A STUDY OF THE HEMAGGLUTININS OF CHLAMYDIA

A Thesis Presented to

the Department of Medical Microbiology

Faculty of Medicine

University of Manitoba

In Partial Fulfillment of

the Requirements for the Degree

Master of Science

---

by

Michael Neuman

A STUDY OF THE HEMAGGLUTININS OF CHLAMYDIA

BY

MICHAEL NEUMAN

A thesis submitted to the Faculty of Graduate Studies of
the University of Manitoba in partial fulfillment of the requirements
of the degree of

MASTER OF SCIENCE

ACKNOWLEDGEMENTS

The author would like to express his sincere gratitude to Dr. N. Kordova, Professor, Medical Microbiology, Faculty of Medicine, for her instruction and patient supervision of the research described herein.

TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

Chlamydia are highly successful parasites of man and economically important animals producing severe and fatal diseases in some but the majority being relatively minor. Worldwide in distribution, the trachoma organism remains the leading cause of blindness; trachoma being one of the three most serious diseases of mankind (Burnet, 1971). In addition, recent research has established the role of Chlamydia trachomatis in urethritis, epididymitis, cervicitis, salpingitis, perihepatitis, pharyngitis, pneumonitis and endocarditis (Schacter, 1978)

Chlamydiae belong to a large group of gram-negative procaryotes capable of agglutinating sensitive erythrocytes. The biological role of the chlamydial hemagglutinin is more complex than that of some free-living gram-negative bacteria (Salit and Gottlich, 1977) because of the complexity of the envelope of the chlamydial particles, the parasite's unique developmental cycle and its obligate intracellullar habitat. Studies performed in this laboratory have shown that the infectious form of chlamydiae, the elementary bodies, are dissimiliar to gram-negative bacteria in their nucleoid and cytoplasmic organization, as well as in their inner envelope structure (Costerton et al, 1975, Costerton et al, 1976a, Costerton et al, 1976b). In addition, chlamydial elementary bodies differ in having structurally unique projections arranged hexagonally on their surface. These structures originate in the intermediate layer between the cell wall and membrane (Matsumoto, 1973 Matsumoto, 1975, Stokes, 1978). It is noteworthy that only elementary

bodies, unlike reticulate bodies agglutinate red blood cells (Tamura and Manire, 1974) and are toxic (Manire and Meyer, 1950). Although the hemagglutinin is contained in the cell envelope (Tamura and Manire, 1974, Zakay-Rones et al, 1968), it is demonstrable in vitro only after sonication of the elementary bodies (Tamura and Manire, 1974, Zakay-Rones et al 1968). The hemagglutinin is also released with lysis of chlamydiae during the infectious process within host cells (Gogolak, 1954, Gogolak and Ross, 1955, Litwin, 1959). This "soluble" hemagglutinin can be completely separated from chlamydial particles by differential centrifugation (Gogolak, 1954, Gogolak and Ross, 1955, Hilleman et al, 1951). In a concentrated crude form it presents a convenient source of material for studies of hemagglutination (Barron and Riera, 1969, Gogolak, 1954, Hilleman et al, 1951, Jenkin et al, 1970) and has been used for the chemical characterization of the hemagglutinin (Jenkin et al, 1970, Sayed and Wilt, 1971). Earlier studies performed in this laboratory have shown that crude concentrated hemagglutinating fractions from L-cells infected with cytopathic chlamydiae agglutinate and damage mouse macrophages in vitro and cause the release of lysosomal acid phosphatase in these cells. The cytopathic properties of the soluble hemagglutinin (also shared by intact elementary bodies) are absent in homogenates of non-infected L-cells (Kordova and Wilt, 1972). Other authors,(Parikh and Scechmeister, 1964) used fluorocarbon and/or ether extracts of chlamydiae to agglutinate polymorphonuclear leucocytes. Several authors reported that

ether extracts of soluble chlamydial hemagglutinin possess high hemagglutinating activity (Gogolak and Ross, 1955, Parikh and Schechmeister, 1964). Little information is present in the literature however, as to how agglutination of sensitive red cells with extracted hemagglutinin is influenced, for example, by the composition and pH of the reaction medium, salts and temperature. Since hemagglutination-inhibition measures qualitatively different chlamydial antibodies than the complement-fixation reaction (Benedict and Briggs, 1958, Sayed and Wilt, 1971, Turner and Gordon, 1964), the potential value of a sensitive hemagglutinating system seemed to justify further studies of the chlamydial hemagglutinin.

The aim of this project was to identify the most favourable conditions for titration of ether-alcohol extracted hemagglutinin and to compare extracts prepared from allantoic fluids of chick embryos and from supernatants of L-cells after infection with the agent of psittacosis (C. psittaci 6BC) and the trachoma organism (C. trachomatis TW3). Several aspects were investigated: production and assay of the hemagglutinin; characteristics of its adsorption to sensitive erythrocytes and stability of the red cell receptor sites to various physicochemical treatments. In addition, it seemed of interest to determine whether extracted hemagglutinin would agglutinate human red cells that do not possess receptor sites for union with crude chlamydial hemagglutinin (Hilleman et al, 1951). Finally, whether and how the extracted hemagglutinins of two variant 6BC strains with different toxicity for macrophages would interact with susceptible erythrocytes having had receptor sites removed or destroyed was investigated. The biological properties

of the hemagglutinin have been supplemented by preliminary chemical investigations; the ether-ethanol extracted hemagglutinin and control preparations were examined by thin-layer chromatography.

LITERATURE REVIEW

I TAXONOMY

Chlamydia comprise a broad group of procaryotic obligate intracellullar parasites which share a common structure, a common developmental cycle in susceptible host cells and a common so-called group antigen. Because of the unique developmental cycle that differentiates them from all other micro-organisms, the chlamydiae have been placed in their own order, the Chlamydiales (Page, 1974). Currently, the genus Chlamydia is subdivided into two species on the basis of two biological properties:

1. Chlamydia differ in their susceptibility to sulfonamide; C. psittaci usually being sensitive and C. trachomatis being resistant.

2. They differ in the kind of intracytoplasmic inclusions produced in the infected host cell. Whereas C. trachomatis produces inclusions containing glycogen, those produced by C. psittaci contain none. Despite overlapping of several characteristics, DNA hybridization experiments have demonstrated that C. psittaci and C. trachomatis are distinct agents (Kingsbury and Weiss, 1968). High homology has been reported between strains of C. trachomatis in contrast to variable (40 to 70%) homology between C. psittaci strains. C. trachomatis strains are human pathogens with man as the sole natural host while C. psittaci infects and produces diseases in a wide range of mammalian and avian species including man. In contrast to C. trachomatis, man is only an accidental host for C. psittaci.

II. INTERACTION OF CHLAMYDIA WITH ERYTHROCYTES

Agglutination of Sensitive Erythrocytes by the Hemagglutinin of Different Chlamydial Agents

The chlamydial hemagglutinin was first reported in 1950 by Haig and Hilleman in association with the meningopneumonitis agent of C. psittaci which had been thought to be a large virus at that time. Hilleman, Haig and Helmond (1951) first described that the hemagglutinin is of smaller size than the complement-fixing infectious chlamydial particles and that the two are readily separated by centrifugation. Subsequently, feline and murine pneumonitis agents (Gogolak, 1954) and the 6BC strain (Gogolak and Ross, 1955) of C. psittaci, as well as the Bour and T'ang strains of C. trachomatis (Zakay-Rones et al, 1968) were shown to produce a hemagglutinin in the allantoic fluid of infected chicken embryos. Currently, it is generally accepted that all chlamydial agents produce a hemagglutinin for certain avian and murine erythrocytes (Storz, 1971).

Agglutination by Chlamydial Hemagglutinin of Red Cells in Relation to Animal Species

Hilleman, Haig and Helmond (1951) reported that the hemagglutinin produced by the meningopneumonitis agent could agglutinate mouse erythrocytes but not pigeon, quail, starling, rabbit, hamster, guinea pig, sheep and human "O" type erythrocytes. Barron and co-workers (1965) showed that individual chickens whose erythrocytes were sensitive to vaccinia hemagglutinin were also susceptible to C. psittaci 6BC hemagglutinin. However,

antisera produced against vaccinia and chlamydiae were not cross-reactive in hemagglutination inhibition. A correlation between the strain of chicken and the susceptibility of its erythrocytes to chlamydial hemagglutinin was also observed (Barron et al, 1965). The frequency of positive reactions for purebred White Leghorns was significantly greater than for Rhode Island Reds. Within the group of positive chickens, a broad range of hemagglutinating titers was observed reflecting differences in sensitivity. In a more recent study, Barron and Riera (1969) confirmed the earlier reported high frequency of positive reactions by erythrocytes from White Leghorns (94.7%) in contrast to New Hampshire Reds (10.0%). Titers of sensitive chicken erythrocytes were slightly higher than of susceptible murine erythrocytes in parallel examinations of the hemagglutinin produced by the 6BC, meningopneumonitis, feline pneumonitis and mouse pneumonitis strains of C. psittaci. The authors found that the hemagglutinin of C. psittaci strains in parallel with the vaccinia hemagglutinin agglutinated turkey erythrocytes but failed to agglutinate dog, sheep, human and peacock red blood cells. They demonstrated that the absorption of psittacosis hemagglutinin with either sensitive mouse or chicken erythrocytes resulted in removal of hemagglutinating activity for both chicken and mouse erythrocytes therefore suggesting that only one type of hemagglutinin was present.

Agglutination of Sensitive Erythrocytes in Relation to "Particulate" and "Soluble" Chlamydial Hemagglutinins

Initial studies on the chlamydial hemagglutinin were performed using crude allantoic fluid of chlamydiae-infected chicken embryos or supernat-

ants of infected allantoic fluids from which chlamydial particles had been removed by centrifugation (Hilleman, Haig and Helmon, 1951). Hemagglutinating activity was associated with a component not readily sedimented at 100,000 g for 75 minutes and distinct from the sedimented chlamydial particles. Gogolak and Ross (1955) proposed that the chlamydial hemagglutinin "represented either incomplete virus material which is not incorporated into the integral elementary body or one of the end products of virus degradation". Zakay-Rones et al (1968) demonstrated that the purified elementary bodies of two strains of the trachoma agent contain a hemagglutinin which is demonstrable only after ultrasonic treatment. Tamura and Manire showed in 1974 that the hemagglutinating titer of C. psittaci elementary bodies increased linearly with time of sonication. The hemagglutinating activity was associated with the cell envelope of chlamydial elementary bodies whereas very little hemagglutinating activity was associated with the cell envelope of the non-infectious reticulate body. The authors suggested "that the active hemagglutinin preparations are in fact, intact envelopes (of elementary bodies) in a solubilized or minute particulate state".

