JOURNAL OF BACTERIOLOGY, Apr. 1974, p. 144-148

Vol. 118, No. 1
Printed in U.S.A.

Hemagglutinin in Cell Walls of *Chlamydia psittaci*

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Received for publication 10 September 1973

Intact purified elementary bodies (EB) of *Chlamydia psittaci* agglutinate chicken erythrocytes in low titer, whereas homogenates of EB and of EB cell walls agglutinate at much higher titers depending on the extent of disruption by shaking and sonication. The hemagglutinin is contained in the cell envelope and can be purified with cell wall fractions. Treatment of cell wall with sodium dodecyl sulfate completely inactivated the hemagglutinin. Purified hemagglutinin was found to have an identical polypeptide composition to EB cell walls. Preparations of purified reticulate forms, the reproductive intracellular form of the organism, were almost totally devoid of hemagglutinin.

Numerous investigators have reported studies on a hemagglutinin in organisms of the genus *Chlamydia* grown in the allantoic cavity of the chicken embryo (1-4) and in L-cells (6). In each case, the hemagglutinin was recovered from the soluble fraction remaining after removal of infectious organisms by differential centrifugation. In 1968, Zakay-Rones and associates purified two strains of *C. trachomatis* elementary bodies (EB) from chicken embryo yolk sacs and demonstrated a hemagglutinin present after the EB were broken up by sonication. The hemagglutinin titer was dependent on the number of EB in the preparation and on the length of sonic treatment. Inhibition of hemagglutinin was demonstrable with specific antiserum.

In the studies to be reported here, the presence of a similar hemagglutinin in the EB of *C. psittaci* and its relationship to the cell envelope will be described.

MATERIALS AND METHODS

C. psittaci strain meningopneumonitis (MP) was used throughout this experiment. The organisms were grown in L-cell suspension cultures.

L-cells were routinely cultivated on a rotary shaker at 100 rpm. Cells were grown in 250- or 1,000-ml flasks or in 5-liter spinner bottles. For infection the cells were then transferred to a 14-liter fermentor and infected with MP organisms. The medium used for cell growth and cell maintenance after infection was the same as in a previous report (9).

For infection of cells, infected cultures were centrifuged at 300 × *g* for 10 min to remove cells and cell debris, and the supernatant fluid obtained was inoculated into cell culture in a 14-liter fermentor. The culture was then diluted two times with new cell maintenance medium and incubated at 37 C for 2 days. Growth of MP organisms in cells was examined in smears stained by Giemsa or Macchiavello stains, and when well-developed MP were observed in almost all cells, the culture was harvested. EB were purified from the culture fluid and cells by the methods previously reported (9).

For preparation of purified reticulate bodies (RB), cells in suspension culture were infected with MP organisms at a multiplicity of 10 to 50 plaque-forming units per cell, and the culture was harvested at 18 h after infection. Details of infection and purification of RB were the same as previously reported (11).

The procedures for purification of EB and RB cell walls were the same as in previous reports (5, 10).

Suspensions of chicken erythrocytes were prepared in the following manner. Blood from white Leghorn chickens was collected in Alsever solution, and the cells were washed three times with a diluent consisting of 1 part McIlvain buffer (pH 6.9 to 7.0) and 4 parts 0.85% saline. Unless indicated otherwise, this diluent was used throughout the study. The volume of cells was measured after centrifugation in a graduated tube in a horizontal rotor at 2,000 rpm for 10 min, and sufficient amount of the diluent was added to the cells to make a 10% suspension. This chicken blood cell suspension could be stored in a refrigerator for 3 to 4 days. Before use, the suspension was diluted to make a 1% suspension. Mouse erythrocytes which were used in some experiments were prepared in the same manner except blood was collected fresh for each assay.

The microtiter plate method developed by Takatsy (8) and improved by Sever (7) was used. Twenty-five microliter amounts of serial twofold dilutions of samples to be tested were prepared in wells of microtiter plates by using a microloop. Then 25 μliters each of diluent buffer and a 1% suspension of chicken erythrocytes were added to each well, and the plates were incubated at 37 C for 30 to 40 min. The end point was taken as the highest dilution of sample which gave complete hemagglutination. Each 25 μliters of

the end-point dilution was considered to contain 1 unit of antigen.

