SPECIFICITY OF ANTIBODIES TO THE PSITTACOSIS-LYMPHOGRANULOMA VENEREUM GROUP ANTIGEN

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the Faculty of Medicine

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by

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ABSTRACT

SPECIFICITY OF ANTIBODIES TO THE PSITTACOSIS-LYMPHOGRANULOMA VENEREUM GROUP ANTIGEN

Studies carried out in this laboratory from 1957 to 1962 on Eskimo sera revealed a high incidence of antibodies to the psittacosis-lymphogranuloma venereum (L.G.V.) group antigen. The present investigation was carried out to assess the specificity of these antibodies. Serological studies using the complement fixation test showed that:

a) The Eskimo sera reacted equally well with either the psittacosis or lymphogranuloma venereum group antigens.

b) When group and specific antigens were prepared from psittacosis and meningopneumonitis agents, the group antigens reacted with Eskimo sera; the specific antigen gave no reaction.

c) Sera from inhabitants of Southern Manitoba with clinical psittacosis confirmed in the laboratory, reacted with both the group and specific psittacosis antigens, but not with the specific meningopneumonitis antigen.

d) Eskimo sera absorbed with group antigen did not react with live suspensions of ornithosis, psittacosis and meningopneumonitis.

e) Antibodies to the psittacosis group antigen could not be absorbed with a series of different bacteria.

Previous clinical examination demonstrated no evidence of lymphogranuloma venereum, trachoma, or inclusion conjunctivitis in this population.

Conclusions:

This evidence suggests that the antibodies in Eskimo sera are not caused by the agents of psittacosis, ornithosis, meningopneumonitis, lymphogranuloma venereum, trachoma or inclusion conjunctivitis, but are probably caused by a related agent which is confined to the North and has not yet been identified.

DEDICATION

To Kaysie, for without her loyalty, encouragement and assistance this thesis would not have been.

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INTRODUCTION

INTRODUCTION

During the winter of 1966-1967, an outbreak of meningitis occurred among the Eskimos on the west coast of Hudson Bay. Fifty percent of the children involved came from the community of Eskimo Point, and a medical team was sent there in an attempt to establish the exact cause of this illness. Many of the individuals in the settlement had arrived within recent years, having formerly led a nomadic existence and depended on caribou for virtually their total diet.

All Eskimo residents of the community were examined physically, and one hundred were given an intensive examination which included the taking of specimens for serological, bacteriological, virological and parasitological study. No viruses were isolated; examination of the stool samples failed to show Salmonella sp., or Shigella sp., and only four showed any indication of parasites. The findings concerning bacteria are reported in Appendix "C" of this work.

The present experiments are confined to a serological study of the serum samples, with particular regard to the psittacosis-lymphogranuloma venereum group antibodies found therein. The presence of the latter was expected, since the work of Hildes, Wilt et al (1,2) had revealed a high incidence of these antibodies among Eskimos. Provided that the present samples contained a sufficiently high incidence and

titre of psittacosis-lymphogranuloma venereum group antibodies, an excellent opportunity was afforded to study the specificity of the group antibodies as found in Eskimo sera.

CHAPTER I

REVIEW OF THE LITERATURE

CHAPTER I

REVIEW OF THE LITERATURE

During a survey of antibodies to viral agents in serum collected from Eskimos of the Eastern Arctic region, Hildes, Wilt et al (25, 26) made the unexpected discovery that such sera contained a high percentage of psittacosis group antibodies. Subsequent investigation by Wilt, Hildes et al (76, 27) revealed that an incidence of 15% to 80% occurred in Arctic Indians and Eskimos (Figure 1). At the same time, of sera from residents of southern Manitoba (1792) suffering from respiratory disease, only 1% showed such antibodies. In 1965, Eddie et al (17) found that 60% of the sera from the Aleuts of the Pribiloff Islands contained psittacosis group antibodies. Stallman, in Australia, discovered an incidence of only 3.7% in the normal population (68).

Attempts to isolate the causative agent (28) from birds inhabiting the west coast of Hudson Bay have proven unsuccessful, although sera from many of these birds were found to contain psittacosis group antibodies. However, in 1965, Eddie et al (17) were able to isolate a psittacosis-like agent from two fur seals on the Pribiloff Islands.

Reed and Hildes (61) carried out a survey of ophthalmic diseases among residents of the west coast of

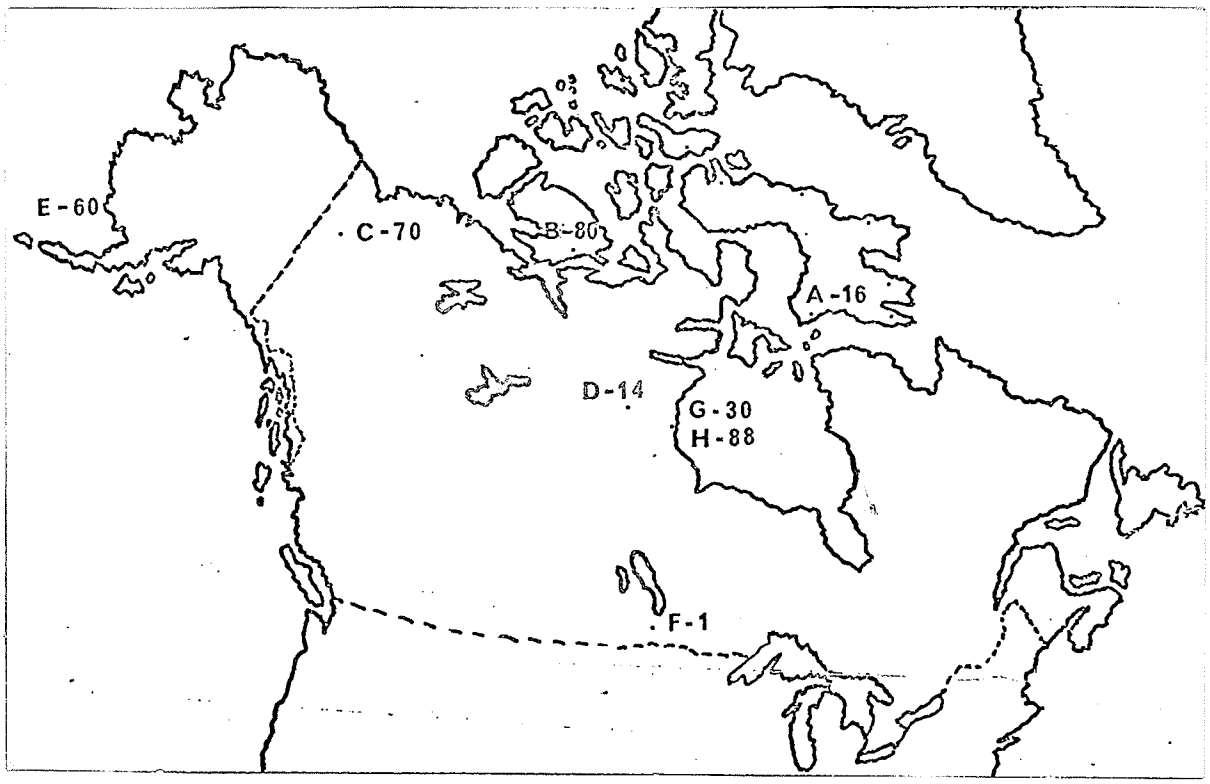


Figure 1

Distribution of Psittacosis Group Antibody in Eskimos and others - Percentage Incidence

1957

A. Baffin Island

B. Cambridge Bay

C. Old Crow

D. Garry Lake (Inland Eskimo)

F. Southern Manitoba

G. Eskimo Point

1965

E. Pribiloff Islands

1967

H. Eskimo Point

Hudson Bay, and found that 7% of the population had corneal scarring from a tuberculo-protein allergic reaction causing phlyctenular keratoconjunctivitis. No evidence of trachoma or inclusion conjunctivitis was recorded.

The typical inguinal scars caused by venereal infection with the agent of lymphogranuloma venereum have not been reported among Eskimos.

The agents of the psittacosis-lymphogranuloma venereum group can be divided into three broad categories according to the disease they cause:

I. a) Psittacosis and ornithosis, affecting birds and man; pneumonitis of man.
   b) Meningopneumonitis isolated from ferrets, sometimes affecting man.
   c) Pneumonitis affecting most mammalian species other than man.

II. a) Trachoma and inclusion conjunctivitis affecting man.
   b) Inclusion conjunctivitis of animals.

III. a) Lymphogranuloma venereum affecting man.
   b) Venereal infections of animals, not affecting man.

CHARACTERISTICS OF THE PSITTACOSIS-LYMPHOGRANULOMA VENEREUM GROUP OF ORGANISMS

History

According to Thygeson (74) in his history of trachoma, Halberstaedter and Prowazek in 1907 found cytoplasmic inclusions (H-P inclusion bodies) in the epithelial cells from experimental trachoma in apes and later in man. They gave them the name "Chlamydozoa" (or "mantle-bodies") when giemsa staining showed they consisted of small reddish elementary particles embedded in a bluish matrix (mantle). Two years later Lindner (74, 44) found such inclusions in most cases of gonococcal-free ophthalmia neonatorium, and reproduced the disease in a macaque with genital secretions from a woman whose baby had the disease. He was able to demonstrate inclusions in the material from the diseased conjunctiva. His work revealed that the blue matrix of Halberstaedter and Prowazek was in reality a conglomerate of large coccoidal bodies, and as these were the first to appear in the developing inclusion he called them "initial bodies".

Nicolle (74) (1913) showed that the agent was filterable; he was able to reproduce trachoma in an ape by using a filtrate of corneal washings from a case of human trachoma.

It was not until 1929 that psittacosis, a hitherto obscure disease, came into prominence. Outbreaks of the disease occurred in twelve countries, stimulating work to find the agent responsible. In 1930, Levinthal (42), Coles (11) and Lillie (43) simultaneously reported the discovery of minute spherical bodies within reticuloendothelial cells from sections of spleen. Coles (11), in his study, used a filtrate from psittacosis-infected mouse spleen in which he was able to demonstrate free coccoidal forms, constituting what was probably the first isolation of a psittacosis-like agent. Bedson and Bland (4) in 1932 suggested that the psittacosis agent had a developmental cycle in the cytoplasm of the host cell, since when impression smears of infected lung or spleen were stained with giemsa stain large (0.5-0.8 microns) bluish-staining coccoidal forms were seen as well as small (0.2-0.3 microns) elementary bodies staining bluish-red. Some of the larger forms appeared to be in the process of division, suggesting that they developed into small elementary bodies. Later, Thygeson (74) was able to show the same intracellular cycle for inclusion blennorrhea which he renamed inclusion conjunctivitis. Rake et al (58) found the growth and serological relationships of psittacosis to be similar to those of lymphogranuloma venereum, and later proved (59) the relationship serologically between the agents of trachoma and inclusion conjunctivitis (TRIC agents) and the psittacosis-

lymphogranuloma venereum group.

Identification and classification. According to Bergey (8) the psittacosis agents belong to the family "Chlamydiaceae", and are characterized as being small, coccoid microorganisms with a characteristic developmental cycle which stain with analine dyes and are gram negative. They have not been cultivated in cell free media, and are obligate intracellular cytoplasmic parasites or saprophytes found in various warm-blooded animals, where they are usually pathogenic.

Although the actual description of these micro-organisms is accepted, the term "Chlamydia" or "Chlamydiaceae" has not received wide acceptance. Meyer (31) uses the term "Bedsonia", a name originated in honor of Bedson who did so much of the early work concerning this group. In Zinsser (77) we have reference to "Bedsonia", or simply to "the psittacosis group".

Despite this controversy, the agents of the psittacosis group are accepted as being closely allied to bacteria and rickettsia, and belonging with the organisms smaller than bacteria but larger than viruses.

Species affected. Following on the recognition of the causative agent of psittacosis, isolation of many different Chlamydial species has been reported from a large number of birds: pigeon and dove, chicken, duck, willet, turkey, muttonbird, American herring gull and laughing gull (31);

fulmar and petrel (24); and egret (63). Agents identified as Chlamydial have been isolated from the following diseases of mammals: meningopneumonitis (18), murine pneumonitis, hamster pneumonitis, feline pneumonitis, opossum pneumonitis; bovine pneumonitis, encephalitis, enteritis and epizootic abortion (31); enzootic abortion (51), pneumonitis (31) and polyarthritis (48) in sheep; pneumonitis in goats (31); and inclusion conjunctivitis in guinea pigs (31), as well as human psittacosis, trachoma and inclusion conjunctivitis. The first laboratory isolation of an agent of trachoma or inclusion conjunctivitis was reported in 1957, when T'ang et al (69) grew the agent of trachoma in the yolk-sac of the developing chick embryo. These results were later confirmed and further documented by Collier and Sowa (12).

Mode of transmission. Lymphogranuloma venereum is most frequently spread through venereal contact, but the portal of entry is sometimes through the conjunctiva (36); similarly, inclusion conjunctivitis, a venereal infection, spreads to the newborn infant during the birth process and to adult eyes through fingers and fomites.

Psittacosis and ornithosis are spread from bird to bird by respiratory droplets. Most human infections occur in people coming into close contact with droplets, dried droppings, or feathers from such diseased birds (36).

Barwell (1) reported that an agent, isolated from a laboratory worker, was indistinguishable from the agent causing enzootic abortion of ewes. Transmission of Chlamydia from mammal to mammal (17) has never been proven but is believed to occur. Eddie et al (17) believe that such infections may spread from animal to man.

Geographic distribution. Chlamydial agents have been identified in virtually all countries. Thus, this genus is world wide in distribution. In 1965, Eddie and associates (17) succeeded in isolating Chlamydia from fur seals in the Pribiloff Islands, Alaska. Their serological studies indicate that a high percentage of fur seals (58% of 233) have antibodies to the agents of the psittacosis group, the pups showing the lowest percentage of antibodies and the young adults the highest. Among birds inhabiting the islands, only two of 105 tested showed antibodies -- a fulmar and a gull. Of the human population, about 60% were found to have antibodies to the group antigen, but only 18% at a level high enough to be significant. Eddie et al (17) believe that in the absence of avian-borne infections, the mammalian-borne agent may be responsible for inducing antibodies in this group of people.

Haagen and Mauer (24) in 1938 repeatedly isolated from fulmars and petrels the agent causing a psittacosis-like infection of humans in the Faroe Islands, while Terzin

in Yugoslavia (72) found 53% of the dogs in the Banat region to have antibodies to the psittacosis group antigen.

Hildes et al (28), feeling that the agent responsible for psittacosis antibodies in Eskimos might be found in geese migrating from their winter habitat in southern Texas to their nesting area at Eskimo Point, examined sera and specimens collected during both the northward and southward migrations. They found that of the sera, 71% of those collected from northbound birds and 12% of those collected from southbound birds reacted with the common group Chlamydial antigen using the indirect complement fixation test. Attempts to isolate an agent of this group were unsuccessful. The agent causing ornithosis has been isolated from pigeons (49) in many areas of the United States, and an ornithosis-like agent from egrets in the State of Louisiana (63).

Propagation

Chlamydial agents were propagated in mice by early workers (5). Smadel et al (67), using the methods described by Cox (13), for cultivation of rickettsia, succeeded in cultivating psittacosis-like agents in the yolk sac of the developing chick embryo. It has since been discovered that some difficulties do arise in the cultivation of Chlamydia in embryonated eggs; for example, during the summer and early autumn, trachoma virus fails to multiply in this medium (34). Many Chlamydia have been found to

develop in tissue culture, a wide variety of primary cultures and cell lines being susceptible (55). Among the primary cultures, human amnion, monkey kidney cells, foetal mouse and chick embryo fibroblasts have proven suitable for propagating Chlamydial agents. Some of the suitable cells are Chang's human liver, Hela and L cell lines (55).

Morphology and Developmental Cycle

It was simultaneously reported by Levinthal (42), Coles (11) and Lillie (43) that minute spherical bodies were to be found in the reticuloendothelial cells from the spleen of patients who had succumbed during an outbreak of psittacosis. Coles (11) was able to infect mice with a filtrate from these spleens and he demonstrated free coccoidal forms in such preparations. By using similar methods, Bedson and Bland (4) were able to present evidence that a developmental cycle was carried out by these agents within the cytoplasm of the cell.

Morphology. The particles seen in such preparations were very pleomorphic (4), varying from coccoidal forms about one micron in diameter which stained blue with giemsa stain, to small forms 0.2 to 0.3 microns in diameter staining reddish-blue. The particles multiplied within the cytoplasm of the mammalian cell, and the larger forms appeared to multiply by binary fission (4). However, removal of the larger forms by filtration apparently did not affect the

infectivity of the suspension. According to Meyer (31), a similar cycle could be demonstrated in avian tissue infected with psittacosis-like agents.

