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Differentiation of Hemagglutinins in Tissues Infected with *Chlamydia*

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Abstract. Crude, soluble, chlamydial hemagglutinin was prepared from allantoic fluid harvested from embryonated chick eggs and the supernatant fluid of mouse L cells infected with either *Chalamydia psittaci* strain 6BC or *Chlamydia trachomatis* strain TW-3. Control non-hemagglutinating specimens of uninfected allantoic fluid and mouse L cells were also prepared. The six preparations were separated by ether-ethanol extraction into lipid-rich and lipid-depleted fractions. Complement-fixing activity was found in the lipid-rich (but not in the lipid-depleted) fraction of infected preparations. In contrast, lipid-rich fractions of infected and uninfected preparations had similar agglutinating activity when sensitive erythrocytes of white Leghorn chickens were used. The lipid-rich fraction of infected and uninfected preparations was separated by thin-layer chromatography (TLC) into seven components with similar R_f values, hemagglutinating patterns, and chemical composition (lipid, protein, and carbohydrate). The highest hemagglutination titers of normal and infected preparations were found in a TLC fraction with similar R_f values and contained lipid, protein, and carbohydrate. This TLC fraction from *C. psittaci* 6BC preparations was used in hemagglutination-inhibition studies. The results indicated that chlamydial hemagglutinin extracted by ether-ethanol and separated by TLC contained, in addition to specific hemagglutinin, nonspecific tissue-lipid hemagglutinin(s) identical to that found in normal preparations.

Chlamydiae are obligate, intracellular parasites that, like many other microbes, agglutinate sensitive erythrocytes. The range of red blood cells susceptible to chlamydial hemagglutinin, however, is limited to mouse erythrocytes [8] and certain sensitive chicken erythrocytes [1,2,22]. The chlamydial hemagglutinin is contained in the surface envelope of infectious elementary bodies [20,22] but is demonstrable in vitro only after sonication of the organisms [20,22]. The hemagglutinin is also released when chlamydiae lyse during the infectious process within host cells [5,6,13]. This so-called soluble hemagglutinin (HA) can be completely separated from chlamydial particles by differential centrifugation of infected host material [5,6,8]. Most studies on the hemagglutinating activity of chlamydiae have been performed on crude concentrated fractions obtained from the allantoic fluid of infected chick embryos after removal of chlamydial particles by differential centrifugation [2,5,8,10]. A major problem in the characterization of chlamydial soluble hemagglutinin is its heterogeneity. Jenkin and co-workers [10] compared the lipid composition of the concentrated, actively hemagglutinating fraction of allantoic fluid from the chlamydiae-infected chick embryo with the lipid composition of concentrated normal allantoic fluid, which had no hemagglutinating activity. These investigators showed definite differences in the proportion of some fatty acids in a number of lipid classes, but "the percentage of total lipid and the fatty acids of the total lipid fraction were essentially similar in normal and infected tissues". Using crude hemagglutinin from allantoic fluid of infected eggs, earlier investigators [6] reported an association of phospholipid, particularly phosphatidylcholine, with chlamydial hemagglutinating activity. However, Jenkin et al. [10] demonstrated that phosphatidylcholine was essentially the same in both uninfected and infected eggs. No direct evidence is available for the chemical nature of chlamydial hemagglutinin, since highly purified preparations were devoid of serological activity [10,20].

Our interest in the biological properties of chlamydial hemagglutinin was reinforced when we ob-

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served that crude, concentrated, hemagglutinating fractions from L cells infected with cytopathic *Chlamydia psittaci* strain 6BC agglutinated and damaged mouse macrophages in vitro and caused release of lysosomal acid phosphatase in these cells; homogenates of uninfected L cells caused neither damage nor agglutination of these cells [12]. Earlier authors [15] used fluorocarbon extracts and/or ether extracts of *C. psittaci* to agglutinate polymorphonuclear leucocytes; such extracts showed high hemagglutinating [6,15] and complement-fixing [7,15] activity. The goal of our present studies was to expand the knowledge of the biological activities of chlamydial hemagglutinin. In this report, we examined ether-ethanol extracts of infected and normal tissues separated by thin-layer chromatography (TLC) to determine chlamydiae-specific and nonspecific hemagglutinating activities. In addition, some preliminary analyses were done on the lipid-rich fractions separated by TLC.