The serum to be tested was inactivated at 56 C for 30 min. Serial twofold dilution of serum was then made in buffer saline in 25-μliter amounts in each well of the microtiter plates. A 4- to 6-unit amount of hemagglutinin contained in a 25-μliter volume of buffered saline was added to each well followed without delay by 25 μliters of a 1% suspension of chicken erythrocytes. The plates were incubated at 37 C for 40 min, and the titration end point was taken as the highest dilution of serum which gave complete inhibition of hemagglutination.

All rabbit and chicken sera that we tested contained a nonspecific hemagglutinin inhibitor. To separate this from specific antibody, 10-fold dilutions of antiserum with physiological saline were mixed with the same amount of saturated ammonium sulfate under cooling in an ice-water bath, and the precipitate produced was separated by centrifugation. The precipitate was dissolved in physiological saline and the ammonium sulfate precipitations were repeated twice. The resulting precipitate was dissolved in physiological saline, and the solution was dialyzed overnight at 4 C against physiological saline. Normal rabbit or chicken serum was fractionated with ammonium sulfate in the same way, and the removal of nonspecific inhibitor was ascertained.

New Zealand rabbits were given 10 injections of 0.5 ml each of purified EB suspension (approximately 1.25 mg dry weight/ml) into the ear vein at 3- to 4-day intervals. Six days after the last injection the animals were bled, and their sera were harvested and heat inactivated at 56 C for 30 min.

For polyacrylamide gel electrophoresis, the samples were suspended in 1.0% sodium dodecyl sulfate (SDS), 1% mercaptoethanol, and 8 M urea mixture and heated at 100 C for 10 min. The clear solutions obtained were mixed with one-fifth volume of 50% sucrose and layered on a polyacrylamide gel column. The polyacrylamide gels are composed of 10% acrylamide, 0.27% *N,N'*-bis-methylene acrylamide, 0.1% SDS, 0.1 M phosphate buffer, pH 7.2. Methods for preparing gels and the techniques for electrophoresis and gel staining are given elsewhere (13).

RESULTS

When purified suspensions of intact EB were mixed with chicken erythrocytes, very little hemagglutination was observed (Table 1). However, EB homogenized in a Mickle shaker with glass beads agglutinated the chicken red blood cells, and longer homogenizations resulted in increased titers.

A further sonication of the homogenate after 1 h of treatment in a Mickle shaker resulted in further increase of the activity. Treatment of the Mickle homogenate in a Bronson sonifier at maximum power for 10 min resulted in a further eightfold rise in hemagglutinin titer. One hemagglutinin unit corresponded to 2.4×10^6 particles of original EB suspension. EB homogenates with a titer of 128 at 37 C agglutinated only at a titer of 64 at 25 C and a titer of 8 at 4 C, showing the reaction to be temperature dependent.

TABLE 1. *Appearance of hemagglutinin in purified MP-EB after homogenization by Mickle shaker*[a]

Periods of homogenization by Mickle shaker (min)	Hemagglutination titer
0	16
5	64
10	128
15	128
20	128
30	256
45	256
60	512

[a] Purified MP-EB (2 mg dry weight) were suspended in 4.0 ml of McIlvain buffer (pH 7.0)-physiological saline (1:4 mixture), and the suspension was mixed with 4 g of no. 16 glass beads. The mixture was treated in a Mickle shaker for designated periods, and the supernatants were tested for hemagglutinin titer.

Hemagglutination of mouse erythrocytes with EB homogenates was also observed. In this case, longer incubation periods at 37 C (1.5 to 2.0 h) than in the case of chicken cells was required for final reading.

When EB homogenates were incubated twice with the suspensions of chicken erythrocytes at 37 C for 2 h, the hemagglutinin was completely adsorbed from the supernatant.