Developmental cycle. The developmental cycle of Chlamydial agents was detailed by the studies of Litwin with the electron microscope (45), by Officer and Brown (55) using foetal mouse lung and Chang's human liver cells examined by the electron microscope, and by Pollard et al (56, 57) using human amnion tissue culture cells with fluorescent staining. Their studies revealed variations in the length of the growth cycle between the various members of the group (45).

Each of these three methods showed what has been called a two-phase growth cycle (45). The first phase lasted up to twenty hours; the second, from twenty to thirty hours. The first phase started with penetration of the cell by the infective particle (45).

Phase I: The attachment of the agents to cells was complete by two hours after infection and was a very inefficient process in comparison with that involving viral agents (55). Only 2% to 5% of mammalian cells in tissue culture were affected by the 6 B.C. strain of psittacosis, and there appeared to be little alteration of the monolayer over a long period. The Borg strain (55), by contrast, completely destroyed the monolayer by the eighth to tenth day through lysis of the cells.

The infective particle was identified by Pollard and Tanami (56, 57), using acridine orange staining in amnion cell cultures, as being 0.2 to 0.3 microns in size, and staining yellow-green, that is, containing DNA. On entering the host cell, the particle seemed to become surrounded by RNA, forming a red matrix with the same stain. This was the initial body (45). By fifteen hours, a vacuole appeared in the cytoplasm surrounding the particle, and several particles up to one micron in size were soon to be found in a single vacuole, indicating that division had taken place. As the virus matured (56), the vacuole enlarged to many times the size of the particles filling it. The electron densities (45) and the red matrix (56) were destroyed when treated with RNAse, indicating that the particles contained large quantities of RNA prior to twenty hours. The infective dose for chick embryo yolk sac was two hundred to one thousand particles (45).

Phase II: The second or logarithmic growth phase occurred between twenty and thirty hours, according to Litwin (45). The vacuole at twenty hours was larger and contained many more particles of a somewhat smaller size than the vacuole at fifteen to eighteen hours (45). The acridine orange fluorescence altered from red to orange at this stage (56). Small particles were first seen in the cytoplasm of infected cells when impression smears made at twenty-two to twenty-three hours were examined under the

light microscope, and both large and small particles could be demonstrated in the vacuole by use of acridine orange stain (56). By the twenty-fifth hour, Litwin (45) observed particles which were apparently dividing by binary fission, and this was the only form of reproduction observed at any time during the growth cycle. At thirty hours, the cells contained two morphologically distinct particles, one dense-centered, about 0.2 microns in diameter, the other with no central organization, measuring about 0.5 microns. Both yellow-green and red-orange fluorescing particles were observed by Pollard at this stage (56). The small particles which contained DNA and stained yellow-green were undoubtedly the small particles described by Litwin, which he believed to be a resting form with no further function within the cell (45).

Particles separated from the cell during phase one (up to twenty hours) had low or no infectivity, while the particles released at thirty hours were of both small and large size and of high infectivity (45). The only identifiable material within the cell during the early phase were the small DNA-containing particles (56).

Metabolism

Both Litwin (45) and Pollard, et al (57) felt that the enlargement of the vacuole was caused by enzymatic action and indicated active metabolism by the large particle. The

latter group was able to halt maturation of the agent by the addition of aminopterin (an antimetabolite) to cultures prior to 15 hours after infection. If aminopterin and folic acid were added to the tissue culture at the same time, some maturation would occur. Aminopterin added after 15 hours did not affect the production of mature elementary bodies (57). It became apparent that the large forms were non-infective but carried on an active metabolism, while the small forms or elementary bodies were infective but had no active metabolism.

Chemical Composition

Jenkins (37) in 1960 reported the successful separation of cell walls from the agents causing meningo-pneumonitis and feline pneumonitis. He was able by repeated washings to obtain a purified suspension of these cell walls, which he analyzed chemically and compared with a chemical analysis of the intact particles. (So far, separation of the small and large forms of Chlamydial agents for chemical analysis has not been reported.) The protein content of both preparations was approximately the same, 30-35%. The carbohydrate content in both cases was about 1.5%, and contained a trace of muramic acid. The difference in composition was found in the ribonucleic acid, deoxyribonucleic acid and phospholipid content. In the intact particle the nucleic acid fractions totalled approximately 5-6% (RNA 2.5%,

DNA 3.4%), while the cell walls contained less than 0.5%. The phospholipid of the intact particle was 7.5%, as compared to 1.5% in the cell walls. The only common amino acids missing from the intact particles were arginine and histidine, according to Moulder (53), and his studies indicated that these were not required for growth. The phospholipids in both preparations were mainly made up of neutral fat (37), but no cholesterol was detectable by filter paper chromatography in either case. The small amount of muramic acid found in the cell walls of the meningopneumonitis agent (37), and also in the intact particle, could well be a definite link to the bacteria, especially since Escherichia coli when similarly treated yielded extremely thin and fragile cell walls (37), although Staphylococcus aureus was unaffected. It seemed likely, then, that the Chlamydial agents were related to the rickettsia and gram negative bacteria. There was strong evidence that these agents produced enzymes (37, 57, 53) and could synthesize protein and nucleic acid, which were specific for the agent but dependent on the host cell for their precursors.

The Effect of Physical and Chemical Agents

Freezing and thawing (57) reduced the infectivity of Chlamydial agents by 50% per cycle. The nucleic acid was stable at $0^{o}C.$ (53); even though the DNA remained firmly attached to the particle at $37^{o}C.$, the RNA was broken down

to nucleosides and released into the medium within four to eight hours, infectivity being lost by eight hours. Inactivation (31) also occurred after five minutes at 56°C., or thirty minutes under ultraviolet light. Storage (31) even at low temperatures slowly reduced the infectivity; this difficulty could be overcome, to some degree, by using a medium composed of skimmed milk containing 7.5% dextrose. Storage in glycerol was unsatisfactory, as Chlamydial agents rapidly lost potency in the medium. The agents were stable when stored in the lyophilized state or at temperatures lower than -60°C.

Meyer and Eddie in Horsfal and Tamm (31) stated that chemical inactivation of the particles was brought about by formalin (0.1%) or phenol (0.5%) (31) in 24 to 48 hours. Ethanol and ether at room temperature destroyed infectivity in thirty minutes. The normal pH range for infective particles was between 7 and 8. Chlamydial agents were susceptible to a great range of antibiotics (32) and a careful selection of one which would not damage the particle had to be made during isolation procedures. Among the antibiotics (32, 33) which inhibited the growth of Chlamydia were penicillin, the sulphonamides, the tetracyclines, erythromycin and chloramphenicol.

ANTIGENS

Bedson (5) was able to demonstrate that a crude

suspension of mouse spleen containing elementary bodies of psittacosis would give a specific complement fixation reaction with anti-psittacosis serum. However, when washed, the suspension had a much reduced titre. After centrifugation for one hour at 10,000 rpm, the pellet retained all of the infectivity but only fifty percent of the complement fixing ability. The supernatant when tested was found to contain the remainder of the complement fixing antigen. Bedson (5) suggested at this time that crude suspensions contained something, other than formed active virus, giving specific complement fixation with psittacosis antibody. The effect of heat was not to reduce the activity of the antigen but to enhance it, in some cases as much as thirty-two fold.

When Bedson (5) absorbed antiserum with the unheated antigen he found that the serum no longer fixed complement with a crude suspension of the elementary bodies of psittacosis. The titre, however, was virtually unchanged under similar circumstances when the serum was absorbed with boiled antigen, although all antibodies to the boiled antigen were removed. He felt that there were at least two antigens contained in the agent of psittacosis, one heat stable, the other heat labile, both capable of specific reaction with antiserum and complement. Precipitation of coagulated proteins, brought about by boiling a crude suspension of mouse spleen, left a clear supernatant which

precipitated a psittacosis antiserum and was capable of inducing an allergic reaction when injected into the skin of a sensitized guinea pig. The clear supernatant left after the coagulation of proteins did not fix complement with antiserum. Bedson (6) in 1937 proved that the enhanced group antigen with the complement fixation test was the most valuable tool for diagnosis. The yolk sac-grown psittacosis agent (67) provided a group antigen which was cleaner and more practical than that produced in mouse spleen.

An antigenic relationship between psittacosis, lymphogranuloma venereum and meningopneumonitis was demonstrated by Rake et al (58), using complement fixation. This relationship later proved to be due to the heat stable antigenic component reported by Bedson. Antisera to heated preparations of these three agents were virtually identical, making impossible a specific diagnosis through serology using group antigen (58). Demonstration (7) of the type-specific antigen was accomplished by absorption of antisera with heated antigens, which removed all group antibodies. When the serum was tested with the homologous antigen, complement fixation occurred, while no reaction took place when the heterologous antigen was used.

Barwell (1) was able to produce a type specific complement fixation antigen when he treated unheated psittacosis and lymphogranuloma venereum agents with potassium periodate. This treatment destroyed the group antigen

but left the specific antigen unaffected. His work was duplicated by Sigel and Pollikoff (65) with the agent of meningopneumonitis. In addition, they were able to show that a loosely-bound group specific antigen remained in the supernatant during washing procedures, and was not affected by the periodate treatment. They suggested that there was a soluble group antigen, a theory not inconsistent with the original work of Bedson (5).

Danish workers (75) extracted a group antigen with ether from the agent of ornithosis, and found it to be associated with a phosphatide. Ross and Gogolak (62) reported in 1957 the production of group and specific antigens by disrupting the agents of meningopneumonitis and feline pneumonitis with sonic vibration and extracting the disrupted particles with ether and alkali. The ether-soluble fraction contained group serological activity, while the alkali-soluble fraction contained both group and specific activity by the complement fixation test. Potassium periodate treatment of the alkali-soluble fraction removed the group activity, and a specific antigen remained which was unstable in storage.

Isolation of cell walls (37) from yolk sac-produced feline pneumonitis and meningopneumonitis agents was accomplished by treatment of washed suspensions of the virus with sodium deoxycholate and trypsin. These cell walls appeared as empty sacs when viewed under the electron microscope.

The cell walls were antigenically species specific (38) by the complement fixation test. Unlike other type specific antigens they were resistant not only to potassium periodate, but also to heat for thirty minutes at 100°C. The production of cell wall material was modified by Fraser and Berman (19), who emulsified the entire egg contents and clarified the resulting suspension by centrifugation and treatment with fluorocarbon. This provided a larger volume of antigen which was then treated with deoxycholate and trypsin.

CROSS ANTIGENICITY

One of the major problems in dealing with infections associated with the Chlamydial group of agents has been the difficulty in identifying the specific etiological agent (70). Isolation of the agent is often impossible, and serological tests used for screening are performed with antigens which are group specific rather than type specific.

Serological tests. Of all the tests tried, the complement fixation test first used by Bedson and Western (3) is still the most important diagnostic tool available for identification of Chlamydial group antibodies. It is however, only group specific.

The serum (29) and toxin (60) neutralization tests, although more specific, were hampered by the low potency (46) of the neutralizing antibodies in the patient's serum.

Hemagglutination inhibition (30, 9) and immuno-fluorescence (10, 54) revealed group specificity only. The former had a high level of non-specific reactivity; the latter was cumbersome.

Intra-group crossing. It would seem likely that there are chlamydia which affect nearly every species of mammal and birds (31), and that all of these carry with them the common group antigen. Identification of inter-strain relationships came from Rake et al (58), and Meyer (49), who established the association between psittacosis, ornithosis, meningopneumonitis, human pneumonitis and lymphogranuloma venereum. The addition of feline pneumonitis to this list by Thomas and Kolb (73) was soon followed by identification of agents in many domestic and laboratory animal infections (31). In 1942 Rake et al (59) were able to show that the trachoma and inclusion conjunctivitis agents both carried the common Chlamydial group antigen.

Unrelated crossing. Serological diagnosis is further confused by reports indicating the discovery of the group antigen in sources other than Chlamydial agents (71, 39, 64). Untreated egg yolk was found to contain antigen which gave a low titre reaction with anti-Chlamydial serum (71). Egg yolk elicited in rabbits a high titre of anti-bodies which reacted with the Chlamydial group antigen by complement fixation. Ethanol enhanced the yolk antigen.

Reports (39, 64) of finding the common group antigen of the psittacosis-lymphogranuloma venereum group in some bacteria, particularily Bacillus anitratum (Herellea) and related organisms, have caused further problems. Studies (39, 64) of the antigenic character of Bacillus anitratum have indicated that the bacterial antigen reacted with anti-ornithosis serum, but that ornithosis antigen did not react by complement fixation with antibodies stimulated by Bacillus anitratum. Egg-produced antigens of psittacosis-like agents contaminated by bacteria could invalidate results obtained with anti-ornithosis sera.

Dane (15) in Australia, using the complement fixation test, found significant titres to psittacosis in five cases serologically diagnosed as Q fever. In the Canadian north, working among Eskimos, Greenberg and Blake (23) in 1958 found a thirteen percent incidence of antibodies to tularemia, while the same year Hildes (25) reported a 15% incidence of Chlamydial antibodies. There seems to be no indication that enteric infections, brucellosis and typhus (6, 23) have any serological relationship with the Chlamydial group of agents.

IMMUNITY

In humans immunity to the Chlamydial group is a poorly studied phenomenon, probably because wide-spread outbreaks are uncommon. Over the years, cases have been

more frequently found among workers in certain occupations -- poultry plant and aviary employees, duck breeders, etc. At the present time Meyer and Eddie (50) consider ornithosis and psittacosis to be occupational diseases. These investigators commented, in a review (50), on the apparent resistance to psittacosis which is exhibited by people who work with pet birds. When examination was made of breeders, owners and dealers who had handled diseased birds for six months or more, eighty percent were found to have complement fixation titres in excess of 1:8, with only about eighteen percent having shown symptoms of influenza-like illness with pneumonia during the previous 10 years.

An outbreak of ornithosis in poultry-plant workers (21) in Wisconsin did not confer permanent immunity on those infected, antibodies remaining at a fairly high titre among many of the workers, but dropping in 18 months below detectable level in others.

It seemed likely that immunity to psittacosis-like infections was shortlived, but that constant exposure might maintain a high antibody level. Meyer et al (50) suggested that it was probably persistent infection in the liver or spleen of the individual that maintained the antibody level.

Officer and Brown (55), using primary tissue cultures of mammalian cells, were able to infect with psittacosis only 2% to 5% of the cells at any one time. As the maintenance media contained a significant quantity of elementary bodies,

they believed that the agent was shed by leaking from the infected cells. That this could possibly occur in vivo was suggested by the findings of Meyer and Eddie (50) that "a 36 year old biochemist who recovered from severe psittacosis and had a complement fixation titre of 1:256, shed that virus in the sputum for 10 years".

SUB-CLINICAL INFECTION

There is strong evidence that sub-clinical psittacosis-like infections do exist in humans. In an outbreak of an ornithosis-like disease among poultry workers in Wisconsin (21), there were 19 cases of which 10 were diagnosed by clinical and serological means, while nine others showed a rise in titre by complement fixation and were diagnosed as positive although the infection was inapparent. Jawetz (35) found in California sub-clinical cases, caused by agents of trachoma and inclusion conjunctivitis, which never exhibited symptoms of the disease. In Australia, Dane (14) found an incidence of psittacosis antibodies in the normal population where no association with illness was discovered. Stallman et al (68) reported that 27% of the non-specific urethritis patients in Queensland had antibodies to the Chlamydial group antigen. Hildes and co-workers (25) found that fifteen percent of the Eskimos in the Eastern Arctic had antibodies to the psittacosis-lymphogranuloma venereum group antigen, with no apparent

symptoms of illness. Excellent evidence of the sub-clinical form of the disease was presented by Fraser et al (20) when they surveyed 2,200 boys recruited to the Navy. During the first three months, 247 showed a rise in titre of psittacosis-lymphogranuloma venereum group antibodies, but of these 101 exhibited no evidence of illness.

Breeders of ducks in various parts of the world (50) have been reported to have an antibody incidence of 50%, while among control groups from the same areas less than 5% showed antibodies. Complement fixation antibodies (50, 68) at an incidence of 25% to 50% were found in persons whose occupations brought them into contact with birds, bird products or droppings.

Of interest was the German report (50) that a bookkeeper and an office clerk at a duck-producing plant had high titres of psittacosis antibodies, although they had no actual contact with the birds.

From these findings, continuing contact with psittacosis-like agents of low virulence, or continuing inapparent infection, would seem to be the major reason for immunity.

CHAPTER II

THE PROBLEM AND THE APPROACH

CHAPTER II

THE PROBLEM

It has been known for several years that there is a high incidence of psittacosis antibodies in Eskimo sera; but the antigen or agent causing these antibodies has not yet been discovered. As cross-reactions within the psittacosis-lymphogranuloma venereum group are almost complete, (58), the specific cause of psittacosis group antibodies found in any sera is difficult to establish. In addition, the fact that the psittacosis group antigen can be found in several sources other than the psittacosis group (71, 39, 64) makes this specificity even more problematic.

