HEMOLYSIS AND MORPHOLOGICAL CHANGES IN RAT ERYTHROCYTES WITH MERCURIALS

Reiko TANAKA and Kengo NAKAI
Department of Pharmacology, Akita University School of Medicine, Akita 010, Japan
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Abstract—Effects of mercurials on rat erythrocytes were studied morphologically using an electron microscope. In the scanning study, the normal biconcave shape of the erythrocytes was changed to rugged surface spherocytes when mercuric chloride was added to the erythrocyte suspension. Methylmercuric chloride produced an irregularity of cell shape with spicules including the final stage of spherocytes. p-Chloromercuribenzoic acid formed crenated cells with protrusion, then spherocytes. By a carbon replica technique, it was revealed that control erythrocytes had a granular surface structure; however the surface of mercuric chloride-treated and methyl-mercuric chloride-treated erythrocytes appeared less granulated. By a negative staining technique, severe damage was observed on the erythrocytes lysed by mercurials. As a decrease in content of reduced glutathione, inhibition of glucose-6-phosphate dehydrogenase activity and formation of methemoglobin in erythrocytes treated with mercurials to induce hemolysis were not observed, it was concluded that the hemolysis induced by mercurials was not due to a disturbance in erythrocyte metabolism but rather to the direct action of mercurials on the cell membrane.

In in vivo studies, a high erythrocyte: plasma ratio of methylmercury in human blood has been reported by Suzuki et al (1) and Swensson et al (2). Similar results have been reported in rat blood by Gage (3) and Norseth and Clarkson (4).

In in vitro studies, the hemolytic effect of mercurials was shown by Benesch and Benesch (5) and Sheets et al (6). On the other hand, a rapid and virtually complete uptake of methylmercury by human and rat erythrocytes was reported by White and Rothstein (7). From these studies, it was suggested that certain changes, biochemical and morphological, occurred in the erythrocytes treated with mercurials; however the direct action of mercurials on the erythrocytes remained to be determined.

In the present report, hemolysis and morphological changes of erythrocytes by mercurials including methylmercury were investigated.

MATERIALS AND METHODS

Preparation of erythrocytes

Fresh blood samples were obtained from male rats (Sprague-Dawley strain) by cardiac puncture and bleeding from the carotid artery using acid-citrate-dextrose or heparin as an anticoagulant. After the centrifugation (670 g×10 min), the bulky layer was removed and the erythrocytes were washed three times with phosphate buffered saline (PBS, pH 7.3). After the last centrifugation, the erythrocytes were resuspended in PBS, each sample con-
taining a concentration of mercurials, and incubated at 37°C under gentle shaking. The effects of mercuric chloride (HgCl₂), methylmercuric chloride (MMC) and p-chloromercuribenzoic acid (PCMB) were examined.

MMC and PCMB solutions, insoluble in PBS, were prepared as follows: MMC was first dissolved in 5 mM NaCO₃ saline then diluted to each concentration with PBS. PCMB was first dissolved in 0.1 N NaOH then diluted to each concentration with PBS adjusting to pH 7.3 with 0.1 N HCl. HgCl₂ was simply dissolved in PBS.

**Determination of the degree of hemolysis**

After the incubation, erythrocyte suspensions were centrifugated at 1500 g for 10 min and the supernatants were separated to determine the degree of hemolysis. The supernatants were diluted with four volumes of 0.4% ammonium hydroxide and the absorbance at 540 nm was measured. Relative ratio of absorbance of diluted supernatant to that of diluted original suspension was expressed as a percentage of hemolysis.

**Osmotic fragility**

A 0.1 ml of the washed erythrocyte (4% in PBS) was added to 4.0 ml of phosphate buffer (10 mM, pH 7.3) containing NaCl in different concentrations and kept for 60 min at a room temperature of 18°–23°C. Percentage of hemolysis was determined as described.

