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A *Chlamydia* from the Peritoneal Cavity of Mice

ROBERT K. GERLOFF AND REX O. WATSON

U.S. Department of Health, Education, and Welfare, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory, Hamilton, Montana 59840

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During continuous intraperitoneal passage of liver and spleen suspension in normal stock mice, a syndrome developed which involved ascites and certain other visceral changes but seldom clinical illness and never fatality. From these mice, a chlamydia was established in yolk sacs of chick embryos and in tissue cultures. This agent readily infects mice when inoculated intranasally but is without effect intracerebrally. It has very low pathogenicity for guinea pigs and is resistant to sodium sulfadiazine. These characteristics, together with results of serum neutralization tests, indicate that the agent is different from the Nigg and DeBurgh strains of mouse pneumonitis.

From 1937 to 1945, several isolations of pneumotropic microbial agents, apparently latent in stocks of normal white mice, were reported. Some (e.g., 7) are true viruses, and others belong to the genus *Chlamydia* (11). Many of the chlamydial isolates (e.g., 4, 5, 8, 10) are similar, and at least three of them probably represent the same agent, as suggested by Cox (1). However, the one reported by DeBurgh et al. (2) is different.

The present paper describes the isolation of another chlamydia, designated hereafter as strain 26, from normal mice. The isolation was accomplished not by serial passage of lung but by a long series of intraperitoneal (IP) passages of liver and spleen. These passages were performed in an attempt to uncover endemic infections possibily occurring in the colony of white mice maintained at this laboratory. This colony was started from stock obtained in 1938 from the Rockefeller Foundation, New York, N.Y. It has been propagated as a random-bred strain since that time.

MATERIALS AND METHODS

Cultivation in mice and embryonated eggs. White mice, age 21 days, were routinely inoculated with 0.5 ml of suspension IP, 0.03 ml intracerebrally (IC), and 0.03 to 0.05 ml intranasally. Yolk sacs of chick embryos, age 6 days, were given 0.5 ml. Tissues were homogenized in phosphate-buffered saline, *p*H 7.4, except for the use of physiological saline when the homogenate was to be inoculated IC. Constant watch for bacterial contamination was maintained by examination of Gram-stained tissue smears and by inoculation of thioglycolate broth with tissue suspension. Tissue smears were examined for chlamydial elementary bodies after staining by either the Macchiavello or Gimenez (3) method. Strain 26 was preserved by storage of infected mouse organs or yolk sac at −65 C, either whole or as 50% suspension in sterile skim milk, *p*H 7.4.

Primary isolation of the agent. The agent was recovered by serial IP passages of mouse liver and spleen. However, for closer simulation of the conditions of experiments for which these passages of normal mouse tissue were to serve as control, the initial four mice were inoculated IP with 10% (w/v) suspension of pooled yolk sacs from two normal 13-day-old chick embryos. On the 9th day, the spleens and a similar mass of liver from two of these animals were pooled and passed in 10% concentration to four other mice IP. Ten days later, liver and spleen from two of the last mice were passed to four mice IP and four mice IC.

The method of the last passage was thereafter used for many serial transfers of liver and spleen through groups of mice at 7-day, occasionally 6- or 8-day, intervals. Mice not sacrificed for passage were observed 2 to 3 weeks longer and, at the time of discard, were periodically autopsied for gross pathological examination. When a chlamydia became evident, transfers of infectious material to mice intranasally and to embryonated eggs were included.

Cultivation in cell cultures. For several purposes, monolayers of mouse fibroblasts, Earle L line, on 12-mm glass cover slips were infected with strain 26 by a centrifugation method adapted from that of Weiss and Dressler (12). After incubation for 42 to 48 hr at 37 C, the monolayers were rinsed with Hanks balanced salt solution, fixed with methanol, and stained by the Gimenez method. Inclusions per unit of area in the monolayer could then be counted and morphology of the inclusions studied.

Complement-fixation (CF) tests. The two group-specific chlamydial antigens used were prepared from boiled, concentrated suspensions of elementary bodies of ornithosis agent (strain D383 of pigeon origin). Rocky Mountain spotted fever and Q fever antigens served as controls.

Neutralization tests. Neutralizing antisera were

prepared in roosters by the method of Hilleman (6). Two birds were immunized against each of these chlamydiae: strain 26; McKercher strain (9) of sheep pneumonitis; and the DeBurgh (2) and Nigg (10) strains of mouse pneumonitis.

