Preliminary characterization of a chlamydial agent isolated from embryonated snow goose eggs in northern Canada

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Chlamydia-like, streptomycin-resistant parasites have been isolated from a high percentage of embryonated snow goose eggs collected at various stages of incubation; the agents stained specifically by immunofluorescence with antiserum against psittacosis and multiplied intensively in the yolk sacs of chicken embryos and induced in mice the production of antibodies that reacted in complement-fixing (CF) tests with a psittacosis group antigen. The isolates showed a high infectivity, but a low pathogenicity for chick embryos and mice. High doses of the isolate produced no cytopathic effect (CPE) in cultured mouse peritoneal macrophages and L cells. Release of lysosomal acid phosphatase did not occur in the inoculated macrophages throughout the time of observation.

Introduction

In 1958 Hildes et al. (12) conducted a sero-logical survey on the residents of 11 villages on Baffin Island for antibodies to chlamydial agents; 15\% of persons tested had complement-fixing antibodies to the psittacosis–human pneumonia antigen. Since 1958, residents of villages in other parts of the Canadian North have been tested for antibodies to Chlamydiae and all have demonstrated a varying, but sometimes high prevalence of antibodies to this chlamydial antigen (13, 14, 37, 39). Chlamydial antibodies have also been detected in sera from snow and Canada geese (12, 37, 38), as well as from muskrats in the Canadian Arctic (32). Chlamydial agents were isolated from muskrats and snowshoe hares during a die-off of these species in Saskatchewan (32) and in a different study from northern fur seals on the Pribilof Islands (5). Investigations have been carried out to determine the medical significance of chlamydial antibodies in residents of the eastern Arctic, as well as the source or possible reservoir of the stimulating antigen. Sera collected in 1963, 1967, and 1970 from residents as well as snow geese and caribou at Eskimo Point (Northwest Territories) demonstrated the presence of antibodies to the chlamydial antigen (38, 39). Epidemiological observations indicate that the residents of this area have a close contact with both snow geese and caribou, and a project was launched to collect fresh material from these species and transport it to the Winnipeg laboratory for attempted isolation and identification of Chlamydiae. The present report deals with studies carried out on the agents isolated from embryonated snow goose eggs, collected at various stages of incubation. Pilot experiments are also included on isolation of Chlamydiae from organs of adult snow geese obtained from the Eskimo Point area.

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Materials and Methods

Snow Goose Eggs
A total of 118 eggs were received in the laboratory. Upon arrival, the eggs were candled and nonfertile eggs and dead embryos were discarded; the rest of the eggs were incubated at 37°C. Eleven embryos which died during incubation were examined and the yolk sacs and embryos free from bacterial and mycoplasmal contamination were preserved at -70°C. The rest of the embryos, irrespective of age, were opened after 14 days of incubation and their tissues were prepared for examination immediately.

Processing of Freshly Harvested Egg Materials
Yolk sacs and embryos of 25 eggs were processed separately. Yolk sac tissue explants were prepared using a modification of a previously described method (36); the embryo was minced and the tissue was placed over a layer of chicken plasma in Falcon flasks. Both yolk sac and embryo tissue explants were maintained in 25-ml Falcon flasks and in parallel, on flying glass cover slips in Leighton tubes for 14 days at 37°C in medium 199 containing 0.1% NaHCO₃, 200 µg streptomycin per milliliter, and 10% heat-inactivated calf serum; the medium was changed every 4 days (chicken plasma, medium 199 and calf serum were purchased from Grand Island Biological Company, Grand Island, New York). Tissues grown on cover slips were examined before and after cultivation in situ by Giemsa and immunofluorescence. Tissues grown in Falcon flasks were pooled, examined for bacterial and mycoplasmal contamination, and processed for identification of the isolate in chicken embryos and mice. The material was kept at -70°C until processed.

Processing of Material from Goose Embryos
As a rule, chlamydial isolates must undergo several blind passages through chicken embryos before the agent can be identified. To shorten the process, a modified in vitro chicken yolk sac tissue technique was used as described earlier (36). For this purpose, chicken yolk sac tissue explants grown in 25-ml Falcon flasks were inoculated with yolk sac and embryo tissue suspensions obtained from the eggs in which the embryos died during incubation. Positive results were usually first recognized by the presence of Chlamydiaceae-like bodies in these cultures at 10 to 14 days after inoculation. Tissues which showed rich yields of the agents were pooled together with growth medium, frozen and thawed 3 times, and centrifuged at low speed. The supernatants were filtered through a Millipore filter with a mean pore diameter of 0.22 µ and used for inoculation of chicken embryos and mice.

