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Histamine immunohistochemistry is superior to the conventional heparin-based routine staining methodology for investigations of human skin mast cells

OLLE JOHANSSON[1], MARKKU VIRTANEN[1,2], MARITA HILLIGES[1] and QINER YANG[1]

[1]*Experimental Dermatology Unit, Department of Neuroscience, Karolinska Institute, 17177 Stockholm, Sweden, and* [2]*Department of Psychology, Uppsala University, Uppsala, Sweden*

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Summary

Conventional studies of mast cells are limited by methodological restrictions such as a selective fixative-dependent routine staining blockage. This is thought to depend on the biochemical differences of the mast cell granule contents suggesting a cellular heterogeneity. Investigations of human mast cells, using routine methods, also suffer from the problem of a low signal-to-noise ratio.

In the present study, normal human skin was used to compare an immunohistochemical method for histamine with two recommended mast-cell fixatives and a new commercial fixative in combination with three routine stains. Mast cells were found throughout the dermis with all the routine stains used. However, immunohistochemistry gave profoundly better results. Small structures, such as thin cytoplasmatic extensions and single granules, were readily detectable. Double-staining (immunohistochemistry followed by routine staining) revealed differences in staining capacity. All immunoreactive cells were not stained by routine stains and sometimes the opposite was also seen. This supports earlier reported evidence of heterogeneity, not only between skin and intestinal mast cells but also among skin mast cells themselves. Furthermore, by focusing on histamine, instead of heparin, we probably overcame the problems of the selective fixative-dependent routine staining blockage. Finally, the immunofluorescence technique provides a high signal-to-noise ratio and is an excellent method for making high-quality microphotographs of human mast cells.

In conclusion, we have found histamine immunohistochemistry (a) to be easy to perform, (b) to show cytoplasmic details better of the, sometimes, dendritic-type mast cells, (c) to result in a higher signal-to-noise ratio, i.e. a better detectability, resulting in a higher number of cells being evident, and (d) to reveal the presence of histamine, instead of heparin, thus being more relevant to all kinds of histamine-related scientific endeavours. However, routine methods occasionally revealed single cells not visualized by the histamine immunohistochemistry.

Introduction

The mast cell is a characteristic granular tissue cell that can be found especially along the epithelial linings of the body facing the external environment, such as the skin, and the respiratory and gastrointestinal tracts. Mast cells are known to be involved in a variety of physiological and pathological conditions such as immediate hypersensitivity (Ishizaka & Ishizaka, 1984), delayed hypersensitivity (Askenase & Van Loveren, 1983; Galli & Dvorak, 1984), cell growth regulation (Persinger *et al.*, 1983; Marks *et al.*, 1986), neoplasia defense (Henderson *et al.*, 1981), and the sensations of pain and itch (Keele, 1970). Elevated numbers of mast cells in the skin are found in different forms of urticaria (Juhlin, 1979), keloids (Kischer *et al.*, 1978), and scleroderma (Claman, 1985).

The first papers on mast cells were written by the legendary Paul Ehrlich in 1877 and 1879. In these, he described the characteristic metachromatic reaction of these cells when stained histologically with basic aniline dyes. Since then this method has been the one mostly used for studying mast cells in both basic science and in

clinical applications. The metachromatic reaction is known to occur between the basic dye and acidic proteoglycans (Holmgren & Wilander, 1937). The major proteoglycan component in cutaneous mast cells is the glycosaminoglycan heparin (Metcalfe *et al.*, 1980), mast cells being the major bodily source of this glycoconjugate (cf. Church *et al.*, 1989), whereas mast cells of the gastrointestinal tract contain mostly chondroitin sulphate (Eliakim *et al.*, 1986).

Mast cells also contain histamine (Riley & West, 1953). Histamine is known to be located in the mast cell granulae (Uvnäs, 1967), where anionic histamine is linked to the cationic carboxylated group of heparin (Uvnäs *et al.*, 1970). There are also reports of skin histamine outside mast cells, in endothelial cells (El-Ackad & Brody, 1975), and in epidermal cells (Søndergaard & Zachariae, 1968).