**Scanning electron microscopy**

Washed erythrocytes were fixed overnight by 2.5% glutaraldehyde at 4°C. After washing with distilled water, fixed erythrocytes were placed on a glass slide and dried at a room temperature of 18°–23°C, then coated with carbon and gold-palladium in a vacuum evaporator. Photomicrographs were taken at magnifications of 3,000 and 10,000 using a scanning electron microscope (JSM-U3).

**Carbon replica**

Fixed and washed erythrocytes as described above, were placed on a freshly cleaved mica surface and dried at a room temperature of 18°–23°C, then coated with carbon-platinum in a vacuum evaporator at an angle of 30 degrees. Replicas were floated out from the mica surface by immersion in 4.0 N NaOH at 65°C and 1.0 N HCl at room temperature for 24 hr respectively. Finally, the replicas were washed twice with distilled water and examined under a JEM-100 B electron microscope.

**Negative staining**

Erythrocyte ghosts produced by hypotonic hemolysis and mercurials hemolysis were fixed and washed in the same way as for the scanning electron microscopic study. Resuspended ghosts in distilled water were placed on a specimen grid, coated with collodion film strengthened by carbon. The specimen was stained negatively with 2.0% phosphotungustic acid, adjusted to pH 6.4 using 1.0 N KOH then examined under a JEM-100 B electron microscope.

**RESULTS**

**Hemolysis**

Time course of hemolysis of rat erythrocytes by HgCl₂, MMC and PCMB is shown in
Fig. 1. Each point represents the mean from three to five experiments. HgCl₂ showed the most hemolytic activity compared with MMC and PCMB. The erythrocytes treated with 0.2 mM HgCl₂ were completely hemolyzed in a period of 20 min; however, other erythrocytes treated with 0.2 mM PCMB and 0.2 mM MMC showed 20% and 15% hemolysis in a period of 20 min, respectively. Complete hemolysis of the erythrocytes, treated with the same concentration of PCMB and MMC were observed in periods of 45 min and 60 min, respectively. HgCl₂ and PCMB showed a proportional lytic activity with the 0.1 mM and 0.2

![Graph showing hemolysis of rat erythrocytes incubated with HgCl₂, MMC, and PCMB at 37°C. Cell concentration is 0.5%.

- H-0.2: 0.2 mM HgCl₂ (● ● ●) H-0.1: 0.1 mM HgCl₂ (▲ ▲ ▲)
- M-0.2: 0.2 mM MMC (● ● ●) M-0.1: 0.1 mM MMC (▲ ▲ ▲)
- P-0.2: 0.2 mM PCMB (● ● ●) P-0.1: 0.1 mM PCMB (▲ ▲ ▲)
mM concentrations. However, 0.1 mM MMC showed no hemolysis while 0.2 mM of the same compound, lysed completely in 60 min.

**Morphological change and osmotic fragility**

Scanning electron micrographs of rat erythrocytes incubated with 0.2 mM MMC at 37°C for 0, 10, 20 and 30 min are shown in Fig. 2. Osmotic fragility of treated cells increased with incubation time. At the same time, progressive morphological changes were observed in a scanning electron microscopic study.

**Change in shape of erythrocytes in a hemolytic process**

The shape of cells in the hemolytic process by mercurials was different depending on

| % Hemolysis | 10-20% | 30% | 90% |
|-------------|--------|-----|-----|
| HgCl₂       | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| MMC         | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
| PCMB        | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) |

**Fig. 3.** Changes in shape of rat erythrocytes in the different grades of hemolysis.

**Fig. 4.** Typical changes in shape of rat erythrocytes by mercurials.

A: Control (30 min in PBS),
B: 0.1 mM HgCl₂ 30 min,
C: 0.2 mM MMC 30 min,
D: 0.2 mM PCMB 30 min.
the compounds as shown in Fig. 3. In the presence of HgCl₂, the cells were changed to spherocytes with rugged cell surfaces at the stage of a high degree of hemolysis (more than 20% hemolysis). MMC produced an irregularity of cell shape with spicules and finally spherocytes were observed. By treatment with PCMB, the cells became crenated with protrusion. Spherocytes were observed at the final stage of hemolysis by PCMB. Typical changes in the shape of erythrocytes induced by mercurials are shown in Fig. 4.

