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Plaque Reduction Technique for Demonstrating Neutralizing Antibodies for *Chlamydia*

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Some members of the genus *Chlamydia* (*Bedsonia* or psittacosis-lymphogranuloma venereum-trachoma-inclusion conjunctivitis group of microorganisms) produce plaques in monolayers of mouse fibroblast (L-929) cells. Hyperimmune chicken antisera may be capable of specifically reducing plaque counts. When the test was applied to chlamydiae isolated from avian species, the results indicated that different isolates with a common source had similar antigenic reactivity. The plaque reduction test is a potentially useful method for serotyping chlamydiae. The difficult aspect of the method appears to be a readily reproducible means of producing neutralizing antiserum.

The genus *Chlamydia* (*Bedsonia* or psittacosis-lymphogranuloma venereum-trachoma group) includes a large group of obligate intracellular parasites that infect man, birds, and mammals. These agents cause pneumonitis, psittacosis, enteritis, abortion, trachoma, lymphogranuloma venereum, inclusion conjunctivitis, encephalomyelitis, and arthritis. Despite the extensive host range of the chlamydiae and the variety of disease syndromes they produce, there is no convenient method for typing isolates. A recent proposal (13) would divide this large genus into only two species, differentiated by inclusion type and sulfonamide sensitivity, criteria that would place many isolates of diverse origins and different biological properties in the same species. There is a need for a test that can be used to determine the antigenic relationships between the various agents. Conceivably such a test may make possible more precise identification and useful grouping of the chlamydiae.

Subgroups can be separated through the study of pathogenicity patterns in experimental animals (9, 12, 17), in vivo toxin neutralization or prevention (4, 8, 16), cross-immunity tests (1, 3, 18), and infectivity neutralization tests in mice and eggs (6, 11). The complement fixation (CF) test in general diagnostic use measures a common group antigen. Purified specific antigens have not yet been extracted. Chlamydial cell walls, when used as antigens in the CF test, are strain-specific (5, 7). Fraser and Berman (5) used cell wall antigen in this way and were able to divide 29 different mammalian isolates into 7 serological subgroups. They could not distinguish between the avian isolates. The complexity of these procedures probably accounts for the small number of chlamydial isolates that have been subjected to analysis.

Some workers have used plaque reduction in efforts to demonstrate antigenic specificity. Chapell and Manire (Bacteriol. Proc., p. 118, 1965) showed the specificity of rabbit antiserum prepared against the meningopneumonitis agent. A reduction in plaque count was obtained when this antiserum was mixed with the homologous agent before inoculation onto cells. No reduction was obtained when 6BC (a California parakeet isolate) and a mouse pneumonitis strain were tested with this serum. Piraino obtained effective plaque reduction with pigeon antiserum and found that CF and neutralizing antibodies were distinct (14).

This paper reports the results obtained by using antichlamydial rooster serum in a plaque reduction test. The rooster was the serum source because this and other laboratories had confirmed the early report of Hilleman and Gordon (6) that the chicken was capable of producing neutralizing antisera. Our results indicate that the plaque reduction technique may be suitable for antigenic grouping of chlamydiae.

MATERIALS AND METHODS

Preparation of antisera. Adult White Rock roosters obtained from Iowa or California-grown white Leghorn roosters from farms known to be free from ornithosis were used for antisera production. Although the roosters were under observation, their blood sera were tested by the indirect complement fixation (ICF) (10), CF (10), and plaque reduction tests. Only well-conditioned roosters with negative

serum tests were used to prepare antisera, and four to six birds were immunized with each isolate.

Yolk sac (YS) suspensions of chlamydiae were used as immunizing antigens. A 20% suspension of YS material (microscopically rich in elementary bodies) was prepared with nutrient broth in tissue grinders. The suspensions were held overnight at 4 C to allow separation of the fat and sedimentation of the coarse tissue material. The supernatant was used as the immunizing antigen. Purification by fractional centrifugation and other manipulations were avoided to minimize loss of antigen. Bacterial sterility was established by inoculation of thioglycolate broth and blood-agar media.

When noninfective suspensions were required for rooster immunization, Formalin was added to a final concentration of 0.1%. These suspensions were incubated at 37 C for 24 to 48 hr and tested for absence of infective particles by mouse inoculation.

To determine an effective regimen for producing neutralizing antibodies, several immunization schedules were tested.