In the neutralization tests, serial twofold dilutions of antiserum were mixed with suspension of chlamydia diluted to appropriate concentration in tissue culture medium. After incubation at 37 C for 1 hr, 1-ml quantities were transferred to flat-bottomed tubes, cover slips with cell monolayers were added, and the tubes were centrifuged as described. A chlamydial concentration was selected that would produce either an optimal number of inclusions for counting or a visible cytopathic effect. Reduction in number of inclusions was preferred as an indicator of neutralization of sheep pneumonitis and mouse pneumonitis (either strain), whereas prevention of cytopathogenicity was preferred for strain 26. When inclusions per unit of area were counted, the unit varied according to the concentration of inclusions in the monolayer; however, the same unit was used for all tests with the same agent. Cytopathic effects were graded from 0 to 4, zero meaning no visible effect and 4 meaning virtually complete destruction.

Sulfadiazine sensitivity tests. Cell monolayers on cover slips were infected in the manner previously described, except that the following quantities of sodium sulfadiazine per ml were included in the medium: 2.0, 0.2, 0.02, 0.002, and 0.00 mg. Stained monolayers were examined for cytopathic effects and inclusions.

Tests for glycogen. Inclusions produced by strain 26 in cell monolayers were tested for glycogen by staining with iodine (11). Similar cultures of lymphogranuloma venereum, strain JH, and meningopneumonitis agent, strain Cal 10, were used as positive and negative controls, respectively.

RESULTS

Primary isolation; establishment in mice and embryonated eggs. During the entire study, 64 serial mouse liver and spleen passages were performed. Abnormality was first seen in animals of the 29th passage in which one of the two IP-inoculated mice had ascites, a condition observed again in a mouse of the 33rd passage. Thereafter, pathological changes increased in extent and variety until, at the 46th and subsequent passages, the following abnormalities were usually present: (i) two- to threefold enlargement of the spleen; (ii) enlargement of the liver with margins of lobes appearing rounded rather than blade-like; (iii) white, sero-fibrinous exudate on the liver and spleen; (iv) ascites with up to 2 ml of peritoneal fluid per 28-day-old mouse. Histopathological examinations were not done.

Clinical signs of illness were seldom noted, even in mice showing the greatest pathological change at autopsy. Spontaneous deaths never occurred in the IP-inoculated groups; two in the IC groups were of doubtful significance. Basophilic bodies resembling chlamydial inclusions and elementary bodies were first seen in smears of peritoneal fluid from mice of the 50th passage.

Limited attempts to establish an agent in embryonated eggs by inoculation with mouse liver and spleen suspension or peritoneal fluid from passages 19, 52, and 54 were unsuccessful. However, in the 59th mouse liver and spleen passage, peritoneal fluid from a mouse showing all the forenamed pathological characteristics was inoculated intranasally into six mice. Two were sacrificed on the 7th day and four on the 14th day. Lungs of one mouse in the second group were moderately consolidated, but the others appeared normal. Subsequent intranasal passage of the diseased lungs to other mice resulted in rapid establishment of an infectious agent, with mortality rate of about 50% and consolidation of lungs in all animals inoculated. Furthermore, with infected mouse lung as inoculum, the agent was readily established in the yolk sacs of embryonated eggs. Typical chlamydial inclusions and elementary bodies were found in smears of both mouse lung and yolk sac. Infection of mice by the intranasal route was accomplished in a similar manner with peritoneal fluid from mice of the 63rd liver and spleen passage.

Comparison of mouse susceptibility by various routes. Groups of six mice were inoculated IP, IC, and intranasally with the following dosages, respectively, of infected yolk sac, expressed as chick embryo LD_{50} : 10^7, $10^{5.8}$, and $10^{5.8}$. During the next 14 days, three mice in the intranasal group died and elementary bodies were seen in lung smears, but mice in the other two groups maintained normal appearance.

In marked contrast, mouse pneumonitis DeBurgh agent was highly lethal for mice after IC inoculation; the LD_{50} constituted less than 10 chick embryo LD_{50} . Mice were more resistant to IP and intranasal inoculation, since 10^7 and $10^{5.8}$ chick embryo LD_{50} by these routes killed only four of six and three of six mice, respectively.

Inoculation of guinea pigs and demonstration of chlamydial CF antibody. Two guinea pigs were immunized by a series of three fairly massive doses of infected yolk sac and mouse lung suspension given IP. Only very slight febrile response (temperature 40 C or higher) was detected. Sera collected from the two animals at 7 days after the final injection had chlamydial CF antibody titers of 1:32 to 1:64 with both antigens; tests with the control antigens were negative.

Growth in cell cultures. Growth characteristics of the three aforementioned murine strains of chlamydiae in cell monolayers were very distinc-

tive. All three agents, inoculated onto monolayers in sufficiently high concentration, produced cytotoxic effects visible in 16 hr. A dilution just beyond the range of cytotoxicity could be selected in which mouse pneumonitis DeBurgh would grow very luxuriantly and produce large numbers of so-called diffuse inclusions (F. B. Gordon and A. L. Quan, Bacteriol. Proc., p. 148, 1962). At a comparable dilution past the cytotoxic range, mouse pneumonitis Nigg produced fewer inclusions and they were of the rigid type (Gordon and Quan, Bacteriol. Proc., p. 148, 1962). Strain 26 was extremely cytotoxic and inclusions were sparse in any dilution past the toxic range. In this respect, it resembled sheep pneumonitis much more than it did the two murine agents. A typical inclusion produced by strain 26 is shown in Fig. 1. Inclusions of this organism would probably be classified as diffuse, although they did not exhibit the plasticity and highly variable shapes observed consistently, for example, with mouse pneumonitis DeBurgh and with meningopneumonitis agent.

