DETECTION OF HISTAMINE IN RAT MAST CELL GRANULES BY ORCEIN-WATER BLUE STAIN

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A new method of staining histamine in rat mast cell granules in formalin fixed paraffin section was developed. Mast cells of several organs of formalin fixed paraffin sections were stained well in red with orcein-water blue as well as in purple with toluidine blue. But only mast cells in dermis showed a different appearance in orcein-water blue stain and toluidine blue stain. The results of an electrophoretic study, diamine oxidase treatment and compound 48/80 treatment of rat lead to the conclusion that the substance stained in red with orcein may be histamine. It was shown that histamine in rat mast cell granule is fixed even by formalin, a finding contrary to previous studies.

The mast cell contains many substances in its granules. Histamine is one of the principal substances in granules. The histochemical demonstration of histamine is significant in the study of mast cells. It has been considered that histamine in tissue easily dissolves out by formalin fixation, thus known staining methods require a complicated technique in fixation or staining because of the solubility of histamine (3, 4, 7).

In the present study, we obtained evidence that histamine can be detected even in formalin fixation. This article reports on the effectiveness of orcein in staining histamine in formalin fixed paraffin sections.

MATERIALS AND METHODS

Adult male Wister rats weighing 200–300 g were anesthetized with ether and exanguinated by cutting the carotid arteries, except when otherwise specified. The tongue, skin, lung, stomach and ileum were removed and fixed overnight or longer in 10% formalin. All specimens were embedded in paraffin, cut in 5 μ m sections with the microtome, deparaffinized and used for staining.

Staining methods

Orcein-water blue stain: The orcein-water blue solution was made by dissolving 0.98 g of orcein (Wako Chem. Co., Ltd., Japan) in 60 ml of 100% ethyl alcohol to which was added 1 ml of concentrated HCl mixed with 0.02 g of water blue (also Wako Co.) dissolved in 40 ml of distilled water (2). The solution was stirred thoroughly, filtrated and used for staining. Sections were treated with orcein-water blue solution for 10 min and differentiated by 60% alcohol with 1% concentrated HCl, dehydrated, cleared and mounted.

Toluidine blue stain: Sections were treated with 1% toluidine blue in 60% alcohol for 10 min, differentiated by 90% alcohol, dehydrated, cleared and mounted.

O-phthalaldehyde stain: Sections were treated with 1% *o*-phthalaldehyde in 60% alcohol with or without 1% concentrated HCl for 10 min, differentiated by 60% alcohol, dehydrated, cleared and mounted (3).

Diazotized *p*-bromoaniline stain described by Lagunoff *et al.* (4) after HCl treatment or no treatment was applied to rat sections.

Agarose gel electrophoresis: 40–100 mg of authentic histamine, heparin, chondroitin sulfate, hyaluronic acid, histidine and lysin were dissolved in 2 ml of distilled water, and serotonin was dissolved in 2 ml of 1 N HCl. Each solution was mixed with an equal amount of 1% orcein in 60% alcohol and the mixtures were electrophoresed for 1 hr in 1% agarose gel (0.05 M Veronal buffer, pH 8.6, 3 mA/cm) and stained with toluidine blue.

Oxidative deamination by diamine oxidase: A section of rat tissue was reacted with 0.1 U/ml of diamine oxidase (1) (Sigma Co., USA) in 0.012 M of sodium phosphate buffer (pH 7.2) overnight and was stained with orcein-water blue. The control was treated with the same buffer overnight and stained with orcein-water blue at the same time.

Treatment with compound 48/80: Animals were injected with compound 48/80 (6, 8) in the caudal vein (5 mg/Kg or 0.5 mg/Kg), and after death from anaphylactic reaction their tongues were removed and fixed in 10% formalin. These specimens were subjected to examination. The rats who lived after compound 48/80 injection were treated with the same process applied to non-treated rats.

RESULTS

Comparison of orcein-water blue stain with-toluidine blue stain on mast cells:

FIG. 1. Serially cut sections of the tongue of a rat stained with toluidine blue (TB) (1a) and orcein-water blue (OW) (1b); Mast cells are stained well with both stains. $\times 400$

FIG. 2. The same section of skin stained with toluidine blue (TB) (2a) after orcein-water blue stain (OW) (2b): Mast cells in dermis are stained weakly in red with orcein-water blue, but stained well with toluidine blue. $\times 400$

Serially cut sections of all the specimens were stained with orcein-water blue and toluidine blue and the mast cells were examined. It was found that mast cells in the tongue, lung, stomach and ileum stained well in red and those in dermis weakly in red with orcein-water blue. But there was no difference between dermis and other

FIG. 3. The electrophoretic pattern of the mixtures of orcein and histamine, serotonin, heparin, chondroitine sulfate, hyarulonic acid and lysin; binding of histamine and lysin with orcein is observed.