An immunohistochemical method for histamine has been described by Panula and colleagues (Panula *et al.*, 1984). This was originally developed for studies of histaminergic neurons in the central nervous system, but recently we have shown that it is also an excellent method for dermatological purposes (Johansson *et al.*, 1992), since it gives a very distinct labelling of mast cells. In the present investigation, we wanted to investigate whether histamine immunohistochemistry has any advantages over routine stains when it comes to performance, sensitivity, detectability, reproducibility, and evaluative capacity. In order to compare the recommended routine methods for mast cells and the indirect immunofluorescence technique, we used routine and immunohistochemical methods as well as a double-staining approach combining these two methods.

Materials and methods

Tissue from normal human skin was sampled from healthy volunteers (medical and dental students). Routine punch biopsies (3 mm) were taken under local anaesthesia (Xylocaine 20 mg ml^{-1}; Astra, Sweden). Two adjacent biopsies were taken from each forearm. One of the biopsies was then fixed in 4% carbodiimide (1-ethyl-3-3-dimethylaminopropyl-carbodiimide; Sigma Chem. Comp., USA), diluted in a phosphate buffer (pH 7.4), for 2 h at 4°C (Panula *et al.*, 1988). The other biopsy was fixed in one of the recommended mast cell fixatives: IFAA (isotonic formalin–acetic acid) = 0.6% formaldehyde–0.5% acetic acid, for 24 h at 4°C (Enerbäck, 1966; Enerback *et al.*, 1986); Carnoy's fluid 60:30:10 by vol. = absolute ethanol–chloroform–glacial acetic acid for 24 h at 4°C (Romeis, 1948); or in a new commercial fixative, OmniFix = 98% water + alcohols (ethanol, methanol) and 2% inert polymers (Zymed Lab. Inc., USA) for 2 h at 4°C with a postfixation in 70% ethanol for 20 h at 4°C. After fixation, all tissues were sectioned on a Microm cryostat to yield 5 or 14 μm-thick sections. The glass slides were coated with chromalum:gelatin (0.5 g:5 g l^{-1}).

The methods used for routine staining were: Toluidine Blue (0.5%), pH 0.5, for 30 min, rinse 3 × 5 min in water (cf. Olafsson *et al.*, 1986); Astra Blue (1%), pH 0.3, for 30 min, rinse 3 × 5 min in 0.7 N HCl, followed by 3 × 5 min in water (cf. Strobel *et al.*, 1981); Alcian Blue (1%)–Safranine (0.5%), pH 1.0, for 30 min, rinse 3 × 5 min in water (cf. Markey *et al.*, 1989). All stainings were performed at room temperature. The routine-stained sections were mounted either directly in phosphate-buffered saline (PBS):glycerine (1:4 v/v) or, after dehydration, in Entellan (Merck, Germany).

For immunohistochemistry, the indirect procedure (Coons, 1958) was employed. Before antibody incubation, all sections were rinsed in PBS. Thereafter, the primary rabbit histamine antiserum (1:2000; Milab AB, Sweden) was applied and the sections were incubated overnight at 4°C in a humid atmosphere. They were then rinsed in PBS and incubated with the secondary antiserum (1:80; Tetramethylrhodamine-isothiocyanate isomer R (TRITC)-conjugated goat anti-rabbit IgG; Boehringer-Mannheim, Germany). All antisera were diluted in 0.3% Triton X-100 (Hartman *et al.*, 1972). The mounting medium for the sections was 0.1% *p*-phenylenediamine (to prevent fading; Johnson & Nogueira Araujo, 1981) diluted in PBS and glycerine (1:10).

The antiserum specificity was tested with controls in which the serum was pre-adsorbed with 0.1 mM histamine dihydrochloride (Sigma Chem. Comp., USA). In this context, Milab AB has shown that the antiserum does not cross-react with noradrenalin, serotonin, vasoactive intestinal polypeptide, glucagon or histidine. Other sections were incubated with the secondary antiserum only. For further technical aspects concerning the immunohistochemistry, see Johansson (1985) and Johansson *et al.* (1992).