The actual diseases caused by agents of the psittacosis group have in the past proved serious where ever they have occurred, but such infections have not been reported among Eskimos. Since there is evidence, however, to indicate that sub-clinical infections do exist in other parts of the world (50), they may therefore also be present among the Eskimo people; such infections could be responsible for the psittacosis antibodies in the sera.

Also, there is reason to suspect that an antigen unrelated to the psittacosis group might be involved. The dependence of virtually all Eskimos on animals and animal products for food and clothing broadens the range of unrelated antigens to be considered as possible sources of

infection producing the antibodies under study.

Eskimo sera, then, can serve here as a model in an attempt to provide more concrete evidence regarding the specificity of psittacosis-lymphogranuloma venereum group antibodies.

THE APPROACH

In 1936, using a suspension of live psittacosis elementary bodies, Bedson (5) was able to show that these elementary bodies contained two separate antigens. In his work, he inactivated the suspension with heat at 100°C. for 30 minutes. He found that a suspension so treated reacted at a higher dilution with positive psittacosis serum than did a suspension of live elementary bodies. He absorbed the psittacosis positive serum with heat inactivated suspensions until no antibodies to the absorbing antigen could be demonstrated. When this absorbed serum was tested with a suspension of live psittacosis agents, the titre of the serum was similar to the original titre found using live elementary bodies.

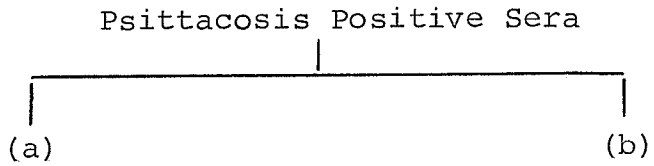
Later it was shown (58) that the heat-labile antigen was type-specific, while the heat stable antigen was common to all of the psittacosis group of agents.

Several workers (71, 39, 64) were able to demonstrate that the common heat-stable psittacosis group antigen was found in such sources as the bacterium Bacillus anitratum

(Herellea) (39, 64) and egg yolk (71).

The discovery, by Barwell (1), that the group antigen could be destroyed using potassium periodate, leaving the specific antigen in the suspension of elementary bodies, provided a method of specifically identifying psittacosis antibodies.

To approach the problem of the specificity of the psittacosis-lymphogranuloma venereum group antibodies in Eskimo sera, it was necessary to design experiments in two major areas: a) using psittacosis positive sera and agents of the psittacosis-lymphogranuloma group, and b) using psittacosis positive sera and antigens unrelated to the psittacosis-lymphogranuloma venereum group.



1) Examination for specific antibodies to psittacosis group agents.

2) Absorption of the psittacosis group antibodies; examination for the remaining specific antibodies to the psittacosis group of agents.

1) Examination for correlation to:
   a) bacterial antigens
   b) Q fever, Mycoplasma
   c) cold agglutinins, Strep. MG. and egg protein.

2) Absorption with bacterial and non-specific antigens: examination for removal of the group antibodies.

Sera from Eskimos and non-Eskimos living in an Arctic community were examined by the complement fixation test for the presence of psittacosis-lymphogranuloma venereum group antibodies. Examination of the sera shown to be positive was carried out in order to establish if possible the specificity of the psittacosis-lymphogranuloma venereum group antibodies. For reference purposes, psittacosis-positive sera were obtained from the Manitoba Provincial Virus Laboratory and from Markham Laboratories, Chicago, Illinois. Antisera to the main agents under study -- psittacosis, meningopneumonitis and ornithosis -- were produced in rabbits.

Antigens specific to the agents of psittacosis and meningopneumonitis were prepared using the methods of Barwell (1), and the sera described above were examined for specific antibodies. Further, the sera were absorbed to remove the psittacosis group antibodies, using the methods of Bedson et al (7), and were tested by complement fixation using live agents of psittacosis, meningopneumonitis and ornithosis. These tests should show the type-specific antibodies to the agents named, if such exist in Eskimo sera.

Because of the presence of the psittacosis-lymphogranuloma venereum group antigen in unrelated sources, Eskimo sera were examined for antibodies to a variety of bacterial and rickettsial pathogens which had animal reservoirs. In addition, the sera were examined for

antibodies to non-specific antigens.

Eskimo sera were absorbed using live suspensions of bacteria which had been isolated from or known to exist in the group of Eskimos from whom the sera were obtained, and non-specific antigens. These absorption experiments paralleled those carried out using the psittacosis-lymphogranuloma venereum group antigen.

Experiments were designed to provide evidence as to the specific nature of the psittacosis-lymphogranuloma antibodies found in Eskimo sera.

CHAPTER III

METHODS

CHAPTER III

METHODS

This chapter deals with the essential methods used throughout the experimental work of this study. However, the portion which describes the materials, where they came from, and how they were handled, is recorded in Appendices A, B and C. Included in Appendix A is a list of E-numbers for the Eskimos from whom serum was acquired.

SEROLOGICAL TESTS

Complement fixation test. The complement fixation test was carried out using a modified Kolmer technique (16), in perspex plates. Serial two-fold dilutions from 1:4 to 1:128 were used with inactivated serum in 0.1 ml volumes, except in the case of second tests, where the serum was known to react at 1:128 or greater. To each dilution was added 0.1 ml of antigen containing 2 antigenic units, calculated on the basis of a checkerboard titration with known antiserum (one antigenic unit is the highest dilution of antigen that gives complete fixation with the highest dilution of antiserum). Complement, 2½ HD_{50} in 0.1 ml was added to each well. After incubation for 18 hours at 4°C. the plates were placed in a waterbath at 37°C. for a warming period of 5 minutes, and 0.1 ml of 2% sensitized sheep erythrocytes was added to each well. After thorough mixing

the plates were returned to the 37°C. waterbath for 15 minutes; they were removed and further mixed by rotation before being replaced in the waterbath for another 15 minutes. At this time the plates were removed and the 50% end point was read.

A full set of controls was used for each test and these included, in addition to sensitized sheep red cells:

1) Anticomplementary serum control (complement, saline and serum)
2) A serum antigen medium control (complement, antigen media and serum)
3) A serum antihemolytic control (saline, saline and serum)
4) An antigen anticomplementary control (complement, saline and antigen)
5) An antigen antihemolytic control (saline, saline and antigen)
6) A red blood cell control (saline, saline and saline)
7) Complement control 1 (complement, saline and antigen)
8) Complement control 2 (½ of complement from 7 in 0.1 ml, saline and antigen)
9) Complement control 3 (½ of complement from 8 in 0.1 ml, saline and antigen)
10) Known positive serum
11) Known negative serum

Residual Antibiotic in Allantoic Fluid

Allantoic fluid from chick embryos of the same

batch as those used for the cultivation of psittacosis, meningopneumonitis and ornithosis was tested for residual tetracycline antibiotics. Serial doubling dilutions of allantoic fluid from 1:1 to 1:64 were made in 1 ml volumes. To each was added 0.05 ml of a 1:10 dilution of a culture of Aerobacter aerogenes, which was sensitive to 6 micrograms of tetracycline. The cultures were incubated for 18 hours at 37°C. and read for inhibition of growth.

No inhibition of growth was recorded.

INOCULATION AND HARVEST OF EMBRYONATED EGGS

The inoculation and harvest of embryonated eggs was carried out following procedures reported in Diagnostic Procedures for Viral and Rickettsial Diseases, American Public Health Association, (41). Each embryo was harvested individually and the allantoic fluid or yolk sacs stored in "bijou bottles". From each bottle one drop was transferred to thioglycollate media, and a further drop was used to make smears on two glass slides.

Sterility tests of harvests

From the individual thioglycollates, inoculated during the harvest procedure and incubated for five days, one drop was transferred to each of two separate blood agar plates, the first being incubated aerobically, the other anaerobically, for 48 hours. All harvests which proved to be contaminated with bacteria were discarded.

Smears for microscopic examination

Two smears were made during the harvest of yolk sac and allantoic fluid antigens. These were fixed and stained using both Castenada's and Macchiavello's stains. Figure 2 shows the uninfected yolk sac cells; figures 3 and 4 show infected allantoic fluid stained with Castenada's and Macchiavello's stain; while figure 5 is a demonstration of intracellular elementary bodies. All photomicrographs were taken at a magnification of 900 times.

ANTIGEN PREPARATION

Infected Allantoic Fluid

It was necessary to prepare antigens using viable agents of psittacosis, ornithosis and meningopneumonitis. The infected allantoic fluid was centrifuged at 18,000 times gravity (g) for 30 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended to 25% of the original volume with veronal buffered saline (V.B.S.) pH 7.4. For each 2 ml of this suspension ½ ml of 1% trypsin was added, to give a final concentration of 2 mg of trypsin per ml. The mixture was left for 1 hour at room temperature, with occasional agitation (see flow diagram figure 9). After 1 hour the suspension was centrifuged at 170 g for 5 minutes; the resulting pellet was discarded, and the supernatant was centrifuged at 18,000 g for 30 minutes, at 4°C. The supernatant was discarded, and the pellet resuspended

to 25% of the original volume. This operation was performed three times, to wash and purify the elementary bodies (65). Following the third wash, the pellet was resuspended in VBS to 10% of the original volume. A smear of this suspension was examined under the light microscope after staining with Macchiavello's stain. Figure 6 shows massed clumps of particles derived from three washings of infected allantoic fluid.

Further examination of the final pellet was carried out using the electron microscope. Preparations negatively stained with phosphotungstic acid were examined. Figure 7 shows elementary bodies and the larger (500 to 800 millimicrons) particles of meningopneumonitis. It should be noted that the small elementary bodies have a dense centre which is a concentration of nucleic acids. The larger forms have a more diffuse centre and are similar to the particles which have been shown to divide by binary fission. The area marked was examined at a higher magnification (Figure 8).

Infected yolk sac

After harvest the yolk sacs were washed free of yolk material. Three yolk sacs were placed in a universal bottle with 6 ml of phosphate buffer pH 7.6 (Appendix D); this constituted a hypotonic solution. The universal bottle was refrigerated at 4°C. for 18 hours, and the supernatant was decanted to a separate container. The yolk sacs and the supernatant were treated separately.

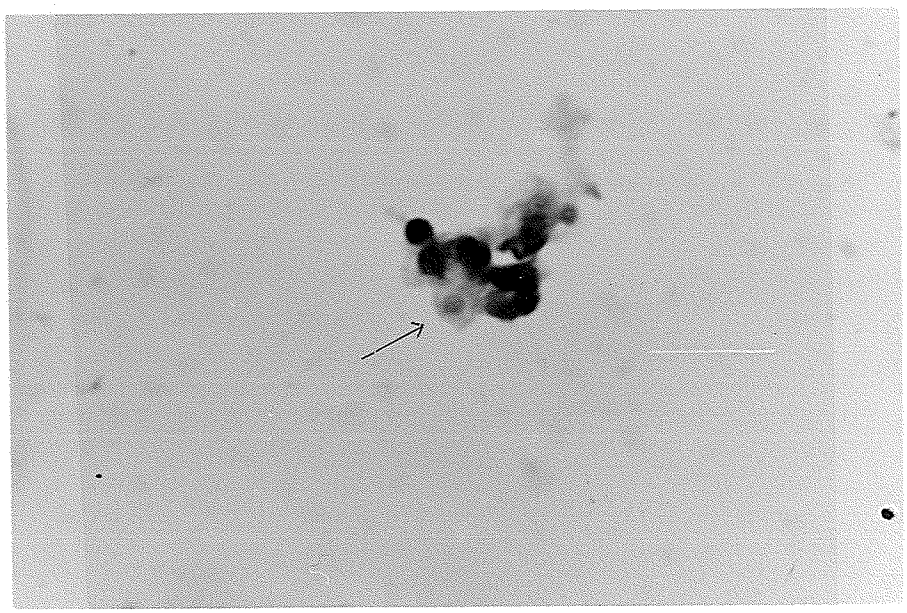


Figure 2

Uninfected yolk sac cells, which have been stained blue with Castenada's stain, showing the clear cytoplasm (x 900).



Figure 3

Meningopneumonitis particles in allantoic fluid, stained blue with Castenada's stain (x 900).

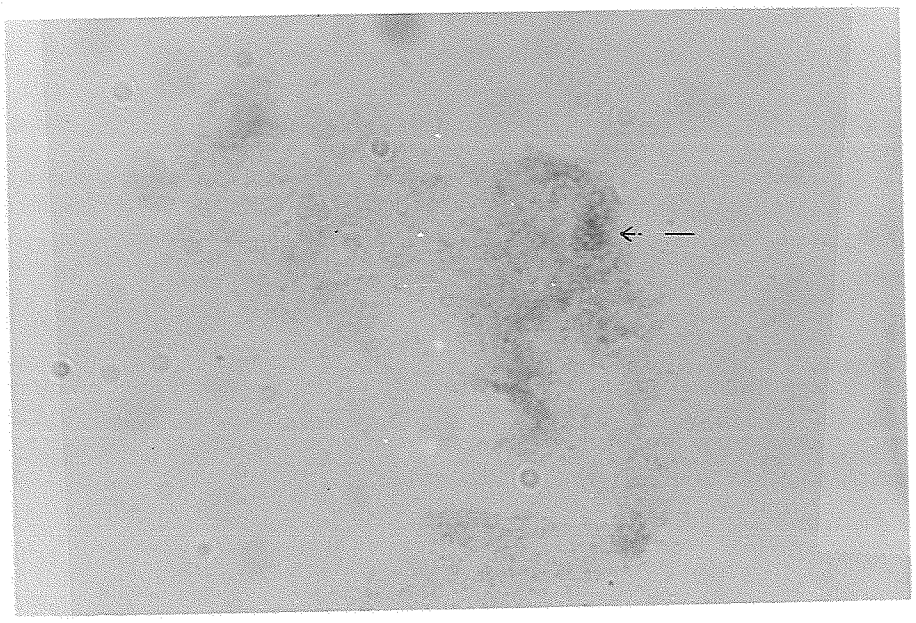


Figure 4

Psittacosis 6BC particles in allantoic fluid, stained red with Macchiavello's stain (x 900).

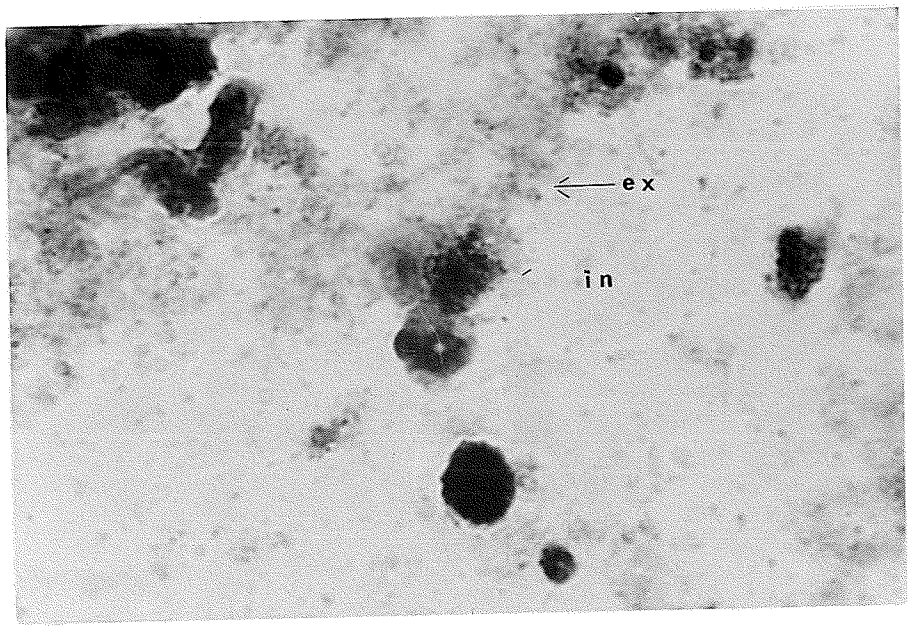


Figure 5

Ornithosis particles in an impression smear of yolk sac, showing intracellular (in) and extracellular (ex) agents, stained blue with Castenada's stain (x 900).