Hemagglutinating Activity of Chlamydial Hemagglutinins

There seems to be agreement in the literature that crude hemagglutinin preparations have relatively low agglutinating activities with both sensitive mouse and chicken erythrocytes. Hilleman et al

(1951) reported that less than one-fifth of crude allantoic fluids of infected chick embryos agglutinated sensitive mouse erythrocytes to titers as high as 1:32. Gogolak (1954), in working with sensitive mouse erythrocytes, reported that 62% of crude allantoic fluids of C. psittaci-infected chicken embryos did not produce demonstrable hemagglutinin. An amount of only 1 ml of hemagglutinin (with a titer of 1:256) could be concentrated from allantoic fluids of 200 infected chicken embryos. Riera and Barron (1970) reported that pools of allantoic fluid from which elementary bodies had been removed agglutinated sensitive chicken erythrocytes to titers of 1:32 to 1:64; one hundred-fold concentrated preparations hemagglutinated to 1:128 to 1:256. Jenkin et al (1970) reported that concentrated hemagglutinin preparations from allantoic fluids from infected chicken embryos agglutinated sensitive erythrocytes to a titer of 1:512. It has been suggested, without any proof however, that some unidentified non-specific inhibitors of chlamydial hemagglutination are present in crude hemagglutination preparations from allantoic fluid (Gogolak, 1954).

Inhibition of Chlamydial Hemagglutination by Specific Antisera

The hemagglutination reaction is inhibited by specific antisera that are cross-reactive beween as well as within both chlamydial species. Specific inhibition of the meningopneumonitis (C. psittaci) hemagglutinin by homologous anti-serum was first described by Hilleman et al (1951); the antiserum when diluted as much as 1:320 inhibited four units of the hemagglutinin while control serum inhibited only at a 1:20

dilution. Gogolak and Ross (1955) reported that antisera to C. psittaci 6BC inhibited 6BC and C. psittaci feline pneumonitis hemagglutinin to identical titers. Barron et al (1965) reported that anti-C. trachomatis serum was more inhibitory to hemagglutination by C. psittaci 6BC than the homologous antiserum. Anti-C. psittaci and anti-C. trachomatis sera were equally inhibitory to the hemagglutinin dissociated from C. trachomatis elementary bodies by ultrasonic treatment (Zakay-Rones et al, 1968). Barron et al (1965) suggested that the chlamydial hemagglutinin might be a group-specific antigen common to the members of Chlamydia. The hemagglutination inhibition test for measuring chlamydial antibody is not routinely used since it is subject to non-specific reactions whose nature is largely not known.

Non-specific Inhibitors of Chlamydial Hemagglutination

Non-specific inhibitors of chlamydial hemagglutination in normal sera of roosters, pigeons, rabbits and man were first reported by Hilleman et al (1951). Barron and co-workers (1965) observed that normal rabbit sera contained a non-specific hemagglutination inhibitor which reacted more strongly with chicken than mouse erythrocytes. Barron and Riera (1969) examined serum from normal (control non-infected) White Leghorn and New Hampshire Red chickens for chlamydial antibodies using the hemagglutination-inhibition test; titers ranged from 1:4 to 1:16. Tamura and Manire (1974) reported that all rabbit and chicken sera tested contained a non-specific inhibitor. This was removed by fractionation of the sera with ammonium sulfate. The nature of the non- spec-

ific hemagglutination-inhibitor in avian and mammalian sera is not known. Several investigators (Hilleman et al, 1951, Barron et al, 1965, Gogolak, 1954) reported that allantoic fluids from infected chicken embryos hemagglutinate when diluted but not in undiluted form. The presence of a non-specific hemagglutination-inhibitor in allantoic fluid of uninfected and chlamydiae-infected chickens has been suggested by several authors but its nature has not been elucidated.

Antigenic Structure of Chlamydiae and Chlamydial Hemagglutinin

Chlamydiae possess a complex antigenic structure which has been studied by a number of earlier investigators (Ross and Gogolak, 1957a, Ross and Gogolak, 1957b, Peters, 1967). Both chlamydial species share a major common antigenic component which appears to be associated with the cell wall (Reeve and Taverne, 1962). This antigen is complement-fixing, heat-stable and ether-soluble, and it may be extracted or partially solubilized from chlamydial particles by boiling or treating with acid, alkali, deoxycholate, ether or ether-alcohol. It appears that lecithin may be a carrier since some of the highly purified antigens were inactive in the complement-fixation test until lecithin was added. The antigen was inactivated by periodate (Ross and Gogolak, 1957b). More recent studies (Dhir et al, 1972) have identified the active moiety as an acidic polysaccharide 2-keto-3-deoxyoctonic acid. The species-specific antigen, common to all C. trachomatis immunotypes but not to C. psittaci, is heat-labile (Schacter and Dawson, 1978). Jenkin, Ross and Moulder (1961) , however, reported that

the species-specific complement-fixing antigen of the meningopneumonitis agent (C. psittaci) was heat stable (100°C for 30 minutes). More recently, Caldwell, Kuo and Kerry (1975) used two dimensional electrophoresis for the antigenic analysis of Chlamydiae. Antigens of chlamydiae were found to be solubilized with the non-ionic detergent Triton X-100 Nineteen and sixteen distinct components were observed for C. trachomatis and C. psittaci respectively. The Triton solubilized antigens of chlamydiae differ between species. In 1980, Hourihan, Rotta and McDonald first reported the isolation and purification of a type specific antigen from C. trachomatis serotype A. Separate fractions were tested for type specificity utilizing the IgG prepared from exhaustively cross-absorbed hyperimmune sera from rabbits immunized with homologous organisms. The purified type specific antigen has a M.W. of 30,000 to 32,000 daltons.

The progress of research in antigenic and chemical analysis of the chlamydial soluble hemagglutinin has been greatly impeded by limitations in the production of sufficient quantities of highly purified preparations. Gogolak and Ross (1954) carried out the first extensive study on a chlamydial hemagglutinin obtained from the allantoic fluid of infected embryonated eggs. The authors described a closer association of the antigen with elementary than reticular bodies and a group-specific reactivity. Jenkin et al (1970) used chemical procedures for isolation and characterization of the hemagglutinin from allantoic fluid from infected chick embryos and reported that highly purified preparations were devoid of serological activity. In 1971, Sayed and Wilt purified a soluble C. psittaci hemagglutinin from overlying media of infected L-cells (but

free from intact chlamydial particles) by chromatography and electrophoresis. The purified antigen had a molecular weight of 100,000 to 120,000 daltons and reacted more strongly with homologous than with heterologous strains. The authors suggested that there might be more than one antigen in the preparation not separable on the basis of molecular weight, density or electrophoretic mobility in polyacrylamide gel; a possible alternative might be the presence of a group- and type-specific activity on the same molecule.

The hemagglutinin is contained in the cell envelope of chlamydial elementary bodies and can be obtained by sonication of the particles (Zakay-Rones et al, 1968). Tamura and Manire (1974) therefore attempted to purify the hemagglutinating-active fraction from purified cell wall preparations of C. psittaci elementary bodies. The authors reported that sodium dodecyl sulfate (SDS) completely inactivated the hemagglutinating activity of the purified preparation. The removal of lipid by the SDS apparently accounted for the loss of hemagglutinating activity. The authors concluded that it remains unclear whether the hemagglutinin is located in the cell walls or in cytoplasmic membranes of the chlamydial elementary bodies.

Because highly purified preparations of hemagglutinin were devoid of serological activity, the chemical nature of the hemagglutinin remains unknown (Jenkin et al, 1971, Tamura and Manire, 1974).

Separation and Purification of Hemagglutinating-active Preparations from Chlamydiae-infected Host Material but Free of Chlamydial Particles

Numerous recent investigators (Jenkin, 1970, Zakay-Rones et al, 1968, Tamura and Manire, 1974) confirmed the early observation of Haig and Hilleman (1950) and Hilleman et al (1951) that intact infectious chlamydial particles agglutinate sensitive erythrocytes in very low titers. Most earlier studies on the hemagglutinating activity of chlamydiae have been performed, therefore, on the so-called "soluble" hemagglutinin that can be completely separated from chlamydial particles by differential centrifugation of infected host material (Hilleman et al, 1951). Earlier investigators (Hilleman et al, 1951, Gogolak, 1954, Gogolak and Ross, 1955 Jenkin et al, 1970) used allantoic fluid of infected chick embryos after removal of chlamydial particles for preparation of the hemagglutinin. More recent investigators (Sayed and Wilt, 1971, Kordova et al, 1972, Kordova et al, 1981) used overlying media from C. psittaci-infected cultured mammalian cells from which chlamydiae were removed by differential centrifugation for preparation of hemagglutinin. Such crude "soluble" hemagglutinin derived from either allantoic fluid or cell culture had relatively high serological activity when compared to that of intact infectious chlamydial elementary bodies.

Different methods have been used for purification of the crude soluble hemagglutinin. Gogolak and Ross (1955) carried out the first extensive study on a C. psittaci soluble hemagglutinin obtained from the allantoic fluid of infected embryonated eggs. They used an ether-

ethanol mixture for extraction. The extracted antigen had a two-fold increased activity relative to the equivalent volume of non-extracted preparation with a hemagglutination titer of 1:256.

Sayed and Wilt (1971) isolated a hemagglutinogen from the soluble antigens of C. psittaci 6BC grown in mouse L-cell fibroblasts by linear sucrose density gradient centrifugation. This hemagglutinin was shown to have at least six contaminating proteins, however, by polyacrylamide disc electrophoresis. Fractionation of the crude soluble antigens by Sephadex gel filtration produced three peaks with the hemagglutinating activity present in a fraction corresponding to a molecular weight of 100,000 to 120,000 daltons. Five bands were visualized after disc electrophoresis of this hemagglutinating fraction. Antigen eluted from an actively hemagglutinating subfraction obtained in this way contained 55% of the activity of the original crude soluble antigen preparation.

More recent studies in this laboratory (Kordova, Wilt and Herchl, 1981) used crude soluble chlamydial hemagglutinin from allantoic fluid harvested from embryonated chick eggs and the supernatant fluid of L-cells infected with either C. psittaci 6BC or C. trachomatis TW3. The crude preparations were separated by ether-ethanol extraction (originally used by Gogolak and Ross, 1955) into lipid-rich and lipid depleted fractions. The lipid-rich fraction was further separated by thin layer chromatography into seven components, one of which was associated with chlamydiae-specific hemagglutinating activity. This fraction contained lipid, protein and carbohydrate.

Preparation of Hemagglutinating-active Fractions from Infectious Chlamydial Particles

In 1968, Zakay-Rones and associates purified two strains of C. trachomatis elementary bodies from chicken embryo yolk sacs and demonstrated hemagglutinating activity after the elementary bodies were broken up by sonication. The hemagglutinin titers were dependant on the number of elementary bodies in the preparation and on the length of time of ultrasonic treatment. Inhibition of hemagglutination was demonstrable with specific anti-serum.

