin preparations. Following paired immunofluorescence serosal mast cell granules were labelled with both FITC and TRITC (Fig. 2), although no MIMCP could be detected in those cells by ELISA (data not shown).

The percentage of BMMC expressing MIMCP as judged by fluorescent labelling was compared with the concentrations of MIMCP per 10^6 BMMC by simple regression analysis (Fig. 3). After the sum of BMMC labelled with FITC alone and of dual fluorescing cells (x) was plotted against MIMCP concentrations (y), the regression equation for all the cell cultures shown in Table 1 was $y = 7·09 + 1·88x$ and $r = 0·86$ ($P < 0·001$). These results confirm that, for each culture of BMMC, the number of FITC-labelled cells is proportional to the concentration of MIMCP per 10^6 BMMC.

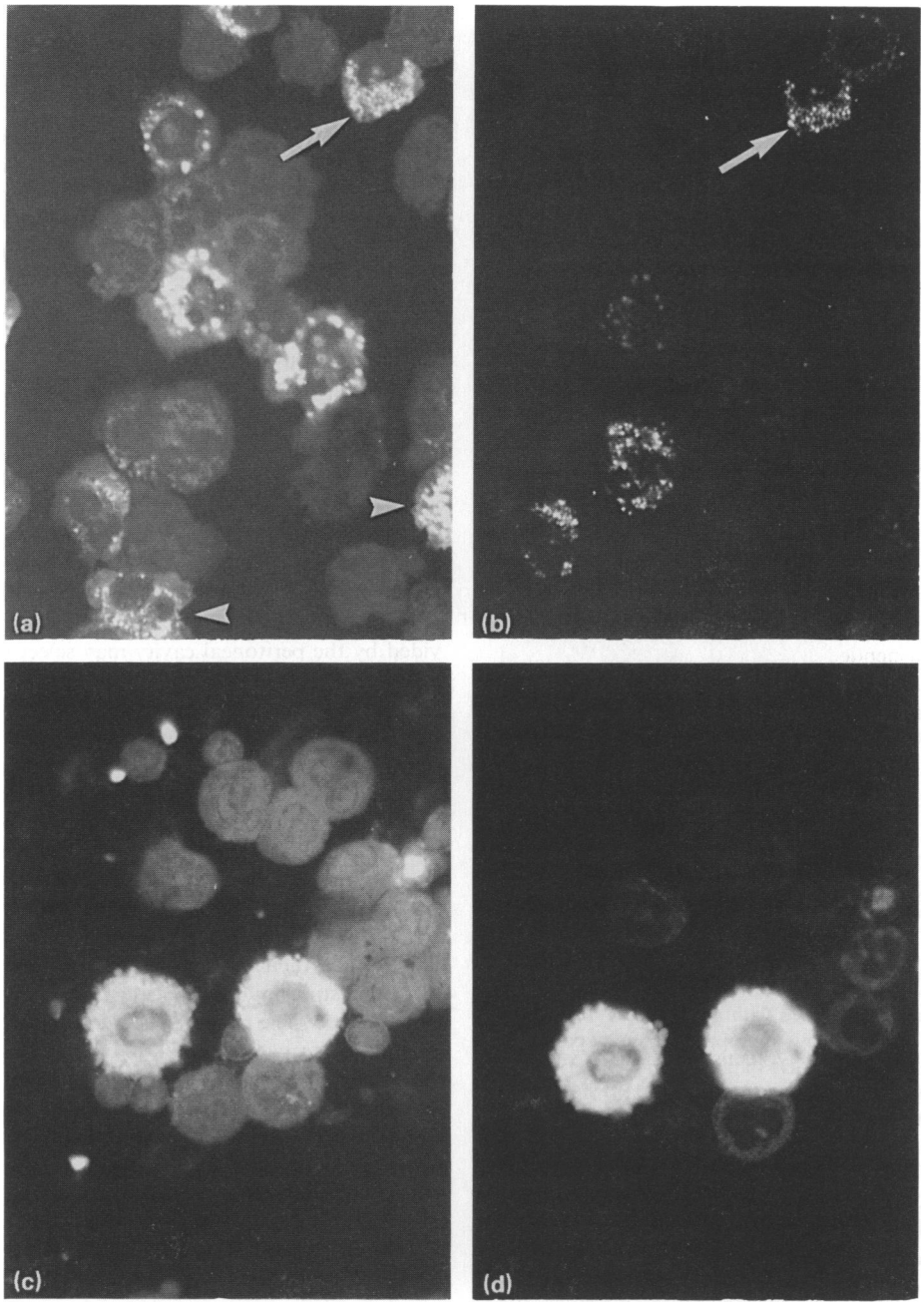

Figure 2. Immunofluorescence patterns of proteinase expression in BMMC and SMC. The BMMC contain cells which exhibit FITC fluorescence with anti-MIMCP (a) and TRITC fluorescence with anti-RMCP I (b). Note the cells which fluoresce with both anti-MIMCP and anti-RMCP I (arrows) and cells which fluoresce with anti-MIMPC alone (arrowheads). Many other BMMC lack fluorescence. Serosal mast cells from peritoneal lavage exhibit both FITC fluorescence with anti-MIMCP (c) and TRITC fluorescence with anti-RMCP I (d) ($\times$ 469).