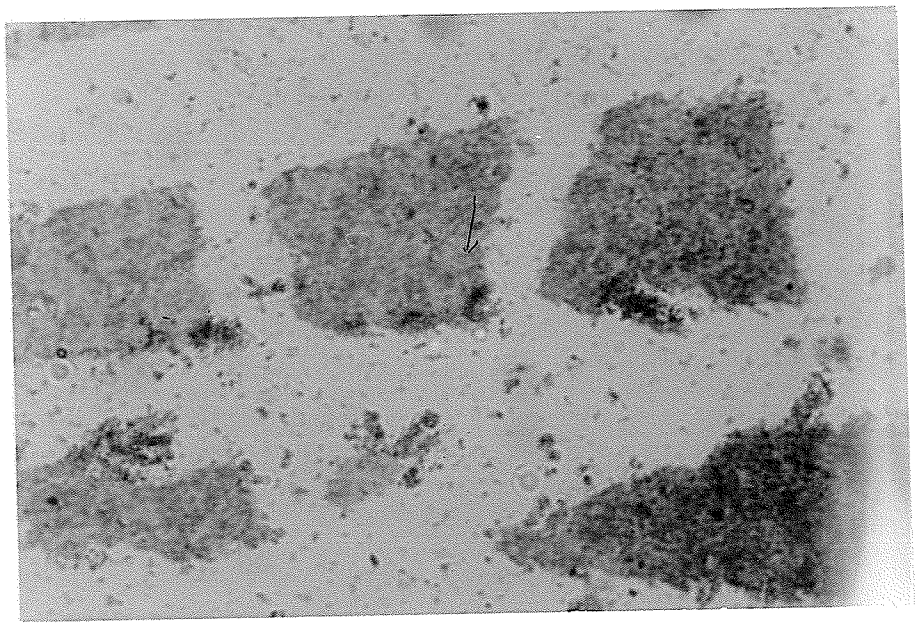


Figure 6

Washed and concentrated psittacosis 6BC particles, in masses, making individual particles unrecognizable, stained red with Macchiavello's stain (x 900).

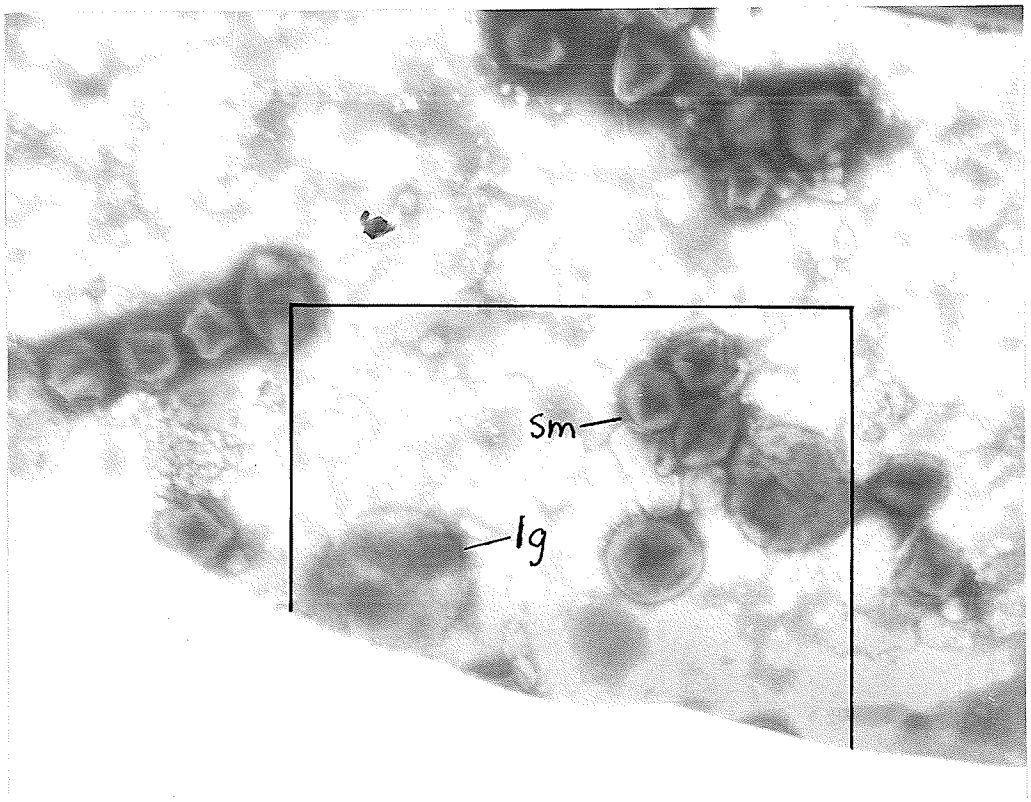


Figure 7

Electron micrograph showing large (lg) and small (sm) particles of meningopneumonitis, stained with 2% phosphotungsic acid (x 20,000).

Size: small 350 millimicrons
large 6-800 millimicrons

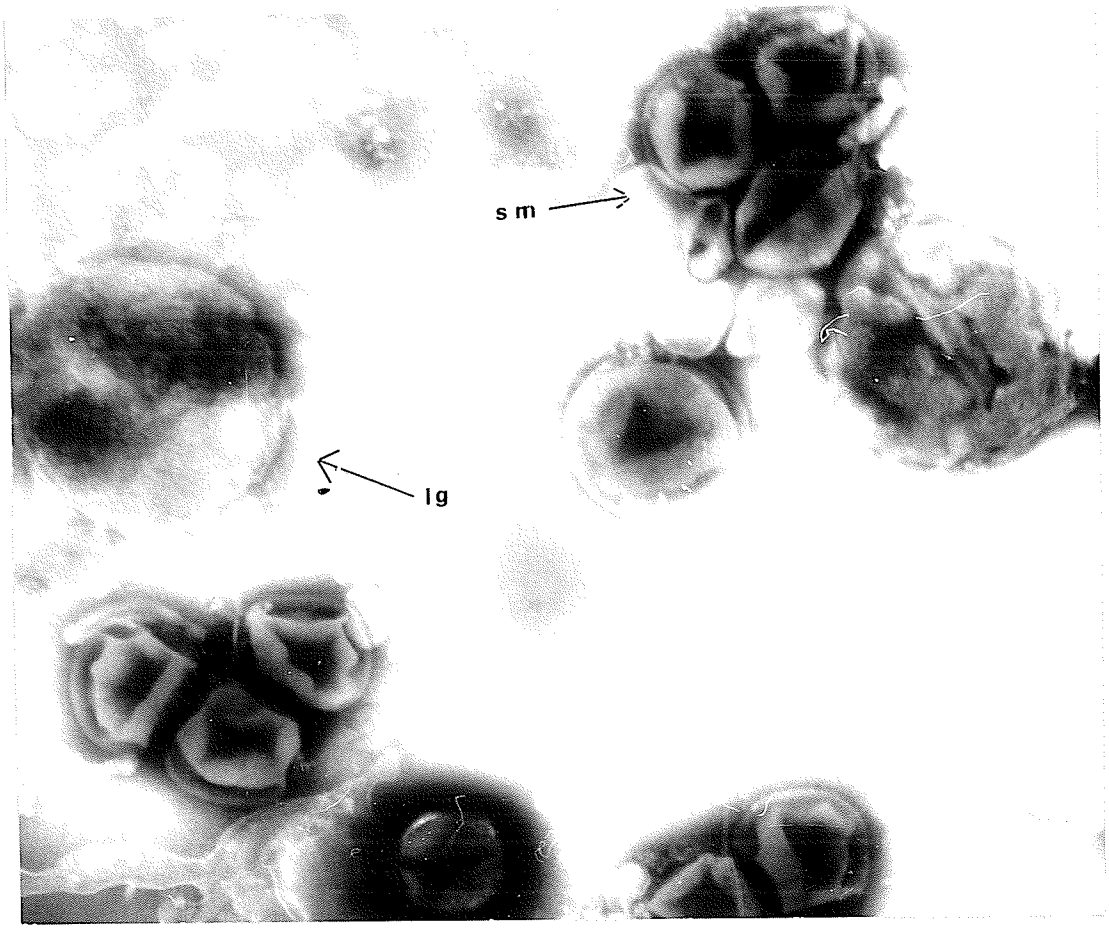
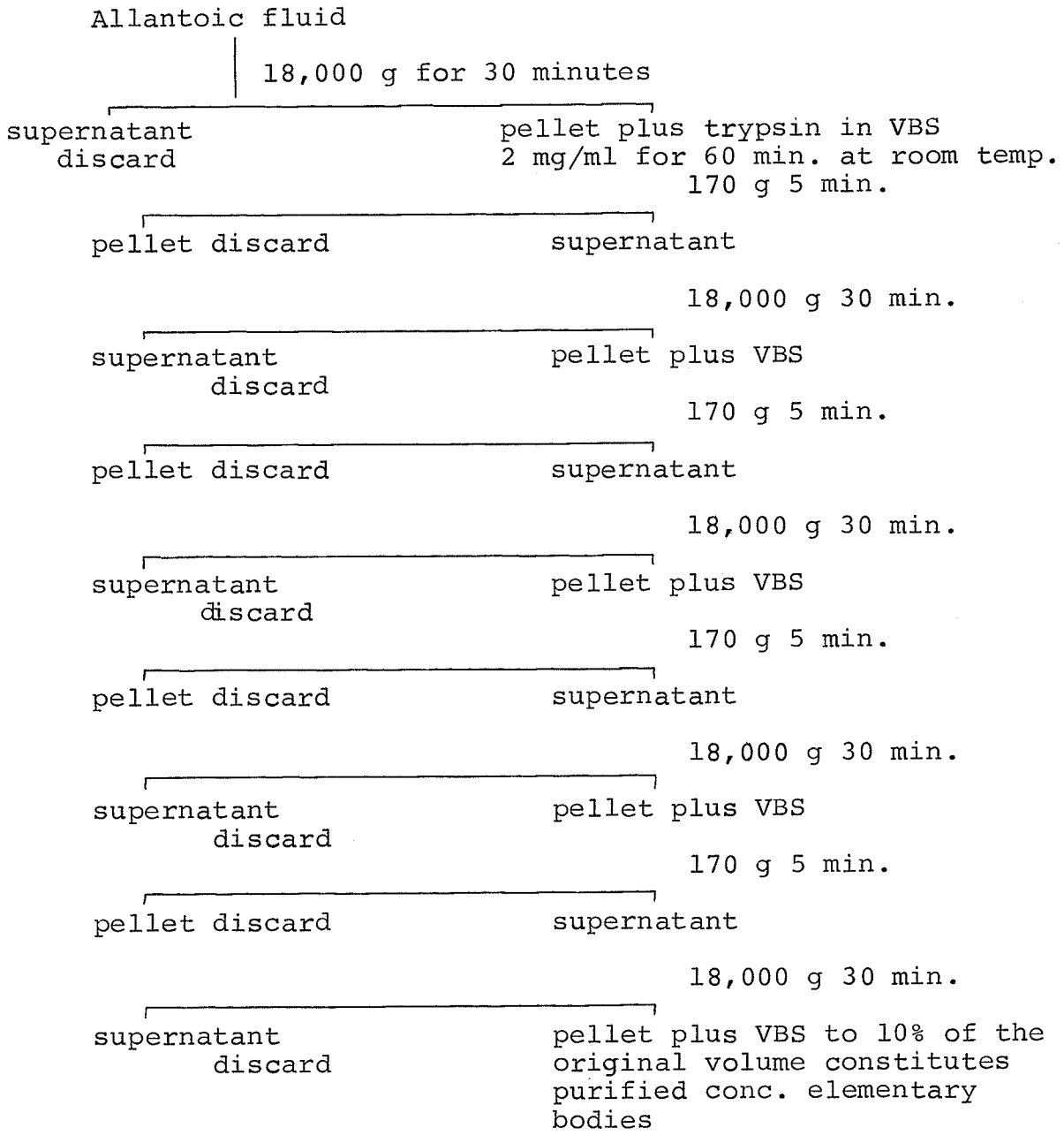


Figure 8

Electron micrograph of area marked in Figure 7 (at increased magnification), showing the large (lg) and small (sm) particles of meningopneumonitis stained with 2% phosphotungsic acid (x 46,000).

Figure 9

Purification of Psittacosis-like agents propagated in allantoic fluid



Supernatant. The supernatant was centrifuged at 170 g for 5 minutes, and the pellet discarded. The remaining supernatant was centrifuged at 18,000 g for 30 minutes. The supernatant from this centrifugation was discarded and the pellet resuspended to 50% of the original volume in VBS. This suspension was washed three times using the technique of low and high speed centrifugation as shown in the flow diagram figure 10. The pellet left after the final centrifugation was resuspended to 10% of the original volume, and a smear was examined for purity using the light microscope.

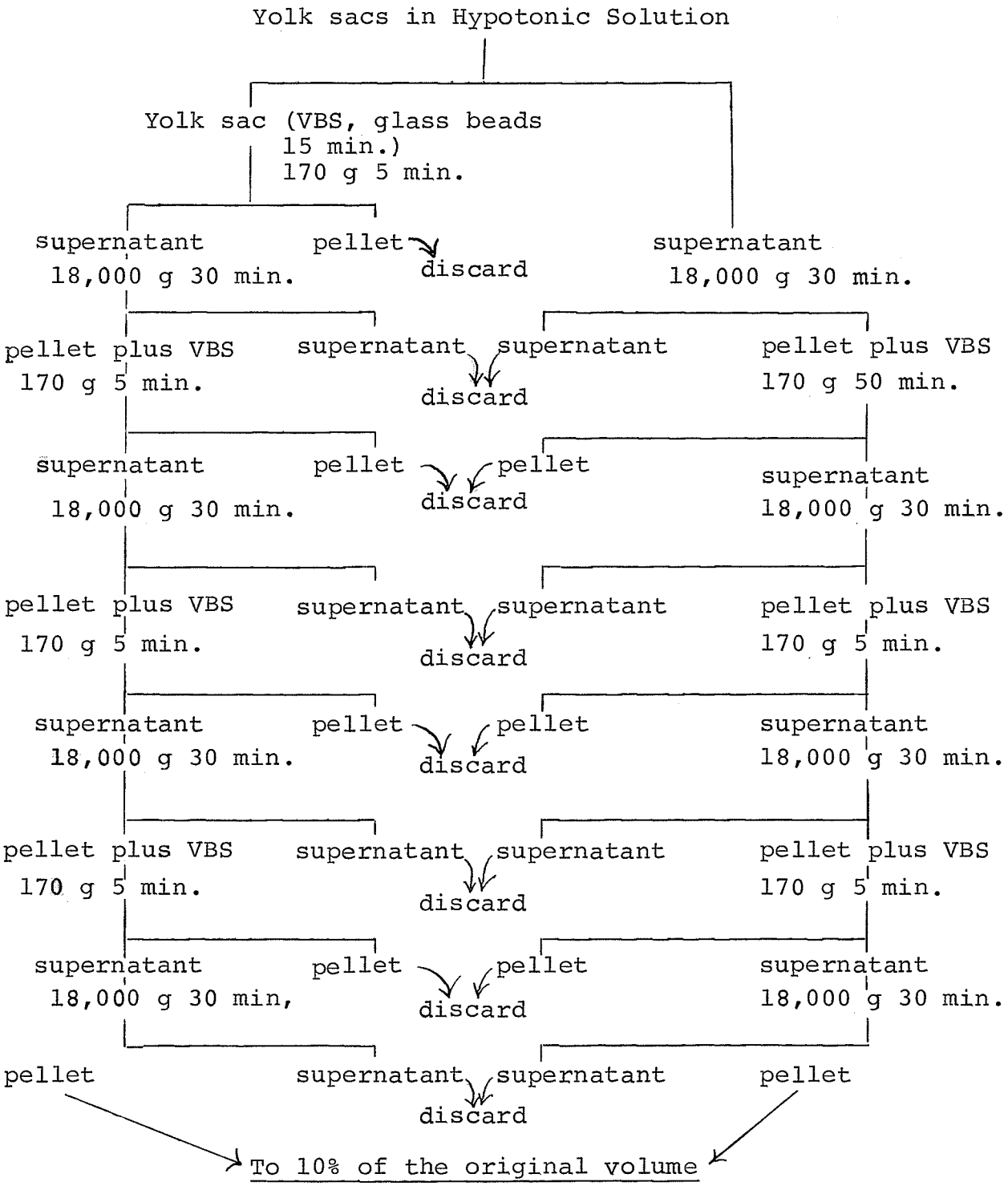
Yolk sac. To the yolk sacs left in each universal bottle 6 ml of VBS and 2 ml of sterile glass beads were added. The screw cap was tightly sealed with sealing tape. The bottles were placed on a vortex test tube mixer, and agitated at medium speed for 15 minutes at 4°C. The supernatant was centrifuged at 170 g for 10 minutes; the pellet was discarded, and the remaining supernatant was centrifuged at 18,000 g for 30 minutes at 4°C. The elementary bodies in the pellet were washed three times and through three cycles of low and high speed centrifugation. Figure 10 is a flow diagram describing this sequence. The final pellet was resuspended at 10% of the original volume.

Specific antigen production

A suspension of elementary bodies with a

Figure 10

Purification of Psittacosis-like agents propagated in yolk sac



complement fixation titre greater than 1:8, titrated with known positive antisera, was used for specific antigen production. This was divided into two equal portions, which were centrifuged at 18,000 g for 30 minutes at 4°C.; the supernatant was discarded. One pellet was resuspended to its original volume in VBS, as shown in Figure 11. The second pellet was resuspended to 50% of its original volume in freshly prepared 0.005 M potassium periodate, and held in a 37°C. waterbath for 30 minutes; then 5% glucose was added to bring the suspension back to its original volume (Figure 11). The suspensions constituted live group and specific antigens.