Tamura and Manire (1974) investigated the hemagglutinin in the cell envelope of C. psittaci elementary bodies grown in L-cells. They partially purified chlamydial cell envelopes, fragmented them with ultrasound and centrifuged the resulting homogenate on sucrose density gradients. The density of the hemagglutinating-active fraction obtained was found to be in the range of 1.182 to 1.209. The density of this hemagglutinin was somewhat less than that reported by Sayed and Wilt (1971) for the hemagglutinin prepared from soluble hemagglutinin fractions of fractions of supernatants of infected L-cells. Tamura and Manire attempted to separate the active hemagglutinin from partially purified cell wall fractions by various detergents; such treatment, however, resulted in loss of all hemagglutinating activity.

Studies performed in this laboratory (Kordova, Wilt and Herchl, 1981) showed that it is possible to extract active hemagglutinin from infectious chlamydial particles by direct ether-ethan-

ol treatment without prior mechanical fragmentation; the chlamydiae-specific hemagglutinating-active fraction from either infectious chlamydial particles or from "soluble" (chlamydiae-free) hemagglutinin localized in similiar spots on the thin-layer chromatogram. The fraction with chlamydiae-specific activity was composed of lipid, protein and carbohydrate.

Chemical Characterization of the Hemagglutinating-active Fraction

No direct information is available as to the chemical nature of chlamydial hemagglutinin since highly purified preparations were devoid of serological activity (Jenkin et al, 1970, Tamura and Manire, 1974).

In 1955, Gogolak and Ross associated lecithin in the crude soluble C. psittaci 6BC hemagglutinin with hemagglutinating activity because phospholipase treatment destroyed this activity. In 1970, however, Jenkin et al showed that phosphatidyl choline was present in essentially the same amount in both the chlamydial "soluble" hemagglutinin harvested from infected eggs as in preparations of concentrated normal allantoic fluids. According to the authors, "the association of specific lipids to the hemagglutinating activity of chlamydiae is yet to be ascertained".

In this laboratory, lipid extracts of normal uninfected tissues, and of tissues infected with C. psittaci and C. trachomatis were examined by thin-layer chromatography (Kordova, Wilt and

Herchl, 1981). The results of these studies indicated that there are two hemagglutinins in chlamydiae infected tissues. The one hemagglutinin that was present in infected tissues is probably identical to the hemagglutinin which was found in normal tissues. This non-specific tissue hemagglutinin differed from chlamydial hemagglutinin in that the chlamydial hemagglutinin was not inhibited by dilute normal serum. The chlamydial hemagglutinin contained protein and carbohydrate besides lipid; it remains to be shown whether the specific chlamydial hemagglutinin is a glycolipid or a glycoprotein.

For several years, the biological and chemical properties of the chlamydial soluble hemagglutinin have been compared to that of poxvirus hemagglutinin (Gogolak and Ross, 1955, Barron et al, 1965 , Schechmeister and Parikh, 1962). At that time, it had been suggested (Burnet, 1946) that poxvirus hemagglutinin is composed of a virus antigen in combination with a phospholipid which is responsible for attachment to the erythrocyte. More recent work (Ikuta et al, 1979) has shown that in poxviruses a number of specific glycoproteins may carry both hemagglutinin-antigen specificity and erythrocyte-binding capacity.

Stability of the Chlamydial Hemagglutinin to Physical, Chemical and Enzymatic Agents

Only fragmentary data is available in the literature on the stability of the chlamydial hemagglutinin. Hilleman and collabor-

ators (1951) found that storage of crude soluble C. psittaci hemagglutinin from allantoic fluid of embryonated eggs in either 0.5% formalin or 0.1% phenol caused the loss of all hemagglutinating activity; a two- to four-fold decrease of activity was seen after storage at 4°C; heating at 56°C for 30 minutes resulted in an eight-fold decrease of titer while boiling abolished hemagglutinating activity. Gogolak (1954) using crude soluble hemagglutinin of another strain of C. psittaci reported similiar effects under similiar conditions: formalin and phenol completely destroyed the hemagglutinating activity, while exposure to a temperature of 56°C for 30 minutes resulted in an eight-fold reduction in titer. Ultraviolet light failed to affect the hemagglutinating activity.

Gogolak and Ross (1955) reported that treatment of crude soluble hemagglutinin of C. psittaci with lecithinase A resulted in almost complete destruction of hemagglutinating activity; the authors suggested that lecithin is essential in the hemagglutinating phenomenon caused by chlamydial hemagglutinin. Zakay-Rones et al (1968) reported that trypsin (0.1 to 0.5% w/v) did not reduce the hemagglutinating activity of sonicated C. trachomatis elementary bodies whereas chloroform and heat (56°C, 30 minutes) inactivated this preparation.

Effect of Environmental and Chemical Conditions on the Hemagglutination Reaction

Limited information is available about the effect of environ-

mental conditions on the hemagglutination reaction. Hilleman et al (1950), using a preparation composed of both soluble and particulate antigens of meningopneumonitis agent (C. psittaci) reported that hemagglutination took place in a narrow range (± 1 pH unit) about neutrality. Gogolak (1954) states only that "wide deviations on both sides of the neutrality point resulted in little or no hemagglutination" of mouse erythrocytes with crude soluble hemagglutinin of C. psittaci. Hilleman et al (1950) observed that unbuffered crude infectious allantoic fluid (pH 8.0 to 8.6) produced partial hemagglutination at intermediate dilutions and none at low and higher dilutions. The maximal titers occurring at this intermediate dilution were restored by neutralization of the allantoic fluid after having been reduced by dilution or concentration from optimal concentration. The authors suggested that this effect was due to reduced calcium binding to protein at pH 7.0 relative to that at higher pH. Therefore, they suggested that at neutrality the union of hemagglutinin with red cells through binding of calcium with either hemagglutinin or receptor sites on the red blood cell would not be prevented. In support of this proposal, they dialyzed the crude allantoic preparation against normal saline, increasing its titer, and reversed this "activation" by introducing calcium and magnesium chloride into the dialyzed preparation. McIlvaine-buffered saline (pH 7.0) was thereafter used as a diluent by several authors (Hilleman et al, 1950, Gogolak, 1954, Gogolak and Ross, 1955, Barron et al, 1965, Barron and Riera, 1969, Riera and Barron, 1970, Sayed and Wilt, 1971, Tamura and Manire, 1974)

because of the citrate present to bind divalent cations. Barron et al (1965) demonstrated that magnesium sulfate inhibited hemagglutination of chicken and mouse erythrocytes by C. psittaci 6BC hemagglutinin supporting the earlier observations of Hilleman et al (1951).

Optimal temperatures for the hemagglutination reaction appear to be between room temperature and 37°C. Barron et al (1965) observed similar results at these temperatures for chicken and mouse erythrocytes using the "soluble" hemagglutinin of C. psittaci 6BC strain. In the cold, weak patterns of agglutination were qualitatively described while generally the titers were lower than at 37°C or room temperature. The same laboratory (Zakay-Rones et al, 1968) later investigated hemagglutination of chicken erythrocytes by sonicated C. trachomatis elementary bodies in relation to the effect of temperature. Identical titers at 20°C and 37°C were reported whereas they were greatly reduced at 4°C.

III. AGGLUTINATION OF WHITE BLOOD CELLS BY C. PSITTACI PREPARATIONS AND BY ITS CELL ENVELOPE

In a series of studies, Parikh and Schechmeister (1961 - 1967) described that leucoagglutination of rabbit polymorphonuclear cells by C. psittaci (meningopneumonitis agent) appears to be similiar to hemagglutination in that the union of the receptor sites of the blood cells and antigenic sites on the organisms has been shown to be involved in both reactions. Strongest chlamydiae-induced leucoagglutination occurred at $37^{o}C$; approximately 62 intact chlamydial particles per leucocyte were required for clumping of leucocytes. The leuco agglutinating activity of intact meningopneumonitis elementary bodies was abolished by pretreatment with trypsin (.25%), mercuric chloride ($5 \times 10^{-5}M$) or p-chloromercuribenzoic acid ($10^{-4}M$). Active leucoagglutinin was demonstrated in a fluorocarbon extract (FEM) of chlamydial particles, an ether extract of FEM and a chlamydial cell wall preparation. These leuco-agglutinins were isolated by continuous flow electrophoresis and column chromatography and have been shown to have precipitating and leucoagglutinating activity; only 2 of 7 chlamydial precipitinogens were involved in leuco-agglutination.

Kordova and Wilt (1972) stated that concentrated chlamydiae-free "soluble" hemagglutinin from culture medium of C. psittaci-infected L-cells also caused agglutination of mouse monocytes.

MATERIALS AND METHODS

CHLAMYDIAL STRAINS

The 6BC strain of C. psittaci was originally obtained as a lyophilized suspension of infected yolk sacs of chick embryos (CE) from the American Type Culture Collection (ATCC). Upon receipt, the agent has been passaged in the yolk sacs of six-day old chick embryos. Stock 20% yolk sac suspensions were prepared using Eagle's Minimal Essential Medium (MEM). The mean infectious titer (ID_{50}) of 6BC in yolk sacs of CE and in L-cells was $10^{8.0}$/ ml.

Strain TW3 of C. trachomatis originated from the American Type Culture Collection. Prior to receipt, it had undergone 24 passages in the yolk sac of CE in the laboratory of Dr. San-pin Wang (University of Washington, Seattle, Washington). Upon receipt as a lyophilized 40% suspension of infected yolk sac, it was further passaged in the manner of C. psittaci 6BC. The ID_{50} of TW3 in the yolk sac of CE was $10^{9.0}$/ml. and in L-cells it was $10^{5.0}$/ml.

Yolk sac suspensions of both C. psittaci 6BC and C. trachomatis TW3 were each used to infect monolayer cultures of mouse fibroblasts (L-cells) and the allantoic fluid of nine-day old chick embryos.

CELL CULTURE

L-929 cells (mouse fibroblasts) were originally obtained from the American Type Culture Collection (Bethesda, Maryland,

U.S.A.). The cells were maintained as monolayers using Eagle' MEM (Flow Laboratories, Virginia, U.S.A.) containing 200 ug/ml of Streptomycin sulfate (Allen and Hanbury's, Toronto, Canada), 10% heat-inactivated calf serum (Grand Island Biological Co., New York, U.S.A.) supplemented with L-Glutamine (Flow Laboratories, Virginia, U.S.A.) as growth medium. The cells were grown before and after infection at $37^{o}C$ in the same medium. Viability of the cells was routinely determined by the trypan blue exclusion method and the cultures were periodically checked for mycoplasma and bacterial contamination.

INFECTION OF L-CELLS

L-cells were inoculated in the following manner: Cells were grown in 75 cm^2 Falcon flasks (Becton-Dickenson and Co., Oxnard California) or in 500 ml Roux bottles until confluent monolayers had developed on the vessel surfaces (10 to 20 X 10^6 cells). Immediately prior to inoculation, culture medium was removed, the monolayers were washed with Dulbecco's Phosphate Buffered Saline Solution (PBS), and were rinsed with 30 ug/ml DEAE-dextran (Pharmacia, Uppsala, Sweden) for one minute. This solution was aspirated, the monolayers were rinsed with Eagle's MEM and appropriate dilutions (usually 10^{-1} or 10^{-2}) of stock inoculum were applied. Following one hour of adsorption, inoculum was removed and the monolayers were replenished with Eagle's MEM.

