Increased Reactivity of *Schistosoma japonicum* Eggs in the Circumoval Precipitin Test after Hydrochloric Acid Treatment

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**Summary:** The effect of a hydrochloric acid solution on the circumoval precipitin (COP) reactivity of *Schistosoma japonicum* eggs was studied. When eggs, recovered by trypsin digestion from tissues of an 8 week-infected rabbit, were treated with a pepsin HCl solution, they had significantly higher reactivity than the saline-treated eggs (control). A similar effect was obtained with HCl solution (0.008 N), alone. In contrast, a sodium hydroxide solution (0.05 N) decreased the reactivity. Treatment with different concentrations of HCl (0.01 to 6 N) for 2 days revealed that the reactivity was highest at 0.1 N, and was diminished by the higher concentrations (3 and 6 N). Preservation in an HCl solution (0.01 N) yielded a peak reactivity at 2 days. While the exact role of the HCl solution was not studied, this study contributes to the preparation of egg specimens and to our understanding of the low reactivity of eggs.

**Key words:** *Schistosoma japonicum* — eggs — circumoval precipitin (COP) test — HCl treatment — pepsin

**Introduction**

The circumoval precipitin (COP) test reported by Oliver-Gonzalez (1954), is widely accepted for the immunodiagnosis of a schistosome infection. In addition to simplicity, sensitivity and specificity, the stability of the egg antigens to formalin (Yogore et al. 1968) and to heat (Kamiya, 1980) are advantageous for use of the test in epidemiological surveys. A tedious microscopic examination in areas of low endemicity is the main drawback of this test (Hillyer and Rivera-Marrero, 1982). Kamachi (1973) reported that positive reactions occurred in only 1.6% of the eggs incubated with sera from individuals with schistosomiasis japonica. This paper demonstrates that COP reactivity of eggs can be improved by treatment with an HCl solution.

**Materials and Methods**

**Eggs**

The livers and intestines of infected rabbits exposed to *Schistosoma japonicum* cercariae (Japanese strain) 8 or 20 weeks previously, were used as the egg source. In most experiments eggs were harvested by trypsin digestion from a rabbit after 8 weeks of infection. The trypsin digestion was performed according to the method of Yokogawa and Sano (1966). The tissues were digested by 0.1% trypsin (Merck, 2000 E/G) in 1/15 M Na₂HPO₄ (approximately pH 8.4) at 37 °C for 4 hours. In some experiments, the egg suspension was also submitted to pepsin digestion according to the method of Smithers (1960). The fluid consisted of 0.1% pepsin (1:1000 Ishizu Co. Ltd.) in 0.85% NaCl and was adjusted to pH 1.8 by adding 0.1 N HCl.
At a final step in the isolation, the egg suspension was filtered through a gauze and two sieves (100 and 200 mesh), and then thoroughly washed with 0.85% NaCl by repeated light centrifugation (500–750 rpm). Lyophilized eggs were used in all experiments.

**COP test**

The COP test was performed using a simplified technique. Vinyl tape with two circular perforations (diameter 10 mm), 10 mm apart, was applied to a slide glass. Ten μl of antiserum and the same volume of egg suspension, containing around 100 eggs, were poured into the wells. After shaking, the slide was covered with cellophane tape, instead of a coverslip. Care was taken that the solution did not leak from the wells, and that the cellophane tape had adhered tightly to the slide. Two test slides were prepared for each sample. The slides were stored at room temperature for 2 days, the 200 eggs were observed under a light microscope. Preliminary tests yielded no significant differences from the original method of Oliver-Gonzalz (1954). The precipitates were categorized into types I, II and III according to Yokogawa et al. (1967) and the reactivity of the eggs was assessed by the COP index, as proposed by Tanaka (1976).

**Acid and alkali treatment**

Varying concentrations of HCl (0.008 N–6 N) in 0.85% NaCl were used. Alkali treatment was carried out with 0.02 M, pH 8.0 phosphate buffered saline (PBS) or 0.005 N NaOH (pH 11.0) in 0.85% NaCl. 0.85% NaCl (pH 6.4) was used as the control. Eggs were left in the solutions for a given time and then were washed with saline until the pH returned to approximately 6.4. Then the eggs were used for the COP test.

**Antisera**

Sera from 10 infected rabbits were used. To avoid changes in antibody titer during the course of the experiments, each serum sample was separated into small volumes and stored at -70°C.

**Results**

When the eggs, which were harvested by trypsin digestion from a rabbit infected for 8 weeks, were exposed to pepsin-HCl or HCl solution (0.008 N HCl in both solutions); they had a higher reactivity than non-treated eggs (control) (Fig. 1). The difference in reactivity could be due to the hydrogen ion concentrations, thus eggs were exposed to different pH's (pH 1.8, 6.4, 8.0, 11.0) for 2 days (Fig. 2). An HCl solution (pH 1.8) increased the reactivity, but a NaOH solution (pH 11.0) decreased it. A solution at pH 8.0 (PBS) produced no noticeable change from the control (0.85% NaCl, pH 6.4).

The effect of the HCl solution was confirmed by testing the sera from 10 infected rabbits (Fig. 3). When the eggs were treated with 0.008 N HCl (pH 1.8) for 2 hours, an increase in the reactivity was

![Fig. 1. Comparative circumoval precipitin (COP) reactivity of S. japonicum eggs treated with HCl (0.008 N) or pepsin-HCl solution](image-url)
COP reactivity increased with HCl

Fig. 2. Comparative circumoval precipitin (COP) reactivity of *S. japonicum* eggs treated with different pH solutions (Cont, 0.85% NaCl, pH 6.4; HCl, 0.008 N, pH 1.8; PBS, phosphate buffered saline, 0.02 M, pH 8.0; NaOH, 0.005 N, pH 11.0) observed in 7 of 10 serum samples. The rank sum test showed a significant difference between the reactivity of treated and nontreated eggs (p<0.025). Although 3 serum samples had a lower reactivity for HCl-treated eggs, the reductions were small.

