

Characterization of a monoclonal antibody recognizing mast cells

T. de Oliveira Nogueira¹ and J.E. Aubin²

¹School of Dentistry of São José dos Campos, São Paulo, UNESP, Brazil and

²Department of Anatomy and Cell Biology, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada

Summary. Immunohistochemical screening for monoclonal antibodies prepared by immunization of mice with a rat osteoblastic cell population led to identification of one antibody that reacted against a small population of cells present in the soft connective tissue compartment of 21 days fetal rat calvaria. The morphology of the cells and the immunohistochemical staining characteristics (a distinct intracellular granular pattern) suggested that the antibody might be reacting specifically against mast cells. We used combined histochemistry and immunohistochemistry to further characterize this antibody, designated RCJ102. Cryosections containing calvaria bone, soft connective tissues and skin were prepared from the top of the head of 21 days fetal rats, and from adult rats cryosections of lung, muscle, adipose tissue and small intestine were prepared. Some sections were labelled by indirect immunofluorescence with RCJ102; corresponding sections were labelled histochemically with toluidine blue. There was a direct correspondence between mast cells identified histochemically and cells labelling with RCJ102 in all tissues except intestine, in which the mast cell detectable by histochemistry were not labelled by RCJ102. These results suggest that the RCJ102 antibody will be a valuable new reagent for further elucidation of the heterogeneity described between connective tissue and intestinal mucosal mast cells.

Key words: Mast cells, Heterogeneity, Immunohistochemistry, Connective tissue, Intestine

Introduction

Mast cells (MC) are ordinarily distributed throughout connective tissue, where they are often situated adjacent to blood and lymphatic vessels, near or within nerves and beneath epithelial surfaces, such as those of the respiratory and gastrointestinal tracts and skin. MC are

identifiable morphologically and histochemically via the metachromatic staining properties of their granules, i.e. dyes such as toluidine blue and alcian blue label the proteoglycans within the granules. These negatively charged proteoglycans are thought to form complexes with positively charged proteases and histamine (Enerback, 1981; Befus et al., 1985; Pearce, 1986).

It is becoming increasingly clear that MC from different tissues and species are biochemically and functionally heterogeneous, although the significance of the heterogeneity is not yet understood. This diversity is particularly evident in rodents in which there are at least two distinct types of MC: the connective tissue type (CTMC) or «typical mast cell» and the mucosal mast cell (MMC) or «atypical mast cell» (Enerback, 1981; Befus et al., 1985; Pearce, 1986). Heterogeneity of MC also occurs in mice but in humans conclusive evidence does not exist, although some results suggest that similar heterogeneity is present (Kitamura, 1989). The two types of MC may show differences in relation to anatomical distribution, morphology, histochemical staining properties, content of glycosaminoglycans, content of neutral proteases or levels of tryptase and chymase, content of histamine, staining with berberine sulphate, staining with safranin O, response to compound 48/80 and other drugs, content of proteases I and II, and T-lymphocyte and thymus dependence of proliferation (Enerback, 1981; Befus et al., 1985; Pearce, 1986; Irani et al., 1986; Galli, 1990).

With respect to morphology and histochemical properties, the CTMC are large, contain densely packed and intensely violet metachromatic granules, while MMC are smaller, vary widely in shape, are not so densely granulated and exhibit a more reddish metachromasia with toluidine blue (Enerback, 1981; Kitamura and Fujita, 1989). The ability to document MMC is very sensitive to fixation (Enerback, 1981). MC of almost all species contain histamine (Pearce, 1986) and in some species also 5-hydroxytryptamine (Befus et al., 1985), but histochemical observations have suggested that MMC in rats contain less histamine than the CTMC (Enerback, 1981). Rat CTMC are rich in heparin but the proteoglycan produced by rat MMC "in

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vivo" is an oversulphated galactosaminoglycan rather than heparin (Enerback, 1981; Kitamura, 1989; Kitamura and Fujita, 1989). Although some species differences are known, rat MMC contain a distinct chymotrypsin-like serine protease designated as rat MC protease II (RMCP II) which is different from the chymotrypsin-like enzyme protease I (RMCP I) of CTMC. Histochemical studies and immunohistochemical analysis have confirmed that RMCP I predominates in CTMC and that RMCP II is located exclusively in cells of MMC phenotypes (Irani et al., 1986).

Monoclonal antibodies (Mab) have also been used to distinguish CTMC and MMC (e.g., Katz et al., 1983; Mayrhofer et al., 1987; Hamman et al., 1994). For example, an antibody YB5.B8 raised against human acute myeloid leukemia cells was found to bind to both MMC and CTMC although some alcian blue positive cells were not labelled, suggesting a small YB5.B8 Mab negative subpopulation of MC that have lost the antigen during differentiation; alternatively that these negative cells are tissue basophils could not be discounted (Mayrhofer et al., 1987).