Western blot analysis of BMMC grown in CM, using anti-MIMCP antibodies, revealed four antigens with MW ranging between 28,000 and 32,000 (Fig. 4). Western blots of purified serosal mast cells contained one major band, with a MW of 28,000, which was detected by both anti-MIMCP and cross-absorbed anti-RMCP I (Fig. 4). Extracts from BMMC also contained a 28,000 MW band which, uniquely, amongst the 28,000–32,000 MW antigens was identified by cross-absorbed anti-RMCP I (Fig. 4).

DISCUSSION

Mouse BMMC, whilst forming an apparently homogeneous population of mast cells by morphological and histochemical criteria, are heterogeneous in their proteinase content as determined by paired immunofluorescence. Cells were identified which lacked proteinases (38–98%), which were detected with anti-MIMCP or anti-RMCP I antibodies, or were labelled with both probes. Antibody to RMCP I identified a 28,000 MW

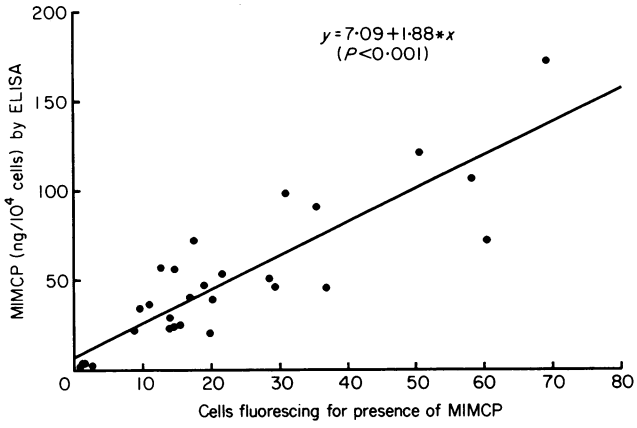

Figure 3. Simple regression analysis showing the relationship between the sum of cells detected by FITC and FITC/TRITC fluorescence (x) and concentrations of MIMCP/10^6 BMMC (y) as determined by ELISA.