The course of infection of monolayers was followed by examination of samples scraped from the monolayer, examination of cells

from Leighton tubes inoculated in parallel with the Falcon flask or of cells released from the culture vessel during infection. Coverslip cultures in Leighton tubes were incubated for varying periods of time after infection following which they were removed, stained with May-Grunwald Giemsa stain (Harleco, Gibbstown, N.J., U.S.A.) and examined for the presence of typical intracytoplasmic inclusions. In addition, L-cells inoculated with C. trachomatis were stained with Iodine-stain and examined to detect typical glycogen-containing inclusions.

EMBRYONATED EGGS

Embryonated eggs were aquired from the Dept. of Animal Science, Faculty of Agriculture, University of Manitoba. They had been obtained from a flock of White Leghorn chickens which had been maintained without antibiotics.

INFECTION OF THE ALLANTOIC FLUID OF CHICK EMBRYOS

Predetermined dilutions of stock suspensions of infected yolk sacs in Eagle's MEM (with the addition of 200 µg/ml Streptomycin) were inoculated into the allantoic fluid of nine-day old chick embryos. These were incubated in a Janesway upright incubator at 37°C and were candled daily to determine mortality. Chick embryos dying within the first three days following infection were discarded. Embryos dying on the fourth to seventh days following inoculation and those which survived on the seventh day after inoculation were refrigerated overnight. Their allantoic fluids were collected, pooled

and routinely tested for bacterial contamination.

PREPARATION OF "PARTICULATE" AND "SOLUBLE" HEMAGGLUTININ FROM CHLAMYDIA INFECTED L-CELLS

Chlamydial particles (i.e. "particulate"hemagglutinin) were harvested from both the maintenance medium of infected cells as well as from infected monolayers. The culture fluids (which contained chlamydiae spontaneously released from cells) were periodically collected, pooled and frozen at -70°C prior to further processing.

Growth of chlamydiae in cells was examined in appropriately stained smears. When well developed typical inclusions were observed in almost all cells, the culture was scraped from the surface of the vessel, cells were concentrated by centrifugation at 270g for 15 minutes and homogenized by a ground glass homogenizer in an ice bath. The homogenate was centrifuged at 270g for 15 min. in a Sorvall RC2B centrifuge using an SS-34 angle head rotor; the pellet which contained cell debris was discarded. The supernatant was centrifuged at 8000g for 1 hour using the angle head rotor to sediment chlamydiae. Organisms from maintenance culture fluids were obtained by a similiar method.

For separation of the crude "soluble" hemagglutinin, the supernatant fluid was further centrifuged at 100,000g for 90 minutes in a Beckman L5-65 ultracentrifuge using a T42.1 rotor.

The sedimented fraction was examined for hemagglutinating activity.

Supernatants of control non-infected L-cell monolayers were treated identically and examined by the hemagglutination test.

PREPARATION OF PARTICULATE AND SOLUBLE HEMAGGLUTININ FROM INFECTED ALLANTOIC FLUIDS

Allantoic fluids were collected from groups of infected chick embryos and centrifuged at 270g using the SS-34 angle head rotor. The flocculent precipitate was discarded and the cleared supernatant was spun at 8000g to pellet chlamydial particles (i.e. particulate hemagglutinin). The supernatant was centrifuged at 100,000g for 90 minutes to sediment the crude soluble hemagglutinin.

ALCOHOL-ETHER EXTRACTIONS OF SOLUBLE HEMAGGLUTININS

Crude soluble hemagglutinin from allantoic fluid and L-cells was alternately resuspended in double-distilled water and centrifuged at 100,000g for a total of three washes. The final pellet was resuspended in a small volume of distilled water. One part of this material was preserved for determination of hemagglutinating and complement-fixing activity. To the other part was added an ether-alcohol mixture in the proportion of three volumes of diethyl ether to one part of 99% ethyl alcohol. Extraction was performed for one hour at room temperature with agitation by a magnetic stirrer. After extraction, the ether-alcohol extract and the ether-insoluble fraction were separated, lyophilized and their dry weight

determined. Unless otherwise indicated, all specimens to be tested were resuspended in McIlvaine's buffered saline (McIlvaine's citrate phosphate buffer, pH 7.0, diluted with four parts of 0.85% NaCl solution) to 1% suspension (w/v). Prior to use for hemagglutination, the suspensions were dispersed with ultrasound.

ERYTHROCYTES

Blood specimens were obtained from adult human donors (blood groups A, B and O), from the tail veins of mice, from baby chicks by decapitation and from the wing

Blood was drawn into an equal volume of Alsever's solution (Flow Laboratories, Rockville, Md., U.S.A.) in which the blood was stored at $4^{o}C$ for up to 4 days prior to use in tests of hemagglutination. Immediately prior to use, the erythrocytes were washed three times in McIlvaine's buffered saline and their concentration was determined by the standard cyanomethoglobin method (Hierholzer and Suggs, 1969). Unless otherwise noted, red cells were made up to desired concentration in this buffer.

HEMAGGLUTINATION ASSAY

Hemagglutination assays were done by the micro-plate method described by Sever (1962) using a Microtiter^{R} kit (Cooke Engineering Co., Alexandria, Virginia, U.S.A.). The standardized hemagglutination test described by Hierholzer *et al* (1969) was employed in which 0.5% erythrocytes were used. All wells initially received 0.05 ml. aliquots of McIlvaine-buffered saline. Prepara-

tions to be assayed were introduced into the first well of a row in 0.05 ml quantities followed by serial two-fold dilution along the row with a micro-diluter. Following dilution, 0.05 ml. of 0.5% erythrocytes were added to all test wells. Plates were incubated at 37°C for 30 minutes prior to examination. The titer was recorded as the highest dilution giving complete hemagglutination.

COMPLEMENT-FIXATION TESTS

These were carried out by the microtiter plate method (Casey, H.L., 1965) in the Cadham Provincial Laboratory in Winnipeg, Manitoba. The Wellcome "Psittacosis complement-fixing antigen" was used as a control.

BUFFERS FOR pH STUDIES

For comparative studies of buffers, McIlvaines-buffered saline was prepared as described above. Dulbecco's PBS was prepared according to the directions described in the Handbook of Biochemistry (Sober, 1970). Dulbecco's PBS without calcium and magnesium was composed of the following: 0.2g KCl, 0.2g KH_2PO_4, 8.0g NaCl, 1.14g Na_2HPO_4; Q.S.A.D. 1 liter doubly-distilled water.

For studies of the comparative effect of pH on hemagglutination, McIlvaine's citrate phosphate buffer was used for pHs 3.0, 5.0, 7.0 and 8.0, and Tris-HCl buffer was used for pH 9.0. These were prepared according to the instructions described by Meynell and Meynell (1965).

CHEMICALS AND ENZYMES

Enzymes and biochemicals were obtained from the sources listed below:

Albumin, Chicken egg, Grade V	Sigma A5503
Fetuin, Type III	Sigma F2379
N-acetyl neuraminic acid, Type IV	Sigma A2751
N-glycolyl neuraminic acid	Sigma G2755
N-acetyl-D-galactosamine	Sigma A4016
N-acetyl-D-glucosamine	Sigma A8625
β-glucuronidase (ex E. coli)	Sigma G1758
α-chymotrypsin (ex bovine pancreas)	Sigma C3142
N-acetyl trypsin (ex bovine pancreas)	Sigma T8378
Neuraminidase (ex Cl. perfringens)	Sigma N3001
Phospholipase C (ex Cl. perfringens)	Sigma P7633

ANTI SERA

Antisera against C. psittaci 6BC (grown in the yolk sacs of eggs) was raised in rabbits. The antiserum obtained reacted by complement fixation to a dilution of 1:32 . The serum was repeatedly adsorbed with non-infected yolk sac material to remove antibodies to yolk sac.

THIN LAYER CHROMATOGRAPHY

Thin layer chromatography was done using Polysilicilic acid gel-impregnated ITLC-SA sheets (Gelman Instrumentation Co.). A mixture of chloroform-ether-ethanol (10:0.2:0.17) was used as the separating solvent. Air-dried sheets were stained for lipids with iodine vapors and proteins were stained with 0.2% Coomassie brilliant blue. For carbohydrates, paper chromatography and silver nitrate reagent were used as described by Trevelyan et al, (1950). TLC fractions were eluted with diethyl ether.

PRETREATMENT OF ERYTHROCYTES WITH ENZYMES AND PERIODATE

Packed red blood cells were washed in the enzyme or chemical solution selected for treatment. They were sedimented at 625g, resuspended in pre-warmed (37^{o}C) enzyme solution and incubated in a total volume of 1.20 ml (25%) for 1 hour at 37^{o}C. They were then washed twice in cold McIlvaine-saline buffer and resuspended in this diluent to a concentration of 4% as determined by the cyanomethemoglobin method (Conrath, 1972); they were further diluted to a working concentration of 0.5% at the time of use. Prior to their use, all pretreated red cells were tested for non-specific hemagglutination.

HEMAGGLUTINATION INHIBITION EXPERIMENTS

Tests of inhibitors of hemagglutination were made by the standardized hemagglutination-inhibition test (Hierholzer and Suggs, 1969).

Aliquots (0.025 ml) of test diluent (usually McIlvaine-saline) were delivered to all the test wells of the microtiter plate. The potential inhibitor of a specified concentration was delivered in 0.025 ml quantities to the initial wells of the row. It was then serially diluted by microdiluter in two-fold dilutions along the row. Four units of hemagglutinin (in 0.025 ml) were added to each test well. Finally, 0.05 ml of 0.5% erythrocytes were added and the plates were incubated at $37^{o}C$.

RESULTS

Because highly purified preparations of the chlamydial hemagglutinin are devoid of serological activity, the detailed composition of the lipid-containing hemagglutinin remains unknown (Jenkin et al, 1970, Tamura and Manire, 1974). Increased hemagglutinating activity of chlamydial preparations following extraction with lipid solvents has been described earlier (Gogolak and Ross, 1955, Parikh and Schechmeister, 1964), however little is known, for example, as to how environmental conditions influence the interaction of extracted chlamydial hemagglutinin with sensitive erythrocytes. The nature of the erythrocytes' receptor sites for chlamydial hemagglutinin is largely not known.

For the present work we have studied the factors which affect the reaction of ether-ethanol extracted chlamydial hemagglutinin with sensitive erythrocytes. In the course of the investigation we observed that the union between extracted chlamydial hemagglutinin and the sensitive red cell was different from that between crude hemagglutinin and the same type of erythrocyte. We performed initial studies on factors influencing the red cell receptor sites for the chlamydial hemagglutinin. By including extracts of control uninfected tissues in the studies, it was found that extracts of both chlamydial hemagglutinins and uninfected tissues had similiar hemagglutinating activity when sensitive erythrocytes were used. Studies were performed to determine the chlamydiae-specific and nonspecific hemagglutinating activities in these

extracted tissue preparations and some preliminary analyses were done on the composition of these specimens.

