Vol. 20: 95–100, 1994 | DISEASES OF AQUATIC ORGANISMS Dis. aquat. Org. | Published November 10

Mast cells in the swimbladder of Atlantic salmon *Salmo salar*: histochemistry and responses to compound 48/80 and formalin-inactivated *Aeromonas salmonicida*

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ABSTRACT: Observations were made on tissue spreads from the swimbladder of the Atlantic salmon *Salmo salar*. The tissues were fixed in ethyl alcohol and stained with alcoholic thionin. All preparations showed numerous tissue mast cells. The distribution of the mast cells resembled their distribution in mammalian tissues, with a concentration in the vicinity of the vascular network. Mast cell degranulation could be demonstrated at 2 h after intraperitoneal injections of compound 48/80, but in fish killed at 24 and 48 h, no signs of degranulation were found. Intrapentoneal injections of formalin-inactivated *Aeromonas salmonicida* subsp. *salmonicida* produced an inflammatory reaction and degranulation of the mast cells of the swimbladder.

KEY WORDS: Mast cells · Inflammation · Histochemistry · Atlantic salmon

INTRODUCTION

In mammalian species, the tissue mast cells are storage sites for preformed mediators of the inflammatory process. The most well-known of these mediators is histamine, which is stored in the mast cell granules (Spector & Willoughby 1963). Tissue injury, whatever the cause, activates the mast cells, degranulation occurs and released histamine produces vasodilatation and increased permeability of the microvasculature. The mast cells are similarly involved in anaphylactic hypersensitivity reactions (Pearce 1989).

Mast cells were originally described by Ehrlich (1877, 1879). Their major morphological characteristic is the presence of cytoplasmic granules which stain metachromatically with blue cationic dyes. There has been much controversy with regard to the presence of mast cells in tissues of fish. The original descriptions of their presence in teleosts date back more than half a century (Michels 1938). Later studies have questioned these findings, largely based on the fact that there are few reports demonstrating the classical metachromasia of mast cells in fish (Ellis 1982, Ellis et al. 1989). Cells of another type, the eosinophilic granular cells (Roberts et al. 1971), which have similar tissue distribution to that of mast cells in mammals, have been considered as mast cell analogues (Ellis 1985). In view of the important role of tissue mast cells in disease and defence processes of mammals, our knowledge of these cells in fish is surprisingly low, and the tissue mast cell has not as yet been implicated in any recognized defence mechanism in fish.

The purpose of the present study was to demonstrate the presence of mast cells in tissues of the salmon by use of classical histochemical techniques, and evaluate their reaction to compound 48/80 and suspensions of formalin-inactivated pathogenic bacteria.

MATERIALS AND METHODS

Observations were made on tissues from 65 Atlantic salmon *Salmo salar*. Young individuals, which were

in the process of parr/smolt transformation, with body weights of 30 to 50 g, were removed from their freshwater holding tank (water temperatures of 7 to 10°C) and killed by a blow to the head. The ventral aorta was cut, the abdominal cavity opened and the swimbladder pulled out with a forceps. Stretched preparations from the swimbladder were made and immediately submerged in absolute ethyl alcohol, 10% phosphate-buffered formalin (pH 7.2) or 10% formol-alcohol (concentrated formalin dissolved in ethyl alcohol, 1:9) for fixation. For comparison, stretched preparations from the cheek pouch of golden hamster *Mesocricetus auratus* were fixed in similar solutions. The hamsters were anaesthetized with pentobarbital sodium (25 µg g^{-1} body weight) and killed by bleeding immediately before removal of the cheek pouch.

Air-drying of fresh preparations before staining (see below) was also tried in tissue from both cheek pouch and swimbladder.

All preparations were stained with a 0.1% solution of thionin (pH 4.1) in 80% ethyl alcohol, rinsed in alcohol, coverslipped with Eukitt and observed under the microscope.

In addition, stretched preparations from swimbladder that were fixed in 10% formalin were subjected to standard procedures for paraffin wax embedment, and stained with haematoxylin and eosin.

Vital staining of mast cells was performed by applying a 0.1% solution of toluidine blue in physiological saline on fresh tissue spreads.