We report a Mab designated RCJ102 that labels CTMC but not MMC providing a useful reagent to allow rapid identification of the two cell types.

Materials and methods

Production of monoclonal antibodies

In a series of injections done to raise mouse monoclonal antibodies against rat osteogenic cells and selected according to their abilities to label subpopulations of cells in bone or surrounding tissues (Turksen and Aubin, 1991), we identified one antibody, designated RCJ102, whose characterization is reported here.

Tissue preparation for histochemistry and immunohistochemistry

Rat calvaria from 3-21 days fetal Wistar rats were prepared with surrounding tissues intact, as described by Turksen and Aubin (1991). At the same time the following tissues were obtained from the adult female rats: muscle from the hind limbs, lung, adipose tissue from the inguinal region and the small intestine. The specimens measured about 1.0 cm³ and were trimmed, fixed in 4% formaldehyde and embedded in paraffin or glycol methacrylate (JB4 Polysciences, Inc. Warrington, PA 18976) by standard procedures. Some specimens were snap frozen in liquid nitrogen and embedded in Tissue Tek Oct 4583 Compound. For the intestine, Motas's fixative was used for fixation before embedding in paraffin and Carnoy's fixative was used for frozen sections that were also treated with ethanol (0.074% concentrated HCl 36% at 4 °C and rinsed in 95% ethanol before staining. Sections were stained with

haematoxylin-eosin, toluidine blue (0.025%, pH 4.5) or Giemsa stain or immunostained with Mab RCJ102 for indirect immunofluorescence.