The hemagglutinin titer in EB homogenates decreased sharply within 10 min of incubation at 56 C. When the homogenate of EB was stored at 4 C, no decrease in titer was observed after 24 h, but it decreased by about 75% after 5 days. At −76 C no loss of the activity was detected after 2 months if the homogenate was sonicated 2 min before titration, indicating that the hemagglutinin in the homogenate of EB was relatively stable under storage in a refrigerator or freezer.

To determine the specificity of the hemagglutinin in EB homogenate, a hemagglutination inhibition test by immune serum was done. Antisera obtained by injection of purified EB to rabbit inhibited hemagglutination at dilutions ranging from 128 to 512. However, all normal and preimmunized rabbit sera tested contained nonspecific inhibitor of hemagglutination at titers of 64 to 128. Various methods to eliminate the nonspecific inhibitor were tested. Preabsorption of sera with chicken red blood cells and heat inactivation at 56 C for 30 min were of no

effect. When samples of preimmune sera and antisera were fractionated with ammonium sulfate, the titer of nonspecific inhibitor in preimmune serum decreased from 128 of original serum to 16 after two precipitations with ammonium sulfate, but hemagglutination inhibition activity in immune serum decreased only from 512 of original serum to 256 during this procedure (Table 2).

Our previous report (5) demonstrated that almost all internal materials of EB were eluted out after 5 min of treatment in a Mickle shaker with glass beads, with only the cell wall and cytoplasmic membranes remaining. Further shaking results in fragmentation of these structures. The results of the above experiments showing an increase of hemagglutinin titer after continuous treatment in a Mickle shaker for 20 min suggested that the hemagglutinin was probably associated with the cell envelopes. Complete fragmentation of the envelopes might allow for separation of individual hemagglutinin particles or at least the release of hemagglutinin from the envelopes.

TABLE 2. *Removal of nonspecific inhibitor of hemagglutination in rabbit serum by ammonium sulfate fractionation*

Fractions	Hemagglutination inhibition titer
Preimmune serum	
Original	128
Precipitate of first ammonium sulfate treatment	32
Precipitate of second ammonium sulfate treatment	16
Immune antiserum	
Original	512
Precipitate of first ammonium sulfate treatment	256
Precipitate of second ammonium sulfate treatment	256

To determine the location of hemagglutinin in EB, the activity of the material in each step of cell wall purification from purified EB was analyzed. In brief, the procedures for EB cell wall purification involved homogenization of EB in a Mickle shaker with glass beads for 5 min, fractionation of the homogenate by sucrose density gradient centrifugation, and treatment of the cell wall fraction with trypsin and finally with SDS. During these procedures the fraction after trypsin treatment contained both cell walls and cytoplasmic membranes, whereas the final product contained only cell wall fraction (12). For analysis of hemagglutinin, all fractions were dialyzed in McIlvain buffer-physiological saline described above and sonicated 20 min for fragmentation. As shown in Table 3, cell wall fractions obtained after sucrose density gradient centrifugation and trypsin treatment contained more than 75% of the hemagglutinin of the EB homogenate. However, after SDS treatment, no activity was found in either the cell wall fraction or its supernatant fluid. This indicates that the hemagglutinin is associated with envelopes of EB. However, it was not

TABLE 3. *Hemagglutinin titer at each step in purification of EB cell walls*[a]

Sample	Total (ml)	Protein (μg/ml)	Total protein (μg)	Hemagglutinin titer units/0.025 ml	Total hemagglutinin units
Whole EB	3.5	159.4	558	48	6,720
EB homogenate obtained after Mickle shaker treatment for 5 min	6.5	87.2	567	24	6,240
Cell wall fraction obtained after sucrose density gradient centrifugation	3.0	60.8	182	48	5,760
Cell wall after trypsin treatment	2.0	90.4	181	64	5,120
Cell wall after SDS treatment	2.0	39.5	79	2	160