The results of studies described in detail below have been summarized in recently published papers (Neuman et al, 1981a , Neuman et al, 1981b, Kordova et al, 1981).

Sensitivity of Chicken and Mouse Erythrocytes to Crude Chlamydial Hemagglutinin

In early studies of chlamydial hemagglutination, mouse erythrocytes were used but Barron et al (1968) later showed that erythrocytes of some chickens are more sensitive than those of mice. Mouse and chick erythrocytes were therefore first compared for sensitivity to crude soluble hemagglutinin recovered from fractions of allantoic fluid of chick embryos and supernatant fluid from L-cells remaining after removal of infectious chlamydial particles. Erythrocytes of mice showed a low hemagglutination-titer (1:8) in each of five trials and were not used for further studies. Red blood cells of adult White Leghorn chickens were significantly more sensitive (average hemagglutination titer 1:64) than those of one-day old White Leghorns (1:2); erythrocytes of adult Leghorns were markedly more sensitive than erythrocytes of New Hampshire Red chickens (1:8). Erythrocytes of several individual adult White Leghorn chickens tested agglutinated chlamydial hemagglutinin to essentially the same titer as vaccinia virus (Connaught Laboratories Ltd. vaccine strain) hemagglutinin . Only erythrocytes of selected adult White Leghorn chickens were used for the studies reported below.

Factors in the Production of Chlamydial Hemagglutinin

Most earlier studies on chlamydial hemagglutination were performed with crude concentrated fractions obtained from the allantoic fluid of infected chick embryos after the removal of chlamydial particles. As mentioned above, highly purified hemagglutinin was devoid of serological activity and, on the other hand, intact chlamydial particles had very low hemagglutinating activity. Experiments were performed to determine the activity of various chlamydial preparations. The hemagglutinating activities of partialy purified chlamydial organisms and of crude soluble hemagglutinin prior to and after treatment with ether-alcohol is summarized in Table 1.

As expected from the results of Hilleman et al (1951), chlamydial organisms agglutinated to low titers. As to the crude soluble hemagglutinin, specimens of TW-3 from L-cells consistently showed somewhat lower activity than that of 6BC from L-cells. This might be explained by the lower mean infectivity (ID_{50}) of the trachoma agent (ID_{50} was 10^5) than that of the psittacosis organism (ID_{50} was 10^8) for L cells. Crude soluble hemagglutinin from allantoic fluid of chicken embryos in which both chlamydiae grew equally well showed similar activity.

Invariably, ether-ethanol extracted preparations showed a 40 to 80 fold higher hemagglutinating activity than crude soluble hemagglutinin before treatment. Specimens of ether-ethanol insoluble fractions had low hemagglutination titers.

TABLE 1

Hemagglutinating activity of chlamydial particles and of crude soluble hemagglutinin before and after ether-ethanol extraction

Chlamydial species Strain	Source	Chlamydial Particles	Crude Hemagglutinin prior to extraction[a]	Crude Hemagglutinin after extraction[b]
C. psittaci 6BC	L-cells	4*	128	256
C. psittaci 6BC	All. fl.	8	128-256	2048
C. trachomatis TW3	L-cells	4	64	256
C. trachomatis TW3	All. fl.	16	128	1024

* Figures indicate reciprocals of highest dilution of partially purified (by differential centrifugation) chlamydial particles and of hemagglutinin specimens producing complete agglutination of sensitive erythrocytes of selected adult White Leghorn chickens following incubation at 37°C for 30 min.

a. 1% and b. 0.1% solution of hemagglutinin in McIlvaine's Saline

Ether extraction of infected tissues is sometimes used to obtain complement-fixing chlamydial antigen (Schacter and Downs, 1978). We examined the ether-ethanol extracted hemagglutinin in regard to complement-fixing activity and found that it had higher titers (1:64) than specimens of crude hemagglutinin (1:16) and of the ether insoluble fraction (1:8) as seen in Table 3.

Stability of the Hemagglutinin under Conditions of Storage

Since there is considerable difficulty in obtaining sufficient amounts of crude hemagglutinin at one time (Jenkin et al, 1970), of necessity, our preparations were stored for various periods of time. Decreased hemagglutinating activity of ether-ethanol treated specimens following storage in a refrigerator ($4^{o}C$) and a freezer ($-70^{o}C$) were observed when compared to freshly prepared extracted hemagglutinin. If, however, specimens were sonicated shortly before titration, there was no significant decrease in hemagglutinating activity after 3 and up to 9 months of storage at $4^{o}C$ and $-70^{o}C$. We therefore sonicated all specimens of hemagglutinin immediately before use.

Effects of Buffers on Hemagglutination Activity

The aim of these and subsequent experiments was to identify the most favorable conditions for the titration of ether-ethanol extracted hemagglutinin; a standard hemagglutination titer of 1:64 was used for testing variations of the conditions. For each assay antigen dilutions and erythrocytes were prepared in the buffer being tested.

Table 2

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]

Chlamydial species and strain	Source	HA and CF titers Prior to extraction[b] HA	CF	After extraction[c] HA	CF
C. psittaci 6BC	L cells	128	16	1024	64
C. psittaci 6BC	Allantoic Fluid	256	32	2048	64
C. trachomatis TW-3	L cells	64	8	256	32
C. trachomatis TW-3	Allantoic Fluid	128	16	1024	32
Control Noninfected	L cells	0	0	1024	0
Control Noninfected	Allantoic Fluid	0	0	512	0

Figures indicate reciprocals of highest dilutions of chlamydial hemagglutinin specimens and of control non-infected specimens producing complete agglutination of sensitive erythrocytes of selected White Leghorn chickens following incubation at 37° for 30 minutes.

a.) Results from the lipid rich fraction

b.) 10 mg/ml and c.) 1 mg/ml of specimen in McIlvaine's buffered saline

Dulbecco's phosphate buffered saline (PBS) containing Ca^{+2} and Mg^{+2}, PBS without Ca and Mg ions and McIlvaine's buffered saline were compared; (McIlvaine's buffer contains citrate that effectively binds Ca and Mg cations). In the presence of McIlvaine-buffered saline as well as in that of PBS (devoid of Ca and Mg ions) at pH 7.0, the hemagglutinating titer was consistently higher (1:64 to 1:128) than in the presence of calcium and magnesium ions (1:16).

The efficiency of McIlvaine's buffer at different pHs was tested (Table 3). There was no difference in hemagglutination titer between pH 7 and 8 (1:64 to 1:128). Two- to four-fold reductions of titers were observed when the test buffer pH was 5 (1:32 to 1:64). At pH 3 and pH 9 (tris-HCl buffer), the erythrocytes were unsuitable for use. McIlvaine's buffered saline at pH 7 was chosen for further use.

Effects of Electrolytes and Non-Electrolytes on Hemagglutination

Isotonic solutions (0.25M) of glucose and sucrose as non-electrolyte test diluents and isotonic (0.15M) $MgCl_2$ and KCl as electrolytic test diluents were compared (Table 4). Hemagglutination failed to take place in non-electrolyte solutions. Titers were also reduced relative to the McIlvaine's buffered saline control (1:64 - 1:128). Use of KCl with a monovalent ion pair resulted in a four-fold decrease in titer (1:16 - 1:32) whereas use of $MgCl_2$ with a divalent cation resulted in an 8-fold reduction (1:8 - 1:16)

TABLE 3

Effect of pH of McIlvaine's citrate-phosphate buffer on hemagglutination by ether-ethanol extracts of soluble hemagglutinins[a]

Chlamydial species Strain	Source	pH 5.0	pH 7.0	pH 8.0
C. psittaci 6BC	L-cells	32	64	64
C. psittaci 6BC	All. fl	64	128	64
C. trachomatis TW-3	L-cells	64	64	64
C. trachomatis TW-3	All. fl.	64	64	64

a. At pH 3 and 9 the erythrocytes were unsuitable for use

TABLE 4

Effect of isotonic electrolyte and non-electrolyte solutions on hemagglutination by alcohol-ether extracts of soluble hemagglutinins

Solution	Concentration (M)	*C. psittaci* L-cell	*C. psittaci* Allantoic
Glucose	.25	0	0
Sucrose	.25	0	0
McIlvaine-saline		64	128
KCl	.15	16	32
$MgCl_2$	.15	8	16

The Influence of Temperature on Hemagglutination

There is little knowledge concerning the effect of temperature on hemagglutination by ether-ethanol extracted preparations, therefore experiments were performed to test the influence of temperature on this reaction. The values shown in Table 5 represent the averages of three independant trials. The reactions observed after 45 minutes over the range of $4^{o}C$ to $37^{o}C$ tested. We observed that the agglutinating pattern developed more slowly at $4^{o}C$ appearing weak after 45 minutes but developing completely with further incubation for an additional 2 hours.

Enzymic Inactivation of Erythrocyte Receptors

While there is no information on the nature of the erythrocyte receptor site(s) for the chlamydial hemagglutinin, earlier workers found that trypsin and neuraminidase destroys the receptor sites of mouse L cells and human HeLa cells for the ligand(s) of chlamydial agents (Byrne, 1978, Byrne and Moulder, 1978, Kuo et al, 1973).

Experiments were performed to determine whether and how the extracted hemagglutinin of the psittacosis agent would interact with susceptible chicken erythrocytes having some of the receptor sites removed or destroyed. In these experiments, two variant 6BC strains with different cytotoxicity for mouse macrophages and mouse fibroblasts (L cells) were compared (Table 6).

TABLE 5

Effect of temperature on hemagglutination by ether-ethanol extracted hemagglutinins[a]

Chlamydial species strain	Source	Temperature		
		4°C	20°C	37°C
C. psittaci 6BC	L-cells	64[b]	64	64
C. psittaci 6BC	All. fl.	64	64	128
C. trachomatis TW-3	L-cells	64	64	64
C. trachomatis TW-3	All. fl.	64	64	64

a. All specimens were diluted to a standard titer of 64

b. Figures indicate reciprocals of the highest dilutions producing complete agglutination of sensitive erythrocytes of selected adult White Leghorn chickens following incubation at 37°C for 45 minutes

Concentrations of hemagglutinin producing a titer of 64 in reaction with untreated erythrocytes were used. Treatments directed at proteins on the red cell membrane included trypsin and chymotrypsin. At a concentration of 1 μg/ml of N-acetyl trypsin or 1 μg/ml of α-chymotrypsin, the treated red cells showed a definite increase in susceptibility to hemagglutination when compared to non-treated cells. The hemagglutination titers of the cytopathic 6BC grown in L-cells were higher than 6BC grown *in ovo*.

Neuraminidase removes sialic acid residues from the cell surface and in this respect acts similiarly to trypsin (Gottschalk *et al*, 1972). After treatment of erythrocytes with 1 U /ml of neuraminidase, the red cells showed a similiar increase in hemagglutination as red cells treated with trypsin or chymotrypsin: the titer of 6BC derived from L-cells in this experiment was also higher than that of 6BC derived from egg.