Methods Used in Analysis of Behavior of Goose Isolates
The different indicator host systems included three subpassages of the isolates in 3-week-old mice and three subpassages of the isolates in 6-day-old chicken embryos. The agent obtained from the third subpassage in chicken embryos was partially purified by differential centrifugation (11). The resultant pellet was resuspended in growth medium and assayed in mouse macrophages in vitro for infectivity, toxicity, and lysosomal host cell response by methods previously described (1, 18); in yolk sac tissue explants for infectivity; and in L cells for infectivity and cytopathic effect by techniques previously described (19).

Mice
Three-week-old pathogen-free mice of the CR strain (Canadian Research Animal Farms, Bradford, Ontario) were used. Females weighing 20-30 g were inoculated by the intraperitoneal route. In the experiments proper, mice were inoculated with filtered pools of the goose isolates pregrown in chick yolk sac tissue in vitro as described above, in an amount of 0.5 ml/mouse. After 7-14 days, the animals were bled and dissected, and their blood and organs examined. The goose isolates were subpassaged three times in mice.

Mouse Controls
Since it is known that mice may have a latent infection with Chlamydiaceae (9), all mice were bled before inoculation and the blood samples were pooled and examined in parallel with blood samples taken 7-14 days after inoculation. Additional controls included a group of noninfected mice which were killed, their organs removed, homogenized, and inoculated intraperitoneally into mice. Three subpassages of these inocula were performed in mice which were bled and dissected, and their blood and tissues examined the same as the mice inoculated with the goose material.

Chicken Embryos
Chicken embryos were received from flocks maintained on an antibiotic-free diet (Department of Agriculture, University of Manitoba). Six-day-old embryos were inoculated via the yolk sac with pooled, filtered goose isolates (see above), resuspended in growth medium containing 200 µg streptomycin/ml in an amount of 0.25 ml/egg. Yolk sacs were kept at 37°C, harvested 10 days after inoculation, stained, and examined as below. The isolates were subpassaged three times in chicken embryos.

Light and Immunofluorescent Microscopic Identification
Light and immunofluorescent microscopic identification of the goose isolates was performed by Giemsa and/or Gimenez (7) staining as well as by the indirect immunofluorescent method described previously (35).

Tests for Complement-fixing (CF) Chlamydial Antibodies
Tests for complement-fixing (CF) chlamydial antibodies in the sera of control and infected mice were kindly performed by Dr. H. Sayed of our department (16).

The Antigen
The antigen used in all serological tests was a commercial (Markham Laboratory, Chicago, Illinois) psittacosis–human pneumonitis group antigen.

Immune Serum
A commercial (Markham Laboratory, Chicago, Illinois) psittacosis antiserum prepared in humans was used. Preparations of goat antihuman globulin and goat antihuman gammaglobulin sera were purchased from Grand Island Biological Company, Grand Island, New York.

Response of in vitro Macrophages
The effect of the goose isolates on mouse macrophages in vitro was assayed using methods described in detail.
elsewhere (18, 20). Briefly, the agent was collected from chicken yolk sacs (third subpassage), partially purified by differential centrifugation, resuspended in growth medium containing 200 μg streptomycin sulfate per milliliter, and assayed for infectivity in freshly harvested, nonactivated mouse peritoneal mononuclear cells. After this, with a high concentration of the agent per cell (10 × TCID₉₀/mouse cell) the viability, cytomorphology, and lysosomal activation of macrophages were determined.

**L Cells**

L cells (clone 929) were purchased from the American Type Culture Collection and cultivated as previously described (19).

**Infectivity of Goose Isolates for L Cells**

Infectivity of the goose isolates for L cells was determined using the partially purified agent and previously described methods of inoculation (19, 21).