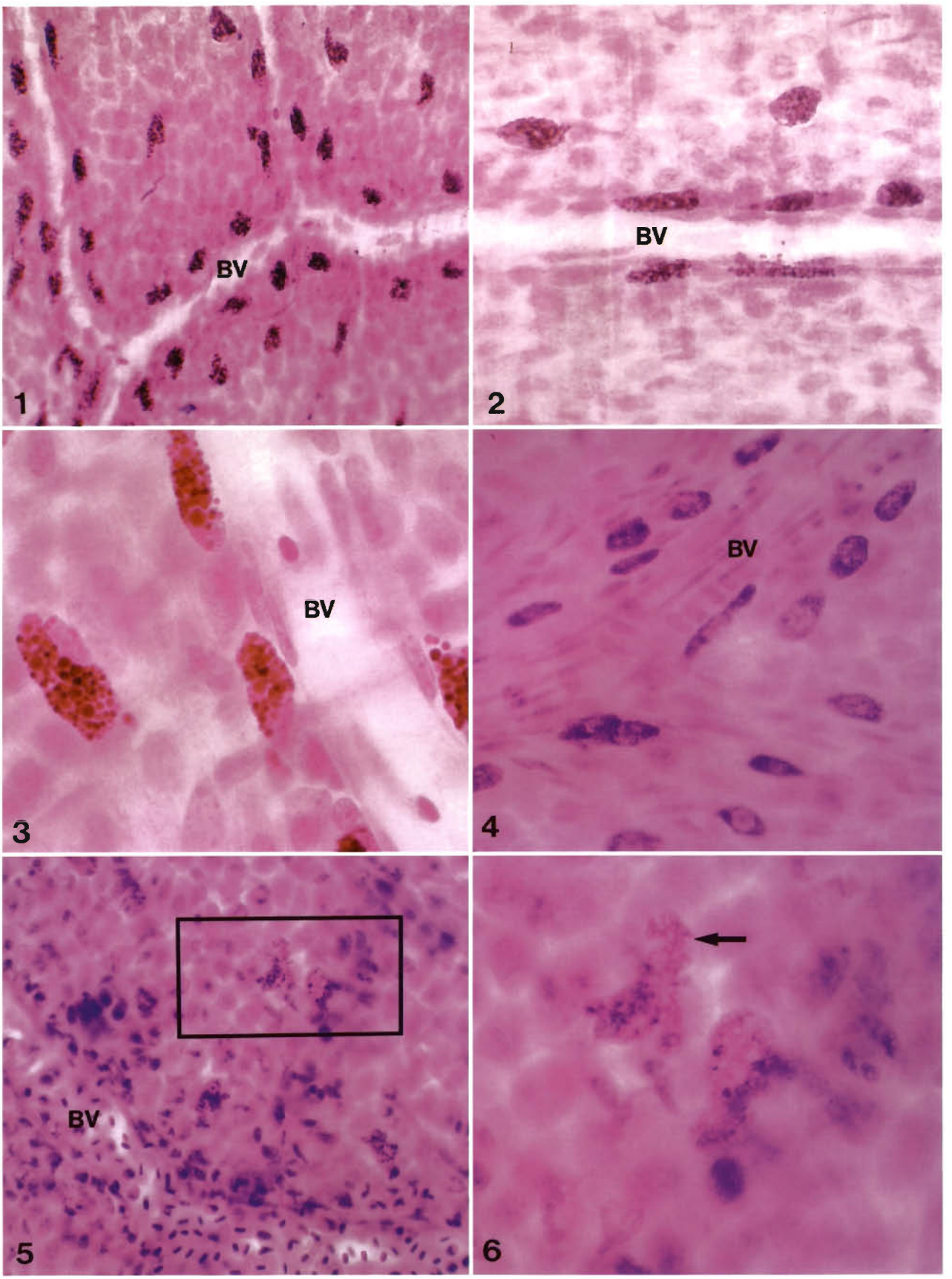
Observations were also made on the effects of intraperitoneal administration of the synthetic polyamine compound 48/80, a mast cell degranulating agent (Paton 1957), and formalin-inactivated suspensions of *Aeromonas salmonicida* subsp. *salmonicida*, on the tissue mast cells of the swimbladder. Bacterial suspensions were washed by centrifugation in sterile phosphate-buffered saline after inactivation and resuspended in the same solution at a concentration of 10^{10} cfu ml^{-1}, which were tested by intraperitoneal injections of 0.2 ml. Compound 48/80 (Sigma), dissolved in physiological saline (1.5 mg ml^{-1}), was tested at a dosage of 8 µg g^{-1} body weight. In both cases, injections of similar volumes of saline were given to fish used as controls. All intraperitoneal injections were performed under tricaine methanesulfonate anaesthesia (MS 222, 70 mg l^{-1}).

