

Short communication

Carnoy's fixative reduces the number of chymase-positive cells in immunocytochemical staining of cord-blood-derived human cultured mast cells

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KleinJan et al. (*Allergy* 1996;51:614-20) reported that Carnoy's fixative reduced the number of chymase-positive mast cells in the nasal mucosa. Therefore, in the present study, we investigated whether Carnoy's fixative reduces the number of chymase-positive cells from cord-blood-derived human cultured mast cells when compared with other types of fixatives. Human mast cells were obtained by culturing cord-blood-derived CD34-positive cells in the presence of stem cell factor and interleukin-6. Staining procedures of the cells in fixation with Carnoy's fixative and with other fixatives gave no differences among the number of tryptase-positive cells, whereas fixation with Carnoy's fixative for 15 min gave a significant decrease in the number of chymase-positive cells compared with acetone for 10 min. The number of chymase-positive cells decreased in a time-dependent manner under fixation with Carnoy's fixative, indicating that Carnoy's fixative had a negative effect on the number of chymase-positive cells from cord-blood-derived human cultured mast cells.

**N. Kanbe¹, M. Kurosawa¹,
Y. Miyachi¹, M. Kanbe²,
D. Kempuraj³, H. Tachimoto³,
H. Saito³**

¹Department of Dermatology, and ²Second Department of Pathology, Gunma University School of Medicine, Maebashi; ³Division of Allergy, National Children's Medical Research Center, Tokyo, Japan

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Motohiro Kurosawa, MD, PhD, Pharm D
Department of Dermatology
Gunma University School of Medicine
3-39-22 Showa-machi
Maebashi 371-8511
Japan

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Recent advances in studies on mast cells led us to obtain human cultured mast cells from cord-blood-derived progenitor cells in the presence of stem cell factor (SCF) and interleukin-6 (IL-6) (1-4). The advantage is that a relatively large number of the cells are accessible, and that degeneration which may occur during the purification process from surgical specimens can be avoided. In fact, we have shown that these cells were functionally matured, containing about 9 pg of histamine per cell, and had functional IgE receptors (1, 3). Thus, they may contribute a great deal to mast-cell research and could be a useful model of human mast cells *in vitro*.

On the other hand, the heterogeneity of mast cells is recognized in the tissues (5, 6). Irani et al. (7, 8) developed highly effective monoclonal antibodies (mAbs) against tryptase or chymase, and

subdivided human mast cells into at least two types on the basis of distinct neutral protease compositions. MC_T, which is considered analogous to the mucosal type in rodent mast cells, contains only tryptase, while MC_{TC}, analogous to the connective-tissue type, contains both tryptase and chymase. After introduction of these mAbs, many investigators used Carnoy's fixative to stain human mast cells. However, KleinJan et al. (9) recently reported that fixation with Carnoy's fixative reduced the number of chymase-positive cells from biopsy specimens of the nasal mucosa. Therefore, in this study, we investigated the effect of Carnoy's fixative on the number of tryptase-positive or chymase-positive cells in immunocytochemical staining of cord-blood-derived human cultured mast cells, and confirmed that Carnoy's fixative

had a negative effect on the number of chymase-positive human mast cells.

Material and methods

Human mast-cell culture

Mononuclear cells (MNCs) were obtained from heparinized umbilical cord blood after centrifugation with Ficoll-paque (Pharmacia Biotech, Uppsala, Sweden). Then, the CD34-positive cells were separated by MACS column with the CD34 progenitor cell isolation kit (Miltenyi Biotec GmbH, Bergisch Galdbach, Germany), according to the manufacturer's instructions. Briefly, MNCs were incubated with human immunoglobulin as an Fc receptor blocking reagent and a hapten-conjugated anti-CD34 mAb at 6°C for 15 min. After washing in phosphate-buffered saline (PBS), the cells were further incubated with colloidal superparamagnetic MACS MicroBeads conjugated to an antihapten antibody at 6°C for 15 min. After washing, resuspended cells were applied to the separation column, which possesses a strong magnetic field in the magnetic cell separation system, and the positive fraction was collected. These cells were suspended in Media I (Dulbecco's modified Eagle's medium component plus 10 µg/ml insulin, 10 µg/ml transferrin, 5×10^{-5} M 2-mercaptoethanol, 25 mM HEPES, and 2.6 ng/ml NaSeO₃; IBL Men-eki Seibutsu Kenkyujo, Gunma, Japan), containing 10% heat-inactivated fetal calf serum (FCS; Cytosystems, Castle Hill, Australia), 100 ng/ml human recombinant SCF, and 50 ng/ml human recombinant IL-6 (kindly provided by Kirin Brewery Co., Maebashi, Japan) at 37°C in a 5% CO₂ humidified incubator. Nonadherent cells were passaged in the same medium weekly. The purity of mast cells was determined by staining with May-Grünwald and Giemsa reagents (Muto Pure Chemicals, Tokyo, Japan), and toluidine blue (Sigma Chemical Co., St Louis, MO, USA) and by immunocytochemical staining for mast-cell proteases, as described below. This study complied with the review board of Gunma University School of Medicine.