Controls

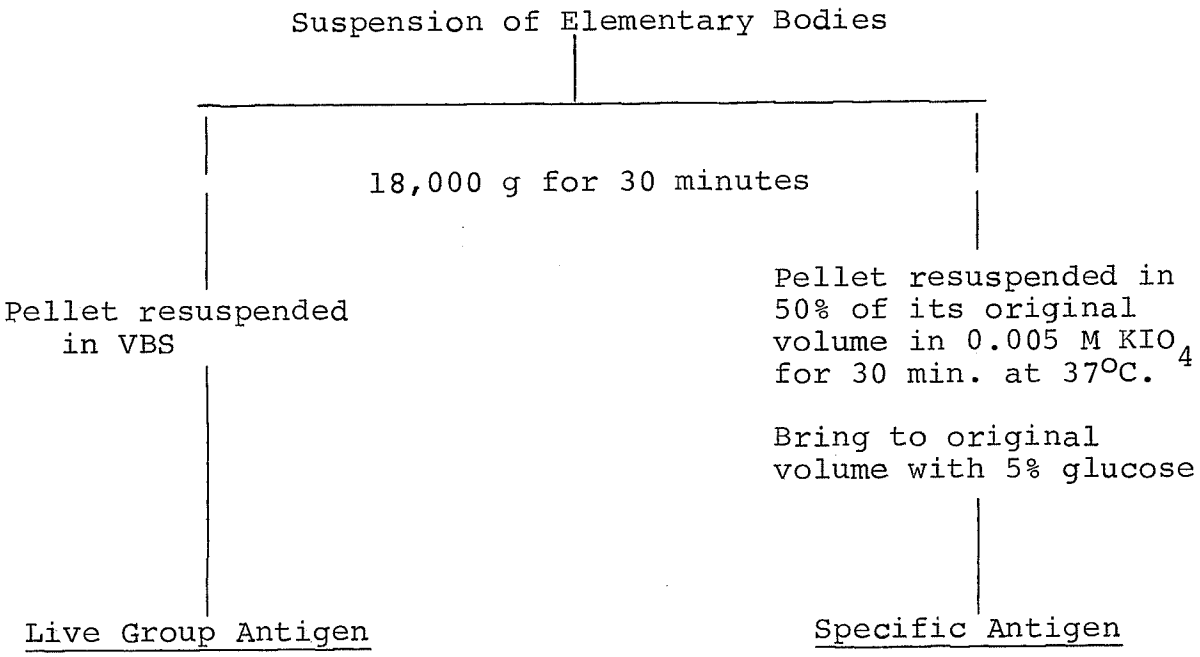
From each lot of embryonated eggs, 20% was kept for controls. Of this portion, 10% was inoculated with uninfected yolk sac suspension or with uninfected allantoic fluid; the rest was left uninoculated. These controls were harvested and examined in precisely the same manner and at the same time as those used for the propagation of psittacosis-like agents (Figure 9).

Commercial tissue antigen. Produced from allantoic fluid (Markham Laboratory).

Yolk sac tissue antigen. Yolk sac control antigen, consisting of uninfected yolk sac, harvested at the same time

Figure 11

Preparation of Group and Specific Antigens From Viable Psittacosis-Like Agents



Live group and specific antigens together equal the original volume of elementary bodies

KIO_4 potassium periodate

VBS veronal buffered saline

as chick embryos infected to produce ornithosis antigens, and treated in a similar manner.

Yolk sac trypsin treated tissue antigen. A control antigen produced through the treatment of uninfected yolk sacs and allantoic membranes by shaking with glass beads. The supernatant left when this material had been centrifuged for 10 minutes at 170 g was treated with trypsin in a final concentration of 2 mg per ml.

Allantoic fluid tissue antigen. Allantoic fluid antigen produced using the same method as was used for the preparation of psittacosis and meningopneumonitis antigens (Figure 9).

ANTISERA PRODUCTION

Antisera specific to psittacosis, meningopneumonitis and ornithosis were produced in rabbits whose pre-inoculation sera were negative to the psittacosis-lymphogranuloma venereum group antigen. A single intravenous injection of 2 ml was made using one of the previously prepared purified suspensions of elementary bodies. The serum collected at 14 days proved to contain an adequate titre of psittacosis-lymphogranuloma venereum group antibodies. A titre of 1:128 or greater was considered to be adequate.

ABSORPTION

The technique employed was essentially that described by Bedson et al (7) in 1949. The absorbing antigen was prepared as a heavy suspension. A volume equal to the volume of serum to be absorbed was centrifuged at 18,000 g for 5 to 30 minutes, depending on the size of the antigenic particles. The supernatant was discarded and the pellet resuspended in the serum to be absorbed. This was left at room temperature for 1 hour, with frequent agitation, then kept at $4^{\circ}C$. for 18 hours. Removal of the antigen from the serum was accomplished by centrifugation at 18,000 g for 30 minutes. The absorbed sera were tested for residual antibodies by complement fixation.

CHAPTER IV

RESULTS

CHAPTER IV

RESULTS

This chapter describes the results of tests conducted to prove that specificity of the psittacosis-lymphogranuloma venereum group antibody as found in Eskimo sera, in accordance with the following plan:

I) To prove the existence of psittacosis-lymphogranuloma venereum group antibody in Eskimo sera.

II) To prove that type-specific antigens of psittacosis and meningopneumonitis were, in fact, antigens by using antisera prepared in rabbits as well as known positive human sera.

III) To discover the reactions in Eskimo sera to type-specific antigens of psittacosis and meningopneumonitis.

IV) To learn whether a type-specific antibody to psittacosis, ornithosis or meningopneumonitis remained in Eskimo sera following absorption with psittacosis-lymphogranuloma venereum group antigen.

V) To prove that antigens other than those of the psittacosis-lymphogranuloma venereum group were not responsible for the antibody under study in Eskimo sera.

I. Complement Fixation Tests Using Eskimo Sera Collected at Eskimo Point in 1967 with Psittacosis-Lymphogranuloma Venereum Group Antigens

In order to prove the presence of psittacosis-lymphogranuloma venereum group antibodies in the Eskimo sera

tests were conducted using heat-inactivated group antigens from different sources (Appendix A). As shown in Table I, Figure 12, 95 Eskimo and 5 non-Eskimo serum samples were tested, of which 88 were positive by complement fixation to a commercial psittacosis group antigen (Markham Laboratories); 64 showed antibodies to a titre of 1:16 or greater. This table gives the number of sera at each two-fold dilution from $<$1:4 to 1:256, with the mean at 1:32.

Comparable complement fixation test results were obtained with each serum sample, using a second psittacosis group antigen (Laboratory of Hygiene). As shown in Table III the latter proved slightly less active than the first antigen, and a two-fold drop in antibody titre was seen in all tests.

Results of complement fixation tests using lymphogranuloma venereum group antigen (Markham Laboratories) are shown in Table II. Of 54 samples tested, 46 had antibody titres identical to those found using psittacosis group commercial antigen. Only three showed a higher titre, while five were lower.

If these antigens were indeed produced from agents obtained from different sources, the overall results would indicate that cross reaction within the group was virtually complete.

Comparison of complement fixation tests with paired sera, 1963 and 1967, using commercial psittacosis antigen. A number of serum samples collected at Eskimo Point in 1963 had been stored in this laboratory. Of these, it was possible to pair 32 with samples collected in 1967 from the same persons. This provided the basic material for the comparison test.

To examine the sera for adverse effects of prolonged storage, a positive psittacosis serum of known titre (1:128), taken in 1963 and stored, was re-tested with Markham antigen. The titre of complement fixing antibody was found to be unchanged, indicating that prolonged storage did not adversely affect these antibodies.

Figure 12 demonstrates this comparison, showing that by 1967 there had been a rise in titre and incidence of psittacosis-lymphogranuloma venereum group antibodies in sera from persons who in 1963 had a low level of antibodies. When examination was made of the sera from people with titres of less than 1:4 in 1963, it was seen that 17 showed a rise in titre by 1967 -- at least 10 of these to a titre of 1:16 or greater. The serum samples collected in 1963 showed that 19% of the people tested had low level antibodies to psittacosis, while only 9% had antibodies at a titre of 1:16 or greater. By comparison, in 1967 among the same group of individuals the incidence was 81%; of these, 53% exhibited titres of 1:16 or greater to the psittacosis antigen.

Observations. Examination of sera from Eskimo and non-Eskimo residents of Eskimo Point, N.W.T., revealed a high incidence of antibodies to the psittacosis-lymphogranuloma venereum group antigen. These antibodies reacted equally well to group antigens prepared from both psittacosis and lymphogranuloma venereum.

Serum samples taken from residents of Eskimo Point in 1963 were tested at the same time as samples taken from the same individuals in 1967. A marked increase in the titre and incidence of the psittacosis antibodies was found to have occurred during this four-year period.

It was possible to show that psittacosis-lymphogranuloma venereum group antibodies existed at a significant titre in 65% of the sera under examination.

TABLE I

Complement Fixation Test Titres with 100 Eskimo Sera and a Commercial Psittacosis Antigen*

Titre of Sera	$<$1:4	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Number of Sera	12	13	11	14	29	8	11	2

* Markham Laboratories, Chicago, Illinois

TABLE II

Comparison of Complement Fixation Tests of Eskimo Sera Using Group Antigen Prepared from Psittacosis and from Lymphogranuloma Venereum

*Psittacosis		*Lymphogranuloms venereum		
		Number of sera		
Titre	Number of Sera	No change	**Rise	**Fall
<4***	7	7	0	0
4	7	6	1	0
8	6	5	1	0
16	8	6	1	1
32	16	15	0	1
64	3	2	0	1
128	5	3	0	2
256	2	2	0	0
	54	46	3	5

* Commercial antigens (Markham Lab.)

** Four fold or greater increase or decrease

*** Reciprocal of the serum dilution

TABLE III

Complement Fixation Tests With 100 Eskimo Sera and Psittacosis Antigen obtained from the Laboratory of Hygiene*

Dilutions of sera	< 1:4	1:4	1:8	1:16	1:32	1:64	1:128
Number of sera	22	13	15	29	9	6	6

* Laboratory of Hygiene, Ottawa, Ontario.

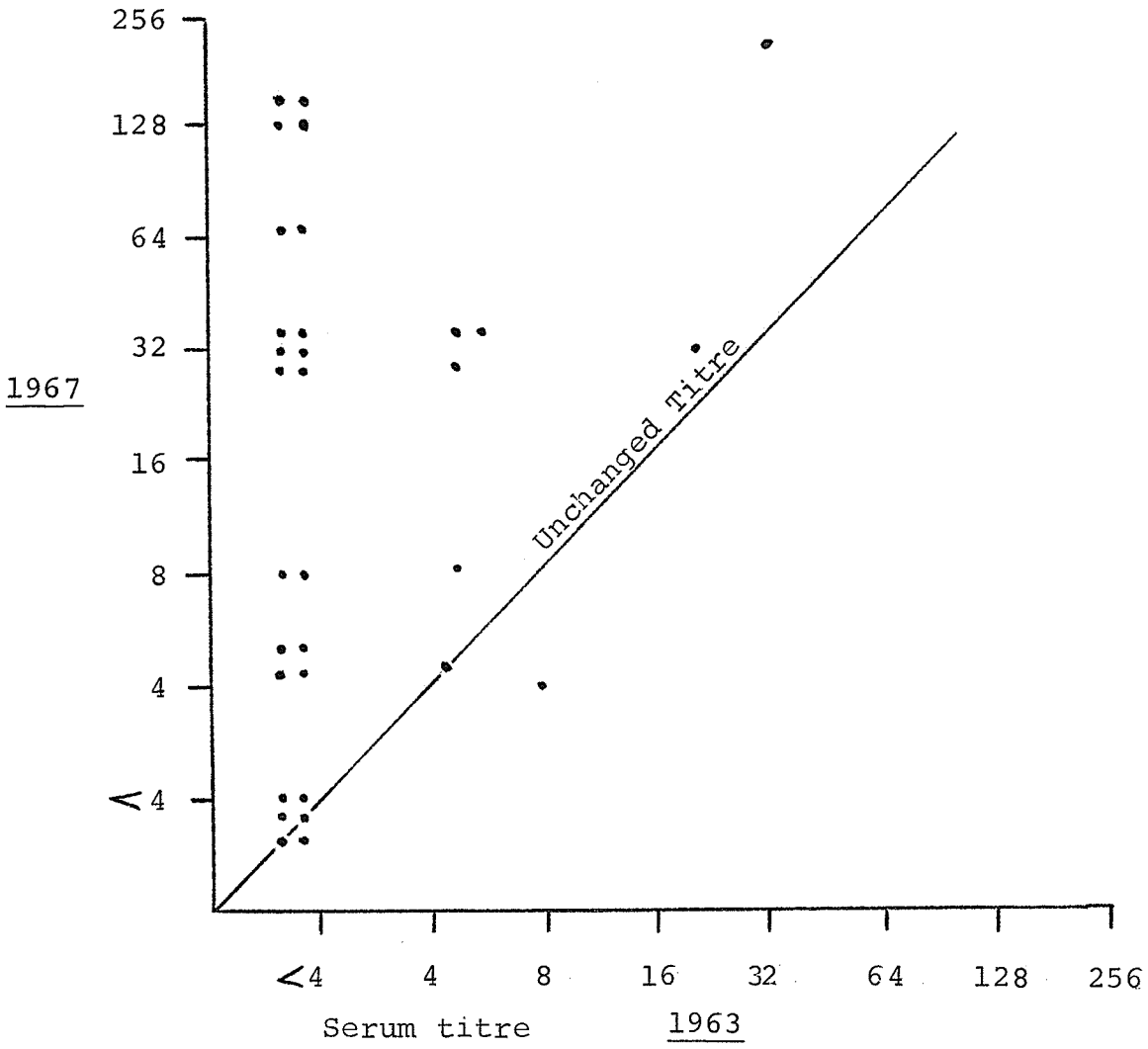


Figure 12

Comparison of Complement Fixation Tests: Paired Eskimo Sera from 1963 and 1967 with Psittacosis Group Antigen.

II. Experiments to Prove That the Type Specific Antigens for Psittacosis and Meningopneumonitis Prepared During the Course of This Work Were in Fact Antigens.

Antisera were produced in rabbits by inoculating intravenously 2 ml of purified and highly concentrated live suspensions of elementary bodies from psittacosis, meningopneumonitis or ornithosis. Pre-immunization serum was negative by complement fixation to the psittacosis-lymphogranuloma venereum group antigen. After 14 days, test sera contained an adequate complement fixation titre to the homologous antigens, and the animals were bled.

Tables IV, V, VI, VII and VIII demonstrate the results of tests employing these sera. Data in Table IV show the reactions of these sera with commercial group antigen (Markham Laboratories) as well as the homologous antigens.

The agents used to immunize rabbits were produced in embryonated eggs. To ensure the specificity of this antibody, each of the rabbit-produced sera was tested by complement fixation using as antigens uninfected allantoic fluid and yolk sac suspensions. These control antigens were produced in conjunction with the various psittacosis-like agents. Such antigens were prepared by submitting the allantoic fluid and yolk sacs to the same procedures as were used for the purification of the psittacosis-lymphogranuloma venereum agents produced in embryonated eggs.

Table V shows these reactions, and also the results of complement fixation tests using commercial human antisera and Eskimo sera tested with these same control antigens. No reaction could be recorded with either of the latter sera. A considerable reaction was recorded with all of the control antigens and the rabbit antisera. These reactions varied from 1:4 with ornithosis antisera and commercial antigen media, to 1:128 with trypsin-treated yolk sac suspension and all of the rabbit-produced antisera.

Each of the rabbit-produced antisera, as well as Eskimo serum and commercial antiserum, was absorbed using a mixture of the control antigens. The absorption procedure was repeated each day for seven days, after which time the tests described in Table V were repeated. The results are given in Table VI.

Virtually all of the egg-induced antibody was removed from the rabbit antisera. However, the meningopneumonitis and the ornithosis rabbit-produced antisera reacted at a dilution of 1:4 with the yolk sac trypsin treated antigen. The psittacosis antisera reacted at a dilution of 1:8 with the same antigen. These antisera still retained an antibody reaction with commercial psittacosis antigen (Markham), but at a much reduced titre (Table VI). Eskimo serum and commercial antiserum did not react with tissue antigens, and the titre to the psittacosis antigen remained the same (Table VI).

a) Absorbed rabbit antisera with live group and specific antigens. Tests using absorbed rabbit-produced antisera and live group and specific antigens of meningopneumonitis and psittacosis revealed that these antibodies were specific for the agents by which they had been induced. In addition to the above results, Table VII shows that both the unabsorbed and the absorbed ornithosis antisera exhibited no reactivity with the specific antigens of psittacosis and meningopneumonitis.

Rabbit antisera produced against meningopneumonitis, and absorbed to be free of antibodies to chick embryo tissue antigens, reacted with meningopneumonitis group antigen to a titre of 1:64. Similarly they reacted with psittacosis group antigen to a titre of 1:64. When these antisera were tested with specific antigens of meningopneumonitis and psittacosis, they reacted to a titre of 1:32 and 1:8 respectively, indicating that the specific antigen produced from live agents of meningopneumonitis was indeed an antigen.