RESULTS

During observations on alcohol-fixed tissue spreads from the swimbladder stained with thionin, numerous mast cells were revealed through the metachromatic staining of their cytoplasmic granules. The presence of mast cells in rows along blood vessels was particularly striking (Fig. 1). Surrounding tissues took a pale blue colour. Along the smallest blood vessels the mast cells had an elongated form (Fig. 2), whereas at some distance from the blood vessels the cells had round or oval cell body. Distinct cytoplasmic protrusions were seen in some of the cells. Inside the cells the granules were occasionally distorted and the cells showed signs of vacuolization. Otherwise the granules were relatively uniform in size, densely packed in the cytoplasm and stained in a deep purple-red tone, i.e. β-chromatic metachromasia (Fig. 3). A few cells with sparse granulation tended to show a more red colour. The nucleus of most mast cells had an eccentric location. In the elongated cells, the nucleus was often found near one of their ends. There were marked local variations in distribution of mast cells within the swimbladder, some areas showing very few cells.

The mast cells in the cheek pouch of the hamster showed striking similarities to those of the salmon swimbladder with respect to staining properties, and location in relation to the blood vessels, but the nucleus of the hamster mast cell was usually found near the centre of the cell (Fig. 4). All preparations had to be handled with great care before fixation to preserve the mast cells intact. Air-drying of preparations from the Atlantic salmon before staining with thionin led to swelling and vacuolization of the mast cell granules, with remnants of metachromatically stained granular material concentrated near the periphery of the vacuoles. Similarly stained mast cells in air-dried preparations from the hamster cheek pouch also showed signs of degranulation, but were far more resistant than those in the swimbladder.

Figs. 1 to 6. *Salmo salar* and *Mesocricetus auratus*. Atlantic salmon swimbladder (Figs. 1 to 3, 5 & 6) and golden hamster cheek pouch (Fig. 4), tissue spread. Alcoholic thionin. Fig. 1. Mast cells located in the vicinity of the microvasculature. BV: blood vessel. ×333. Fig. 2. Elongated mast cells adjacent to small blood vessels (BV). ×533. Fig. 3. Note the individual mast cell granules. ×840. Fig. 4. Golden hamster cheek pouch. Note similarity in mast cell distribution when compared to Figs. 1 & 2. Some mast cells out of focus due to thickness of preparation. ×533. Fig. 5. Inflamed tissue following intraperitoneal injection of formalin-inactivated suspension of *Aeromonas salmonicida*, 48 h post injection. Note haemoconcentration in the small blood vessel (BV) compared to Figs. 1 to 3. Detail in frame shown in Fig. 6. ×333. Fig. 6. Detail from Fig. 5 showing vacuolization and disappearance of granular material from mast cells (arrow). ×840


BV
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Vital staining with toluidine blue revealed presence of mast cells in the hamster as well as in the salmon, but in the latter species tissue mast cells appeared to be considerably less numerous in vitally stained preparations compared to alcohol-fixed ones. The cytoplasmic granules of the mast cells developed their metachromatic staining reaction within a few minutes after application of toluidine blue. However, in the salmon the granules showed rapid swelling and vacuolization. In the course of a period of about 45 min, the metachromatically stained granular material seemed to become dissolved. The remnants of the mast cell often looked like a cluster of vacuoles.

