Antigens of *Bordetella pertussis*

IV. Effect of Heat, Merthiolate, and Formaldehyde on Histamine-Sensitizing Factor and Protective Activity of Soluble Extracts from *Bordetella pertussis*

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**Abstract**

MUNOZ, J. (Rocky Mountain Laboratory, Hamilton, Mont.), AND B. M. HESTEKIN. Antigens of *Bordetella pertussis*. IV. Effect of heat, Merthiolate, and formaldehyde on histamine-sensitizing factor and protective activity of soluble extracts from *Bordetella pertussis*. J. Bacteriol. 91:2175–2179. 1966.—Both histamine-sensitizing and protective activities of soluble preparations from *Bordetella pertussis* cells are destroyed by heating at 80 C for 0.5 hr. The histamine-sensitizing activity appeared to be more susceptible to inactivation by heat than the protective activity. Formaldehyde in a final concentration of 0.5% rapidly diminished the histamine-sensitizing ability of saline extract (SE) held at 37 C. The protective activity was clearly more resistant to inactivation by formaldehyde at similar temperature. The inactivating action of formaldehyde was slower when the concentration of SE was increased or when the mixture was kept at 2 to 5 C. Merthiolate in a final concentration of 1:10,000 had no demonstrable deleterious effects on either protective or histamine-sensitizing activity of SE.

The protective antigen(s) of *Bordetella pertussis* in whole cells is destroyed by heating aqueous suspensions to boiling temperatures. At least seven heat-labile antigens have been recognized in *B. pertussis* strains (1, 4), but no definite relationship between protective activity and any of these heat-labile substances has been established. According to the results of Eldering, Eveland, and Kendrick (3), antigen 1, common to all freshly isolated strains of *B. pertussis*, might well be involved in protection. However, Preston (15, 16, 17), Preston and Evans (18), and Chalvardjian (2) presented evidence which suggests that development of immunity may involve more than one antigen.

No detailed study on the effect of temperature on the protective activity of soluble preparations has been reported. Whole cell pertussis vaccines can be heated at 56 C for 1 hr without destroying their protective activity (7). Maitland, Kohn, and Macdonald (9) showed that the histamine-sensitizing factor (HSF) of *B. pertussis* was destroyed by heating whole cells at 80 C for 0.5 hr. Kind (8) confirmed this observation and, in addition, found that heated preparations also lost their ability to increase the susceptibility of mice to anaphylaxis. Others have obtained similar results (6, 13). Niwa found that HSF in culture supernatant fluids was not inactivated by heating at 56 C for 5 min, but was inactivated at 70 C for 30 min. The factor was also destroyed by shaking in the air at 37 C for 30 min (9, 13).

Formaldehyde has been used in the preparation of experimental whole cell pertussis vaccines with only slight loss of potency over long periods of storage (7, 14). The deleterious effect of formaldehyde on protective activity of vaccines is recognized by others (5), and National Institutes of Health minimal requirements for pertussis vaccine of 1 May 1963 state that formaldehyde adversely affects potency. Recently it has been shown that formaldehyde-treated *B. pertussis* cells no longer produced histamine sensitization in mice but still retained their ability to interfere with sensitization of mice to histamine by untreated cells (6, 10).

Since no comparison has been made on the effect of heat, formaldehyde, and Merthiolate on the protective and HSF activities of soluble preparations from *B. pertussis*, the present work was undertaken.

**Materials and Methods**

*Mice.* Mice of both sexes (equal number of each sex always used), weighing from 18 to 22 g, of the Swiss-Webster strain (CFW) raised in this laboratory or purchased from Carworth Farms, New City, N.Y., were employed for HSF assays. The protective ac-
activity was determined in female mice, weighing 14 to 16 g, of the Rocky Mountain Laboratory strain (RML).

*B. pertussis*. The cells and saline extract (SE) from *B. pertussis* were prepared as previously described (11).

Protection and histamine-sensitization tests were performed by the methods previously described (12). Histamine diphosphate was employed, but the amounts given are expressed as histamine base.

**RESULTS**

Effect of heat on protective and histamine-sensitizing activity of SE. A solution of SE containing 15 mg in 37.5 ml of 0.067 M phosphate-buffered saline (pH 7.3) was made. Samples of this material were either unheated or heated for 0.5 hr at 60, 65, 70, 75, or 80 C. For each treatment, three groups of 10 CFW mice each and three groups of 15 RML mice each (except for 60 C treatment) were inoculated intraperitoneally (ip) with 0.2 ml containing either 20, 40, or 80 μg of SE. The CFW mice were challenged ip with 0.5 mg of histamine 4 days later, and the RML mice were challenged intracranially with 40,000 living *B. pertussis* cells 14 days later. The results of these experiments showed that SE heated at 60 C for 0.5 hr lost little histamine-sensitizing activity, although heating at 70 C for 0.5 hr destroyed most of the HSF activity (Fig. 1). Results obtained with 20 and 40 μg of heated SE showed marked reduction of both HSF and protective activity, whereas those obtained with the 80-μg dosage indicated that the HSF activity was destroyed more readily by heat than the protective activity (Fig. 1 and 2). Preparations heated at 80 C for 0.5 hr had no significant protective or HSF activities at the highest level tested (80 μg).