These tests were repeated using rabbit-produced psittacosis antisera. A group antigen reaction of 1:128 was achieved. The specific antigen of meningopneumonitis gave a titre of 1:16; the specific antigen of psittacosis gave a titre of 1:64, indicating that the specific antigen produced from live agents of psittacosis was indeed an antigen.

b) Sera from non-Eskimo patients, having psittacosis-like infection, with psittacosis and meningo-pneumonitis group and specific antigens. In order to evaluate further the specific antigens, sera from patients with respiratory disease diagnosed as psittacosis on clinical and serological grounds were obtained from the Provincial Laboratory of Manitoba, Virology Section, and examined for group and specific activity to both psittacosis and meningo-pneumonitis. Three of these sera, (VLG, VLM, VLL) were from persons known to have had contact with psittacine birds (parrots, budgerigars, etc.). With the others the contact was unknown.

Examination of Table VIII shows that of the patients described, the ones who had had contact with psittacine birds also had specific antibodies to psittacosis. The other sera were negative to both psittacosis and meningo-pneumonitis specific antigens. In the commercial positive control serum the antibodies appear to have been caused by psittacosis. The serum labelled VLT was collected from a patient in Churchill, Man., with atypical pneumonia; and since Churchill is the closest non-Eskimo community to Eskimo Point, it is of interest to note that his psittacosis-group serological reaction was identical to that of the Eskimos.

Observations. Antisera to psittacosis, meningo-pneumonitis and ornithosis, prepared in rabbits, were found

to contain a high titre of antibodies to antigens produced from uninfected chick embryos (the growth media for these agents). It was possible to remove these antibodies by absorption of the sera seven times, using uninfected chick embryo antigens. These antisera were found to be specific for the homologous agents.

Examination of sera from persons in southern Manitoba who had psittacosis diagnosed by clinical and serological means revealed that those known to have had contact with sick psittacine birds gave a positive reaction to the type-specific psittacosis antigen, but a negative reaction to the type-specific meningopneumonitis antigen.

Antisera produced in rabbits confirmed that type-specific antigens prepared from elementary bodies of psittacosis and meningopneumonitis were, in fact, antigens.

TABLE IV

Rabbit Antisera -- Complement Fixation with Commercial Group and Homologous Live Group Antigen

Serum Produced Against	Commercial Group Antigen	Homologous Group Antigen
Meningopneumonitis	*256	256
Psittacosis	1024	512
Ornithosis	256	256

* Reciprocal of the serum dilution

TABLE V

Rabbit Antisera -- Complement Fixation With Control Antigens

Antiserum	Psittacosis Group	Commercial Antigen Media	Yolk Sac Antigen Media	Yolk Sac Plus Trypsin	Allantoic Antigen Media
Meningopneumonitis	*256	64	32	128	32
Ornithosis	256	4	64	128	<4
Psittacosis	1024	64	64	128	16
Eskimo Sera	256	<4	<4	<4	<4
Markham Lab. Antisera	64	<4	<4	<4	<4

* Reciprocal of the serum dilution

TABLE VI

Rabbit Antisera Absorbed With Control Antigens -

Complement Fixation Test With Control Antigens

Antiserum	Commercial Psittacosis Antigen	Commercial Antigen Media	Yolk Sac Antigen	Yolk Sac Plus Trypsin	Normal Allantoic Antigen
Meningopneu-monitis	*64	< 4	< 4	4	< 4
Ornithosis	16	< 4	< 4	4	< 4
Psittacosis	256	< 4	< 4	8	< 4
Eskimo Sera	256	< 4	< 4	< 4	< 4
Markham Lab. Antisera	64	< 4	< 4	< 4	< 4

* Reciprocal of the serum dilution

TABLE VII

Rabbit Produced Antisera Absorbed With Control Antigens:- Tested by Complement Fixation with Live Group and Specific Antigens of Psittacosis and Meningopneumonitis

Serum	Meningopneumonitis		Psittacosis	
	Group	Specific	Group	Specific
Meningo-pneumonitis	64*	32	64	<16***
Ornithosis	16	<8**	32	<8**
Psittacosis	256	16	128	64

* Reciprocal of the Serum Dilution

** Reacted at 1:4 with tissue antigens

*** Reacted with tissue antigen at 1:8

TABLE VIII

Non-Eskimo Sera From Patients With Clinically and Serologically Diagnosed Psittacosis, Reacted Against Group and Specific Antigens of Psittacosis and Meningopneumonitis, By Complement Fixation

Serum	Psittacosis		Meningopneumonitis	
	Group	Specific	Group	Specific
VLG*	128**	16	64	<4
VLM	64	16	64	<4
VLL	256	64	128	<4
VLH	64	4***	64	4***
VLK	32	<4	32	<4
VLT	256	4***	128	4***
VLF****	<4	<4	<4	<4
Pos. Control*****	64	8	32	<4

* VL refers to the virus laboratory
** Reciprocal of the serum dilution
*** Reacted with tissue antigens at 1:4
**** Serum positive to adenovirus
***** Markham laboratories

III. Eskimo Sera With Psittacosis and Meningopneumonitis Group and Specific Antigens

Group and specific antigens of psittacosis and meningopneumonitis were tested by complement fixation with dilutions of Eskimo sera. Table IX shows seven representative results, indicating that no matter what the level of group activity in the serum, from 1:4 to 1:256, no specific reaction occurred with meningopneumonitis or psittacosis. Very few results were obtainable with ornithosis specific antigen, as the yolk sac-produced antigen proved anti-complementary.

The results obtained using specific antigens with Eskimo sera indicate that the psittacosis-lymphogranuloma venereum group antibodies found in the sera were not caused by the agents of psittacosis or meningopneumonitis.

Observations. Psittacosis and meningopneumonitis type-specific antigens did not react with Eskimo sera. However, the ornithosis specific antigen was anti-complementary. The ornithosis live group antigen was not anti-complementary; it was assumed therefore that an unfavorable reaction had occurred between potassium periodate (used to prepare specific antigens) and some impurity in the suspension of elementary bodies. The reactions between Eskimo sera and live group antigens of psittacosis, meningopneumonitis and ornithosis were at identical titres.

The psittacosis-lymphogranuloma venereum group antibodies in Eskimo sera were apparently not caused specifically by psittacosis or meningopneumonitis.

TABLE IX

Representative Results of Complement Fixation Tests Using Group and Specific Meningopneumonitis and Psittacosis Antigens with Eskimo Sera

Sera No.	Antigens					
	Meningopneumonitis		Psittacosis		Ornithosis	
	group	specific	group	specific	group	specific
64	256*	<4	256	<4	256	4
63	128	<4	128	<4	64	4
23	64	<4	32	<4	32	4
53	64	<4	32	<4	32	4
11	64	<4	128	<4		
22	16	<4	16	<4		
56**	<8	<8	<8	<8		

* Reciprocal of the serum dilution

** Anti-complementary at 1:4

IV. Eskimo Sera Absorbed to Remove Psittacosis-Lymphogranuloma Venereum Group Antibodies

Eskimo sera were absorbed to remove antibodies to the psittacosis-lymphogranuloma venereum group. Two different types of absorption were used: one with inactivated group antigen from a commercial source (Markham), the other with a live suspension of meningopneumonitis organisms. The results of these absorptions are shown in Tables X and XI.

a) Eskimo sera absorbed with commercial group antigen. Eskimo sera were absorbed three times with a psittacosis group antigen, and the absorbed sera tested by complement fixation using live agents of psittacosis, meningopneumonitis and ornithosis. Two typical reactions are shown in Table X, which illustrates the titres obtained by both unabsorbed and absorbed sera, tested at the same time. This table shows the complete removal, by absorption, of all antibodies to the absorbing antigen and the three testing agents.

b) Eskimo sera absorbed with live group antigen. To evaluate further the antibody to the psittacosis-lymphogranuloma venereum group which exists in Eskimo sera, these sera were absorbed six times with a suspension of live meningopneumonitis agents. If such antibodies were caused by either ornithosis or psittacosis, specific antibodies to the live agents of these organisms should still remain

in Eskimo sera. Examination of Table XI shows that all antibodies to ornithosis and psittacosis was removed by absorption. In two of the sera, a low-level reaction was recorded both to the absorbing antigen and to a suspension of psittacosis agents. This in all probability reflects an incomplete absorption.

Observations. Eskimo sera were absorbed to remove the common group antibodies. After absorption these sera did not react with the agents of psittacosis, meningopneumonitis or ornithosis. Live elementary bodies contain both a group and a type-specific antigen; therefore, since absorption had removed the group antibodies from the sera, reaction should have occurred between the specific antigenic component of the agent and the specific antibody remaining in the sera. Since no reaction occurred, it must be assumed that none of these agents was responsible for the psittacosis group antibodies found in Eskimo sera.

TABLE X

Eskimo Sera Absorbed With Psittacosis Group Antigen and Tested by Complement Fixation With Live Agents of Meningopneumonitis, Psittacosis and Ornithosis

Serum*	Absorbing antigen	Live Agents		
		meningo-pneumonitis	ornith-osis	psitt-acosis
Unabsorbed 15	32**	32	16	16
Absorbed	<4	<4	<4	<4
Unabsorbed 53	64	32	64	64
Absorbed	<4	<4	<4	<4

* Representative absorptions

** Reciprocal of the serum dilution

TABLE XI

Eskimo Sera Absorbed With Live Meningopneumonitis Antigen and Tested by Complement Fixation With Live Agents of Meningopneumonitis, Psittacosis and Ornithosis

Serum*	Antigens		
	meningo-pneumonitis**	psittacosis	ornithosis
Unabsorbed 22	16	32	16
Absorbed	<4	<4	<4
Unabsorbed 51	64	64	32
Absorbed	4***	4	<4
Unabsorbed 11	32	64	32
Absorbed	4	4	<4

* Representative groups

** Absorbing antigen

*** The 1:4 reactions are indicative of antibody left following absorption

V. Reactions of Eskimo Sera With Antigens Other Than Those of the Psittacosis-Lymphogranuloma Venereum Group

Cross-reactions have been reported between the psittacosis-lymphogranuloma venereum group and other antigens: Bacillus anitratum (Herellea 39, 64) and egg yolk (71). Selected Eskimo sera were examined for antibodies to bacterial and other antigens in an attempt to correlate the results with the findings concerning the psittacosis-lymphogranuloma venereum group antibodies. It has been shown that many bacterial antigens are not related to the antibodies under study (6); also antibodies to viral agents failed to correlate with the psittacosis-lymphogranuloma venereum group antibody (26, 25). Organisms causing diseases transmissible from animals to man were considered as likely to cross-react, in view of the fact that the Eskimo people have frequent contact with animals and birds, their diet often consists of uncooked meat, and the women customarily chew hides to soften them for use in making articles of clothing.

Most typhus patients have agglutinins to OX-19 (Weil-Felix) (31); other rickettsial diseases also exhibit cross-reactions with different Proteus strains (OX-2, OX-K). Tests were therefore carried out to establish possible cross-reaction between the psittacosis group antibodies and Proteus OX-19. Because Q fever (Coxiella burneti), which is caused by an animal-associated rickettsia, does not react with any Weil-Felix antigen, complement fixation tests were performed

to look for a correlation between Q fever and the psittacosis antibodies in Eskimo sera. A number of mycoplasma species have been isolated from Eskimos; since such organisms have been incriminated in atypical pneumonias, as have psittacosis agents, a thorough examination of Eskimo sera for complement fixation antibodies to Mycoplasma pneumoniae was carried out.

a) Psittacosis-positive Eskimo sera with bacterial antigens. The next group of tables, XII, XIII and XIV, deal with agglutinating bacterial antibodies found in psittacosis-positive Eskimo sera.

Ten Eskimo sera (10% of the samples collected) positive to the psittacosis group antibody were examined for brucellosis, using both the plate and tube agglutination tests (antigen: Difco Laboratories, Detroit, Michigan, U.S.A.). All agglutination tests were negative. As is shown in Table XII, the level of antibodies to the psittacosis group antigen was compared with the agglutination titre of brucellosis. The brucellosis titre proved to be less than 1:10, despite variations in the psittacosis titre from less than 1:4 to 1:256.

When thirteen of the Eskimo sera were tested by tube agglutination for tularemia (antigen: Lederle Laboratories, Pearl River, N.Y., U.S.A.) low levels of agglutinating antibody were found in three sera. As illustrated in Table XIII, which examines results of complement fixation tests with psittacosis and compares the psittacosis titre with the

tularemia agglutination titre, ten of the sera having antibodies to psittacosis, ranging from titres of 1:4 to 1:256, exhibited no antibodies to tularemia. The highest agglutinating titre was 1:40, and this serum had a complement fixation titre of 1:4. An agglutinating titre against tularemia of 1:20 was found in one serum, which gave a psittacosis reaction of 1:256. Since four other sera, however, had psittacosis reactions of 1:128 and 1:256, with no apparent agglutinating antibodies to tularemia, it would appear that the antibodies to psittacosis as found in Eskimo sera were unrelated to Pasteurella tularensis.

Proteus sp. were isolated from the ears and throats of several residents of Eskimo Point (see Appendix). Weil-Felix agglutination tests using Proteus OX-19 (Lederle Laboratories) were carried out on 57 Eskimo sera, and the results were compared with those obtained when the same tests were performed on sera from patients in the Winnipeg General Hospital. Of the 57 Eskimo sera tested with Proteus OX-19, 2 reacted to a titre of 1:80; 24 had agglutination titres of less than 1:10; while the majority of those showing positive reactions had titres ranging from 1:10 to 1:20. When 100 tests were carried out on sera from patients in the Winnipeg General Hospital, 53% showed antibodies at a level of less than 1:10; 27% at a level of greater than 1:20, while only 20% showed antibodies at 1:10 to 1:20. A comparison of the

of the two sets of tests is shown in Table XIV, where the Weil-Felix test results concerning Eskimo sera have been converted into percentages. This table demonstrates in general that the percentage incidence of reaction using Eskimo sera is similar to that using non-Eskimo sera from patients in hospital. Past evidence indicates that residents in the Winnipeg area rarely exhibit antibodies to psittacosis (76).

A comparison between the incidence of psittacosis group antibodies as found in Eskimo sera and the incidence of Weil-Felix (Proteus OX-19) agglutinating antibodies indicates that the two types of antibodies coexist in Eskimo sera, but are unrelated to one another. Of four samples having a psittacosis titre of 1:4, only 2 (or 50%) had low level agglutinating antibodies to Weil-Felix antigen. Of six samples showing a psittacosis titre of 1:128, three (or 50%) had low-level agglutinating antibodies to the Weil-Felix antigen. Regardless of the dilution at which psittacosis group antigen reacted, from 1:4 to 1:256, the incidence of agglutinating antibodies to Proteus OX-19 remained at approximately fifty percent.

TABLE XII

Brucella Antibody in Eskimo Sera Compared to Psittacosis Group Antibody in the Same Sera

Psittacosis		Brucellosis	
Number of Sera	C.F. Titre	Number of Sera	Agglutination Titre
3	<4*	3	<10**
1	4	1	<10
1	16	1	<10
3	32	3	<10
1	128	1	<10
1	256	1	<10
10		10	

* Reciprocal of serum dilution

** Serum dilution, final dilution reported at 1:20

TABLE XIII

Tularemia Agglutinating Antibody in Eskimo Sera Compared with Psittacosis Group C.F. Antibody in the Same Sera

Psittacosis		Tularemia		
Number of Sera Tested	C.F. Titre	Number of Sera Negative (< 1:10)	Number of Sera Positive	Agglutination Titre
3	*4	2	1	*40
1	16	1	0	
4	32	3	1	20
3	128	3	0	
2	256	1	1	20
13		10	3	

* Reciprocal of the serum dilution

TABLE XIV

Comparison of Weil-Felix Test -- Eskimo Sera and Sera From Patients at the Winnipeg General Hospital

Serum	Dilutions			
	<*10	10	20	>20
Eskimo	**42	26	19	13
Winnipeg General Hospital	53	7	13	27

* Reciprocal of serum dilutions

** Percent

b) Comparison of complement fixation tests for psittacosis, Q fever and Mycoplasma. This series of tests was carried out by complement fixation, making comparisons readily discernible, as shown in Tables XV and XVI. In Table XV we have the results of 25 complement fixation tests for Q fever (Markham Laboratories) as compared with 25 complement fixation tests for psittacosis group antibodies in the same Eskimo sera. Only four of the sera reacted with the Q fever antigen, all at a dilution of 1:4. Three of these reactions occurred in sera having a titre of 1:32 to the psittacosis antigen. The other reacted in a serum showing a titre of 1:8 to psittacosis. Eskimo sera exhibiting psittacosis antibodies at dilutions of 1:64, 1:128 and 1:256 showed no antibodies to Q fever.