Exposure of tissue spreads of the salmon swimbladder to watery fixatives (10% formalin) caused disappearance of the ability of the mast cells to stain metachromatically with alcoholic thionin. Granular material dissolved, as apparent from a metachromatic halo around the cell border. After 30 min, only a few of the mast cells retained their characteristic staining reaction, and after 1 h not a single mast cell could be identified. The process of loss of metachromatic dye-binding material showed resemblance to the process already described in fresh tissue spreads stained with toluidine blue. When exposed to 10% formol-alcohol, swimbladder tissue spreads also lost their ability to stain metachromatically with alcoholic thionin, but this process was slower, single mast cells were occasionally found after 2 h. The mast cells of the hamster cheek pouch retained their staining characteristics with alcoholic thionin after fixation for 24 h in watery as well as alcoholic solutions of formalin.

Formalin-containing fixatives did not seem to be destructive to the cellular structures of the swimbladder mast cells when evaluated light microscopically. After staining with alcoholic thionin the cells now appeared as if their slightly basophilic cytoplasm were occupied by numerous colourless vacuoles, constituting the remnants of the granules of the mast cell. Even after previous alcohol fixation and staining with alcoholic thionin, watery fixatives removed the metachromatic dye-binding components from the mast cell granules.

Samples from the swimbladder fixed in 10% formalin, embedded in paraffin wax and stained with haematoxylin and eosin showed numerous cells answering to the description of the eosinophilic granular cell (Roberts et al. 1971). The location of these cells, as well as their structure, was analogous to that described for mast cells in alcohol-fixed preparations stained with alcoholic thionin.

No effects on the mast cells could be demonstrated in alcohol-fixed, thionin-stained swimbladder tissue spreads from Atlantic salmon killed 24 and 48 h after injection of compound 48/80 and control injections of saline. In fish killed 2 h after injection of compound 48/80 release of granular material from the mast cell was evident, but absent in control fish. The changes partly resembled the process described for mast cells after vital staining with toluidine blue or exposure of tissue spreads to aqueous solutions of formalin, with the formation of cytoplasmic vacuoles. Fusion of 2 or more adjacent granules occurred, and there seemed to be a release of granular material to the cytoplasm, giving it a red colour which made it difficult to distinguish the contour of the individual granules of the mast cell. However, there was one marked difference from the responses described after exposure of fresh swimbladder tissue spreads to formalin or toluidine blue: intact granules were released to the surroundings of the cells. These granules apparently lost their metachromatic dye-binding material outside the cell, some of them appearing as colourless vacuoles, some with partly retained metachromatic staining properties, and some indistinguishable from intact granules inside unaffected mast cells.

In ethanol-fixed and thionin-stained preparations from Atlantic salmon injected with formalin-inactivated culture of *Aeromonas salmonicida*, the mast cells, particularly those in the vicinity of small blood vessels, also showed degranulation (Figs. 5 & 6). Furthermore, an ongoing inflammatory reaction was evident, which in addition to release of granular material from the mast cells also showed aggregation of red blood cells, partial stasis and accumulation of leucocytes in the microvasculature of the swimbladder (Fig. 5). The signs of inflammation were more extensive at 48 than at 24 h after injection of the formalin-inactivated bacteria.

DISCUSSION

The present study has demonstrated the presence of mast cells, defined as connective tissue cells containing granules that stain metachromatically with certain cationic dyes (Selye 1965), in tissue spreads from the swimbladder of Atlantic salmon.

As already mentioned, there has been much confusion regarding the existence of mast cells in fish tissues (Ellis 1982). Early investigators (Michels 1923) strongly stressed that the granules of mast cells in fish are extremely sensitive to watery solutions, as also shown in the present study. Recent studies applying a variety of fixatives and stains have demonstrated mast cells only occasionally (Roberts et al. 1971, Chiarini-Garcia & Ferreira 1992). Nevertheless, it should be noted that all investigators applying alcoholic solutions for fixation and staining have found mast cells to be present in teleosts (Michels 1923, Duthie 1939, Reite 1965, 1969a). A report on the identification of tissue mast cells in salmonids claimed that these cells, termed 'basophile

(mast) cells', can be identified in tissue specimens fixed in several of cross-linking and precipitating fixatives (Bolton 1933). The definition of the tissue mast cell used in the referred study is, however, different from standard definitions (Selye 1965), metachromasia not being required.