Materials and Methods

Chlamydia psittaci strain 6BC (6BC) and *Chlamydia trachomatis* strain TW-3 (TW-3) were obtained as lyophilized suspensions of infected yolk sac of chick embryo (CE) from the American Type Culture Collection (ATCC, Rockville, Maryland). After receipt, chlamydiae were passaged once in the yolk sac. For preparation of hemagglutinin, chlamydiae were cultivated once in the allantoic fluid of CE and in monolayers of L cells. The mean infectious titer (ID_{50}) of 6BC in CE and in L cells was $10^{8.0}$/ml. The ID_{50} of TW-3 in CE was $10^{8.5}$/ml and in L cells it was $10^{5.0}$/ml. Most experiments were done with 6BC and some with TW-3. Both produce chlamydial soluble hemagglutinin in the allantoic fluid of infected chick embryos [2,8,22] and in infected L cells [16,20].

Production of chlamydial hemagglutinin. Pools of allantoic fluid from 300 infected chick embryos and pools of supernatant fluid (7,000 ml) of infected L cell monolayers were used as sources of chlamydial hemagglutinin because these materials have been reported to be the least contaminated with host-cell debris [14]. Gross debris was sedimented by centrifugation at 1,000 × *g* for 10 min and the chlamydial particles were removed from the supernatant by centrifugation at 10,000 × *g* for 60 min. The chlamydial hemagglutinin was then sedimented at 100,000 × *g* for 1 h, the supernant fluid was removed, and the sediment was resuspended in distilled water, lyophilized, and stored at −70° or at 4°C. Samples were prepared from control allantoic fluid of 300 uninfected chick embryos and uninfected L cells. An amount of 20 ml of uninfected L cells were disrupted by shaking with glass beads and treatment with an ultrasonic disintegrator (Biosonic IV, Bronwill Scientific Inc., Rochester, New York) at 10 KC for 5 min; debris was removed by centrifugation and supernatants were processed as described above.

Extraction of chlamydial hemagglutinin with ether-ethanol. This procedure was chosen since it was used earlier for separation of 6 BC hemagglutinin [6] and hemagglutinins of several enveloped viruses [3]. Freeze-dried hemagglutinin and control uninfected host material were resuspended in 20 ml of McIlvain's buffer-saline solution [8]; 45 ml of an ether-ethanol mixture was added consisting of 3 volumes of diethylether to 1 volume of 99% ethyl alcohol. Extraction was conducted at room temperature for 1 h with agitation by a magnetic stirrer. The lipid-depleted sediment and the ether-ethanol lipid-rich phase were separated, dried in vacuo, and lyophilized, and the dry weight was determined. Specimens were resuspended in McIlvain's buffer-saline to a 1% suspension (wt/vol) and sonically treated immediately before TLC separation. For TLC, an amount of 1.0 to 1.5 mg of the specimen was used.

Thin-layer chromatography. Polysilicilic acid gel impregnated (ITLC-SA) sheets (Gelman Instrument Co., Ann Arbor, Michigan) were used for TLC. The solvent system was a mixture of chloroform-ether-ethanol (10:0.2:0.17). The lipids on the chromatographs were located with iodine, proteins with 0.2% Coomassie brilliant blue, and carbohydrates with silver nitrate [21]. Diethylether was used for the elution of TLC fractions.

Hemagglutination and hemagglutination-inhibition tests. The microtiter-plate method was used [17,19]. Only erythrocytes of white Leghorn chickens sensitive to vaccinia virus hemagglutinin [1,22] were used in all tests. Blood specimens in Alsever's solution were stored at 4°C for a maximum of 4 days before use. Rabbit normal sera and chlamydial antisera against egg-grown and L cell–grown 6BC were prepared as described earlier [11] and used for hemagglutination-inhibition tests [17,19].