[a] Purified EB suspension in McIlvain buffer-physiological saline (1:4 mixture, pH 7.2) was homogenized in a Mickle shaker with glass beads for 5 min. The homogenate obtained was layered on a 5 to 45% sucrose density gradient column and centrifuged at 8,000 rpm for 30 min. Cell wall fraction obtained was diluted with buffer and spun down by high-speed centrifugation. Cell walls obtained in pellet were suspended in 0.2 M tris(hydroxymethyl)aminomethane buffer, pH 7.4, and treated with trypsin (final concentration: 200 μg/ml) for 2 h at 37 C. Cell walls were spun down by centrifugation and resuspended in water. Then the cell walls were treated with 0.5% SDS for 2 h at 37 C. After incubation, cell walls were recovered in pellet by centrifugation, and SDS that remained was washed off by repeating centrifugation. During this purification a sample was taken at each step and sonicated for 20 min to complete homogenization. After sonication hemagglutinin titers were assayed.

shown whether it is associated with the cell walls or the cytoplasmic membranes. All attempts to separate active hemagglutinin from partially purified cell wall fractions by various detergents failed, and such treatment resulted in loss of all activity.

In an effort to further purify the hemagglutinin, partially purified cell walls obtained after trypsin treatment were sonicated for 60 min for complete fragmentation. The homogenate was layered on a stepwise sucrose density gradient and then centrifuged. The hemagglutinin was concentrated at the interface of 40 to 45% sucrose as shown in Fig. 1. When the sonicated homogenate of cell walls in 50% sucrose were interlayered between 55 to 45% sucrose and centrifuged, the same results were obtained. This indicated that the density of hemagglutinin in EB is between 1.182 and 1.209 at 5 C.

When the band of hemagglutinin obtained in the sucrose density gradient centrifugation in Fig. 1 was harvested, diluted three times with physiological saline, and centrifuged at 105,000 $\times$ g for 120 min, and the polypeptide composition in the pellet obtained was analyzed by polyacrylamide gel electrophoresis, the polypeptide pattern observed after electrophoresis was very similar to that of purified cell walls (Fig. 2). These results suggested that hemagglutinin is associated with envelopes of EB of *C. psittaci*, and the active hemagglutinin preparations are, in fact, intact envelopes in a solubilized or minute particular state.

A suspension of purified RB in McIlvain buffer-physiological saline mixture was homogenized by sonication for 20 min without glass

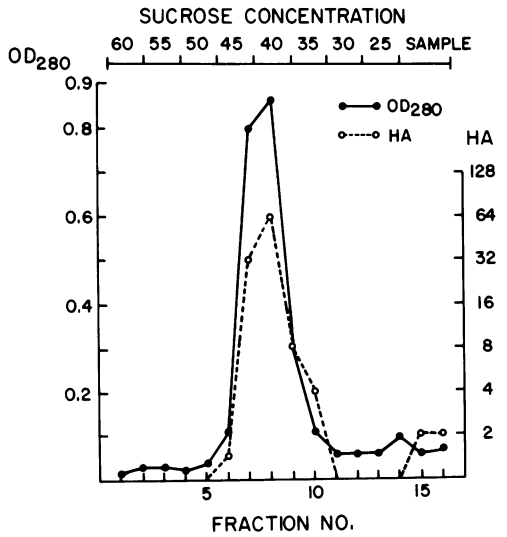

FIG. 1. *Stepwise sucrose density gradient centrifugation of hemagglutinin in EB cell wall homogenates. Partially purified cell walls obtained after trypsin treatment were suspended in McIlvain buffer-physiological saline mixture and sonicated for 60 min. The homogenate (0.5 ml) was layered on a stepwise sucrose density gradient column which was prepared with 0.5 ml each of 60, 55, 50, 45, 40, 35, 30, and 25% sucrose in 5-ml nitrocellulose tubes in the Spinco 39 swinging bucket rotor. The tubes were centrifuged at 38,000 rpm for 15 h. After centrifugation, fractions of 5 drops were collected from a pinhole in the bottom of the centrifuge tube.*