Schedule A. On day 1, 1 ml of formalinized agent was given intramuscularly (im); on days 31, 38, 45, 1 ml of live agent was given im; on day 66, 1 ml of live agent was given intravenously (iv); on day 87, the birds were exsanguinated.

Schedule B. On day 1, 1 ml of formalinized agent was given im; on days 8, 10, 12, 15, 17, and 19, 1 ml of live agent was given im; on day 21, a trial bleeding was performed, and, if a good neutralization titer was obtained, the birds were exsanguinated on day 35. If the antibody response was inadequate, live antigen was given im on days 35, 37, and 39 and a trial bleeding was performed on day 70; if positive neutralization occurred, the birds were exsanguinated on day 85, and, if the response was still inadequate, the pattern of three im inoculations followed by a 31-day rest period was repeated twice before the attempt was terminated.

Schedule C. Schedule C was like Schedule B, except that live antigen was used for the initial inoculation.

Schedule D. On day 1, 1 ml of live antigen was given im; on days 15, 17, 19, 22, 24, 26, 29, 31, 33, 36, 38, and 40, alternate im and intraperitoneal (ip) inoculations of live antigen were given; on day 54 a trial bleeding was performed, and, if serum gave homologous neutralization, the roosters were bled on day 68; if no neutralization occurred, another three ip inoculations were given on days 68, 70, and 72; trial bleedings were made on day 86, and the roosters were either bled out or another series of inoculations was initiated on day 100.

Schedule E. Schedule E was used for chlamydial isolates highly virulent for chickens; on day 1, 1 ml of 10^{-5} live YS suspension was given im; when roosters were clinically ill (usually days 5 to 7), 100 mg of tetracycline was given im followed by tetracycline-impregnated feed; on day 28, the birds were exsanguinated.

Sera collected from prebleedings were also used as control sera. Because the tests required a large volume of normal serum, additional normal rooster serum was obtained from the Grand Island Biological Co. (Grand Island, N.Y.). The results of the ICF and CF tests were negative, and this serum did not inhibit plaque formation.

Serum dilutions were made in phosphate-buffered saline (PBS), *p*H 7.2, enriched with 10% fetal calf serum (enriched PBS) and inactivated at 56 C for 30 min.

Chlamydial isolates. The agents used in this study included strains isolated from turkeys [T-515 (Texas), NJ (New Jersey), and no. 14 (Oregon)], from parrots [AC (Australian), M (Mexico), and B (Brazil)], from parakeets [51C (California)], and from pigeons [SDP (California) and AU46 (California)]. Several chlamydiae of human origin were also used: lymphogranuloma venereum (434-B, 440-LN); fast-growing trachoma-inclusion conjunctivitis (TRIC) strains (T'ang and LB-1).

Plaque assay. The infectivity titrations were performed by plaque assay in mouse fibroblast (L-929) cells (2). Yolk-sac-grown chlamydial pools were prepared and samples were stored at −70 C until use. All agent dilutions were prepared in enriched PBS. Inocula were standardized to yield from 100 to 250 plaque-forming units (PFU) per petri dish when mixed with an equal amount of normal rooster serum. A 1-ml amount of diluted agent was mixed with an equal amount of diluted antiserum and held overnight at 4 C. A parallel combination was made with normal control serum.

Cell sheets were washed twice with PBS. An inoculum of 0.2 ml of the combined agent and serum was allowed to adsorb at 37 C for 2 hr in a CO_2 incubator. Plates were agitated every 30 min to spread the inoculum and keep the cell sheets from drying. Monolayers were then washed twice with PBS, and 5 ml of overlay medium was added. Overlay used in these tests did not contain streptomycin. The plates were incubated at 37 C in a 5 to 10% concentration of CO_2 in air at 70 to 85% relative humidity. LGV and TRIC agents were also incubated at 37 C. On day 7, the plates were fed another 5 ml of overlay medium; on day 13, a third overlay containing 1:10,000 neutral red was added. Plaques were counted on day 14. Serum titers are expressed as that dilution reducing the plaque count to 50% of the control counts. The Reed and Muench formula was used to calculate the 50% end-point titers (15).