Complement-fixation tests were carried out by the microtiter-plate method [4] in the Cadham Provincial Laboratory in Winnipeg, Manitoba. The Wellcome "Psittacosis complement-fixing antigen" (Wellcome Reagents Division, Greenville, North Carolina) was used as control. Other methods are described along with the Results.

Results

Examination showed that the crude concentrated supernatant of L cells and allantoic fluid of chick embryos infected with *Chlamydia psittaci* 6BC (6BC) and *C. trachomatis* TW-3 (TW-3) (but free from chlamydial particles) had hemagglutination titers of 64 to 256. The complement-fixing (CF) titers of these specimens were in the range of 16–32. No hemagglutinating or CF activity was detected in crude concentrated preparations of uninfected L cells and allantoic fluid (Table 1).

When specimens of infected and uninfected tissues were treated with ether-ethanol, two fractions were obtained: an ether-ethanol-soluble, lipid-rich fraction and a lipid-depleted, insoluble fraction. It was of interest that the lipid-rich fraction of both infected and uninfected tissues showed similar high hemagglutination titers. Results from the lipid-depleted fraction of infected tissues showed low hemagglutinating activity. CF activity was detected only in the lipid-rich fraction of infected tissues. CF titers of this fraction were higher than those of crude preparations prior to treatment (see Table 1). Neither hemagglutinating nor CF activity was detected in lipid-depleted fractions of ether-ethanol-treated, uninfected tissues (not shown in Table 1).

When lipid extracts of normal tissues and of tissues infected with 6BC and TW-3 were examined by thin-

Table 1. Hemagglutinating (HA) and complement-fixing (CF) activity of concentrated chlamydial hemagglutinin and of control preparations of L cells and allantoic fluid prior to and after ether-ethanol extraction.[a]

		HA and CF titers			
		Prior to extraction[b]		After extraction[c]	
Chlamydia strains	Source	HA	CF	HA	CF
C. psittaci 6BC	L cells	128	16	1,024	64
C. psittaci 6BC	Allantoic fluid	256	32	2,048	64
C. trachomatis TW-3	L cells	64	8	256	32
C. trachomatis TW-3	Allantoic fluid	128	16	1,024	32
Control uninfected	L cells	0	0	1,024	0
Control uninfected	Allantoic fluid	0	0	512	0

[a] Results from the lipid-rich fraction. Figures indicate reciprocals of highest dilutions of chlamydial hemagglutinin specimens and of control uninfected specimens producing complete agglutination of sensitive erythrocytes of selected adult white Leghorn chickens on incubation at 37°C for 30 min.
[b] Ten milligrams/milliliter of specimens in McIlvain's buffer saline.
[c] One milligram/milliliter of specimens in McIlvain's buffer saline.

layer chromatography (TLC), each of the specimens separated into seven components when stained for lipids by iodine; all specimens displayed a very similar separation pattern. The mean R_f values of the separated fractions of uninfected and infected tissues are shown in Table 2. Each of the seven components contained protein and carbohydrate in addition to lipid. The only difference observed was that fractions 4 and 5 of the specimens from normal L cells and allantoic fluid produced more weakly stained spots by all three staining procedures as compared with the same spots of infected tissues.

Next, fractions separated by TLC were examined for hemagglutinating activity. The results are shown in Table 3. The highest hemagglutinating titers were detected in fraction 4 of both normal and infected preparations; lesser activity was shown in fraction 3, followed by fraction 2. Low levels of activity were invariably found in fractions 1, 5, 6, and 7.