During the 1967 survey, Mycoplasma sp. were isolated from throat swabs of six Eskimos. Because of this finding, 90 sera were examined by complement fixation test using Mycoplasma pneumoniae antigen (Baltimore Biological Laboratory, Baltimore, Maryland, U.S.A.) to determine the incidence of antibodies to this organism. Comparisons of the antibody titres to mycoplasma and to psittacosis in Eskimo sera are shown in Table XVI. If we accept a complement fixation titre of 1:8 as significant of mycoplasma infection, there would seem to be no correlation between the two antibody reactions. Twenty-one of the psittacosis group

and 81 of the mycoplasma group were found to have antibodies at a level of less than 1:8. By comparison, 59 of the psittacosis group had antibodies at a titre of greater than 1:8, while only two reacted to mycoplasma at this level. Nine of the sera reacted at a titre of less than 1:4 to psittacosis, while 52 showed a similar reaction to mycoplasma antigen. It would seem difficult, therefore, to claim that mycoplasma was responsible for the psittacosis group antibodies as found in Eskimo sera.

TABLE XV

Eskimo Sera -- Complement Fixation Reactions

With Psittacosis and Q Fever Antigens

Psittacosis		Q Fever	
Number of Sera	Titre	Number of Sera (<1:4)	Number of Sera (1:4)
2	<4*	2	0
5	4	5	0
2	8	1	1
3	16	3	0
6	32	3	3
4	64	4	0
2	128	2	0
1	256	1	0
25		21	4

* Reciprocal of the serum dilution

TABLE XVI

Eskimo Sera -- Complement Fixation Titres With Psittacosis and Mycoplasma Antigens

	Dilutions		
	<8*	8	>8
Psittacosis	21	11	59
Mycoplasma	81	8	2

* Reciprocal of the serum dilution

c) Eskimo sera examined for antibodies to Cold Agglutinins, Streptococcus MG and to Egg Proteins. All of the Eskimo and non-Eskimo sera were examined by the cold agglutinin test. No positive reactions were found. (All blood collected was kept at room temperature until separation of the serum was completed; therefore cold agglutination could not have occurred at that time.)

No positive agglutination took place between 26 Eskimo sera and the Streptococcus MG antigen (Difco Laboratory). The tube agglutination test was used.

One of the known cross-reactions of the psittacosis-lymphogranuloma venereum group is with common egg yolk (71). When egg yolk is injected into an animal it elicits an antibody response. The antibodies so produced react by complement fixation not only to egg yolk, but also to psittacosis group antigens.

Eskimo sera were tested by complement fixation with uninfected allantoic fluid and yolk sac suspensions, from eggs inoculated with normal allantoic fluid, and harvested at the same time and from the same batch of eggs as were allantoic fluid and yolk sacs infected with psittacosis-like agents. No reaction occurred between any such antigen media and Eskimo sera nor commercial antigen media (Markham) purchased for control purposes.

d) Absorption of Eskimo sera with bacterial and other unrelated antigens. The sections a, b and c deal with possible cross-reactions between the psittacosis antibody of Eskimo sera and some of the agents causing diseases likely to be found in Eskimos. To establish the possible effect of other bacterial antigens, Eskimo sera were absorbed with a series of such agents as were isolated from or known to exist in Eskimos at Eskimo Point. Each organism, as a live suspension, was used for a separate absorption, which was carried out in the manner described in the section under methods. Neisseria gonorrhoeae, meningiditis and catarrhalis, the latter two isolated from Eskimos. Proteus vulgaris, mirabilis and rettgeri all isolated from Eskimos. Hemophilus influenzae, Mycobacterium tuberculosis, Pseudomonas aeruginosa. Since egg yolk is reputed to contain the psittacosis group antigen (71), each of these sera was absorbed with egg yolk, yolk sac suspension and allantoic fluid. Human "A" erythrocytes are reported to contain a heterophil antigen (40), so each serum was treated for 24 hours using human group "A" erythrocytes.

The complement fixation titres of two sera absorbed as above are shown in Table XVII. Unabsorbed sera were tested at the same time as the absorbed sera. Both commercial group (Markham Laboratories) and live group psittacosis antigens were used in these tests, and although differences are recorded in the antibody level shown by the two antigens,

no drop in titre through the process of absorption could be found.

Observations. Brucellosis antibodies were not found in the sera under study. Although agglutinating antibodies to tularemia and Proteus OX-19 (Weil-Felix) were discovered, it was possible to demonstrate that these coexisted with, rather than caused, the psittacosis-lymphogranuloma venereum group antibodies. Mycoplasma and Q fever complement fixing antibodies, though present in Eskimo sera, were not found to be a factor in producing the antibodies to the psittacosis-lymphogranuloma venereum group. This observation was borne out by the failure to find cold agglutinins in the sera under study.

Absorption of Eskimo sera with suspensions of bacteria, allantoic fluid, yolk sac and human "A" erythrocytes failed to reduce the titre of Eskimo sera to the psittacosis-lymphogranuloma venereum group antibody.

The antibody under study in Eskimo sera is not related to tularemia, brucellosis, mycoplasma or Q fever. It could not be removed from the sera by absorption with a wide variety of bacterial and non-specific antigens.

TABLE XVII

Eskimo Sera Absorbed With Bacteria, Heterophil Antigen and Yolk Sac Suspension (Representative Titrations)

Serum Number		Commercial Psittacosis Antigen	Live Group Psittacosis Antigen
15	a) Unabsorbed	*32	16
	b) Absorbed	32	16
53	a) Unabsorbed	64	32
	b) Absorbed	64	32

* Reciprocal of the serum dilutions

CHAPTER V

DISCUSSION

CHAPTER V

DISCUSSION

The high incidence of antibodies to the psittacosis-lymphogranuloma group antigen found in Eskimo sera (25, 76) was something of an enigma. Surveys of normal populations elsewhere in the world showed that only 1% to 7% had antibodies to the psittacosis-lymphogranuloma venereum group. For example, Stallman (68) in Australia found that certain groups of people had a high incidence of antibodies -- persons suffering from non-specific urethritis, poultry workers, and those in contact with wild or domestic psittacine birds -- but in general surveys of an area, only 3.7% of the normal population was involved. These reports showed that among the cases of high Psittacosis group antibodies there is evidence of a common factor: for instance, ornithosis in poultry workers. Such evidence is not indicated in a study of Eskimo sera, as there seems to be no common source. Eddie et al (17) found antibodies and isolated agents of the psittacosis group in fur seals, which seemed related to the high incidence of antibodies in the Aleuts. However, it is difficult to believe that the isolated community of Indians (27) at Old Crow (70%), far from the sea, or the inland caribou-eating Eskimos (14%) could possibly have contact with the fur seal. Similarly the residents of Eskimo Point live in proximity to the nesting grounds of the snow goose, which fact

would indicate ornithosis as a possible source of the antibodies; but many other affected Eskimos do not share this proximity.

The findings of Wilt et al in 1958, in which the high incidence of antibodies to the psittacosis-lymphogranuloma venereum group as found in Eskimos was compared with the low incidence (1%) of these antibodies in residents of southern Manitoba, (including 6 Indians and 20 Metis) would indicate that the antigen responsible for such antibodies is confined to the Arctic regions.

Using the complement fixation test it was possible to demonstrate that persons from Eskimo Point, including non-Eskimos employed in the area, had a much higher incidence of psittacosis antibodies in 1967 than had been revealed in 1958 (Table I). Of the five non-Eskimos surveyed during 1967, four were found to have a titre of 1:16 or greater. This involvement of persons not indigenous to the North was unsuspected, in view of the low incidence of psittacosis antibodies in southern Manitoba.

It was demonstrated that not only the incidence but also the titre of the psittacosis antibodies had dramatically increased in ten years. Comparison of sera from 1963 with sera taken in 1967 from the same persons showed a marked increase in titre and incidence of psittacosis antibodies (Figure 12). Only two persons of this group in 1963 showed a titre of 1:16 or greater, while in 1967, 18 reacted at this

dilution of serum. Dane (15) and Bedson (7) believe that a complement fixing antibody titre in one serum of 1:16 or greater to the psittacosis-lymphogranuloma venereum group antigen is indicative of a positive test. According to this criterion, 64% of the Eskimo sera taken in 1967 reacted to a significant titre; there were therefore available sera with a high incidence of psittacosis antibodies which reacted at a significant titre, and furthermore a large proportion of the antibodies were of recent origin, having occurred within the previous four years.

What is the cause of this antibody? Rake et al (58), and later other workers (59, 51, 73), have demonstrated that cross-reactions within the psittacosis-lymphogranuloma venereum group were virtually complete; it has been possible to show that psittacosis-lymphogranuloma venereum antibodies in Eskimo sera (Table II) exhibited a similar range of cross-reactions. Therefore, any of the agents within the psittacosis-lymphogranuloma venereum group (31) could be responsible for the antibodies found in Eskimos. However, there are sources of the group antigen other than the psittacosis-lymphogranuloma venereum group of agents, including Bacillus anitratum (Herellea) (39, 64) and egg yolk (71). It was difficult, then, to claim specificity for the psittacosis-lymphogranuloma venereum group antibodies in Eskimo sera without eliminating, as possible sources of the group antigen, non-specific egg protein and bacteria.

Egg yolk, allantoic fluid and yolk sac suspensions did not react with Eskimo sera, nor did the absorption of the sera with these substances remove any of the antibodies to the psittacosis group. It must be assumed that these antigens are not responsible for the psittacosis group antibodies unless the goose, gull, and other eggs with which the Eskimo comes into contact contain an antigen not found in hen's eggs.

Bacterial and viral involvement in causing the psittacosis group antibodies might be suspected as a consequence of the present mode of life of the Eskimos (23). These people, traditionally nomadic, are for the first time living in permanent quarters, but are continuing to follow their habitual, primitive sanitary practices. Evidence of poor sanitation (52) is suggested by the bacterial findings in throat swabs (see Appendix) from a high percentage of which gram negative bacilli were recovered. However, there was no indication that such bacteria were other than commensals. As caribou would form the entire diet of this particular group of Eskimos if it were possible, and as they customarily eat this meat raw, frozen or only partially cooked even after storage, a large number of bacterial species can be postulated which might provide a source of psittacosis antibody. Surprisingly enough, considering the Eskimo diet and association with animals, neither Salmonella nor Shigella species were isolated from stool samples in this community,

and were unlikely to be sources of the psittacosis lymphogranuloma venereum group antibodies. It would be well to bear in mind also that the non-Eskimo inhabitants of Eskimo Point displayed as great a reaction to the psittacosis group antigen as did the Eskimos, although their housing conditions, diet and sanitary practices were quite different.

It is evident then that circumstances exist which would permit extensive cross-reactions between the psittacosis group and bacterial antigens. Bedson (6) in 1937 was able to show that sera, from patients with some of the common diseases, did not cross-react with the psittacosis antigen by complement fixation: Streptococcal septicemia, pneumonia of unknown origin, tuberculosis, jaundice, acute myelitis, osteomyelitis, pleurisy, chronic nephritis, and nephritis with terminal infection. Greenberg and Blake (22, 23) found, in their study of Indian and Eskimo sera, that 7% of the Indian and 13% of the Eskimo sera reacted to the tularemia antigen. During the same surveys, 4% of the Indian and 0.3% of the Eskimo sera were found to react to the brucella antigen. To discover whether there was now any better correlation between the antibodies to the psittacosis group and these bacterial antigens, agglutination tests for the latter were carried out.

It was possible to demonstrate antibodies to psittacosis in persons having no antibodies to brucellosis (Table XII). Similarily, tularemia failed to show any

antigenic relationship to the psittacosis-lymphogranuloma venereum group antibodies (Table XII).

A comparison of these findings with those of Greenberg and Blake (23) suggests that the antibodies to the psittacosis-lymphogranuloma venereum group, found in Eskimo sera, are unrelated to Pasteurella tularensis and to Brucella abortus.

Dane (15) published some interesting findings by which he showed that certain patients serologically diagnosed as having Q fever or typhus, also had low level antibodies to psittacosis, but since he made no attempt to correlate these results it was felt that this was an area requiring tests with Eskimo sera. It was noteworthy that of the Q fever patients only 7.8% had psittacosis-lymphogranuloma venereum group antibodies. This relates very well to the findings (Table XV) whereby 16% of 25 Eskimos with psittacosis antibodies had complement fixing antibodies to Q fever. The fact that in the latter the dilution was at a low level (1:4) suggested that the antibodies to psittacosis, with which we are concerned, were not related to Q fever.

Workers in Australia were able to demonstrate (15) in some cases the coexistence of agglutinins to Proteus OX-19 (Weil-Felix) and psittacosis group antibodies. The sera from many of the Eskimos at Eskimo Point also reacted to the Weil-Felix test. Antibodies as revealed by the Weil-Felix

test and antibodies to the psittacosis antigen both existed in Eskimo sera, but were in no way related.

Dane (15) was also able to show that among patients who had suffered from leptospirosis through contact with animals, only 4% showed psittacosis group antibodies. Leptospirosis then would seem an unlikely source of the antibody to the psittacosis group.

The agents of the psittacosis-lymphogranuloma venereum group have recently been classified with the bacteria (8). However, through size and characteristics they belong with the small organisms such as Rickettsia and Mycoplasma. It would seem likely that if a cross-reaction were to exist it would be with one of these small organisms. Representatives of the rickettsia, as shown by Weil-Felix and Q fever tests, do not appear to be involved as a cause of the psittacosis-lymphogranuloma venereum group antibodies.

Of the Eskimo sera 91 were examined for Mycoplasma pneumoniae by the complement fixation test (Table XVI). The results indicate that Mycoplasma are not the agents causing the psittacosis antibody in Eskimo sera. Further, the cold agglutinin tests, which are often used to screen sera for possible Mycoplasma involvement, all proved negative. This does not necessarily mean that some Mycoplasma species might not react; however, since Eskimos suffer from many respiratory infections, the Mycoplasma most likely to cross-

react with the psittacosis group was considered to be Mycoplasma pneumoniae.

Since it was not possible to run tests for all bacteria and other unrelated antigens, it was considered necessary to absorb Eskimo sera with a number of different bacterial antigens, isolated from the same Eskimos whose serum exhibited a high incidence of psittacosis antibodies. In addition, yolk sac and allantoic suspensions as well as human Group "A" erythrocytes were used for absorption -- the latter in an attempt to remove the heterophil antibodies.

No drop in titre was shown between unabsorbed and absorbed sera when these were tested in an identical manner using both a commercial and a live group psittacosis antigen (Table XVII). If any of the bacteria or other antigens used were responsible for the psittacosis group antibodies found in Eskimo sera, there would have been at least a minor reduction in the titre of the absorbed sera. A particular word should be said concerning tuberculosis; all of the persons tested, including non-Eskimos, were Mantoux-positive. As a reduction in psittacosis antibodies did not occur, when sera were absorbed with tubercle bacillus, one can infer that there is no connection between the two.

"The lipopolysaccharide hapten of erythrocytes and of the Forssman heterogenetic antigen is immunologically related to blood group "A" substance," according to Kwapinski (40). For this reason, absorption with human group "A"

erythrocytes was carried out, and it was hoped that any heterophil antigen would thus be removed from Eskimo sera. No lowering of psittacosis titre occurred. The dramatic rise in psittacosis antibodies (Figure 12) from 1963 to 1967 would indicate that infection rather than a heterophil antigen was the cause, since physical conditions had not altered significantly in the community during this period.

Most Eskimos have been inoculated (23) following a vaccination program similar to that carried out in large urban communities in Manitoba. As the people in the latter communities do not show a high incidence of psittacosis antibodies (76), it would seem unlikely that such inoculations could be responsible for psittacosis group antibodies in the residents of Eskimo Point.

The evidence so far presented conveys the impression that the cause of the psittacosis antibodies in Eskimo sera was unrelated to antigens outside the psittacosis-lymphogranuloma venereum group. It was necessary, therefore, to take a closer look at the psittacosis-lymphogranuloma venereum agents.

According to Wilt et al (76), and the observations of physicians examining Eskimos, lymphogranuloma venereum is not known in the Arctic, and certainly no evidence of this disease was discovered at Eskimo Point in 1967. Sigel (66) stated that with this condition there is always a characteristic scarring, and that the lymphogranuloma venereum

antibody titre tends to be in excess of 1:320. Trachoma, according to Reed et al (61) has never been identified as a disease in Arctic regions. In Reed's experience, most corneal scarring, among the people of Eskimo Point was caused by phlyctenular keratoconjunctivitis. Newborn conjunctivitis, the most common form of inclusion conjunctivitis, has not been reported from Eskimo Point. Since the agents of the psittacosis group are susceptible to many antibiotics, it is possible that the drops placed in the eyes of the new-born could conceal infection with this agent. Inclusion conjunctivitis, a mild venereal infection, could be hidden in the community under these circumstances; however, children below the age of puberty show as high an incidence of psittacosis antibodies as do the adults, indicating that the antibodies are not likely to be caused by the agent of inclusion conjunctivitis.