Factors affecting the antigen-antibody combination. The optimal time and temperature for the antigen-antibody interaction were determined. A 6-ml amount of a 10^{-4} dilution of a chlamydial isolate from an Australian Eclectus parrot was prepared in PBS, mixed, and divided between two tubes. To one tube we added 3 ml of a 1:5 dilution of inactivated control rooster serum in enriched PBS, and to the other we added 3 ml of a 1:5 dilution of inactivated antiserum (Parrot 5139) in enriched PBS. These tubes were placed in an ice bath. (Three plates each were infected at 0 hr with inoculum from normal serum and antiserum tubes.) Each combination was then further divided between two tubes. One pair of tubes was held at 4 C and the other was held at 37 C (water bath). Samples were taken at 2, 18, and 26 hr. Standard

procedure was followed for infection, washing, overlay, and counts on the plates.

RESULTS

As Table 1 shows, 2 hr of incubation at 37 C produced a 76% decrease in plaque number in the control serum sample. The original mixtures produced averages of 180 and 179 plaques per petri dish. The plaque count from control serum tubes refrigerated at 4 C remained unchanged through the third sampling, at 26 hr. The best reduction was obtained with the antiserum-agent combinations refrigerated at 4 C overnight (18 and 26 hr). For all further tests, we used approximately 18 hr for convenience.

If cells were infected for 2 hr and washed with PBS and neutralizing antiserum was then added in the overlay medium, plaque reduction was still observed. However, the reduction was only one-half that observed when the same concentration of serum was added to the chlamydiae before infecting the cells, whereas five times as much antiserum is required for use in overlay.

Titrations of homologous serum. A large number of rooster antisera were screened for plaque reduction against the homologous (immunizing) isolate. For routine screening purposes, a 1:10 dilution of serum that reduced the plaque count by > 90% was considered positive. Such sera were then retested to determine their homologous titers. Table 2 presents the end-point dilution of 12 antisera: three against turkey isolates, three against parrot isolates, two against pigeon isolates, one against a parakeet isolate, and three against LGV isolates. No correlation was apparent between neutralizing and ICF antibodies. Repeated immunizations invariably produced high ICF titers. Most immunized roosters did not produce significant neutralizing antibody. In most experiments, only one of the three or four immunized roosters produced measurable neutralizing antibody. For some isolates, high-titered neutralizing antibody titers were not achieved. None of the immunization schedules was consistently superior to the others. Schedule A produced some high-titered sera, but many roosters died after the iv inoculation. Schedule E gave poor yields in terms of volumes of sera, as surviving birds were often debilitated.

TABLE 1. *Effect of time and temperature on neutralization and stability of a chlamydial agent*

Reaction mixture	Temp	Time of incubation			
		0 hr	2 hr	18 hr	26 hr
	C				
Agent + normal serum	4	100[a]	96	98	97
Agent + immune serum	4	100	21	10	4
Agent + normal serum	37	100	24	0	0
Agent + immune serum	37	100	4	0	0

[a] Plaque reduction is expressed as a percentage of the plaque count in the control tubes at 0 hr.

TABLE 2. *Antiserum plaque-reducing titer with homologous isolate*

Serum prepared against	Serum no.	Titer with homologous isolate[a]	Group ICF titer
Texas turkey (T-515)	5127	1:520	1:512
New Jersey turkey (NJ)	Pool B	1:400	1:256
Oregon turkey (No. 14)	59	1:456	1:32
Australian parrot (AC)	Pool	1:20	1:1,024
Mexican parrot (M)	47	1:92	0
Brazilian parrot (B)	5139	1:14	1:256
California parakeet (51C)	63	1:48	1:256
San Diego, Calif., pigeon (SDP)	5133	1:18	1:32
Au46, Calif., pigeon	5140	1:240	1:256
434-B (LGV)	WB410	1:143	1:32
440-LN (LGV)	LB-8	1:38	1:128
440-LN (LGV)	WB510	1:447	1:64

[a] Dilution of serum yielding 50% reduction in plaque count.

In preliminary experiments, the nine antisera prepared against avian chlamydiae were also tested for ability to reduce plaque counts of chlamydial isolates other than homologous strains by either screening or end-point titration against some representative chlamydiae within the Hooper Foundation collection. The isolates included 26 from turkeys, 10 from parrots, 6 from parakeets, and 10 from pigeons. The antisera often did not reduce the plaque counts of various heterologous isolates. The results indicate that chlamydiae from one avian genus may have a closer antigenic relationship to one another than to chlamydiae from other avian hosts (Table 3). Although some differences emerged, the turkey isolates all appeared to be antigenically related, even though the antisera were prepared against

TABLE 3. *Summary of cross-neutralization tests with chlamydiae of avian origin*

Strain isolated from	Sera prepared against isolates from			
	Turkey (3)[a]	Parrot (3)	Parakeet (1)	Pigeon (2)
Turkey.....	26/26[b]	0/6	0/6	1/6
Parrot.....	2/5	10/10	0/3	0/3
Parakeet....	3/6	1/4	4/6	0/4
Pigeon.....	2/2	1/2	0/1	6/10

[a] Numbers in parentheses indicate number of strains used to prepare antisera.