To determine the chlamydial specificity of the hemagglutinin in separated fractions, a hemagglutination-inhibition test using immune serum was carried out. Two specimens of extracts of 6BC derived from supernatant fluid of infected L cells were pre-

Table 2. Thin-layer chromatography of ether-ethanol extracts of concentrated chlamydial hemagglutinin and of control L cells and allantoic fluid.[a]

		R_f values of isolated fractions[b]						
Chlamydia strain	Source	→ 1	2	3	4	5	6	7
C. psittaci 6BC	L cells	0	45.63	71.10	78.90	84.85	92.19	99.06
C. trachomatis TW-3	L cells	0	21.97	51.89	76.69	82.64	91.87	98.68
Control, uninfected	L cells	0	39.65	69.43	80.25	87.72	94.11	99.05
C. psittaci 6BC	Allantoic fluid	0	21.38	52.83	71.07	78.30	88.05	96.86
C. trachomatis TW-3	Allantoic fluid	0	21.27	47.38	73.01	80.86	90.60	98.95
Control, uninfected	Allantoic fluid	0	37.20	60.40	79.54	87.13	94.06	98.68

[a] Thin-layer chromatography with polysilicic acid gel-impregnated sheets and chloroform-ether-ethanol (10:0.2:0.17) solvent system. The air-dried sheets were stained with iodine vapors. Arrow indicates direction of flow. 0 indicates sample application spot (origin).
[b] Each figure represents a mean of three independent runs.

Table 3. Hemagglutinating activity of fractions separated by thin-layer chromatography of extracts of concentrated chlamydial hemagglutinin and of control L cells and allantoic fluid.[a]

		Hemagglutin titers of isolated fractions[b]						
Chlamydia strain	Source	→ 1	2	3	4	5	6	7
C. psittaci 6BC	L cells	4	8	32	64	2	4	4
C. trachomatis TW-3	L cells	4	4	16	32	2	4	2
Control uninfected	L cells	2	8	2	16	2	2	4
C. psittaci 6BC	Allantoic fluid	2	2	8	16	2	2	2
C. trachomatis TW-3	Allantoic fluid	2	4	4	16	2	4	4
Control uninfected	Allantoic fluid	2	4	32	4	2	2	2

[a] Thin-layer chromatography with polysilicic acid gel-impregnated sheets and solvent system chloroform-ether-ethanol (10:0.2:0.17). Separated fractions were eluted and examined for hemagglutinating activity. Arrow indicates direction of flow.
[b] Figures indicate reciprocals of highest dilutions of specimens producing complete hemagglutination of sensitive chicken erythrocytes.

Table 4. The effect of diluted (1:80) *Chlamydia psittaci* 6BC antiserum and normal serum on different hemagglutinating units of isolated fractions of extracted hemagglutinin from supernatant fluid of L cells infected with 6BC.[a]

Fraction no. isolated by TLC	HA titer	Hemagglutinating units/ml of specimen inhibited by:	
		6BC antiserum	Normal serum
4	256	16	4
↑ 3	64	8	8
2	16	4	4

[a] A checkerboard titration was performed to determine the highest decimal dilution of immune and normal rabbit serum which completely inhibited the activity of specimen of antigen in serial twofold dilutions using four wells for each dilution. Arrow indicates direction of flow.

pared and separated by TLC. Fractions 4, 3, and 2 of the two specimens were examined with normal (preimmunized) rabbit serum and 6BC antiserum previously tested by CF and used in immunofluorescent studies [11]. To ensure that the immune serum would contain antibodies against only chlamydiae and none against L cells, antiserum was obtained from rabbits immunized with chlamydiae grown in the yolk sac of chicken embryos. Results from hemagglutination-inhibition tests using a checkerboard titration showed (Table 4) that chlamydial antiserum in a dilution of 1:80 inhibited up to 16 hemagglutinating units of fraction 4 (titer 256) of specimens from infected cells. In contrast, normal serum at a 1:80 dilution inhibited only 4 hemagglutination units of this fraction. Chlamydial antiserum and normal serum in a dilution of 1:80 inhibited hemagglutination of fractions 3 and 2 to similar titers. Because of the low levels of hemagglutinating activity in fractions 1, 5, 6, and 7 (see Table 3), they were not examined by hemagglutination-inhibition tests.