In addition to general species variations in mast cell distribution and the fact that mast cells undergo maturation, i.e. a gradual development from their precursors, variation in morphological, biochemical, and functional characteristics of mature mast cells derived from different anatomical sites has been reported in mammalian species (Huntley 1992). Maximow (1906) was probably the first to recognize that certain mast cells in the intestinal mucosa of rats were atypical in that their staining characteristics differed from those of the mast cells in other anatomical sites. Enerbäck (1966a, b) extended these observations and defined conditions of fixation and staining which discriminated between 'typical' and 'atypical' mast cells. The 'atypical' mast cells, now termed mucosal mast cells, were unusually susceptible to fixation and differed in dye-binding properties from the 'typical' ones, the connective tissue mast cells. Regarding studies on tissue mast cells in fish the observations cited above are important. The observations illustrate that differences, with regard to staining properties, between mast cells from phylogenetically distant species as well as differences between mast cells within a certain species must be expected. Mast cells of the Atlantic salmon swimbladder resemble mammalian mucosal mast cells, both in their staining properties and their susceptibility to fixatives. The glycosaminoglycan component of the granules of mucosal mast cells, which is responsible for the specific metachromatic dye-binding, can become extracted during fixation, or the dye-binding groups blocked by aldehydes (Wingren & Enerbäck 1983).

There is uncertainty as to whether or not the mast cells function as a storage site for histamine in fish (Ellis 1982), despite the fact that several previous studies have conclusively demonstrated that most tissues of fish (lungfish is an exception) contain very low levels of histamine (Reite 1965, 1969a, b, Takaya 1969, Chiu & Lagunoff 1972, Lorenz et al. 1973). By comparing 2 tissues, rainbow trout swimbladder and hamster cheek pouch, both rich in mast cells, the former tissue showed histamine levels of 0.2 to 0.5 $\mu g\ g^{-1}$, while the levels of the latter were 19.8 to 30.5 $\mu g\ g^{-1}$ (Reite 1969a). However, the term 'histaminocyte' as proposed for the mast cell (Riley 1953, Werle & Amann 1956) may have caused confusion.

The mast cells of the swimbladder of Atlantic salmon showed no morphological signs of degranulation 24 and 48 h after injection of the histamine releasing agent compound 48/80. It should be noted, however, that with the great number of granules present in the mast cells, the loss of a few granules could easily escape detection. Recovery may be rapid, however. Intestinal mucosal mast cells of the rat, different from its connective tissue mast cells, are resistant to 48/80 (Pearce et al. 1985). That the tissue mast cells of fish, like those of mammals, are involved in inflammatory reactions became evident after injection of formalin-inactivated *Aeromonas salmonicida* in the Atlantic salmon. The mast cells of the swimbladder in these fish were highly affected.

Ample evidence exists that stores of preformed histamine are not required in the inflammatory process. Mammals depleted of their mast cell stores of histamine by means of compound 48/80 can still display acute inflammation (Spector & Willoughby 1963). Only the rapid phase of the process, occurring within seconds or a few minutes after tissue injury and involving vascular dilatation and increased permeability produced by released histamine, is lacking. However, these phenomena are induced at a somewhat slower rate through other mechanisms, including mast cell products other than histamine. Powerful effects of histamine on vascular smooth muscle appeared late in vertebrate phylogeny, and are absent in fish (except the lungfish) and amphibians (Reite 1969b, c, 1970). Slight vascular effects of histamine combined with low tissue levels makes histamine an unsuitable condidate for a role as mediator in the acute phase of the inflammatory reaction in fish.