With eggs from a rabbit after an infection period of 20 weeks, the reactivity of eggs harvested by trypsin digestion plus pepsin digestion was compared to the reactivity of eggs harvested by trypsin digestion alone. In this case the effect of HCl was more evident (Table 1). In 9 of 10 sera tested, the reactivity of eggs obtained by trypsin plus pepsin digestion (exposed to HCl) was higher than that by trypsin digestion. The general reactivity of both groups was very low, presumably because the eggs were old. When the above two egg groups were treated with an HCl solution (0.01 N) for 2 days and

Fig. 3. Effect of an HCl solution (0.008 N) on circumoval precipitin (COP) reactivity of *S. japonicum* eggs using infected sera from 10 rabbits (open bar, non-treated eggs; hatched bar, HCl-treated eggs)
reacted with randomly sampled sera, the reactivity increased to a greater extent in both groups. The eggs harvested by trypsin and pepsin digestion still had a higher reactivity.

The effect of HCl on the reactivity was tested using eggs placed in HCl solutions for 2 days (Fig. 4). An increase in reactivity was observed at concentrations of 0.01 N and 1 N, and was significantly higher at 0.1 N HCl concentration compared to control (p<0.05 by Mann-Whitney's U test). The concentrations of 3 and 6 N decreased the reactivity and cracked a number of eggs.

The relationship between the reactivity of eggs and the period of preservation in HCl solution (0.008 N) was investigated (Fig. 5). The reactivity increased up to 2 days of preservation and then decreased with some fluctuation. After 28 days, the decrease was apparent. The reactivity on 2 days was significantly higher than that of control (0 day) (p<0.05 by Mann-Whitney's U test).

<table>
<thead>
<tr>
<th>Serum No.</th>
<th>COP index</th>
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<tbody>
<tr>
<td>T</td>
<td>T + P</td>
</tr>
<tr>
<td>1</td>
<td>7.4</td>
</tr>
<tr>
<td>2</td>
<td>4.5</td>
</tr>
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<td>3</td>
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</tr>
<tr>
<td>8</td>
<td>14.1</td>
</tr>
<tr>
<td>Mean</td>
<td>8.9</td>
</tr>
</tbody>
</table>

T: Eggs harvested by trypsin digestion
T+P: Eggs harvested by trypsin digestion and pepsin digestion
T+HCl, T+P+HCl: T or T+P were also treated with an HCl solution (0.01 N) for 2 days

*Fig. 4. Influence of the HCl concentration on circumoval precipitin (COP) reactivity of S. japonicum eggs*
Although there are several types of enzymes that can be used for digestion, the choice is usually related to separation of free eggs from the debris of the host tissues. Yokogawa and Sano (1966) compared trypsin, pepsin and collagenase using tissues from mice infected with *S. japonicum*. The pepsin solution had a detrimental effect on the longevity of the isolated eggs, though no difference was evident in the isolation capacities. The reactivity of eggs using the COP test was not considered in that study. We found a higher COP reactivity in eggs obtained by trypsin plus pepsin digestion than those by trypsin digestion. It was apparent that the higher reactivity was caused by exposure to HCl contained in the pepsin digestion fluid.

The major antigenic substances of *S. japonicum* eggs which are responsible for the COP reaction are reportedly glycoproteins (Long et al. 1981). Owhashi and Ishii (1981) characterized the allergens extracted from *S. japonicum* eggs and found that they were resistant to heat, urea and guanidine, yet were rather sensitive to alkali (NaOH) treatment and trypsin digestion. Similar characteristics of crude *S. japonicum* egg antigens were noted by Fujinaga et al. (1981), who also found that the antigens which dissolved in acid buffer (pH 2.6) were reactive. The present study also showed that HCl treatment of intact eggs was effective for COP reactivity, while alkali treatment was not.

HCl treatment at high concentrations (3 and 6 N) for long durations had an untoward effect on the eggs (Figs. 4 and 5). Similar observations were made by Newsome (1958) using extracts from *S. mansoni* eggs. They compared the reactivity of saline extracted egg antigens with the reactivity of HCl (0.05 N) extracted egg antigens by a precipitin reaction. The reactivity of HCl-extract had disappeared by 4 weeks, although the HCl extract produced heavier flocculation than the saline extract during the initial stages.

Garcia et al. (1981) reported that *S. japonicum* eggs harvested after 65 or more days of infection had a lower reactivity than those harvested at 55 days. Since ultrastructural studies have revealed that egg antigens which cause the COP reaction pass through micropores on egg shells.
in human schistosome eggs (Ford et al. 1980; Demaree and Hillyer, 1981; Han-Min et al. 1982), it is likely that the permeability of antigens decreases with the age of the eggs. Consumption or denaturation of the egg antigens by host tissues may also be involved in the lower reactivity of the old eggs. HCl and subsequent collagenase treatment affect the removal of extracellular materials from the cell surface and the basement membrane (Evan et al. 1976). Therefore it was assumed that the increase in COP reactivity of eggs, especially old eggs, after HCl treatment is caused by the removal of materials present outside the egg shell or inside egg shell, which can act as permeability barriers. The presence of immunoglobulins inside the egg shells of S. japonicum eggs in the infected rabbit or mouse liver tissue has been observed (Hirata, 1984).

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References