Observation and photography was performed by two independent observers in a Nikon Microphot-FXA microscope equipped with light- and dark-field optics.

Results

Using the routine staining methods, mast cells were found with all the fixatives tested (cf. Fig. 1). The best results were obtained with the combination of IFAA or Carnoy's fluid with Toluidine Blue. This resulted in a typical violet metachromatic staining of the mast cell granules and a pale nucleus. Alcian Blue–Safranine and Astra Blue showed similar orthochromatic results, a blue coloration of the granules. No specific red-stained granules were found in the sections stained with Alcian Blue–Safranine. The intensity of the staining was best with Toluidine Blue, followed by Alcian Blue–Safranine and Astra Blue, respectively. The carbodiimide fixative in combination with the routine stains gave equal results completely comparable to the other fixatives. However, a brownish colour of the basal and spinosal keratinocytes was seen with the carbodiimide.

Routine-stained mast cells were found in the whole dermis, but more frequently in the upper part, and also as groups of cells located close to dermal appendages, such as hair follicles, nerves, vessels and eccrine sweat glands. In the latter, mast cells were mostly observed close to the tubular coils of the glands. The general appearance of the cells was flat or ovoid-shaped, especially among the cells located adjacent to nerves and vessels. The choice of mounting media seemed crucial for

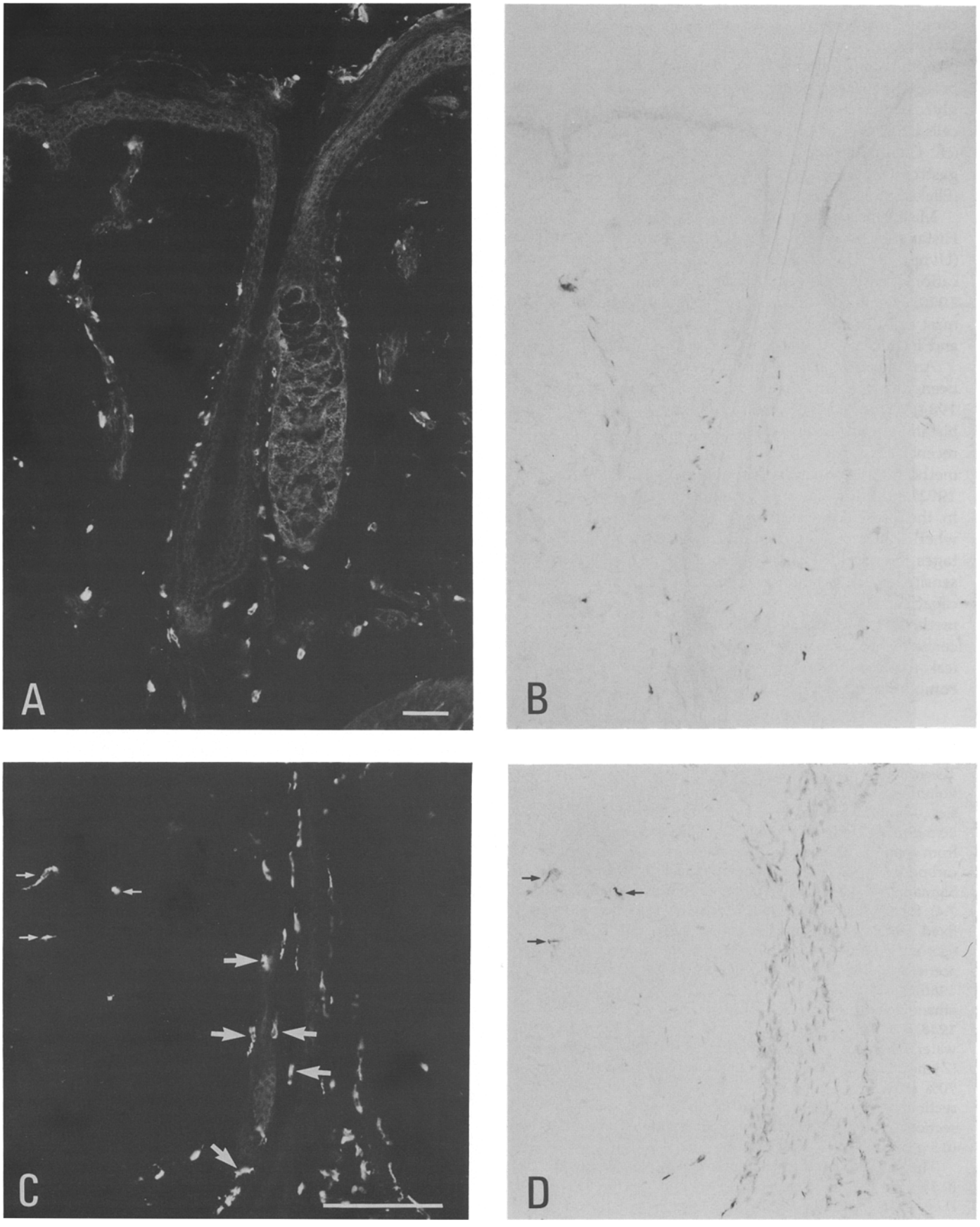

Fig. 1. (A–D) Double-staining using histamine antiserum (A) and Alcian Blue–Safranine (B) or histamine antiserum (C) and Toluidine Blue (D). Small arrows in C and D point to cells clearly seen in both types of preparation, whereas large arrows in C point to cells conclusively labelled only with the immunohistochemical approach. Specimen from male (A, B) or female (C, D) forearm. Bars = 50 μm.

the routine stain. When using *p*-phenylenediamine or glycerine:PBS, the staining disappeared completely and irreversibly within a few days.