**Effect of formaldehyde and Merthiolate on HSF and protective antigen activities in SE.** A solution containing 800 μg/ml of SE in 0.067 M phosphate-buffered saline (pH 7.35) was incubated at 37 C for 1 week in the presence of 0.5% formaldehyde. At the end of 1 week, the material was tested at a level of 20, 40, 80, and 160 μg per mouse. The HSF activity under these conditions was no longer significant at any of the dosage levels tested. A preparation kept under identical conditions, but containing 1:10,000 Merthiolate instead of formaldehyde, had full activity at all concentrations tested (Fig. 3). The protective activity of these same preparations was markedly reduced (see results at 20- and 40-μg levels in Fig. 4) in samples kept in 0.5% formaldehyde, whereas samples kept in the presence of Merthiolate showed full activity even at the 20-μg

![Fig. 2. Effect of heat on mouse protective activity of SE heated to temperature indicated for 0.5 hr.](image)

![Fig. 3. Effect of 0.5% formaldehyde and 1:10,000 Merthiolate on HSF activity of SE stored in a concentration of 800 μg/ml at 37 C for 1 week.](image)
dose level (Fig. 4). It is significant that the HSF activity appeared to be more susceptible to the action of formaldehyde than was the protective activity.

When the concentration of SE was increased from 800 to 1,600 μg/ml, the inactivation of the protective activity by 0.5% formaldehyde was not striking (compare Fig. 4 and 5). It has also been found that storage of SE at 2 to 5°C for 2 weeks in presence of 0.5, 0.25, or 0.125% formaldehyde does not reduce either HSF or protective activity as rapidly as at 37°C.

The following experiment was performed to find the effect of formaldehyde on HSF activity in SE solution stored at 2 to 5°C for longer periods of time than in previous experiments.

A 33.6-mg amount of SE was dissolved in 42 ml of 0.067 M phosphate-buffered saline (pH 7.35), and then 7-ml portions of this solution were mixed with 7 ml of 1, 0.5, or 0.25% formaldehyde in saline. In addition, 7 ml of SE solution plus 7 ml of 1:5,000 Merthiolate were also mixed. The mixtures were stored at 2 to 5°C and were tested at 1, 2, 6, and 27 weeks for their ability to sensitize mice to histamine. As control, a sample of SE was kept frozen and was tested at the same intervals of time. The results are tabulated in Table 1. From these results, it is clear that no detectable loss of HSF activity was noticed during 27 weeks when SE was kept frozen, in 1:10,000 Merthiolate, or in 0.125 to 0.25% formaldehyde. The sample kept in 0.5% formaldehyde showed a decrease in activity only after 27 weeks at 2 to 5°C. The protective activity has been shown to be more stable than HSF in the presence of 0.125, 0.250, or 0.5% formaldehyde at 37°C in experiments of shorter duration, and it is reasonable to assume that this activity would be at least as stable as the HSF activity in the presence of formaldehyde at 2 to 5°C.

**DISCUSSION**

The results presented confirmed that the protective antigen(s) and the HSF of *B. pertussis* are heat-labile. Temperatures as low as 65°C for 0.5 hr inactivate demonstrably the protective and HSF activities of soluble preparations, and a temperature of 80°C for 0.2 hr seems to destroy almost completely both HSF and protective activities. An indication was obtained that the protective activity was slightly more stable than the HSF activity, but since the nature of the two assays is fundamentally different, it is difficult to attach much significance to the differences observed.

In previously published work, it has been stated that both HSF and protective antigen activity appeared to be present in the same molecular entity (11), since attempts to separate the two activities thus far have failed. From the results given in this paper, it seems clear that HSF activity of SE decreased in the presence of formaldehyde at 37°C at a faster rate than did the protective activity. If, as has been postulated, HSF and protective activities are actually part of the same molecule, this action of formaldehyde might be similar to the detoxification of exotoxins with the formation of immunologically active toxoids. The final answer to this problem, however, will come only when HSF is obtained free from other antigenic materials. The inactivating action of formaldehyde was greatly reduced by increasing the concentration of SE employed or by incubating the mixture at 2 to 5°C. HSF or protective activities of SE were not demonstrably affected by 1:10,000 Merthiolate or by keeping the solutions in a frozen state.
This has also been the experience with whole cell vaccines. Kendrick et al. (7) showed that vaccines containing Merthiolate are stable for almost 10 years, and Verwey, Schuchardt, and Ciminera (Bacteriol. Proc., p. 80, 1957) reported that vaccines kept for many years in the presence of Merthiolate were little affected in their ability to protect mice. Similar results have been obtained with respect to HSF activity (Munoz, unpublished data).

As mentioned before, Kind (8) found that the ability of pertussis vaccines to increase the susceptibility of mice to anaphylaxis was also destroyed by heating to 80°C for 0.5 hr. Most likely the HSF activity and the ability to induce hypersensitivity to anaphylaxis are due to the same substance, since highly purified preparations of HSF also have the ability to increase the susceptibility of mice to anaphylaxis (Munoz, unpublished data).

TABLE 1. Stability of HSF activity in formaldehyde at 2 to 5°C

<table>
<thead>
<tr>
<th>Preparation of SE</th>
<th>SE dose (µg/mouse)</th>
<th>Time (weeks) at 2 to 5°C</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
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<tr>
<td>Frozen.............</td>
<td>3.2</td>
<td>0/9*</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>8/10</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>9/10</td>
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<tr>
<td>Merthiolate........</td>
<td>3.2</td>
<td>2/9</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>9/9</td>
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<tr>
<td></td>
<td>80</td>
<td>10/10</td>
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<tr>
<td>Formaldehyde, 0.125%</td>
<td>3.2</td>
<td>3/10</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>10/10</td>
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<tr>
<td></td>
<td>80</td>
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<tr>
<td>Formaldehyde, 0.25%</td>
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<td>4/8</td>
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<td>6/9</td>
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<tr>
<td></td>
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<tr>
<td>Formaldehyde, 0.5%</td>
<td>3.2</td>
<td>3/10</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>10/10</td>
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<td>None</td>
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* Deaths per total number challenged.

LITERATURE CITED