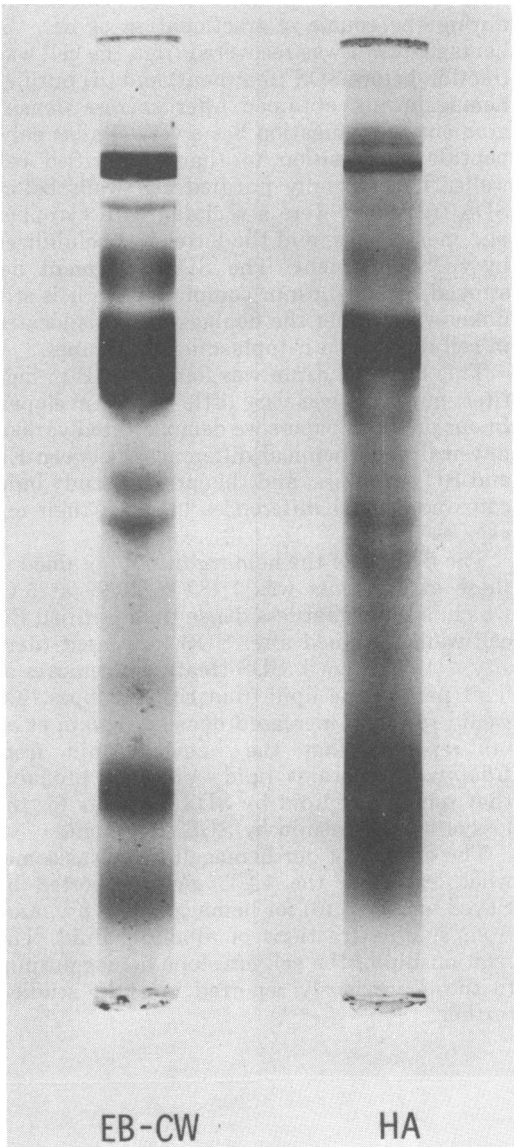

FIG. 2. *Comparison of polypeptide patterns of purified EB cell walls and hemagglutinin by polyacrylamide gel electrophoresis.*

beads, and hemagglutinin in the homogenate was analyzed. In spite of many efforts, no or very little activity was found.

DISCUSSION

A hemagglutinin associated with the EB in *C. trachomatis* has been reported by Zakay-Rones et al. (14). Our studies have demonstrated similar hemagglutinin in EB homogenate of *C. psittaci*. This study has also shown that this hemagglutinin most likely is a part of the envelope of EB from the following evidence: (i) during the course of fractionation of EB, the hemagglutinin was recovered from the cell wall fraction before SDS treatment, and (ii) purified hemagglutinin obtained after sucrose density gradient centrifugation has a very similar polypeptide composition to that of purified cell walls. The partially purified cell walls before SDS treatment were associated with cytoplasmic membranes, and the latter was solubilized by SDS treatment. The SDS treatment destroyed hemagglutinin completely, so it is still unknown whether the hemagglutinin is located in cell walls or in cytoplasmic membranes.

This hemagglutinin was found in EB in high titer, but there was very little in RB envelopes. In our previous papers we demonstrated various physical and chemical differences between EB and RB envelopes, and this present study indicates additional differences between their envelopes.

The density of the hemagglutinin obtained in these experiments was 1.182 to 1.209 at 5 C, which is somewhat less dense than purified EB cell walls obtained after SDS treatment (density = 1.295 g/cm^3). SDS treatment removes at least part of the lipid from EB envelopes that would result in increased density. Jenkin et al. (4) reported that the hemagglutinin from *Chlamydia* contains lipid, and it is probable that removal of lipid by SDS accounts for the loss of hemagglutinin by SDS treatment.

The density of our hemagglutinin was somewhat less than the 1.235 g/cm^3 reported by Sayed and Wilt (6) for hemagglutinin prepared from soluble fractions of allantoic fluid. The relationship of the cell envelope hemagglutinin to those previously reported must be studied further.

ACKNOWLEDGMENT

This work was supported by a Public Health Service grant AI-00868 from the National Institute of Allergy and Infectious Diseases.

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