Immunohistochemical labelling was obtained only in the carbodiimide-fixed tissues (cf. Fig. 1). The immunoreactivity had a very high signal-to-noise ratio, i.e. the detectability was optimal, and was located to the cytoplasm of the cells leaving the nucleus unlabelled. The cells were at the same locations as described above. Some cells showed a typical dendritic morphology with long cytoplasmatic processes (cf. Fig. 2). No specific immunoreactivity was found in nerves or endothelial cells. However, mast cells were sometimes seen very closely adjacent.

Control sections incubated with the secondary antiserum only or with the primary antiserum pre-adsorbed with the antigen showed no immunofluorescence.

Some of the immunohistochemically-labelled sections were later double-stained with the routine methods (Fig. 1), resulting in both meta- and orthochromatic colours, comparable to the above-mentioned routine sections. When comparing corresponding pictures of the same section, differences in immunoreactivity versus staining intensity could be observed between the immunohistochemical and the routine staining method. Cells with a strong immunofluorescence were not necessarily distinctly stained with the routine method, or vice versa. Routine-stained cells were sometimes so weakly labelled that it was difficult to judge whether they actually were specifically stained, or if it was only a background phenomenon. Occasionally, cells detectable only with routine staining methods were evident. This was, however, encountered to a much lower extent (cf. Fig. 1). Upon comparison, cellular structures, such as granules and the cytoplasmatic processes, appeared, however, much more salient with the immunohistochemical technique.

Discussion

A very distinct labelling of mast cells is obtained with the immunofluorescence method for histamine. The specific signal is high and the background is very low. The signal-to-noise ratio is, thus, definitely higher with the immunohistochemical method compared to the routine staining results of immersion-fixed specimens. The accuracy of immunohistochemistry is excellent, thus helping the subcellular morphology of mast cells to be studied. The typical dendritic appearance of some of the mast cells in our studies has not, to our knowledge, been described in papers using routine staining methods.