Monoclonal antibodies

Antibody B7 against chymase and G3 against tryptase were kindly provided by Dr Lawrence B. Schwartz (Department of Medicine, Medical College of Virginia, Richmond, VA, USA). For double staining, biotin-conjugated mouse mAb specific to chymase and alkaline phosphatase-conjugated mouse mAb specific to tryptase were obtained from Chemicon International (Temecula, CA, USA). Mouse-derived anti-human *c-kit* mAb was purchased from Nichirei (Tokyo, Japan).

Fixation

The cultured cells were spun down onto a glass slide with a cytocentrifuge (Cytospin 3; Shandon, Cheshire, UK) at 20 g for 5 min, and dried. The slide was then fixed with Carnoy's fixative (60% ethanol, 30% chloroform, and 10% glacial acetic acid) at room temperature. Fixation time series in Carnoy's fixative were as follows; dip (0.01 min), 1 min, 15 min, 1 h, 6 h, and 24 h. After fixation for each indicated time, the slide was rinsed in PBS, pH 7.4. Control fixation with other fixatives was investigated with acetone, methanol, 4% paraformaldehyde in PBS, and 10% formaldehyde (Wako Pure Chemical Industries, Osaka, Japan).

Immunocytochemical staining procedure

Immunocytochemical staining was performed by the alkaline phosphatase antialkaline phosphatase (APAAP) method. In brief, each slide was incubated with 5% bovine serum albumin (BSA; Sigma) in Tris-buffered saline (TBS), pH 7.6, at 37°C for 10 min. Next, the slide was incubated with B7 mAb (1 µg/ml) or G3 mAb (0.7 µg/ml) in TBS containing 1% BSA overnight at 4°C. After washing in TBS with 0.05% Tween 20 (TTBS), the slide was incubated with rabbit anti-mouse immunoglobulin antibodies (DAKO, Carpinteria, CA, USA) for 30 min. The slide was then washed in TTBS, and incubated with an alkaline phosphatase mouse antialkaline phosphatase antibody immune complex (APAAP complex; DAKO) for 30 min. The slide was washed extensively in TTBS, the color was developed with levamisole and a Fast Red kit (DAKO) which monitored the development of color under the microscope, and positive cells developed a red color. The slide was then rinsed in distilled water, counterstained with 5% methyl green stain solution (Muto Pure Chemicals), and mounted in glycerin-gelatin. Control isotype matched antibodies (mouse IgG1) were also used to check the nonspecific staining if any.

To check *c-kit* expression on the cultured cells, slides were incubated with anti-human *c-kit* mAb (1 µg/ml) for 30 min in the refrigerator. After washing, immunocytochemical staining was performed by the APAAP methods.

Tryptase and chymase were double-stained also by the method described by Irani et al. (8). Briefly, after blockage of the endogenous peroxidase with 0.6% H₂O₂ for 30 min, the slide was incubated with mAb specific to chymase conjugated with biotin at 4°C overnight. The slide was then incubated with horseradish peroxidase-conjugated streptavidin (Sigma) for 1 h at room temperature. The color was developed by H₂O₂ and 9-amino-3-

ethylcarbazole (Sigma), and positively stained cells developed a brown color. The slide was further incubated with antitryptase mAb conjugated with alkaline phosphatase at 4°C overnight. The color was developed by levamisole, Naphthol AS-MX, and Fast Blue (Sigma), and positively stained cells developed a blue color.