Neutralization tests. Sera from the two roosters immunized with each agent gave nearly identical results; those from one serum of each pair, in the highest dilution giving essentially complete neutralization of the homologous agent, were selected for presentation here.

Neutralization of each agent by its homologous antiserum was greater than that by heterologous antisera (Table 1), indicating that the four agents are distinct. Inferences that slight antigenic similarities are indicated by the relative numbers of inclusions should not be drawn from these data. However, in titrations of the antisera, slight antigenic similarity between sheep pneumonitis and strain 26 was suggested by reactions obtained with the lowest serum dilution (1:4).

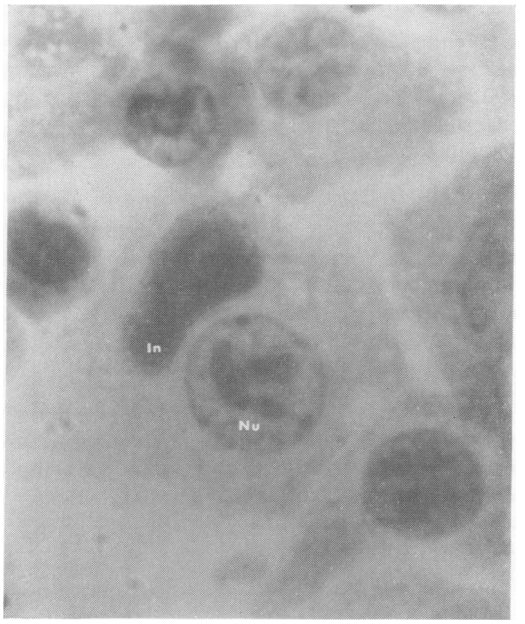

FIG. 1. *Typical inclusion produced by strain 26. In, inclusion; Nu, nucleus of host cell.* ×2,500.

TABLE 1. *Neutralization of strain 26 and three other chlamydiae by homologous and heterologous antisera*

Antiserum	Serum dilution (reciprocal)	Results of tests with the following chlamydiae			
		MoPn (DeBurgh)[a]	MoPn (Nigg)[a]	ShPn[a]	Strain 26[b]
MoPn (DeBurgh)...	8	3	290	180	4
MoPn (Nigg)...	16	167	2	140	3.5
ShPn.....	32	188	200	4	3
Strain 26.....	32	214	147	181	0
Control (without serum)...		195	246	172	4

[a] Figures denote number of inclusions per unit area in cell monolayers. MoPn, mouse pneumonitis; ShPn, sheep pneumonitis.

[b] Figures denote degree of cell destruction, range 0 to 4.

TABLE 2. *Effect of sodium sulfadiazine on growth of three chlamydiae in cell monolayers*

Drug concn (mg/ml)	No. of inclusions per unit area		
	Strain 26	Mouse pneumonitis (Nigg)	Mouse pneumonitis (DeBurgh)
2	24	0	277
0.2	200	0	431
0.02	264	0	473
0.002	248	0	504
0.000	208	650	326

Sensitivity to sulfadiazine. The three murine strains of chlamydiae were tested in cell monolayers for sensitivity to sodium sulfadiazine. Strain 26 and mouse pneumonitis DeBurgh were resistant to a concentration of 0.2 mg/ml in tissue culture medium (Table 2), whereas mouse pneumonitis Nigg was highly susceptible. Inhibition of strain 26 by 2 mg/ml was almost complete and that of DeBurgh was partial. The distinctly lower inclusion count with the latter organism in the absence of drug was due in part to destruction and sloughing of some host cells.

Tests for glycogen. Evidence of glycogen in the inclusions of strain 26 was repeatedly absent in

tests in which lymphogranuloma venereum and meningopneumonitis gave clear-cut positive and negative readings, respectively. This organism is thus tentatively considered as being glycogen-negative. Final conclusion in this regard should await availability of a host cell with which greater numbers of inclusions per unit area can be obtained.