**Results**

The results of the isolation assays and characterization of the embryonated snow goose egg isolates are summarized in Table 1 and illustrated in Figs. 1–4. The light microscope and immunofluorescent examination of embryonated goose yolk sacs and embryo tissues before and after cultivation in vitro revealed many pleomorphic, intracellularly located Chlamydia-like bodies. These were found in more yolk sacs than in embryo tissues, both before and after cultivation in vitro. In explants of embryo tissues, a marked increase in the number of positive samples was observed 14 days after cultivation. Small bodies resembling chlamydial elementary bodies were usually predominant in yolk sac and embryo tissues after prolonged cultivation, whereas large forms of the agent were predominant in the earlier days of cultivation (compare Fig. 1 and Fig. 2).

The chicken embryos inoculated by the yolk sac route were highly susceptible to infection with the snow goose isolates. Eighteen of 24 inoculated chicken yolk sacs contained considerable numbers of Giemsa- or Giménez-stained intracellularly located pleomorphic Chlamydia-like bodies after the first subpassage of the isolates. Despite progressive multiplication of the agent in yolk sacs (TCID₉₀ was 5 × 10⁸/ml), only 4 of 24 embryos died after the third passage, as estimated by the single dilution technique (10⁻²/0.25 ml per egg), although optimum time was allowed between inoculation and harvest of the yolk sacs. No gross pathologic changes were observed by macroscopic examination of the infected chick embryos or yolk sacs. Inocula from dead goose embryos induced in chicken embryos a process of infection similar to that which occurred using inocula from living goose embryos.

The serological results on mice in which the snow goose egg isolates were passaged are summarized in Table 1. Complement-fixing antibodies against the chlamydial antigen were demonstrated in 12 of 19 pooled samples from inoculated mice as compared to only 3 of 36 pooled samples from control mice before inoculation. In addition, three subpassages of control, noninoculated mouse tissues into mice revealed no chlamydial antibodies in any mice.

**TABLE 1**

**Isolation and identification of Chlamydiae from snow goose material**

<table>
<thead>
<tr>
<th>Snow goose specimens</th>
<th>Methods of analysis*</th>
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<td>Staining of snow goose tissues before and after cultivation in vitro</td>
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<td>Positive by Giemsa</td>
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<td>Dead embryos</td>
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<td>Organs of 18 adult birds</td>
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*Controls: 3/36 serum pools of mice were positive for chlamydial antibodies before inoculation (at an average titer of 1:4). 0/3 serum pools of mice were positive after three subpassages of organs of control, non-infected mice.
Although a high percentage of mice inoculated with goose isolates developed chlamydial antibodies, no overt disease was established in the mice during three subpassages, nor were any gross pathological changes observed in their organs. Microscopic examination of impression smears of organs of inoculated mice revealed Giemsa-stained, cytoplasmic, Chlamydiae-like inclusions in the spleens of several animals.

The results of these assays can be summarized as follows: in a high percentage of embryonated snow goose eggs, Chlamydiae-like, streptomycin-resistant parasites were observed; the agents stained specifically by immunofluorescence with a commercial human antiserum against psittacosis; the isolates multiplied intensively in yolk sacs of chicken embryos and induced in mice the production of antibodies that reacted in CF tests with a commercial psittacosis group antigen. While the responsiveness of both chicken embryos and mice to the infectivity of the isolates was high, the infectious process was not accompanied by disease, nor were gross pathological changes observed in the organs of inoculated animals after repeated subpassages, i.e. the isolates showed a high infectivity, but a low pathogenicity for chick embryos and mice.

It seemed of interest to examine the behavior of the goose isolates at the cellular level, in indicator host systems which we have used to differentiate cytopathic from non-cytopathic variant strains of C. psittaci 6BC (18-21). For this purpose, the partially purified inoculum obtained after three subpassages of the goose egg isolates (from dead goose embryos) in the yolk sac of chicken embryos was used, and was assayed in cultured peritoneal mouse macrophages as well as in L cells. Doses as high as yolk sac 1 x TCID<sub>50</sub>/macrophage produced no agglutination of phagocytes and no cytopathic effect (CPE) was observed throughout the 7-day period of observation, although chlamydial pleomorphic bodies were detected by Giemsa staining in over 40% of the infected cells from the third day up to the seventh day after infection (Fig. 3, 3a). Release of lysosomal acid phosphatase did not occur in the inoculated macrophages throughout the time of observation.

Infectivity titrations of the goose isolates in L cells revealed chlamydial inclusion bodies only with higher doses of the inoculum (up to 10<sup>-3</sup>); even the highest doses of the isolate, however, produced no CPE (Fig. 4). Hence, the behavior of the isolates in cultured mammalian cells appeared to be similar to the effect of the egg-attenuated, noncytopathic avian C. psittaci 6BC strain assayed in the same host systems (19, 21).