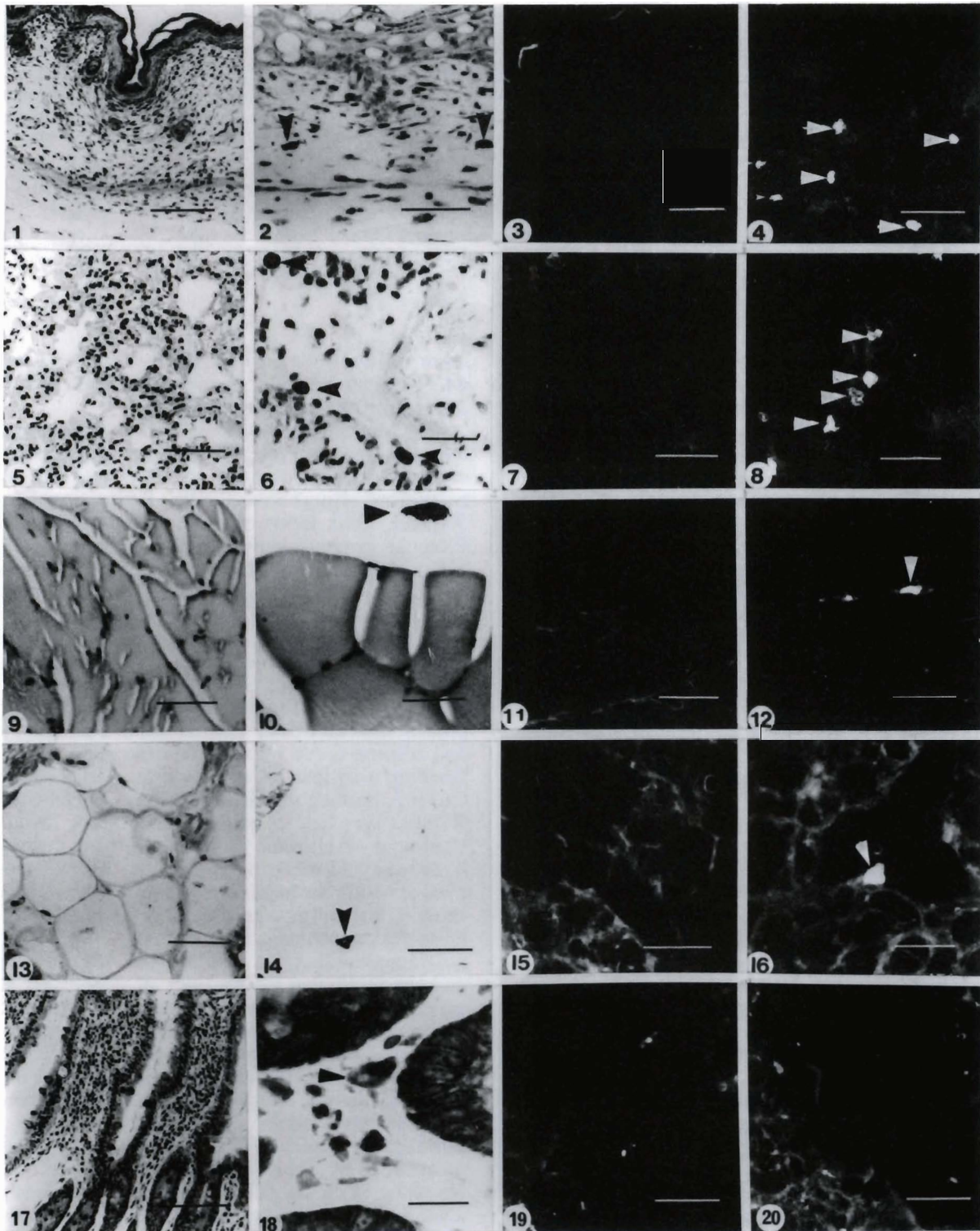
Indirect immunofluorescence

Indirect immunofluorescence was done as described (Turksen and Aubin, 1991). Briefly cryostat sections were air-dried for 30 min at room temperature, fixed in 3.7% formaldehyde, rinsed in PBS for 5 min at room temperature and post-fixed in -20 °C methanol for 5 min. The sections were washed in PBS 3X and then incubated with 3% (wt/vol) BSA (essentially fatty acid free; Sigma Chemical Co.; St. Louis, USA). The sections were incubated with the BSA solution for 1 h at room temperature to block potential non-specific sites, then rinsed in PBS again, overlaid with first antibody, RCJ102, and incubated in a humidified chamber for 35 min at 37 °C. After washing 3 times with PBS, sections were incubated with the second antibody (FITC-labelled-sheep-anti-mouse IgG, Du Pont NEN Products/Boston MA) for 35 min at 37 °C. Sections were rinsed with PBS, mounted in Moviol (Hoescht Ltd., Montreal, PQ) and observed with a Zeiss Photomicroscope III with epifluorescence attachments. Photographs were taken using Kodak (Eastman Kodak Co., Rochester, NY) T-Max 3200 ASA films and developed with Kodak T-Max developer.

Cryostat sections were also utilized for assessing the effects of enzymatic pretreatment on immunohistochemical staining with RCJ102 and histochemical staining with toluidine blue. The enzymes used were heparinase (heparin lyase from *Flavoprotein heparinum*; Sigma), chondroitinase ABC (chondroitin ABC lyase from *Proteus vulgaris*; Sigma), and histaminase (diamino oxidase from porcine kidney; sigma). The enzymes concentration were 40 ng/ml in PBS with 0.5% of BSA. The sections were overlaid with the solutions and left overnight in a humidified chamber at 37 °C. Controls were overlaid with the buffer only.

Results

In the course of screening for mouse monoclonal antibodies raised against rat osteogenic cell populations, one antibody, designated RCJ102, was selected for further study because it labelled a few cells in the connective tissue of the fetal rat head. The cells had a granular appearance, which also characterized the intracellular immunostaining (Fig. 4) and a morphology and distribution reminiscent of the appearance of MC. To characterize further the nature of the cells stained by RCJ102, a variety of rat tissues were screened in addition to calvaria sections: lung, muscle, adipose tissue and small intestine (Figs. 5-20). RCJ102 labelled a few granular cells in frozen sections from all tissues tested (Figs. 4, 8, 12, 16), except the intestinal mucosa (Fig. 20), even after the latter was fixed with Motas's and Carnoy's fixative, fixatives known to be optimal for



Figs. 1-20. Histochemical and immunohistochemical labelling of skin (Figs. 1-4), lung (Figs. 5-8), muscle tissue (Figs. 9-12), adipose tissue (Figs. 13-16) and intestinal mucosa (Figs. 17-20). Figs. 1, 5, 9, 13, and 17 are plastic sections stained by haematoxylin & eosin showing general histological features of each tissue. $\times 182$; bar: 1 cm = 55 μ m. Figs. 2, 6, 10, 14, and 18 are plastic sections stained with toluidine blue showing clearly the mast cell (arrowheads). $\times 284$ or $\times 578$; bars 1 cm = 35 μ m and 17.5 μ m respectively. Figs. 3, 7, 11, 15, and 19 are indirect immunofluorescence staining controls with second antibody alone of frozen sections. $\times 220$; bar 1 cm = 45 μ m. Figs. 4, 8, 12, 16, and 20 are indirect immunofluorescence staining with RCJ102; labelled mast cells are marked with arrowheads. Mast cells in intestinal mucosa did not label with RCJ102 (Fig. 20). $\times 220$; bar = 45 μ m.

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Table 1. Immunostaining with RCJ102 and histochemistry with toluidine blue on frozen sections with and without treatment with heparinase, chondroitinase ABC and histaminase.