β -Glucuronidase and periodate are directed at the carbohydrate moieties on the erythrocyte membrane. Treatment of red cells with glucuronidase at a concentration of 100 U/ml and 1000 U/ml resulted in a slight enhancement of hemagglutination titers observed with both 6BC specimens. At a concentration of 10^{-4}M periodate, the red cells showed a definite increase in susceptibility to hemagglutination with 6BC derived from L-cells; a lesser effect was observed with 6BC derived from egg.

TABLE 6

Effect of pretreatment of sensitive White Leghorn chicken erythrocytes with enzymes and periodate on hemagglutination by soluble chlamydial hemagglutinins* derived from L-cells and allantoic fluid of chick embryos infected with C. psittaci 6BC

Enzyme or Chemical	Concentration or Activity	C. psittaci	
		L-cell	Allantoic Fluid
No pretreatment	-----	64	64
Trypsin	1 ug/ml	2056	256
Chymotrypsin	1 ug/ml	2056	512
Neuraminidase	1 U/ml	1024	256
B-Glucuronidase	100 U/ml	128	64
B-Glucuronidase	1000 U/ml	256	128
Periodate	10^{-4}M	2056	256

* Hemagglutinin was prepared by ether-ethanol extraction of infected tissues; a standard hemagglutination titer of 64 was used.

These findings suggested that trypsin-, chymotrypsin- and neuraminidase-sensitive receptor sites are not essential for binding of the ether-ethanol extracted soluble chlamydial hemagglutinin to chicken erythrocytes; neither are β-glucuronidase- nor periodate-sensitive receptor sites essential. The increased hemagglutination titers seen when erythrocytes were treated with these agents probably reflects more efficient cross-linking of erythrocytes by extracted soluble hemagglutinin.

Effects of Carbohydrates on Hemagglutination of Ether-Ethanol Extracted Chlamydial Hemagglutinin with Chicken Erythrocytes

Carbohydrates are increasingly identified as part of the cell surface receptors for viral and bacterial agents (Ofek et al, 1978). It seemed of interest whether or not some carbohydrates might competitively inhibit the reaction between soluble chlamydial hemagglutinin and sensitive erythrocytes. A preliminary experiment identical in form to the standardized hemagglutination-inhibition test (Hierholzer and Suggs, 1969) was performed (Table 7). The carbohydrate (or glycoprotein) to be tested was substituted for specific antisera ; the aliquots delivered to the initial microtiter plate wells were present in the concentration noted.

Of the carbohydrates tested so far, only fetuin caused inhibition of hemagglutination.

TABLE 7

Effects of carbohydrates on hemagglutination by ether-ethanol extracted chlamydial hemagglutinin*of chicken erythrocytes

Carbohydrate or Glycoprotein	Concentration	Hemagglutination Inhibition Titer
N-acetyl neuraminic acid	1 mg/ml	0
N-acetyl neuraminic acid	10 mg/ml	0
N-glycolyl neuraminic acid	1 mg/ml	0
N-acetyl β-D-mannosamine	1 mg/ml	0
N-acetyl glucosamine	10 mg/ml	0
N-acetyl galactosamine	10 mg/ml	0
Ovomucin	10 mg/ml	0
Fetuin	10 mg/ml	64

* Hemagglutinin derived from C. psittaci 6BC-infected L-cells was used.

Assay of Sensitivity of Human Erythrocytes to Ether-Ethanol Extracted Chlamydial Hemagglutinin

Hilleman et al (1951) reported that crude chlamydial hemagglutinin failed to agglutinate human "O" type erythrocytes. Barron and coworkers (1965) showed that certain chicken erythrocytes that were sensitive to vaccinial hemagglutinin were also susceptible to chlamydial hemagglutinin.

A study was made of the susceptibility of human blood groups A, B and O erythrocytes to ether-ethanol extracted chlamydial hemagglutinin; extracts of non-infected tissues being included as a control. All specimens were assayed in parallel with White Leghorn chicken erythrocytes having been pre-selected for their susceptibility to vaccinial hemagglutinin. The presence or absence of hemagglutination was interpreted as indicating the presence or absence of the specific receptor site for the hemagglutinin. Table 8 summarizes the results of this study.

These data indicate (1) that human blood group A,B and O erythrocytes do not possess receptor sites for crude chlamydial hemagglutinin; they further show that (2) human erythrocytes do not possess receptor sites for ether-ethanol extracted chlamydial hemagglutinin, (3) nor do they possess receptor sites for crude or extracted preparations of normal, non-infected L-cells and allantoic fluid.

Chicken erythrocytes, in contrast to human erythrocytes, were agglutinated by crude chlamydial hemagglutinin, extracted chlamyd-

TABLE 8

Assay of sensitivity of human erythrocytes to ether-ethanol extracted chlamydial hemagglutinin

Preparation	Erythrocytes			
	Human			Chicken
	A	B	O	
Control L cells prior to extraction	-	-	-	-
Control L cell after extraction	-	-	-	1024*
Chlamydial hemagglutinin prior to extraction	-	-	-	128
Chlamydial hemagglutinin after extraction	-	-	-	2048

* Figures represent reciprocals of highest dilution of hemagglutinin specimens producing complete hemagglutination.

- Indicates absence of hemagglutination

ial hemagglutinin and extracted uninfected L-cells. They were agglutinated by ether-ethanol extracted chlamydial hemagglutinin to significantly higher titers than crude hemagglutinin preparations before extraction. Crude preparations of uninfected L-cells failed to agglutinate chicken erythrocytes.

Thin Layer Chromatography of Ether-Ethanol Extracts of Non-Infected and Chlamydia Infected Tissues

Studies conducted in this laboratory have shown that chicken erythrocytes that are susceptible to chlamydia soluble hemagglutinin are also agglutinated by ether-ethanol extracts of control uninfected tissues; these extracts were therefore investigated further to determine chlamydiae-specific and non-specific hemagglutinating activities (Kordova et al, 1981).

Two fractions were obtained by ether-alcohol extraction of both infected and uninfected tissues: an ether-ethanol soluble lipid fraction and a lipid-depleted insoluble fraction. Lipid fractions of both infected and uninfected tissues showed similar high hemagglutinating titers. In contrast, complement-fixing activity was detected only in lipid fractions of infected tissues (Table 2). Neither hemagglutinating nor complement-fixing activity was detected in lipid depleted fractions of uninfected tissue specimens (not shown in Table 2).

Lipid extracts of normal tissues and tissues infected with 6BC and TW-3 were examined by thin-layer chromatography. Each specimen separated into seven components when stained for lipids by iodine; all specimens displayed a similar separation pattern. The mean Rf values of the separated fractions of non-infected and infected tissues are shown in Table 9 . Each of the seven components contained protein and carbohydrate in addition to lipid. The only difference observed was that fraction 4 and 5 of the specimens from normal L cells and allantoic fluid produced more weakly staining spots by all three staining procedures when compared to the same spots of infected tissues.

Each fraction from each specimen was examined for hemagglutinating activity (Table 10). The highest hemagglutinating titers were selected in fraction 4 of both normal and infected tissues; lesser but significant activity was shown in fraction 3, followed by fraction 2. Low levels of activity were found invariably in fractions 1,5,6,and 7.

A hemagglutination inhibition test using immune serum was carried out to determine the chlamydial specificity of the hemagglutinin in the separated fractions. Two specimens of extracts of 6BC derived from infected L-cells were examined with normal rabbit serum and 6BC antiserum. To ensure that the immune serum would contain antibodies only against chlamydiae and none against L-cells, antiserum was obtained from rabbits immunized with chlamydiae

grown in the yolk sac of chick embryos. Hemagglutination tests using a checkerboard titration showed (Table 11) that chlamydial antiserum in a dilution of 1:80 inhibited up to 16 hemagglutinating units of fraction 4 of specimens from infected cells. In contrast, 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, they were not examined by the hemagglutination inhibition test. These results indicate that fraction 4 contained a specific chlamydial hemagglutinin which was inhibited by immune serum but not by normal serum.

TABLE 9

Thin-layer chromatography (TLC) of ether-ethanol extracts of concentrated chlamydial hemagglutinin and of control L cells and allantoic fluid

Chlamydial species and strain	Source	→	R_f values of isolated fractions 1	2	3	4	5	6	7
C. psittaci 6BC	L cells		0	45.63[a]	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 noninfected	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 noninfected	Allantoic Fluid		0	37.20	60.40	79.54	87.13	94.06	98.68

TLC with polysilicic acid gel impregnated sheets (ITLC-SA) 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 samples application spot (origin).
a. each figure represents the mean of three independant runs.

TABLE 10

Hemagglutinating activity of fractions separated by thin-layer chromatography of extracts of concentrated chlamydial hemagglutinin and of control L cells and allantoic fluid

Chlamydial species and strain	Source	Hemagglutinin titers of isolated fractions						
	⟶	1	2	3	4	5	6	7
C. psittaci 6BC	L cells	4	8	32	64	2	4	4
C. trachomatis TW-3	"	4	4	16	32	2	4	2
Control noninfected	"	2	8	2	16	2	2	4
C. psittaci 6BC	All. Fl.	2	2	8	16	2	2	2
C. trachomatis TW-3	"	2	4	4	16	2	4	4
Control noninfected	"	2	4	32	4	2	2	2

TLC with polysilicic acid gel impregnated sheets (ITLC-SA) and chloroform-ether-ethanol (10:0.2:0.17) solvent system. Separated fractions were eluted and examined for hemagglutinating activity.

a.) Figures indicate reciprocals of highest dilutions of specimens producing complete hemagglutination of sensitive chicken erythrocytes.

Arrow indicates direction of flow.

TABLE 11

The effect of diluted (1:80) C. psittaci 6BC antiserum and normal serum on different hemagglutinating units of isolated fractions of extracted hemagglutinin from L cells infected with C. psittaci 6BC

No. of isolated fraction by TLC	HA Titer	Hemagglutinating units/ml of specimen inhibited by:	
		6BC antiserum	Normal serum
4	256	16 HAu	4 HAu
3	64	8 HAu	8 HAu
↑ 2	16	4 HAu	4 HAu

A checkerboard titration was performed to determine the highest decimal dilution of immune and normal rabbit serum which completely inhibited the activity of specimens of antigens in serial 2-fold dilutions.

The arrow indicates the direction of solvent flow. HAu represents hemagglutinating units.

DISCUSSION

Previous investigations have concerned the hemagglutinating activity of several chlamydial agents, the sensitivity of erythrocytes of different species of animals, some inhibitory effects on chlamydial hemagglutinin and some of the conditions affecting hemagglutination. Systematic investigations of the hemagglutinating activity of chlamydiae have not been done and, with notable exceptions (Jenkin et al, 1970, Tamura and Manire, 1974, Sayed and Wilt, 1971) the chlamydial hemagglutinin has been neglected in the last ten years.