The chlamydial hemagglutinin is released by complete fragmentation of infectious elementary bodies [20,22]. In a pilot experiment, chlamydiae grown in L cells were partially purified by differential centrifugation and treated with ether-ethanol in the same way as soluble fractions of infected tissues (but free from chlamydiae). Hemagglutinating activity (titer 256) was detected in the lipid-rich fraction; none was found in the lipid-depleted fraction. An amount of 0.5 mg of the lipid-rich fraction in McIlvain's buffer-saline was applied on ITLC-SA sheets. In two independent runs, five fractions were detected. The highest hemagglutinating activity was found in the 4th fraction; low activity was detected in fraction 3 and none was present in fractions 1 and 5. Diluted (1:80) chlamydial antiserum inhibited 16 hemagglutinating units of fraction 4, while normal serum in the same dilution inhibited only 4 units. These results indicate (i) that it is possible to extract active hemagglutinins from chlamydial particles by direct ether-ethanol treatment without prior mechanical fragmentation, (ii) that the chlamydiae-specific hemagglutinin extracted from either chlamydial particles or from chlamydiae-free infected tissue material localized in similar spots in the chromatogram and (iii) that the fraction with chlamydiae-specific activity was heterogeneous and contained components whose hemagglutinating activity was inhibited by dilute normal serum.

Discussion

It has been recognized for some time that the best source of erythrocytes for chlamydial hemagglutination is red cells agglutinated by vaccinia virus hemagglutinin [1,2,22]; these cells include those from mice and selected fowls [3]. Burnet and Stone [3] and Stone [18] showed that the same range of red cells that are agglutinated by vaccinial hemagglutinin are also agglutinated by tissue lipids and purified phospholipids.

Host-derived lipids in preparations of chlamydial hemagglutinin from infected tissues have been documented [10,14]. Participation of nonspecific host-tissue lipids in chlamydial hemagglutination might have been expected but has not been described previously in chlamydial hemagglutination for one of two reasons: (i) normal allantoic fluid as control had been included but it was tested before extraction with organic solvents and therefore no hemagglutination was found [6,15]; (ii) lipids from chlamydiae-infected and normal tissues have been compared by using two-dimensional TLC [9], but the separated fractions have not been assayed for hemagglutinating activity.

In the present study, TLC of ether-ethanol extracts revealed a separation pattern common to noninfected tissues and tissues infected with two different chlamydial agents. The only difference observed was that spots of fraction 4 and 5 of uninfected tissues were invariably less dense than corresponding spots of chlamydiae-infected tissues. Jenkin [9] has used two-dimensional TLC of phospholipids and re-

ported that HeLa cells showed a phospholipid pattern similar to *Chlamydia trachomatis* TW-3 but with a much lower density observed in all spots. In another study, Jenkin and co-workers [10] reported that the dry weight and total lipids extracted from chlamydiae-infected tissues were higher than in uninfected tissues, which suggests an increase in lipids in chlamydial infection. The TLC method that we have tried has not been sensitive enough to show differences between experimental and control material. Others [14] have stressed that only when the fatty acids of isolated lipids are analyzed do host and *Chlamydia* differences become apparent.

The experimental observations reported in these studies lead to the conclusion that there are two hemagglutinins in chlamydiae-infected tissues. The one hemagglutinin that was present in infected tissues is probably identical to the hemagglutinin found in normal tissues. This nonspecific tissue hemagglutinin differed from chlamydial hemagglutinin (found in fraction 4) in that the chlamydial hemagglutinin was not inhibited by dilute normal serum. In this regard, it is known that the resistance of the virus hemagglutinin and the susceptibility of the nonspecific lipid hemagglutinin to inactivation by dilute normal serum [18] is the basis for the most satisfactory means of differentiation between hemagglutinating tissue lipids and vaccinial hemagglutinin. The situation with regard to the chlamydial hemagglutinin is more complex than that for free-living bacteria because of the complexity of the envelope of the chlamydial particles, the parasite's unique developmental cycle, and its obligately intracellular habitat. Purified hemagglutinin may be important in serodiagnosis. The development of sensitive tests awaits the production of sufficiently large amounts of purified hemagglutinin from chlamydial envelopes.

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