Recently, there has been a great interest in a possible heterogeneity of mast cells (Befus *et al.*, 1986; Irani & Schwartz, 1989). Although the vast majority of studies on this topic have been performed on rodents, there is also some evidence for differences between mast cell populations in man (Lowman *et al.*, 1988). It has long been known that mast cells of the intestinal mucosa show blocking of histological staining if they are fixed with formalin fixatives, something that skin mast cells show to a lesser degree (Enerbäck, 1966a, b). The difference in the glycosaminoglycan content could be the cause. Therefore, fixatives with very low concentrations of formaldehyde are recommended for mast cell studies (Enerback *et al.*, 1986; Olafsson *et al.*, 1986). Others recommend the

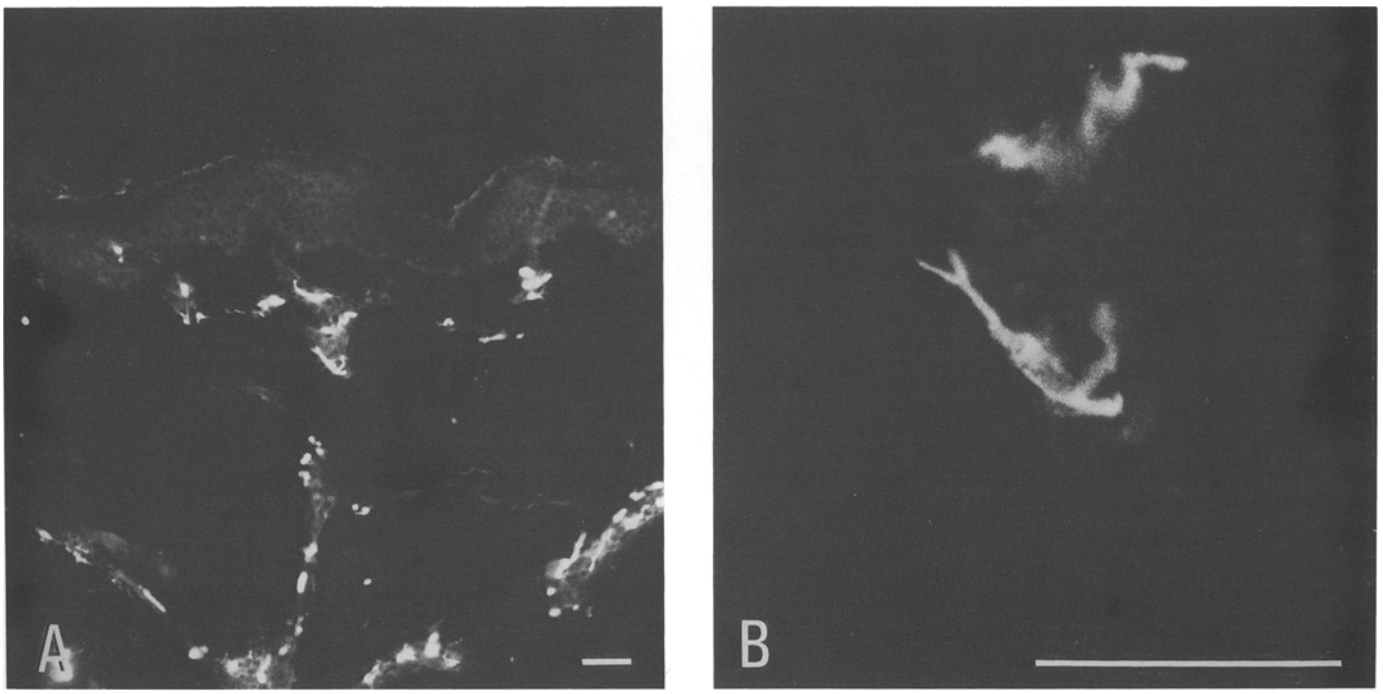

Fig. 2. (A–B) Immunofluorescence micrographs using histamine antiserum. Specimen from male forearm. Note the apparent dendritic morphology of the cells in B. Bars = 50 μm.