Microscopic evaluation

After evaluation of the slides, the stained cells were counted through a grid attached to the eyepiece of the microscope at a magnification of $\times 400$. The counts were done blind by two investigators, and each count of the slide represented the mean of the counts by the two investigators.

Statistics

Statistical analyses were performed with the analysis of variance (ANOVA) followed by Dunnett's multiple comparison test.

Results

General description

In the presence of 100 ng/ml SCF and 50 ng/ml IL-6, mast cells appeared as early as week 2 of the culture. After week 8, all of the cultured cells were metachromatically stained with toluidine blue and appeared homologous in May-Giemsa staining. Immunocytochemical staining showed that more than 90% of the cells were positive for tryptase, indicating that the cultured cells were mast cells. In the present study, we used cells more than 10 weeks old, all of which were metachromatically stained with toluidine blue and were *c-kit* positive.

Acetone fixation in comparison with Carnoy's fixative

On the basis of the investigation by Irani et al. (7, 8), we had routinely examined the phenotype of the cultured mast cells by immunocytochemical staining by fixation with Carnoy's fixative for 15 min at room temperature (1-4).

Therefore, to assess the effect of fixation with Carnoy's fixative in immunocytochemical staining we compared fixation with Carnoy's fixative for 15 min and that with acetone for 10 min, according to the condition reported by KleinJan et al. (9). As shown in Table 1, staining procedures with anti-tryptase mAb showed no differences between the positive cells. On the other hand, the percentage of chymase-positive cells after fixation with acetone

Table 1. Percentage of tryptase-positive or chymase-positive cells from cord-blood-derived human cultured mast cells by single staining with mAb after fixation with either acetone or Carnoy's fixative

mAb used	Fixation	
	Acetone 10 min	Carnoy's fixative 15 min
Antitrypsase	92.8±1.7	90.3±1.7
Antichymase	54.5±5.5	16.6±4.0**

Each value represents mean $\pm$ SEM of eight separate experiments.

** $P < 0.01$ when compared with value in acetone fixation.

for 10 min was 54.5 ± 5.5 (mean $\pm$ SEM, $n=8$), and that with Carnoy's fixative for 15 min was 16.6 ± 4.0 . Fixation with Carnoy's fixative for 15 min gave a significant decrease in the percentage of chymase-positive cells compared with acetone fixation for 10 min ($P<0.01$).

Effects of the fixation time with Carnoy's fixative

The number of chymase-positive cells was decreased in a time-dependent manner in fixation with Carnoy's fixative and was reduced significantly by 1-, 15-, and 60-min fixation (Fig. 1) compared with acetone for 10 min ($P<0.05$ at 1 min; $P<0.01$ at 15 and 60 min). After fixation with Carnoy's fixative for 24 h, all of the cells were negative for chymase. In contrast, the percentages of tryptase-positive cells were not changed significantly, even though, after fixation with Carnoy's fixative for 60 min and 24 h, the staining intensity of tryptase-positive cells became weaker.

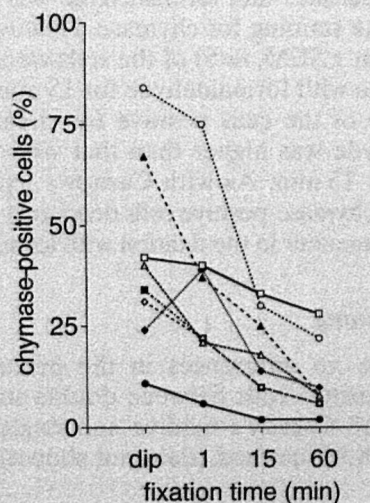


Fig. 1. Effects of fixation time with Carnoy's fixative on number of chymase-positive cells from cord-blood-derived human cultured mast cells. Each sample is plotted with same character ($n=8$).

Table 2. Effects of fixation time with various fixatives on percentage of chymase-positive cells from cord-blood-derived human cultured mast cells

Fixatives	1 min	15 min	1 h	2 h	6 h
Carnoy's fixative	46.5±3.8	25.6±6.0	22.4±4.9	9.4±1.6	0.1±0.1
Acetone	N.D. ^{a)}	47.3±4.5	N.D. ^{b)}	N.D. ^{b)}	N.D. ^{b)}
Methanol	39.8±12.0	47.9±9.2	49.5±11.2	51.7±13.1	45.0±11.7
4% paraformaldehyde in PBS	N.D. ^{a)}	50.7±10.2	51.9±7.1	56.3±11.1	45.9±3.0
10% formaldehyde	N.D. ^{a)}	36.0±7.5	34.5±4.3	17.7±1.0	5.6±2.7

Each value represents mean ± SEM of five separate experiments.