In a pilot experiment, organs of 18 adult snow geese were examined. Birds (females and males) were shot, and portions of liver, spleen, lung, and ovaries were pooled, and processed for inoculation into young mice; the material was subpassaged three times. The results of these experiments are included in Table 1. Three of four pooled samples from the inoculated mice demonstrated CF antibodies against Chlamydiae after the third subpassage. Examination revealed no gross pathological changes in inoculated animals and no deaths of mice were recorded. These experiments indicate that adult birds may carry Chlamydiae without having gross pathological lesions in their organs. The agents induced production of chlamydial antibodies in mice, but failed to produce overt disease or gross pathological effects in the organs of the inoculated animals.

**Discussion**

The transmission of Chlamydiae from bird to egg has been demonstrated by several workers in a number of avian species (3, 4, 22, 24, 31, 33) whereas others have failed to demonstrate this mode of transmission (6, 30, 31, 33). To our knowledge, there are no reports of isolation of Chlamydiae from snow goose embryos, and we
do not know if the relatively high percentage of embryos harboring Chlamydiae reported in this study is representative of the many migratory birds in northern Canada, or whether our findings represent an isolated incidental phenomenon; in considering the present results, as well as the results of earlier serological surveys, (12, 13, 38) we think that congenital chlamydiosis in snow geese might not be uncommon. It is possible, however, that the methods applied in our studies have contributed to the rather high incidence of positive isolations; the method of prolonged in vitro cultivation of tissue explants from healthy carriers of a number of pathogens has proved successful before (17, 34).

In nature, latent chlamydiosis is the predominant type of pathogen–bird relationship; several tissues may be continuously infected without a conspicuous deleterious effect (25, 26, 33). Because of the anatomy of the bird’s reproductive system, microbes can be transferred from the parent to a fertilized egg by a variety of different ways (2). Chlamydiae have been isolated from oviducts and ovaries of affected psittacine birds (26, 24, 33). It has also been reported that after experimental chlamydiosis, egg production in turkeys dropped far below normal levels (33). It seems reasonable to assume that latent chlamydiosis in the reproductive tissues of the parent bird may lead to a situation in which the embryo is not killed, but sustains the latent, non-cytocidal agent. Latent chlamydia infections have been produced experimentally by inoculation of chicken embryos (24, 33). The factors and mechanisms which cause the parasites to remain in the bird in a latent, but potentially virulent state are unknown; nor is it known what causes a non-virulent agent to change into a virulent one.

From the goose eggs that were collected and incubated, several goose embryos survived almost to hatching time without obvious pathological changes, although they harbored Chlamydiae. On the other hand, a considerable number of embryos arrived in the laboratory dead; decomposition of the material prevented further examination. Embryos that died at various intervals after arrival in the laboratory, however, were carefully inspected. No pathologic changes characteristic of fatal chlamydiosis were observed. As described above, sub-passages of material from dead embryos did not behave differently in the indicator host systems from that of material derived from surviving infected embryos. It can be concluded that the chlamydial isolates from both the surviving and dead goose embryos were avirulent for chick embryos and young mice during three sub-passages. High doses of partially purified inocula produced no cytopathic changes in cultured mouse macrophages and in L cell fibroblasts. Several primarily avirulent, egg-grown avian chlamydial strains have been shown to acquire cytopathic properties after repeated passages in cultured mammalian cells. These virulent mammalian strains proved to be also virulent for intact animals (10, 28, 29); relative to this, studies are in progress to follow the future behavior of our goose egg isolates.

Serological surveys and epidemiological studies indicated that northern Canada may be regarded as an endemic area of chlamydiosis. It has been shown that a high percentage of residents have Chlamydiae-induced antibodies, but no manifest diseases (13, 14, 39). Pilot examinations have also shown that transients in the North acquire CF antibodies without associated symptoms (38). Serological studies have suggested that migratory birds may play an important role in sustaining the infection (23–25, 33). The findings reported here lend additional support to this view, as well as to the assumption that transovarial transmission of chlamydial agents in birds may be an important factor in sustaining the epizootiology of chlamydioses in some parts of the world.

Acknowledgments

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