use of fixatives, e.g. Carnoy's or basic acetic lead, that are based on acetic acid and ethanol containing no formaldehyde (Strobel *et al.*, 1981; Marshall *et al.*, 1987; Markey *et al.*, 1989). Additional differences between mast cell types can be seen depending on the histological staining procedures. Using two different orthochromatic routine stains in combination, Alcian Blue and Safranine, Marshall and his colleagues found most dermal mast cells to have blue granules, some with both blue and red and some with only red granules (Marshall *et al.*, 1987). Whether this is a sign of a true difference in cellular biochemistry remains to be solved. A more clear evidence of a biochemically-based heterogeneity between different possible subpopulations comes from immunocytochemical studies on the proteases of mast cell granules (Irani *et al.*, 1986). Lung mast cells mainly contain tryptase (Schwartz *et al.*, 1981), whereas mast cells of skin are found to contain both tryptase and chymase. Quantitative estimates show that the chymase content is generally lower than tryptase, but the chymase content of adult skin mast cells is 25-fold larger than those of lung (Schwartz *et al.*, 1987). The present study also supports the idea of heterogeneity among mast cells, not only between skin and intestinal populations, but also within the skin mast cell population. Our results suggest that the use of routine stains for counting procedures could lead to an underestimation of the cell number and that histamine immunohistochemistry fulfils the task better when the total mast cell number is wanted. From the above, it is evident that, when carrying out or evaluating morphological work on mast cells, methodological problems should be considered as otherwise the findings may possibly be artefacts of the histological or histochemical technique.

It must be noted that there are considerable differences in working with human tissue as compared to the tissues of laboratory animals that are normally used. In parallel tests of rat tissue, we found that their mast cells are more easily stained using routine staining methods than human cells, the latter expressing a large variability in staining ability. This variability was, however, not seen using immunohistochemistry. From the perspective of the intra- and interspecies heterogeneities and the obvious limitations of the common investigation methods, a warning must be raised about transferring results regarding mast cells between species, tissues, methods, physiology and pathology.

It is possible that the problem of selective staining blockade by fixative-dependent routines of intestinal mast cell populations is eliminated when focusing on histamine instead of heparin. It could, consequently, be a way out of the heterogeneity problem when a total estimate of mast cells is wanted. This must, of course, be investigated in future comparative gastrointestinal research projects.

The only working fixative for histamine immunohistochemistry is carbodiimide (1-ethyl-3-3-dimethylaminopropyl-carbodiimide; cf. Panula *et al.*, 1988; Johansson *et al.*, 1992. It should, of course, be remembered that other histamine antibodies could work with other fixatives (Håkanson *et al.*, 1986)). The carbodiimide fixative does not result in a lower cell number using routine stains as compared with the best conventional fixatives. Carbodiimide induces a new peptide bond between free carboxyl and amino groups, without further changing the molecular structure. Formaldehyde, on the other hand, is thought to cross-link the terminal amino groups of proteins. Its possible interaction with the compounds of mast cell granules could be either between the proteoglycans or proteases, or with the histamine molecule itself. The lack of an immune reaction with this and the other fixatives is probably due to an altered antigenicity, undetectable by the antibodies.

Finally, one interesting trend is worth comment. When looking into the dermatological literature on mast cells, we were – as morphologists – surprised by the lack of pictures. The situation is similar even when consulting the leading textbooks in the field. The microphotographs sometimes seen are usually based on electron microscopy, and are in many ways excellent and illustrative, but the much more easily accessible appearances seen by light microscopy are less evident in the literature. Are mast cells today believed to be so well known and completely unfolded that they no longer are worth taking pictures of? Or are the technical problems the reason? Pictorial material is especially important when one talks about normal versus pathological appearances, drug effects, treatment-induced changes, etc. Hopefully, histamine immunohistochemistry will give future investigators a better tool for examining human mast cell-related questions (including pathologies), as well as creating high-standard images of such cells. It lends itself very well to unbiased interpretations and judgements of numerical estimates, such as planimetry, morphometry or stereology. Its relative simplicity makes it possible to use at any research laboratory, and also double-labelling experiments can be performed. From many physiological and pathological points of view, histamine is also the more interesting substance of the mast cell. Therefore, we conclude that histamine immunohistochemistry should be favoured.

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