The chlamydial hemagglutinin is contained in the surface envelope of only the infectious elementary bodies (Zakay-Rones et al, 1968, Tamura and Manire, 1974) and its study is important for several reasons. For example, there is increasing evidence that antibodies directed against surface components of microbes such as their hemagglutinin seem to be important in protecting against disease (Smith, 1977). Therefore, a need exists for a sensitive well-characterized hemagglutinating system. As described previously, crude soluble chlamydial hemagglutinin is low in activity, subject to nonspecific inhibition by factors in normal sera and other tissues, and is generally not well characterized.

Allantoic fluids of infected chick embryos from which chlamydial particles have been removed have been the main source of the soluble hemagglutinin in earlier studies (Hilleman et al, 1951,

Gogolak, 1954, Barron et al, 1965, Jenkin et al, 1970). Supernatants of infected cultured cells and supernatants of allantoic fluids are thought to be a convenient source of chlamydial antigens without a great deal of host material (Jenkin et al, 1970) and we therefore used this material for our studies. Ether extraction of purified chlamydiae and of infected host material to obtain complement-fixing antigens has been used by several earlier authors (Christenson and Volkert, 1955, Hilleman and Nigg, 1948, Parikh and Schechmeister, 1964a, Ross and Gogolak, 1957). Ross and Gogolak (1957) found no complement-fixing antigen in ether-extracts of chlamydial soluble hemagglutinin using fractionation with acetone and methanol but found a highly reactive complement-fixing antigen from elementary bodies using the same procedures. The method of ether-ethanol extraction of hemagglutinating antigens utilized in these studies was developed by adopting and combining the methods described earlier by others (Christenson and Volkert, 1955, Gogolak and Ross, 1955). The complement-fixing activity of our ether-ethanol extracted hemagglutinin obtained from supernatants of infected cultured cells and of allantoic fluids was higher than that of the Wellcome "Psittacosis complement-fixing antigen" which is prepared from suspensions of boiled crude yolk sac of infected chick embryos and represents at this time the only commercial source of chlamydial group-specific antigen.

The specimens of our ether-ethanol extracted hemagglutinin were stable for several months when refrigerated or frozen, provid-

ing the specimens were treated with ultrasound shortly before examination. Collins and Barron (1970) reported that crude soluble chlamydial hemagglutinin was unstable at 5°C for one to two days as determined by immunodiffusion, but that antigenic activity was restored by sonication of the material. This would suggest that the soluble crude hemagglutinin as well as the ether-ethanol extracted preparations reaggregate when stored.

We found that erythrocytes of White Leghorn chickens which were agglutinated by ether-ethanol extracted chlamydial hemagglutinin were also agglutinated to similiar high titers by vaccinia virus hemagglutinin. This is in accordance with earlier reports (Barron et al, 1965, Barron and Riera, 1969, Zakay-Rones et al, 1968)

We observed that erythrocytes of one-day old baby chickens showed a very low susceptibility to our specimens of chlamydial hemagglutinin. While there are no earlier reports on the sensitivity of baby chicken red blood cells to chlamydial hemagglutinin, several earlier authors (Clarke and Nagler, 1943, McCarthy and Helbart, 1960) reported that chicken embryo erythrocytes are not agglutinated by vaccinial hemagglutinin and that optimal susceptibility to it does not develop until approximately six weeks after hatching. More recent studies have shown that susceptibility to vaccinia virus hemagglutinin is correlated with the presence on the erythrocyte of K isoantigen (Brown et al, 1973).

Our specimens of ether-ethanol extracted hemagglutinin showed optimal hemagglutination titers in McIlvaine buffered saline. The results of our experiments are consistent with earlier sudies of Hilleman et al (1951) who however used crude preparations of the chlamydial soluble hemagglutinin.

By using the ether-ethanol extracted hemagglutinin we found that the hemagglutination reaction is independant of the temperature in the range of $4^{o}C$ to $37^{o}C$. Hilleman et al (1951) used crude soluble hemagglutinin and showed that the reaction diminished at $4^{o}C$. Tamura and Manire (1974) reported that homogenates of chlamydial elementary bodies reacted less strongly at $4^{o}C$ than at higher temperatures.

In our experiments, we observed enhanced hemagglutination titers when erythrocytes were pretreated with enzymes and periodate. These results suggest that trypsin-, chymotrypsin-, and neuraminidase-sensitive receptor sites are not essential for the binding of the ether-ethanol extracted soluble chlamydial hemagglutinin; nor are β-glucuronidase or periodate-sensitive receptor sites essential. The increased hemagglutination titers seen when erythrocytes were treated with these agents probably reflects more efficient cross-linking of erythrocytes by extracted soluble hemagglutinin.

We observed differences between the hemagglutination titers of preparations extracted from cytotoxic 6BC (L-cell derived) when compared to those of non-cytotoxic egg-derived 6BC; the cytotoxic 6BC hemagglutinated pretreated sensitive chicken erythrocytes

to higher titers than the non-cytotoxic 6BC. The reasons for the observed differences are not known. It has been reported in the literature (Smith, 1977), however, that virulent strains of microbes adhere to the receptor sites of sensitive cells more strongly than virulent strains, suggesting subtle differences in the microbial surface components responsible for virulence.

Earlier studies by others (Hilleman et al, 1951) showed that human blood group O erythrocytes are not agglutinated by crude chlamydial hemagglutinin. We have found that human blood groups A, B and O erythrocytes do not possess receptor sites for ether-ethanol extracted chlamydial hemagglutinin and that they do not possess receptor sites for crude or extracted preparations of normal, non-infected L-cells and allantoic fluid.

We observed that chicken erythrocytes, in contrast to human erythrocytes, agglutinated ether-ethanol extracted chlamydial hemagglutinin to significantly higher titers than crude chlamydial preparations before extraction. No hemagglutination of chicken erythrocytes occurred with crude preparations of control non-infected tissues, however, the same non-infected control tissues produced high hemagglutination titers after ether-ethanol extraction. In other words, chicken erythrocytes have receptor sites for components of both noninfected and chlamydiae-infected tissues when extracted by ether-ethanol.

Participation of non-specific tissue hemagglutinin(s) in chlamydial hemagglutination has not been described previously. However, earlier investigations by Burnet and Stone (1946) and Stone (1946) showed that extraction of a wide variety of tissues with organic solvents yields lipid material that hemagglutinates the same range of red cells as does the vaccinial hemagglutinin. As to the chlamydial hemagglutinin, it has been recognized that the best source of erythrocytes for hemagglutination is red cells sensitive to vaccinial hemagglutination (Barron et al, 1965).

A major problem in the characterization of chlamydial soluble hemagglutinin is its heterogeneity (Jenkin et al, 1970). A series of experiments was initiated in our laboratory, in an attempt to analyze the chlamydial hemagglutinin. For this purpose thin-layer chromatography of the ether-ethanol extracts of infected and normal tissues was performed and the isolated fractions were tested for chlamydiae-specific and non-specific hemagglutinating activities. The results of these studies indicated that chlamydial hemagglutinin extracted by ether-ethanol and separated by thin-layer chromatography contained, in addition to specific hemagglutinin, nonspecific tissue-lipid hemagglutinin(s) identical to that found in normal tissue preparations. Examinations further showed that separated fractions were heterogeneous and contained lipid, carbohydrate and protein.

We believe that expansion of the studies initiated here should provide sufficient data on the nature of the chlamydial hemagglutinin, its specificity, antigenicity and immunogenicity.

SUMMARY

The properties of the chlamydial hemagglutinin are largely unknown since previous work on purification resulted in loss of serological activities. Our work has focused on examination of ether-ethanol extracted high-titer hemagglutinin from L cells and from allantoic fluid from chick embryos infected with C. psittaci 6BC and C. trachomatis TW-3.

Comparative studies were done on environmental conditions affecting the hemagglutination of sensitive red cells by soluble chlamydial hemagglutinin prior to and after purification of the hemagglutinin with ether-ethanol.

Studies were performed for the development of sensitive techniques for investigation of ether-ethanol purified hemagglutinin.

Experiments were performed on chicken erythrocytes sensitive to hemagglutination to chlamydiae by removing or destroying receptor sites with trypsin, chymotrypsin, β-glucuronidase or periodate; hemagglutination prior to and after treatment of erythrocytes was assayed using extracted soluble hemagglutinin prepared from tissues infected with cytotoxic and noncytopathic 6BC strains. Increased agglutinability of such treated erythrocytes indicated more effic-

ient cross-linking of erythrocytes by extracted hemagglutinin; hemagglutination titers were consistently higher with specimens of cytotoxic 6BC as compared with noncytopathic 6BC.

Studies were performed on human erythrocytes which are not agglutinated by crude chlamydial hemagglutinin (Hilleman et al, 1951). We have shown that human blood group A, B and O erythrocytes do not have receptor sites for ether-ethanol purified hemagglutinin.

The role of non-specific host-tissue hemagglutinin in chlamydial hemagglutination has not been described previously. Studies performed in this laboratory using fractionation by thin-layer chromatography have shown that there are two hemagglutinins present in the specific soluble chlamydial hemagglutinin. Differentiation between non-specific tissue hemagglutinin and chlamydiae-specific hemagglutinin was based on resistance of the specific chlamydial hemagglutinin and the susceptibility of the non-specific tissue hemagglutinin to inactivation by dilute (1:80) normal serum.

Preliminary examination of ether-ethanol extracted chlamydial hemagglutinin by thin-layer chromatography showed that separated fractions were heterogeneous and contained lipid, carbohydrate and protein.

BIBLIOGRAPHY

Barron, A.L., Z. Zakay-Rones and H. Bernkopf. 1965. Hemagglutination of chicken erythrocytes by the agent of psittacosis. Proc. Soc. Exp. Biol. 119, 377

Barron, A.L. and M.C. Riera. 1969. Studies on hemagglutination by Chlamydia. Proc. Soc. Exp. Biol. 131, 1087

Benedict, A.A. and N.T. Briggs. 1958. Separation of psittacosis complement-fixing and hemagglutinating antibodies by starch electrophoresis. Virology 6, 94

Brown, H.S., W.E. Briles and E.R. Brown. 1973. Vaccinia virus hemagglutination receptor in chickens determined by K isoantigen locus. Proc. Soc. Exp. Biol. 142,16

Burnet, F.M. and J.D. Stone. 1946. The hemagglutinins of vaccinia and electromelia viruses. Aust. J. Expt. Biol. 24,1

Burnet, M. 1971. In: Genes, Dreams and Realities, Medical and Technical Publishing

Byrne, G.I. 1978. Kinetics of phagocytosis of Chlamydia psittaci by mouse fibroblasts (L-cells): Separation of the attachment and ingestion stages. Inf. Immun. 19, 607

Byrne, G.I. and J.W. Moulder. 1978. Parasite-specified phagocytosis of Chlamydia psittaci and Chlamydia trachomatis by L and HeLa cells. Inf. Immun. 19, 598

Caldwell, H.D., C.C. Kuo and G.E. Kenny. 1975. Antigenic analysis of chlamydiae by two-dimensional electrophoresis.II. A trachoma-LGV specific antigen. J. Immunol. 115, 969

Casey, H.L. 1965. Adaptation of LBCF method to microtechnique. In: Standardized diagnostic complement-fixation method and adaptation to the micro-test. Public Health Service Monograph No. 74, Washington, D.C.