The other diseases which could be responsible for the psittacosis-lymphogranuloma antibodies found in Eskimo sera are psittacosis, meningopneumonitis and ornithosis. The agents causing these diseases were obtained and propagated in embryonated eggs, with group and specific antigens being prepared in the manner described (Chapter III). Barwell (1), Sigel and Pollikoff (65) produced a successful specific antigen by treating the psittacosis-lymphogranuloma venereum agents with 0.005 M potassium periodate. This chemical destroyed the group antigen, but did not affect the specific antigen

known to exist as a result of the absorption experiments carried out by Bedson (5). The activity of such antigens lasted for only a few months when stored at -20°C.; it was found that periodate reacted unfavorably with most of the yolk sac-produced live antigens.

The only small aliquot of yolk sac-produced ornithosis specific antigen which did not show anticomplementary activity was prepared by treating the infected embryonic tissue with hypotonic solution for 24 hours, followed by centrifugation of the supernatant at low and high speeds (Figure 10). The suspension of elementary bodies after treatment with fluorocarbon, glass beads or trypsin seemed to react unfavorably with potassium periodate. As a result, all antigens which were prepared in this manner were anticomplementary. Further work would be required to discover a satisfactory method of preparing specific antigens using yolk sac propagated elementary bodies.

When the group and specific antigens, prepared from allantoic fluid, were applied to Eskimo sera (Table IX) negative results were achieved with the specific antigens of meningopneumonitis and psittacosis. The ornithosis agents were mainly yolk sac produced and satisfactory specific antigens were not obtained. Concentrated suspensions of live elementary bodies of psittacosis, meningopneumonitis and ornithosis were prepared, and three rabbits were injected intravenously, one with each agent. Serum collected from

these rabbits should contain both group antibodies and antibodies specific to the homologous agent. Tests using the three antisera and tissue antigens prepared from embryonated eggs revealed considerable reaction in the sera to these antigens (Tables IV and V). Such activity was removed by absorption, as shown in Table VI. Live group and specific antigens, both homologous and heterologous, were used to test the rabbit-produced antisera. As shown in Table VII, these specific antigens reacted with their homologous antiserum to significant titres, and only very weakly, if at all, with the heterologous antiserum. At no time did the specific antigen of meningopneumonitis or psittacosis react with the ornithosis antiserum.

To test further the specificity of the periodate-treated fraction, we acquired from the Manitoba Provincial Laboratory, Virology Section, sera diagnosed by clinical, epidemiological and serological methods as being psittacosis-positive. Three of the patients so involved were known to have had contact with psittacine birds, and their sera reacted with the specific antigen for psittacosis but not for meningopneumonitis (Table VIII). The other sera were negative to these specific antigens. It must be assumed that the cause of their antibodies is another agent within the psittacosis group.

Since Hildes et al (28) had discovered that the snow goose exhibited a significant incidence of psittacosis-lymphogranuloma venereum group antibodies, it would be reasonable to expect that antibodies found in Eskimo sera might be caused by ornithosis, which is the agent incriminated as causing disease of non-psittacine birds. To establish the presence of ornithosis specific antibodies, Eskimo sera were absorbed with both commercial and live group antigens. Bedson (5) had been successful in identifying specific antibodies by absorbing the serum with a heat-killed suspension of psittacosis agents and testing the absorbed serum with live antigens. The serum still retained the specific antibodies, which in turn reacted with the specific antigenic component of the live agent. Since it had already been established that meningopneumonitis was not the cause of these group antibodies as found in Eskimo sera, live agents of this species were used for absorption. The results of the two different types of absorption showed that all antibodies to ornithosis, meningopneumonitis and psittacosis were removed from the Eskimo sera. The evidence provided by these absorption experiments indicates that the agents of psittacosis, meningopneumonitis and ornithosis are not responsible for the psittacosis-lymphogranuloma venereum group antibodies under study.

Further work must be carried out in order to discover the source and significance of the psittacosis-lymphogranuloma venereum antibodies:

1) Examination of animals and humans should be carried out to look for the reservoir of the antigen -- caribou, seal, walrus, wolf, fox, dog, fresh water fish as well as humans.

2) The agent isolated from fur seals by Eddie and his associates (18) should be acquired, and employed to examine sera already absorbed with psittacosis group antigen.

3) Reference antisera to known psittacosis-lymphogranuloma venereum agents should be used for the evaluation of any specific antigens produced for study.

4) Some agents of the psittacosis-lymphogranuloma venereum group are known to cause encephalitis in animals (10). Since the westcoast Hudson Bay Eskimos have been involved in an outbreak of meningitis, perhaps more significance should be attached to the antibodies under study than has been hitherto realized.

5) Characterization of this antibody as to whether it is 19s or 7s should shed light on the nature of the antigen.

6) The technique of gel diffusion might prove a valuable aid in identifying the type specific antigen-antibody reactions.

7) Difficulties were encountered in the propagation of the three agents we used. The embryonated eggs came from a flock of birds known to be infected with leukosis virus.

An evaluation of the effect of this virus on the growth of psittacosis agents in eggs could be carried out in tissue culture using chick fibroblasts from eggs of an infected flock, and leukosis-free diploid strains.

8) Tests for C-reactive protein should be run to establish possible evidence of infection in Eskimos having the higher titres of psittacosis-lymphogranuloma venereum group antibodies.

9) The difficulty in establishing the presence of psittacosis group agents in animal tissue might be overcome by the use of impression smears made from tissues obtained from animal sources and stained using the fluorescent antibody technique. In this way, a large number of tissues could be rapidly screened.

10) Only brief mention has been made regarding the live agents and the length of time they have been propagated in artificial media. These agents were all isolated at least 25 years ago, and propagation in embryonated eggs and tissue culture may have mutated the specific antigenic component. Thus, antibodies formed in the past four years against naturally occurring agents might not be detectable with the specific antigens used in this work.

CHAPTER VI

CONCLUSIONS AND SUMMARY

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CONCLUSIONS

A high incidence of antibodies to the psittacosis-lymphogranuloma venereum group antigen was found to exist in sera collected from residents of Eskimo Point, N.W.T. It has been possible to demonstrate that these antibodies are not due to cross-reactions with the following specific agents: Brucella abortus, Pasteurella, Mycoplasma pneumoniae, Proteus OX-19, Q fever, Neisseria meningiditis, Proteus vulgaris, mirabilis, and rettgeri, Hemophilus influenzae, Mycobacterium tuberculosis, Pseudomonas originosa, egg protein, and Forrsman heterogenetic antigen.

Specific agents of psittacosis, meningopneumonitis and ornithosis, which are members of the psittacosis-lymphogranuloma venereum group, are not responsible for the antibodies found in Eskimo sera. However, the latter were probably caused by infection with an organism belonging to the psittacosis-lymphogranuloma venereum group, since it was possible to remove all of the antibodies by absorption with group antigen.

Lack of clinical evidence or sequelae of infection would make it unlikely that these antibodies are caused by the agents of lymphogranuloma venereum, inclusion conjunctivitis or trachoma.

Therefore, antibodies found in Eskimo sera are most likely caused by an unidentified agent belonging to the psittacosis-lymphogranuloma venereum group.

SUMMARY

1) Eskimo sera from Eskimo Point, N.W.T., collected in 1967 showed a high incidence of psittacosis-lymphogranuloma venereum group antibodies.

2) Antigens not specific to the psittacosis-lymphogranuloma group could not be implicated as a source of the antibodies to the psittacosis group as found in Eskimo sera.

3) The type specific antigens of psittacosis, meningopneumonitis and ornithosis did not react specifically with the antibodies in Eskimo sera.

4) The diseases of lymphogranuloma venereum, trachoma and inclusion conjunctivitis have not been clinically identified among Eskimos, so are unlikely to be responsible for the antibodies in Eskimo sera.

5) Birds such as geese and gulls, suspected of infecting Eskimos with ornithosis, do not appear to be the reservoir of the antigen causing the antibodies in Eskimo sera.

6) The psittacosis-lymphogranuloma venereum group antibodies in Eskimo sera are most likely caused by a specific agent within the psittacosis-lymphogranuloma venereum group, as yet, not identified.

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APPENDICES

APPENDIX A

MATERIALS

Sera

Eskimo sera. Serum samples were taken from 95 Eskimos from Eskimo Point, N.W.T., and five samples from non-Eskimo residents. (List of E-numbers follows in this section.)

Eskimo sera taken in 1963 and stored in the Virus Section of the Provincial Laboratory of Manitoba, were made available through the courtesy of W. Stackiw. (List of E-numbers follows in this section.)

Positive sera. Positive psittacosis serum was purchased from Markham Laboratories, Chicago, Illinois, U.S.A.

Serum positive and negative to the psittacosis group antigen was provided by W. Stackiw of the Provincial Virus Laboratory.

Complement. Complement was purchased from the Connaught Laboratory, Toronto, Ontario.

Antigens

Psittacosis. Psittacosis antigen was purchased from Markham Laboratories, Chicago, Illinois, and acquired through the courtesy of Dr. Polley, Laboratory of Hygiene, Ottawa, Ontario.

Tissue antigens. Tissue antigens were purchased from Markham Laboratories, Chicago, Illinois, or prepared from chick embryos during the course of the work.

Pasteurella and Weil-Felix. Pasteurella and Weil-Felix antigens were purchased from Lederle Laboratories, Pearl River, New York, U.S.A.

Q fever and Lymphogranuloma venereum. Q fever and lymphogranuloma venereum were purchased from Markham Laboratories, Chicago, Illinois.

Streptococcus MG. Streptococcus MG was acquired from Difco Laboratories, Detroit, Michigan, U.S.A.

Mycoplasma pneumoniae. Mycoplasma pneumoniae was purchased from Baltimore Biologicals, Baltimore, Maryland, U.S.A.

Bacteria Used For Absorption

Bacteria were grown on suitable semi-solid media. After 24 hours incubation at 37^{o}C. the colonies were washed from the surface using 2 ml of V.B.S. per slope. The suspension of bacteria was centrifuged, washed in V.B.S. and recentrifuged. The pellet so achieved was resuspended in the serum to be absorbed.

Psittacosis-Lymphogranuloma Venereum Group Agents

Psittacosis. The agent of psittacosis 6BC was

obtained from Dr. Labzoffsky of the Ontario Public Health Laboratories, where it had been held for 25 years and passaged alternately in tissue culture and allantoic cavity of the chick embryo.

Meningopneumonitis. The meningopneumonitis Francis strain was obtained in a 10% yolk sac suspension from the American Type Culture Collection. It had originally come from the Squibb Institute of Medical Research.

Ornithosis. The Ornithosis P-4 strain also came from the American Type Culture Collection. This agent was at a passage level of M/11, CE/41 (yolk sac), and had originally been acquired from E.L. Buescher, Army Institute of Research, Walter Reed.

Appendix A

List of E numbers of Eskimos whose sera was used for tests 1967 and 1963.

1967 & 1963

E1-3	E1-175	E1-491
E1-6	E1-223	E1-492
E1-30	E1-231	E1-536
E1-54	E1-277	E1-558
E1-74	E1-296	E1-563
E1-75	E1-311	E1-567
E1-117	E1-316	E1-576
E1-136	E1-342	E1-594
E1-141	E1-403	E1-641
E1-142	E1-406	E1-715
E1-150	E1-490	E1-783

Appendix A

List of E numbers of Eskimos whose sera was used for tests 1967.

1967 only

E1-7	E1-507	E1-775	E3-663
E1-9	E1-519	E1-778	E3-883
E1-35	E1-524	E1-797	E3-911
E1-43	E1-537	E1-802	E3-1078
E1-131	E1-564	E1-803	E3-1081
E1-160	E1-574	E1-820	E3-1106
E1-176	E1-582	E1-826	E3-1143
E1-179	E1-595	E1-854	E3-1205
E1-233	E1-620	E1-855	E3-1254
E1-234	E1-624	E1-871	E3-1405
E1-261	E1-657	E1-876	E3-1614
E1-368	E1-659	E1-879	Non Eskimo
E1-425	E1-725	E1-889	Albert
E1-447	E1-730	E1-925	David
E1-464	E1-750	E3-172	Helen
E1-465	E1-763	E3-231	Tanya
E1-489	E1-764	E3-634	Francine

APPENDIX B

HANDLING OF SPECIMENS TAKEN FROM ESKIMOS

Throat swabs. Three throat swabs were taken from each individual: one was placed in Hank's basic salt solution and frozen, a second was placed in diphasic medium and refrigerated at 4°C., while the third was placed in Stewart's transport medium.

Naso-pharyngeal swabs. Naso-pharyngeal swabs were immediately incubated at 37°C. on Thayer-Martin slopes in candle jars, were subcultured as necessary and kept at a temperature as close as possible to 37°C. during transport.

Stool samples. Stool samples were divided into two portions, one being refrigerated at 4°C. and the other frozen.

Eskimo serum. Serum samples, following separation, were frozen.

During shipping, all specimens collected were kept in the state at which they had been stored.

APPENDIX C

BACTERIA ISOLATED FROM THROAT AND NASO-PHARYNGEAL SWABS

Throat and naso-pharyngeal swabs, for bacterial studies and throat swabs for virus and mycoplasma studies were taken from the group of Eskimos at the same time as the sera were collected. No viruses were isolated in either embryonated eggs or tissue culture (Monkey kidney, chick fibroblasts, human amnion). Six Mycoplasma sp. were isolated; these were not from persons showing a high titre of psittacosis-lymphogranuloma venereum group antibody.

Table XVIII lists the bacteria isolated from greater than 9% of this group of people. A coagulase positive Staphylococcus was carried by 16%, while 11% of the naso-pharyngeal swabs were positive for Neisseria meningiditis type "B". Neisseria sp. other than meningococcus was carried by 64% of the bacterial throat swabs. The most interesting finding with respect to throat swabs was that 69% of all such swabs carried gram negative bacilli (Escherichia coli, Aerobacter aerogenes, Alkaligenes sp., Proteus sp.).

Stool samples did not yield any Salmonella or Shigella sp. Virtually no evidence of parasitism was discovered.

TABLE XVIII

Bacterial Species Isolated From Throat and Naso-pharyngeal Swabs of Eskimos Exhibiting Antibody to the Psittacosis-Lymphogranuloma Venereum Group Antigen

Bacteria	Percentage Incidence
Staphylococcus pyogenes	16
Staphylococcus epidermidis	18
Neisseria meningiditis	11
Neisseria sicca and catarrhalis	64
Streptococcus viridans	71
Escherichia coli	18
Aerobacter aerogenes	22
Alkaligenes sp.	19
Proteus vulgaris, mirabilis, rettgeri	10
Hemophilus influenzae	9
Diphtheroids	34

Other species present in less than 3% of the swabs included Achromobacter, pneumococcus, Sarcina and yeast.

APPENDIX D

FORMULAE

Veronal Buffered Saline

83.8 gm sodium chloride

2.52 gm sodium bicarbonate

3.0 gm sodium 5,5-diethyl barbiturate

4.6 gm 3,5 diethyl barbituric acid

1.0 gm magnesium chloride ($MgCl_2 \cdot 6H_2O$)

0.2 gm calcium chloride ($CaCl_2 \cdot 2H_2O$)

The last three components were dissolved in 500 ml of hot distilled water. The first three components were dissolved in distilled water and the hot solution added. The mixture was cooled and distilled water added until a volume of 2000 ml had been reached. Each day this stock was diluted 1:5 with distilled water. The dilute buffer was used at a pH 7.3 to 7.4.

Macchiavello's Stain

0.25 gm basic fuchsin, 100 ml of distilled water

1 gm methylene blue, 100 ml distilled water

1 gm citric acid in 200 ml distilled water

The smear was fixed with heat and flooded with basic fuchsin for 5 min., washed in distilled water, dipped briefly into citric acid, and immediately washed thoroughly with tap water. The slide was now flooded with methylene blue

for 20 to 30 seconds, washed thoroughly and left to dry.

Castenadas' Stain

Weis' Mordant

100 ml formalin

7.5 ml glacial acetic acid

Castenadas stain and technique

0.1 gm Azure II in 10 ml methanol

90 ml phosphate buffer M/15

5 ml formalin

0.25% safranine for counterstain

The first three ingredients are mixed.

The smear was placed in Weis' mordant for 5 minutes, washed thoroughly, and flooded with Azure II for 20 minutes, then washed and flooded with safranine for 7-10 seconds.

M/50 Phosphate Buffer

sol A) 0.2 M Na H_2PO_4

sol B) 0.2 M Na_2H PO_4

1) For 0.1 M phosphate buffer

Solution A - 13 ml

Solution B - 87 ml

Distilled water - 100 ml

2) For 0.02 M or M/50 phosphate buffer

10 ml of solution 1

40 ml distilled water

Adjust pH to 7.6 with 0.1 Normal, NaOH or 0.1 Normal, HCl.