[b] Number of isolates with positive neutralization test/number tested.

strains isolated from flocks in New Jersey, Texas, and Oregon.

With some sera, an occasional heterologous titer was higher than the homologous titers. Some highly potent antisera appeared to have a very broad spectrum. For example, antiserum 59, against an Oregon turkey isolate, reduced the plaque counts of isolates from 24 turkeys, 1 parrot, 3 parakeets, and 2 pigeons (Table 3). The only negative result with this serum was against a parrot strain. Interestingly, serum against the other two turkey strains did not reduce the plaque count of the Oregon turkey isolate. The evidence for host specificity in Table 3 would be more impressive but for the results with this serum.

DISCUSSION

The plaque neutralization test is not generally used for routine survey or identification because it is time-consuming and exacting. It is widely used, however, in the study of basic properties of viruses and bacteria when accuracy and sensitivity are essential. This is particularly true when one is trying to determine antigenic relationships of agents, to detect antigenic variants, and to analyze agent populations. Since the dose of agent employed is small, 100 to 250 PFU, the method is sensitive for detecting neutralizing antibodies.

When strict attention was paid to certain technical details, the plaque reduction technique proved suitable for assay of neutralizing antibody against chlamydiae. The antigen-antibody mixtures should react at 4 C and not be incubated for 1 or 2 hr at 37 C because thermal inactivation of the agents complicates the interpretation of results. When PBS without serum was used as the diluent for agent and serum, we observed greater plaque count in the antiserum-agent sample with some heterologous isolates than in the control serum-agent sample. This did not happen consistently and complicated the interpretation of results. The use of enriched PBS corrected this problem.

Although only one test was made comparing the neutralization test in mammalian cells with that in eggs or mice, it showed the plaque reduction method to be the most sensitive. When undiluted antiserum was used, the average day of death was somewhat delayed in eggs but no neutralization of infectivity was observed in mice. The plaque reduction method gave a 50% endpoint titer of 1:520 for this antiserum.

Plaques produced by LGV and TRIC agents at 37 C were superior to those at 35 C, although it is generally accepted that these agents grow best at 35 C.

The results of this study, although preliminary, suggest that a systematic antigenic classification of chlamydiae is possible. The major problem to be overcome is the production of reagent-neutralizing antisera. The chicken is the only host that has regularly produced antibodies capable of neutralizing chlamydial infectivity. Many monkey, turkey, pigeon, rabbit, guinea pig, parrot, and human antisera were tested, but none was effective in the neutralization test although all had titers by the CF or ICF test. The techniques of preparing reliable specific antisera have not been developed. Only a few roosters in any series yielded useful sera. The broad spectrum of some highly potent neutralizing antisera is disturbing but may reflect over-immunization and not be a valid reflection of antigenic makeup. The possibility that some of these antisera may be selectively absorbed to increase specificity remains to be explored. Such studies may lead to the techniques for preparation of a battery of typing antisera.

The host range and increased number of chlamydial strains isolated pose a serious problem in classifying them systematically and practically. The plaque reduction technique is potentially a reliable and convenient method for classification and epidemiological studies of these agents. It may be particularly useful in typing chlamydiae isolated from human psittacosis patients when no avian contact can be proved. The test may be of greatest use in following other interspecies transfer of chlamydiae, that is, the relationship of isolates from arthropods, mammals, and birds in the same geographic locale.

Thus, the orderly process of identification would be isolation, propagation, CF test for antigenic classification in the chlamydial group, and then screening with available antisera to determine, if possible, the specific antigenic grouping. When a reduction of plaque count is obtained with one of the test sera, one can more

accurately determine the agent's place in the group by titrating the antiserum to obtain its 50% end-point titer. Where indicated, adsorption studies should be made. Such information would permit a more precise classification of *Chlamydia* than is now possible.

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