DISCUSSION

A critical view of the source of strain 26 must logically consider places of origin other than the mouse colony itself; for example, the yolk sacs injected into the initial group of mice, contamination with other chlamydiae in the laboratory when strain 26 was established, and even origin from humans working in the laboratory. The agent presumably did not arise from yolk sacs, since eggs from the flock of hens that produced the two eggs in question were used in many studies, both chlamydial and rickettsial, before and after this one without evidence of microbial contamination. Furthermore, the fact that 29 liver and spleen passages in mice were necessary to elicit any sign of infection would preclude origin from the eggs. Chlamydiae to be considered as possible cross-contaminants would include two strains of ornithosis and one each of sheep pneumonitis and bovine encephalomyelitis. All four, however, have been differentiated from strain 26 by serum neutralization tests or pathogenicity tests in mice.

The rapidity and severity of mouse infection after intranasal inoculation and failure of limited attempts to establish the agent in eggs directly from mouse liver and spleen would suggest that the agent originated from mouse lung rather than from liver and spleen. However, pathological changes suggestive of an infectious agent were noted in the abdominal viscera of mice before intranasal inoculations were started. Also, the case for origin from the lungs is counteracted by unsuccessful attempts to recover a chlamydia from lungs of mice from the same colony in a study begun 4 months after conclusion of the present one. Twenty-four serial intranasal passages of lung in 10 to 20% suspension failed to produce clinical signs of illness or pathological change, and failed to yield recovery of an infectious agent.

The assumption thus remains that strain 26 agent arose from liver and spleen of normal mice but also possessed very strong pneumotropism. Apparently it was a nearly perfect parasite while in the peritoneal cavity, being able to reside there indefinitely without severe injury to its host. On the contrary, when introduced into the lungs of mice, it became a vicious invader producing disease which usually culminated in death of the host. Even massive doses of agent, produced in the yolk sac where its growth was most luxuriant, failed to affect mice when introduced IP.

Differentiation of strain 26 from chlamydiae previously reported from normal mice was sought in detail, the Nigg and DeBurgh strains being used as prime examples of such other agents. Data obtained show that the three organisms are different. Strains 26 and Nigg can be most readily differentiated on the basis of sulfadiazine sensitivity; strains 26 and DeBurgh on the basis of their different effect on mice by the IC route. Because of its resistance to sodium sulfadiazine and the probable absence of glycogen in its inclusions, strain 26 would presumably be considered a strain of *Chlamydia psittaci* (11).

Whether this agent originated from the mouse colony or, as a remote possibility, from the embryonated eggs is perhaps less important than the fact that another chlamydia has been found latent in a microbial host widely used in biomedical research. Also, the ubiquitous nature of chlamydiae is again emphasized.

The present paper is based on a single isolation. Although the hens which produced the eggs in question are no longer available for study, the desirability of attempting reisolation of strain 26 from the mouse colony at hand and of testing its pathogenicity for mice from other colonies is recognized. On the basis of data presented here, future isolation attempts should include serial IP and intranasal passages of mouse liver and spleen rather than IP and IC passage.

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LITERATURE CITED

1. Cox, H. R. 1947. Psittacosis, ornithosis, and related viruses. Ann. N.Y. Acad. Sci. 48:393–414.
2. DeBurgh, P., A. V. Jackson, and S. E. Williams. 1945. Spontaneous infection of laboratory mice with a psittacosis-like organism. Aust. J. Exp. Biol. Med. Sci. 23:107–110.
3. Gimenez, D. F. 1964. Staining rickettsiae in yolk sac cultures. Stain Technol. 39:135–140.
4. Gönnert, R. 1941. Über ein neues, dem Erreger des Lymphogranuloma inguinale ähnliches Mäusevirus. Klin. Wochenschr. 20:76–78.
5. Gordon, F. B., G. Freeman, and J. M. Clampit. 1938. A pneumonia-producing filterable agent from stock mice. Proc. Soc. Exp. Biol. Med. 39:450–453.
6. Hilleman, M. R. 1945. Immunological studies on the psittacosis-lymphogranuloma group of viral agents. J. Infec. Dis. 76:96–114.
7. Horsfall, F. L., and R. G. Hahn. 1940. A latent virus in nor-

mal mice capable of producing pneumonia in its natural host. J. Exp. Med. 71:391–408.
8. Karr, H. V. 1943. Study of a latent pneumotropic virus of mice. J. Infec. Dis. 72:108–116.
9. McKercher, D. G. 1952. A virus possibly related to the psittacosis-lymphogranuloma-pneumonitis group causing a pneumonia in sheep. Science 115:543–544.
10. Nigg, C., and M. D. Eaton. 1944. Isolation from normal mice of a pneumotropic virus which forms elementary bodies. J. Exp. Med. 79:497–509.
11. Page, L. A. 1968. Proposal for the recognition of two species in the genus *Chlamydia* Jones, Rake, and Stearns, 1945. Int. J. Syst. Bacteriol. 18:51–66.
12. Weiss, E., and H. R. Dressler. 1960. Centrifugation of rickettsiae and viruses onto cells and its effect on infection. Proc. Soc. Exp. Biol. Med. 103:691–695.