Christenson, P.M. and M. Volkert. 1955. Two complement-fixing antigens from infected yolk sacs. Acta. Patha. Microbiol. Scand. 37, 219

Clarke, E. and F.P.O. Nagler. 1943. Haemagglutination by viruses: The range of susceptible cells, with special reference to agglutination by vaccinia virus. Aust. J. Exp. Biol. 21, 103

Collins, A.R. and A.L. Barron. 1970. Demonstration of group and species-specific antigens of chlamydial agents by gel diffusion. J. Infect. Dis. 121, 1

Conrath, T.D. Ed., 1972, Handbook of Microtiter Procedures, p389, Dynatech Corp., Cambridge, Mass., U.S.A.

Costerton, J.W., L. Proffenroth, J.C. Wilt and N. Kordova. 1975. Ultrastructural studies of Chlamydia psittaci 6BC in situ in yolk sac explants and L-cells; a comparison with gram-negative bacteria. Can. J. Microbiol. 21, 1433

Costerton, J.W., L. Proffenroth, J.C. Wilt and N.Kordova. 1976a. Alterations in the ultrastructure of Chlamydia psittaci 6BC harvested from the allantoic fluid of chick embryos. Can. J. Microbiol. 22, 9

Costerton, J.W., L. Proffenroth, J.C. Wilt and N. Kordova. 1976b. Ultrastructural studies of the nucleoids of the pleomorphic forms of Chlamydia psittaci 6BC: a comparison with bacteria. Can. J. Microbiol. 22, 16

Dhir, S.P., G.E. Kenny and J.T. Grayston. 1972. Characterization of the group antigen of Chlamydia trachomatis. Infect. Immun. 4, 725

Gogolak, F.M. 1954. The mouse erythrocyte hemagglutinin of feline pneumonitis virus. J. Inf. Dis. 95, 220

Gogolak, F.M. and M.R. Ross. 1955. The properties and chemical nature of the psittacosis virus hemagglutinin. Virology 1, 474

Gottschalk, A., G. Belyavin and F. Biddle. 1972. In: Gottschalk, A. (Ed.), Glycoproteins: Their Composition, Structure and Function; 2nd Ed., Part B, p. 1082, Elsevier, Amsterdam

Haig, D.A. and M.R. Hilleman. 1950. Hemagglutination by meningopneumonitis virus and its inhibition by immune serum. Fed. Proc. 9, 383

Hierholzer, J.C. and M.T. Suggs. 1969. Standardized virus hemagglutination and hemagglutination-inhibition tests. I: Standardization of erythrocyte suspensions. App. Microbiol. 18, 816

Hierholzer, J.C., M.T. Suggs and E.C. Hall. 1969. Standardized viral hemagglutination and hemagglutination-inhibition tests. II. Description and statistical evaluation. App. Microbiol. 18, 824

Hilleman, M.R. and C. Nigg. 1948. Studies on lymphogranuloma venereum complement-fixing antigens. IV. Fractionation with organic solvents of antigens of the psittacosis-lymphogranuloma venereum group. J. Immunol. 59, 349

Hilleman, M.R., D.A. Haig and R.J. Helmold. 1951. The indirect complement-fixation, hemagglutination and conglutinating complement absorption tests for viruses of the psittacosis-lymphogranuloma venereum group. J. Immunol. 66, 115

Hourihan, J.T., T.R. Rota and A.B. McDonald. 1980. Isolation and purification of a type-specific antigen from Chlamydia trachomatis propagated in tissue culture utilizing molecular shift chromatography. J. Immunol. 124, 2399

Ikuta, K., H. Miyamoto and S. Kato. 1979. Two components specifically responsible for hemagglutination in vaccinia and cowpox viruses. Virology 96, 327

Jenkin, H.M., M.R. Ross and J.W. Moulder. 1961. Species-specific antigens from the cell walls of the agents of meningopneumonitis and feline pneumonitis. J. Immunol. 86 123

Jenkin, H.M., S. Makino, D. Townsend, M.C. Riera and A.L. Barron. 1970. Lipid composition of the hemagglutinating active fraction obtained from chick embryos infected with Chlamydia psittaci 6BC. Inf. Immun. 2, 316

Kingsbury, D.T. and E. Weiss. 1968. Lack of deoxyribonucleic acid homology between species of the genus Chlamydia. J. Bact. 96, 1421

Kordova, N and J.C. Wilt. 1972. Lysosomes and the "toxicity" of Rickettsiales. I. Cytochemical studies of macrophages inoculated in vitro with C. psittaci 6BC. Can. J. Microbiol. 18, 457

Kordova, N., J.C. Wilt and R. Herchl. 1981. Differentiation of hemagglutinins in tissues infected with Chlamydia. submitted for publication

Kuo, C., S. Wang and J.T Grayston. 1973. Effects of polycations, polyanions and neuraminidase on the infectivity of trachoma-inclusion conjunctivitis and lymphogranuloma venereum organisms in HeLa cells; sialic acid residues as possible receptors for trachoma inclusion conjunctivitis agent. Inf. Immun. 8, 74

Litwin, J. 1959. The growth cycle of the psittacosis group of organisms. J. Inf. Dis. 105, 129

Makino, S., H.M. Jenkin, H. Yu and D. Townsend. 1970. Lipid composition of Chlamydia psittaci grown in monkey kidney cells in defined medium. J. Bact. 103, 62

Manire, G.P. and K.F. Meyer. 1950. The toxins of the psittacosis-lymphogranuloma group of agents. III. Differentiation of strains by the toxin-neutralization test. J. Inf. Dis. 86, 241

Matsumoto, A. 1973. Fine structures of cell envelopes of chlamydial organisms as revealed by freeze-etching and negative staining techniques. J. Bact. 116, 1355

Matsumoto, A. and N. Higashi. 1975. Morphology of the envelopes of Chlamydia organisms as revealed by freeze-etching technique and scanning electron microscopy. Ann. Rep. Inst. Vir. Res. Kyoto Univ., 18, 51

McCarthy, K. and D. Helbart. 1960. A comparison of the haemagglutinins of variola, alastrim, vaccinia, cowpox and electromelia viruses. J. Path. Bact. 79, 416

Meynell, E. and G.G. Meynell. 1965. Theory and Practice in Experimental Bacteriology. Cambridge University Press, Cambridge, England

Neuman, M.R., N. Kordova and J.C. Wilt. 1981. Chlamydial hemagglutinin: Interaction of the ether-ethanol extracted fraction with sensitive erythrocytes. Zbl. Bakt. Hyg. 248, 558

Neuman, M.R., N. Kordova and J.C. Wilt. 1981. C. psittaci 6BC soluble hemagglutinin: Factors influencing the red cell receptor sites. Zbl. Bakt. Hyg. 249, 282

Ofek, I., E.H. Beachey and N. Sharon. 1978. Surface sugars of animal cells as determinants of recognition in bacterial adherence. Trends. Biochem. Sci. 3, 159

Page, L.A. 1974. In: Bergey's Manual of Determinative Bacteriology. 8th ed., R.E. Buchanan and R.E. Gibbons (Eds.), The William and Wilkins Co., Baltimore, Md., U.S.A.

Parikh, G.C. and I.L. Schechmeister. 1964a. Comparative immunological, biological and biophysical behaviour of meningopneumonitis viral antigenic preparations. Nature 204, 1013

Parikh, G.C. and I.L. Schechmeister. 1964b. Interaction of meningopneumonitis virus with white blood cells II. Antigenic subunits of meningopneumonitis virus. Virology 22, 177

Parikh, G.C. and I.L. Schechmeister. 1967. Agglutination of white blood cells by meningopneumonitis organisms. J. Path. Bact. 93, 317

Peters, J.H. 1967. Immunologic and antigenic modification of trachoma Bedsoniae. Amer. J. Opthalmol. 63, 1506

Reeve, P. and J. Taverne. 1962. Some properties of the complement-fixing antigens of the agents of trachoma and inclusion blenorrhea and the relationship of the antigens to the developmental cycle. J. Gen. Microbiol. 27, 501

Riera, M.C. and A.L. Barron. 1971. Reactions with hemagglutinin of psittacosis agent in gel diffusion. Proc. Soc. Exp. Biol. Med. 135, 594

Ross, M.R. and F.M. Gogolak. 1957a. The antigenic structure of psittacosis and feline pneumonitis viruses. I. Isolation of complement-fixing antigens with group and species specificity. Virology 3, 343

Ross, M.R. and F.M. Gogolak. 1957b. The antigenic structure of psittacosis and feline pneumonitis viruses. II. Chemical nature of the alkali-soluble antigens. Virology 3, 365

Salit, I.E. and E.C. Gotschlich. 1977. Type I Eschericia coli pili: Characterization of binding to monkey kidney cells. J. Exp. Med. 146, 1182

Sayed, H. and J.C. Wilt. 1971. Purification and properties of a chlamydial hemagglutinogen. Can. J. Microbiol. 17, 1509

Schacter, J. and C.R. Dawson. 1978. In: Human Chlamydial Infections, PSG Publishing Co. Littleton, Mass., U.S.A.

Schechmeister, I.L. and G.C. Parikh. 1961. Agglutination of white blood cells by meningo-pneumonitis virus. Fed. Proc. 20, 437

Schechmeister, I.L. and G.C. Parikh. 1962. Interaction between meningo-pneumonitis virus (MPV) and white blood cells. Fed. Proc. 21, 455

Sever, J.L. 1962. Application of a microtechnique to viral serological investigations. J. Immunol. 88, 320

Smith, H. 1977. Microbial surfaces and pathogenicity. Bact. Rev. 41, 475

Sober, H.A. (Ed.) 1970. Handbook of Biochemistry, Chemical Rubber Co., Cleveland, Ohio, U.S.A.

Stokes, G.V. 1978. Surface projections and internal structure of Chlamydia psittaci. J. Bact. 133, 1514

Stone, J.D. 1946. Lipid haemagglutinins. Aust. J. Exp. Biol. 24, 197

Storz, J. 1971. Chlamydia and Chlamydia-Induced Diseases, C.C. Thomas, Springfield, Ill., U.S.A.

Tamura, A. and G.P. Manire. 1974. Hemagglutinin in the cell walls of Chlamydia psittaci. J. Bact. 118, 144

Trevelyan, W.E., D.P. Procter and T.S. Harrison. 1950. Detection of sugars on paper chromatograms. Nature 166, 444

Turner, W. and F.B. Gordon. 1964. Indirect hemagglutination with trachoma agent and related organisms. J. Bact. 87, 1251

Zakay-Rones, Z.E., E. Katzenelson and R. Levy. 1968. Hemagglutinin of trachoma agent. Israel. J. Med. Sci. 4, 305