Since heterogeneity of mast cells to a large extent is linked to staining characteristics, which in turn are dependent on the nature of the mucopolysaccharide of mast cell granules, a small change in mucopolysaccharide structure or charge may cause the cell to fall outside the definition of a mast cell, but it may still retain most or all of the functional roles of mast cells. This question is highly relevant in view of recent investigations on granular cells in teleosts. Several reports (Ellis 1985, Vallejo & Ellis 1989, Powell et al. 1993) describe that eosinophilic granule cells of the rainbow trout intestine undergo degranulation in response to injection of *Aeromonas salmonicida* products. Intraperitoneal injections of formalin-inactivated *A. salmonicida* produced a similar response in the mast cells of Atlantic salmon swimbladder. The eosinophilic granule cells have a morphology similar to that of the salmonid tissue mast cells described in the present study. Furthermore, like mast cells they are claimed to contain mucopolysaccharides and proteases (Ezeasor & Stokoe 1980). Our results indicate that also in their response to intraperitoneal injections of compound 48/80, the swimbladder mast cells of salmon show close resemblance to responses previously described for eosinophilic granule cells of the rainbow trout intestine (Vallejo & Ellis 1989).

The biological armamentarium associated with the mammalian mast cell appears to have developed in discrete steps during vertebrate evolution (Chiu & Lagunoff 1972, Reite 1972). A definition of mast cells based on the structure of a single component within this armamentarium, as it is found in mammals, may be too narrow to include cells which otherwise are morphologically similar and have the same function. Mast cells and eosinophilic granule cells of fish appear to be closely related. The present results suggest that in Atlantic salmon swimbladder, removal of the metachromatic dye-binding components from the mast cells or blocking of the staining ability of these components by fixatives may alter the characteristics of these cells from those of mast cells to those of eosinophilic granule cells.

LITERATURE CITED

Bolton, L. L. (1933). Basophile (mast) cells in the alimentary canal of salmonid fishes. J. Morph. 54: 549–591

Chiarini-Garcia, H., Ferreira, R. M. A. (1992). Histochemical evidence of heparin in granular cells of *Hoplias malabaricus* Bloch. J. Fish Biol. 41: 155–157

Chiu, H., Lagunoff, D. (1972). Histochemical comparison of vertebrate mast cells. Histochem. J. 4: 135–144

Duthie, E. S. (1939). The origin, development and function of the blood cells in certain marine teleosts. Part. I. Morphology. J. Anat. 73: 396–412

Ehrlich, P. (1877). Beiträge zur Kenntnis der Anilinfärbungen und ihrer Verwendung in der mikroskopischen Technik. Arch. mikroskop. Anat. 13: 263–277

Ehrlich, P. (1879). Beiträge zur Kenntnis der granulierten Bindegewebszellen und der eosinophilen Leukocyten. Arch. Anat. Physiol., Physiol. Abt. 3: 155–169

Ellis, A. E. (1982). Histamine, mast cells and hypersensitivity responses in fish. Dev. comp. Immunol. Suppl. 2: 147–155

Ellis, A. E. (1985). Eosinophilic granular cells (EGC) and histamine responses to *Aeromonas salmonicida* toxins in rainbow trout. Dev. comp. Immunol. 9: 251–260

Ellis, A. E., Roberts, R. J., Tytler, P. (1989). The anatomy and physiology of teleosts. In: Roberts, R. J (ed.) Fish pathology. Baillière Tindall, London, p. 13–55

Enerbäck, L. (1966a). Mast cells in the rat gastrointestinal mucosa. I. Effect of fixation. Acta pathol. microbiol. scand. 66: 289–302

Enerbäck, L. (1966b). Mast cells in the rat gastrointestinal mucosa. II. Dye binding and metachromatic properties. Acta pathol. microbiol. scand. 66: 303–312

Ezeasor, D. N., Stokoe, W. M. (1980). A cytochemical, light and electron microscopic study of the eosinophilic granule cells in the gut of the rainbow trout, *Salmo gairdneri* Richardson. J. Fish Biol. 17: 619–634

Huntley, J. F. (1992). Mast cells and basophils: a review of their heterogeneity and function. J. comp. Pathol. 107: 349–372

Lorenz, W., Matejka, E., Schmal, A., Seidel, W., Reimann, H.-J., Uhlig, R., Mann, G. (1973). A phylogenetic study on the occurrence and distribution of histamine in the gastrointestinal tract and other tissues of man and various animals. Comp. gen. Pharmacol. 4: 229–250