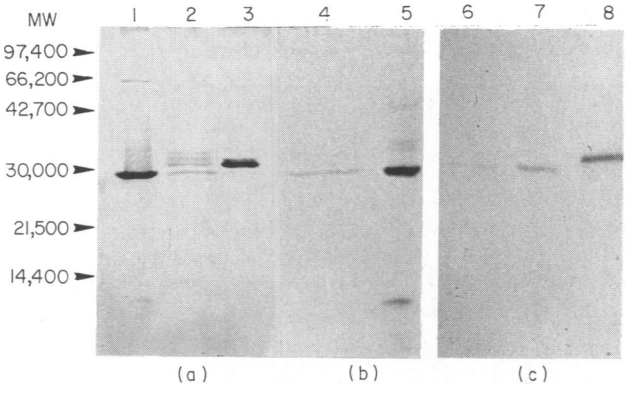

Figure 4. Immunoblot analysis of BMMC and SMC extracted in sample buffer (5×10^4 cells/lane) and probed with anti-MIMCP (a) or anti-RMCP I (b and c) followed by appropriate immunoperoxidase conjugate and diaminobenzidine/H_2O_2 staining. Samples in lanes 1, 5 and 7 are SMC, and in lanes 2, 4 and 6 BMMC. Purified MIMCP (40 ng) and RMCP I (40 ng) were run in lanes 3 and 8, respectively.

putative proteinase present in mouse SMC[29] and, as shown here, some BMMC. Similarly, in addition to MIMCP, which has a MW of 30,000, three other putative proteinases were identified in BMMC by Western blotting with anti-MIMCP. This is in good agreement with earlier studies using [^{3}H]diisopropyl fluorophosphate (DFP) where four neutral serine proteinases, 28,000–31,000 MW were identified in extracts of BMMC.[30]

The 28,000 MW antigen from BMMC was detected with anti-MIMCP on Western blot, showing that it is highly related to MIMCP. It was the only one of the four polypeptides detected with anti-MIMCP which was also detected with anti-RMCP I. On this basis it is very similar or identical to the 28,000 MW antigen previously described in SMC.[22]

One of the limitations of the anti-MIMCP antibody, despite the fact that it is highly specific for MIMCP by ELISA,[27,29] is that it cross-reacts with the RMCP I-like protease from SMC when the latter is denatured by SDS–PAGE or is insolubilized by fixation.[27,29] This is exemplified by the fact that SMC, which lack MIMCP both by ELISA[27] and by Western blotting,[22] are detected immunohistochemically with anti-MIMCP antibodies.[22] Nevertheless, despite these limitations, it is clear that BMMC are a heterogeneous population and that some of the cells may have a proteinase phenotype similar or identical to that of SMC.

The present results also show that expression of mast cell proteinases in cultured murine BMMC, which is very low when compared to that of rat BMMC,[28] is under complex control, probably being depedent on more than one T-cell factor since cells grown in WEHI-conditioned medium, contining IL-3 alone, express less enzyme than BMMC grown in conditioned medium from mitogen-stimulated T cells. It is possible that the BMMC lacking proteinases, even when cultured in the presence of T-cell conditioned medium, do not receive the appropriate maturation signals. These novel findings may, therefore, provide a model for the study of the factors which control mast cell proteinase expression.

The presence of putative SMC in BMMC could explain apparent phenotypic changes, reported by others,[12,13] where BMMC, exhibiting mucosal mast cell-like phenotype as determined by GAG histochemistry and biochemistry, switched to serosal mast cell phenotype when injected intraperitoneally into mast cell-deficient W/W^v mice.[13] The microenvironment provided by the peritoneal cavity may select for the survival and proliferation of those precursor cells committed to differentiate into the SMC proteinase phenotype. A similar selection process may occur *in vitro* where BMMC cultured in the presence of 3T3 fibroblasts expressed glycosaminoglycans characteristic of serosal mast cells.[31] Comparable changes were originally reported by Ginsburg and colleagues,[32] who noted phenotypic variants when mast cells were grown on fibroblast monolayers.

In summary, these immunofluorescence and immunoblotting studies show that mouse BMMC are probably not a homogeneous population since a proportion of the cells resemble SMC in their proteinase content. Similarly, MMC of the gastrointestinal mucosa are heterogeneous in their expression of proteinases.[22] The results raise questions about the true nature of mast cell transdifferentiation in the mouse, based, as it is, on the histochemical and biochemical analysis of GAGs.[12,13] Further study of the regulatory mechanisms of mast cell differentiation is required, with particular emphasis on proteinase expression, before the significance of the transdifferentiation studies can be fully evaluated.

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