FIG. 4. The same plate as in Fig. 3. is stained with toluidine blue. Mucopolysaccharides are stained in different places than orcein.

tissues in toluidine blue stain (Figs. 1, 2).

It was considered that different substances were stained with these staining methods. The substance which was stained in red with orcein-water blue was examined by three experiments.

1. From the results of electrophoresis, it was supposed that histamine and lysin, which is oxidative deaminated by diamine oxidase, were bound to orcein (Fig. 3).

FIG. 5. A section of tongue of a rat stained with orcein-water blue after oxidative deamination: Mast cells are stained weakly in red with orcein-water blue (arrows). $\times 200$

FIG. 6. High magnification of the same section as in Fig. 5. $\times 600$

After toluidine blue staining of the gel plate, heparin, chondroitin sulfate, and hyaluronic acid run in different places than orcein (Fig. 4). The substance which was stained in red with orcein-water blue was assumed to be histamine.

2. Oxidative deamination by diamine oxidase resulted in the mast cells being poorly stained with orcein-water blue (Figs. 5, 6), although the controls were well stained (Figs. 7, 8).

3. In the tongue of rats injected with compound 48/80 (5 mg/Kg), the mast cells

FIG. 7. Control section of oxidative deamination: Mast cells are well stained with orcein-water blue. $\times 200$

FIG. 8. High magnification of the same section as in Fig. 7. $\times 600$

were not stained with orcein-water blue (Fig. 9). In those injected with compound 48/80 (0.5 mg/Kg), the mast cells were stained weakly in red and some granules strongly in red (Fig. 10). However, there was no difference between compound 48/80 injected rats and controls by toluidine blue staining.

From these data, the substance stained in red with orcein-water blue is considered to be histamine.

FIG. 9. Serially cut sections of the tongue of a rat injected with compound 48/80 (5 mg/Kg): Mast cells are not stained with orcein-water blue (OW) (9b), but stained well with toluidine blue (TB) (9a). $\times 400$

FIG. 10. Serially cut sections of the tongue of a rat injected with compound 48/80 (0.5 mg/Kg): Mast cells are stained weakly in red except some granules with orcein-water blue (OW) (10b), but stained well with toluidine blue (TB) (10a). $\times 400$

Effectiveness of HCl on orcein-water blue stain and other histamine staining methods:

Orcein-water blue stain without HCl poorly stained the mast cells.

Mast cells were stained well in yellow fluorochrome with *o*-phthalaldehyde

FIG. 11. *O*-phthalaldehyde stain containing 1% concentrated HCl: Mast cells are well stained. $\times 400$

FIG. 12. *O*-phthalaldehyde stain without HCl: Mast cells are poorly stained except one. $\times 400$

stain containing 1% concentrated HCl (Fig. 11), but were poorly stained without HCl (Fig. 12).

In diazotized *p*-bromoaniline stain, mast cells were not so well stained with or without HCl.

DISCUSSION

In the present study, we showed evidence that histamine in the rat mast cell granule is fixed by formalin and stained with orcein and *o*-phthalaldehyde with HCl treatment. Schauer and Werle used Reinecke salt solution to precipitate histamine in rhombic crystals (7). Lagunoff, Phillips and Benditt fixed frozen and dried tissues with formaldehyde vapor and stained with diazotized *p*-bromoaniline (4). Juhlin and Shelley employed *o*-phthalaldehyde, and its complex with histamine formed yellow fluorochrome in fresh or frozen specimens (3). It has been considered impossible to fix histamine in tissue by formalin, because histamine is freely soluble in water. However, it may be possible to fix histamine in rat mast cell granules by formalin from the results obtained in this study.

The amino group of histamine might play a role in binding with orcein, because the mast cell granules were poorly stained with orcein-water blue after oxidative deamination by diamine oxidase.

Mast cell granules were stained with only orcein, but it was difficult to distinguish the red granules in the brown background. Hence water blue was used to stain the background in pale blue and it became easier to observe the red granules.

Mast cells in formalin fixed paraffin section were reported to be poorly stained with *o*-phthalaldehyde stain or diazotized *p*-bromoaniline stain (3, 4). But, using HCl in *o*-phthalaldehyde stain, we found mast cells were stained well in yellow fluorochrome. And orcein-water blue stain without HCl was a poor stain of mast

Fig. 13. Reaction between histamine and formaldehyde (reference 5).

cells. From these different staining results, it is considered that histamine complex is formed by formalin fixation. If the complex is hydrolysed by HCl, it becomes possible to stain it with dyes. The complex is presumed to make Schiff's base (5) which is made from the amino group of histamine and the aldehyde group of formaldehyde (Fig. 13).

Mast cells were not well stained with diazotized *p*-bromoaniline even after HCl treatment. The poor staining is presumably due to other substances than histamine.

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