TISSUES	ENZYMATIC PRETREATMENT							
	Untreated		Heparinase		Chondroitinase ABC		Histaminase	
	Tol. Blue	RCJ102	Tol. Blue	RCJ102	Tol. Blue	RCJ102	Tol. Blue	RCJ102
Calvaria (skin)	+	+	-	-	+	+	+	-
Lung	+	+	-	-	+	±	+	±
Muscle	+	+	-	-	+	+	+	-
Adipose tissue	+	+	-	+	+	+	+	-
Intestinal mucosa	-	-	ND	ND	ND	ND	ND	ND

ND: not done since the untreated sample was negative.

MMC preservation. The number of cells labelling was more or fewer depending on the tissue labelled and the area of each tissue examined. The tissue with most cells labelling was the connective tissue in the corium region of the skin covering the calvarial bone (Fig. 4). In muscle and adipose tissue (Figs. 12, 16), cells labelled were in the connective tissue septae and often surrounding blood vessels. In the lung, labelled cells were around the blood vessels and bronchioles. Intestinal mucosa was always negative (Fig. 20).

In corresponding serial frozen sections, toluidine blue was used to compare the distribution of histochemically stained cells with immunolabelled cells. There was a 1:1 correspondence between toluidine blue metachromatically stained cells (Figs. 2, 6, 10, 14) with morphology typical of MC and cells labelling with RCJ102 (Figs. 4, 8, 12, 16), again in all tissues except intestine. Evidence of MMC in the intestinal mucosa in frozen sections was obtained after fixation with Carnoy's and staining with Giemsa stain; these cells did not label with RCJ102.

Pretreatments with heparinase, chondroitinase ABC and histamine were done to determine whether these enzymes altered either the toluidine blue staining or the antibody staining (Table 1). Chondroitinase ABC treatment had no effect in any tissue except lung in which RCJ102 labelling intensity diminished; toluidine blue stain was also unaffected in all tissues. Histaminase obliterated RCJ102 staining in skin, muscle and adipose tissue and markedly decreased staining in lung but did not affect toluidine blue staining. Heparinase treatment of the sections obliterated RCJ102 staining of cells in skin, lung and muscle, but not in adipose tissue; no toluidine blue staining remained in any tissue (Table 1).

Discussion

The antibody we have prepared, RCJ102, labels MC in skin, lung, muscle and adipose tissue and not intestinal mucosa suggesting that this antibody differentiates between CTMC and MMC. The intestinal mucosal MC, which is considered to be a MMC, while clearly evident in paraffin and plastic sections with toluidine blue and in frozen sections with Giemsa stain

were unlabelled with RCJ102. This suggests that RCJ102 can be used as an intracellular marker for distinguishing CTMC from MMC.

Toluidine blue is a cationic dye that stains negatively-charged proteoglycans which are thought to form complexes with positively-charged proteases and histamine (Enerback, 1966a, 1981; Enerback and Wringer, 1980; Befus et al., 1982; Zengh et al., 1991). Proteoglycans are known to be a major constituent in MC and CTMC contain a large amount of heparin that has more negative charge density than the oversulphated chondroitin sulphate of the MMC (Stevens et al., 1988). Also CTMC contain a large amount of histamine in contrast to MMC which contain only a small amount (Enerback, 1966a,b; Enerback and Lowhagen, 1979; Kitamura and Fujita, 1989). After heparinase pretreatment on frozen sections of the skin, lung, muscle and adipose tissue toluidine blue staining and RCJ102 labelling were lost, except in adipose tissue, where some RCJ102 labelling remained (Table 1). The labelling with RCJ102 was lost in adipose tissue after histaminase treatment. Preliminary immunoblotting results with histamine and heparin suggest that RCJ102 does not bind directly to heparin or histamine, however the intracellular epitope/antigen may be bound to one of these macromolecules.

Understanding the regulation of MC development and the biological significance of phenotypic heterogeneity now represent central goals of MC research. The presence of heterogeneity in MC populations has been suggested to result from their unique differentiation process. Since MC precursors differentiate invading particular tissues, their phenotype can be influenced by the tissue environment in which differentiation occurs, although the mechanism by which the phenotype is determined by tissue factor(s) remains to be clarified. However, MMC may change to CTMC in an appropriate anatomical environment (Katz et al., 1983; Kobayashi et al., 1986; Kitamura, 1989; Martin et al., 1990). The phenotypic changes occur in the opposite direction as well (Kitamura et al., 1987). The MC phenotypic variation, theoretically, might reflect the following mechanisms acting alone or in combination: a) the existence of distinct mast cell lineages, b) the process of

cellular maturation, c) the functional status of the cell and d) the acquisition of molecules derived from other cell types (Combs et al., 1965; Befus et al., 1985; Nakano et al., 1985; Stevens et al., 1988; Kitamura and Fujita, 1989; Galli, 1990; Martin et al., 1990). In any case, it is well established that many differences do exist between the two types of MC, CTMC and MMC, including now their staining with RCJ102, which may prove a useful marker for further characterization of the differences in the two MC types.

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