N.D.^{a)}: not detected because cells were not fixed.

N.D.^{b)}: not detected because cells were destroyed.

Effects of other fixatives

Acetone or paraformaldehyde is often used for the fixation of frozen tissue, and methanol or paraformaldehyde usually for immunocytochemical staining. Therefore, the effect of other fixatives on immunocytochemical staining of the mast cells for chymase was investigated (Table 2). With acetone, 1-min treatment was not sufficient for fixation, and treatment of more than 60 min destroyed the cells. With methanol, the number of chymase-positive cells was not significantly changed over the fixation time, and the percentages of the chymase-positive cells with the fixations for 1 min, 15 min, 1 h, 2 h, and 6 h, were 39.8±12.0 (mean ± SEM, $n=5$), 47.9±9.2, 49.5±11.2, 51.7±13.1, and 45.0±11.7, respectively. With paraformaldehyde, 1-min treatment was not sufficient to fix the cells. When the cells were fixed with 4% paraformaldehyde for more than 15 min, the cells maintained their structures well. With the fixations for 15 min, 1 h, 2 h, and 6 h, 50.7±10.2 (mean ± SEM, $n=5$), 51.9±7.1, 56.3±11.1, and 45.9±3.0% of the cells were positive for chymase, respectively. The data sheet enclosed in the antichymase mAb from the company indicates that formaldehyde was not sufficient for the staining for chymase. However, 36.0±7.5% (mean ± SEM, $n=5$) of the cells were positive for chymase with formaldehyde for 15 min, and the percentage of the cells positive for chymase with formaldehyde was higher than that with Carnoy's fixative for 15 min. As with Carnoy's fixative, the number of chymase-positive cells decreased in a time-dependent manner in the fixation with formaldehyde.

Double staining

There were no differences in the percentage of chymase-positive cells between double staining by fixation with Carnoy's fixative and single staining by the APAAP method (data not shown).

Discussion

Carnoy's fixative contains ethanol, chloroform, and glacial acetic acid. It was found to be one of

the very few common fixatives which preserved the dye-binding of gut mucosal mast cells (10), and has been used frequently for the study of mast cells. For the study of human mast cells, Carnoy's fixative has also been routinely used for staining with mAb against tryptase and chymase (7, 8). In fact, we have routinely examined the phenotype of the cord-blood-derived cultured cells with Carnoy's fixative for 15 min at room temperature, and reported that the percentages of chymase-positive cells from the human cultured mast cells were 18.4±4.4% (1) and 23.6±10.8% (3), and that the population of chymase-positive cells varied among cord-blood specimens.

In immunohistochemical staining of frozen biopsy specimens, however, mild fixatives such as acetone are generally used. Recently, KleinJan et al. (9) reported that fixation with Carnoy's fixative reduced the number of chymase-positive human mast cells. Thus, they stained the sections of biopsy specimens from the nasal mucosa which were fixed with acetone, and showed that fixation with acetone gave a significantly larger number of chymase-positive cells than Carnoy's fixative, suggesting that Carnoy's fixative weakened the binding of the mAb to the respective epitope, as the epitope is either changed or damaged.

In the present study, we confirmed that Carnoy's fixative reduced the number of chymase-positive cells from human cultured mast cells in a time-dependent manner. After fixation with Carnoy's fixative for 15 min, the percentage of chymase-positive cells was decreased to 35% or less, suggesting that there are probably two populations of chymase-positive cells, one sensitive and the other insensitive to Carnoy's fixative, as reported (9).

In the present experiments, we further investigated the effects of other fixatives and found Carnoy's fixative for dip, methanol, or 4% paraformaldehyde to be suitable for the fixation of human cultured mast cells.

In conclusion, in the present study, we found that Carnoy's fixative had a negative effect on the

number of chymase-positive cells from cord-blood-derived human cultured mast cells.

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