**Carbon replica**

The surface of rat erythrocytes is shown in Fig. 5. The control erythrocyte (untreated) showed a granular surface structure; however, the surface of HgCl₂ and MMC treated erythrocytes appeared less granulated. No significant change of surface structure of cells was observed when treatment with PCMB was given.

![Carbon replica images](image)

**Fig. 5.** Electron micrographs of surface of rat erythrocytes (carbon replica technique). Erythrocytes were incubated with HgCl₂, MMC and PCMB at 37 C. Hemolysis of erythrocytes was approximately 50%.

A: Control, B: HgCl₂, C: MMC, D: PCMB.

**Negative staining**

Rat erythrocyte ghosts produced by hypotonic hemolysis showed clear-edged holes of an irregular size. Similar structures were observed on the ghosts produced by mercurial hemolysis; however the size and arrangement of holes were somewhat different depending on the compounds used (Fig. 6).

**DISCUSSION**

One of the effects of mercurials on erythrocytes is metabolic disturbance such as decrease of reduced glutathione (GSH) and inhibition of glucose-6-phosphatase (G6PD) activity which finally result in hemolysis. The other effect is a direct action on cell membrane that
results in a hemolysis. However, as shown in preliminary experiments (8) with the HgCl₂, MMC and PCMB-treated erythrocytes, decrease of GSH content, inhibition of G6PD, and formation of methemoglobin to induce lysis were not detectable at the concentration of the mercurials which induces a hemolysis. However in the cells treated with N-ethylmaleimide (NEM), an alkylating agent capable of combining with sulfhydryl compound (9), a moderate decrease of GSH content, significant inhibition of G6PD activity and slight formation of methemoglobin were detected at the non-hemolytic concentration. Therefore, it was assumed that mercurial induced hemolysis was not due to a disturbance in erythrocyte metabolism but rather to the direct action of mercurials on the cell membrane.

To determine the action of mercurials on the cell membrane, electron microscopic studies were carried out. Using a negative staining technique, severe damage on ghosts produced by mercurials hemolysis was demonstrated in the many clear edged holes clearly observed (Fig. 6). In a scanning electron microscopic observation, erythrocytes treated with HgCl₂, PCMB and MMC showed different changes of shape (Fig. 3, 4) suggesting different mechanisms of action on cells by the compounds.

On the other hand, Libuse (10) suggested that the characteristic shape of erythrocytes was determined by a certain conformation of certain proteins in the membrane, e.g. adenosine triphosphatase (ATPase) and the effect of adenosine triphosphate (ATP) was also considered by other workers to be of some importance (11). The role of ATPase in the maintenance of biconcavity has been supported by Williams (12). Furthermore, the interaction of mercurials with cell membrane components is assumed to have an important effect in inducing hemolysis. William and Donald (13) reported the complex formation of Hg⁺ and solubility
of CH$_3$Hg$^+$ in lipids. In our work, an inorganic mercurial (HgCl$_2$) showed a higher hemolytic activity than organic mercurials (MMC, PCMB) in the same concentration (0.1 mM or 0.2 mM) and such observations suggest that different hemolytic mechanisms are involved.

Hiller and Hoffman (14) and Robert and Wendell (15) reported a granular appearance due to "plaque" on the surface of cell membrane when they used a shadowcasting technique with chromium or platinum, and Van Gastel et al (16) stated that the loss of granularity may be related to the loss of lipid. However, it would hardly seem likely that alteration of membrane structure is due only to a lipid component. As shown in Fig. 6, with HgCl$_2$ and MMC treatment, the surface of the cell membrane appeared much smoother compared with the non-treated cell surface, suggesting alteration in the membrane structure.

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