Maximow, A. (1906). Über die Zellformen des lockeren Bindegewebes. Arch. mikrosk. Anat. Entv. Mech. (Bonn) 67: 680–756

Michels, N. A. (1923). The mast cell in the lower vertebrates. Cellule 33: 338–462

Michels, N. A. (1938). The mast cells. In: Downey, H. (ed.) Handbook of haematology, Vol. I. Hoeber, New York, p. 232–354

Paton, W. D. M. (1957). Histamine release by compounds of simple chemical structure. Pharmacol. Rev. 9: 269–328

Pearce, F. L. (1989). Mast cells: function, differentiation and activation. Curr. Opin. Immunol. 1: 630–636

Pearce, F. L., Ali, H., Barrett, K. E., Befus, A. D., Bienenstock, J., Brostoff, J., Ennis, M., Flint, K. C., Hudspith, B., Johnson, N. M., Leung, K. B. P., Peachell, P. T (1985). Functional characteristics of mucosal and connective tissue mast cells of man, rat and other animals. Int. Arch. Allergy appl. Immunol. 77: 274–276

Powell, M. D., Briand, H. A., Wright, G. M., Burka, J. F. (1993). Rainbow trout (*Oncorhynchus mykiss* Walbaum) intestinal eosinophilic granule cell (EGC) response to *Aeromonas salmonicida* and *Vibrio anguillarum* extracellular products. Fish Shellfish Immunol. 3: 279–289

Reite, O. B. (1965). A phylogenetical approach to the functional significance of tissue mast cell histamine. Nature 206: 1334–1336

Reite, O. B. (1969a). Phylogenetical persistence of the non-mast cell histamine stores of the digestive tract: a comparison with mast cell histamine. Experientia 25: 276–277

Reite, O. B. (1969b). The evolution of vascular smooth muscle responses to histamine and 5-hydroxytryptamine. I. Occurrence of stimulatory actions in fish. Acta physiol. scand. 75: 221–239

Reite, O. B. (1969c). The evolution of vascular smooth muscle response to histamine and 5-hydroxytryptamine. II. Appearance of inhibitory actions of 5-hydroxytryptamine in amphibians. Acta physiol. scand. 77: 36–51

Reite, O. B. (1970). The evolution of vascular smooth muscle response to histamine and 5-hydroxytryptamine. III. Manifestation of dual action of either amine in reptiles. Acta physiol. scand. 78: 213–231

Reite, O. B. (1972). Comparative physiology of histamine. Physiol. Rev. 52: 778–819

Riley, J. F. (1953). Histamine in tissue mast cells. Science 118: 332–333

Roberts, R. J., Young, H., Milne, J. A. (1971). Studies on the skin of plaice (*Pleuronectes platessa* L.). I. The structure and ultrastructure of normal plaice skin. J. Fish. Biol. 4: 87–98

Selye, H. (1965). The mast cells. Butterworths, London

Spector, W. G., Willoughby, D. A. (1963). The inflammatory response. Bact. Rev. 27: 117–154

Takaya, K. (1969). The relationship between mast cells and histamine in phylogeny with special reference to reptiles and birds. Arch. histol. jap. 30: 401–420

Vallejo, A. N., Ellis, A. E. (1989). Ultrastructural study of the response of eosinophil granule cells to *Aeromonas salonicida* extracellular products and histamine liberators in rainbow trout *Salmo gairdneri* Richardson. Dev. comp. Immunol. 13: 133–148

Werle, A., Amann, R. (1956). Zur Physiologie der Mastzellen als Träger des Heparins und Histamins. Klin. Wochenschr. 34: 624–630

Wingren, U., Enerbäck, L. (1983). Mucosal mast cells of the rat intestine: a re-evaluation of fixation and staining properties, with special reference to protein blocking and solubility of the granular glycosaminoglycan. Histochem. J. 15: 571–582

Responsible Subject Editor: C. B. Schreck, Corvallis, Oregon, USA

Manuscript first received: December 21, 1993
Revised version accepted: June 17, 1994