Detection of Chlamydial Antibodies in Animal Sera by Double Diffusion in Gel

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Postinoculation sera collected from pigeons, turkeys, guinea pigs, sheep, a calf, a rabbit, and a horse experimentally infected with various strains of Chlamydia psittaci yielded a high incidence of positive reactions when tested by double diffusion in gel. Antigen was a deoxycholate extract of SA-2 strain of C. trachomatis. Good correlation was obtained with results of complement fixation tests, whereas double diffusion in gel was less sensitive. Immunoelectrophoresis of the antigen revealed presence of two antigens in the extract.

In comparison to other serological methods such as complement fixation (CF) and immunofluorescence, double diffusion in gel (DDG) has not been widely used for the detection of chlamydial antibodies in sera. Recently, deoxycholate extracts of yolk sac-propagated chlamydiae (SA-2 strain of Chlamydia trachomatis) containing group-specific antigen have been shown to produce reaction lines when diffused in agar gel against sera from persons with various chlamydial infections (3). Furthermore, concentrated hemagglutinin prepared from the 6BC strain of C. psittaci has also been shown to form reaction lines when diffused against human antichlamydial sera (6).

Since chlamydiae infect numerous species of birds and mammals, it was of interest to explore the application of the same immunodiffusion method to the detection of chlamydial antibodies in the sera of a variety of animals. Conveniently available from previous experimentation (5; L. A. Page, unpublished data) were sera and serum fractions prepared by sucrose gradient centrifugation obtained from seven species of experimentally infected birds and mammals. The reactions observed by diffusing these samples in gel against extracts of the SA-2 strain are the subject of this report.

MATERIALS AND METHODS

Organisms. The SA-2 strain of C. trachomatis, TRIC/2/HAR-2/OT obtained from S. D. Bell, Jr., Department of Microbiology, Harvard School of Public Health, Boston, was used to prepare antigen. The organisms were propagated in the BGM line of African green monkey kidney cells (2).

Antigen preparation. Heavily infected BGM cells in Blake bottles were scraped into the cell culture medium (200 ml) and centrifuged at 27,000 × g for 30 min in a Sorvall RC2-B centrifuge. The sediment was suspended to ¼ of the original volume in phosphate-buffered saline (PBS), pH 7.2, disrupted for 25 sec with a Branson model W-185C sonifier at a setting of 75 watts. The material was subjected to one -20 C freeze-thaw cycle. Fifteen milliliters of 0.5% trypsin (Nutritional Biochemicals Corp., Cleveland, Ohio, 1-300) was added to the suspension, incubated at 37 C, chilled, and centrifuged at 27,000 × g for 30 min. The pellet was resuspended in PBS and washed three times by centrifugation and resuspension. The final pellet was suspended in PBS to ¼ of the original volume and extracted with sodium deoxycholate as previously described (3). Control antigen from uninfected cell cultures was prepared in a similar manner.

Antisera. Sera from clotted bloods of seven species of birds and mammals bled before and after experimental infection with various strains of C. psittaci were used (Table 1). These sera had been prepared by one of us (Page) and were previously utilized for studies of the biophysical characteristics of chlamydial antibodies (5; L. A. Page, unpublished data). Antisera to SA-2 strain of C. trachomatis (rabbit 258), 6BC (rabbit 226), and meningopneumonitis, Francis (rabbit 269), strains of C. psittaci, and uninfected progenitor cultures of BGM (rabbit 2033) were produced in the laboratory of the senior author. Normal animal sera were obtained from collections of laboratories of the Department of Microbiology, State University of New York at Buffalo.

Method for DDG. A method described previously (3) for diffusing serum and antigen in gel was used. Briefly, 0.8% Ionagar no. 2 (Consolidated Laboratories, Inc., Glenwood, Ill.) dissolved in 0.15 M saline was pipetted onto microscope slides. Serum wells of 4.0-mm diameter and antigen wells of 5.0-mm diam-
eter were cut into the hardened agar. The distance between the edges of antigen and antibody wells was 2.0 mm. Sera were prediffused for 30 min before addition of antigen to wells, and slides were incubated at 4°C in a humidified chamber. Lines of reaction were usually visible after 16 to 24 hr and were recorded photographically after 3 to 4 days. Lines were graded visually according to their intensity and recorded as strong (S), moderate (M), or weak (W).

**Serum inactivation.** Unless otherwise indicated, all sera were heated to 56°C for 30 min prior to diffusion tests because previous tests of unheated sera from apparently normal chickens and sheep had occasionally produced nonspecific precipitin lines that did not appear if the sera were inactivated. Other preliminary tests employing heated and unheated chlamydial antisera demonstrated that 56°C inactivation did not affect formation of lines caused by specific antigen-antibody reactions.

**Antigen and antibody titrations.** Antigen and antibodies were serially diluted in PBS and diffused against each other in a "box-type" titration. The end point in each case was the highest dilution giving a line of reaction.

**Immunoelectrophoresis.** Immunoelectrophoresis of sera was performed using an LKB model 6800A instrument according to the micromethod of Scheidegger (7) with the modification that wells were 5.0 mm in diameter.

**CF method.** Both the direct and indirect procedures employed for this study have been published in detail elsewhere (4). Unless otherwise indicated, the results presented were obtained by the direct method.

**RESULTS**

**DDG reactions with whole sera.** Typical reaction lines appearing between wells containing the chlamydial antigen and adjacent wells containing sera from animals infected with various strains of *C. psittaci* are illustrated in Fig. 1. The lines fused among themselves and also with the lines formed by rabbit antisera to *C. psittaci* and *C. trachomatis* strains. No lines appeared when the antigen was diffused in gel against micromethod to uninfected cell cultures. Also, no lines appeared when extract of BGM cells was diffused against chlamydial antisera.

Results of DDG tests with pre- and postinoculation sera of *Chlamydia*-infected pigeons, turkeys, guinea pigs, sheep, a calf, a rabbit, and a horse are shown in Table 1. Of 10 preinoculation sera, none formed lines of reaction. Lines of varying intensity were observed for 18 of 22 postinoculation sera tested. Three of the nonreacting postinoculation sera were from turkeys inoculated with strains of *B. avium* origin that are known not to multiply in turkeys.

Negative results were obtained by DDG with a serum from a turkey injected with a large dose of killed *Herellea vagincola* (*Bacterium anitratum*) and sera from two turkeys injected with killed *Pasteurella multocida*, strain 1059.

Two of the DDG-positive turkey sera (154 and 756) formed multiple reaction lines; all other positive sera formed single reaction lines.

Positive DDG tests were obtained with 5 of the 21 sera representing consecutive bleedings of a calf (5309) inoculated intravenously over a period of 3 months. The five positive sera represented bleedings between the 45th and 72nd day of the experiment (Table 2).

**Antigen and antibody titrations.** Reactions of serial twofold dilutions of antigen against varying dilution of each of four antisera are summarized in Table 3. Precipitin lines were not observed when the antigen was diluted more than 1:2 and reacted against calf, sheep, and turkey sera, but a 1:8 dilution of antigen still produced a line against undiluted hyperimmune rabbit serum. Serum from sheep 205 which had a (CF) titer of 192 reacted weakly in dilutions up to 1:16 with antigen diluted 1:2.

**Comparison of DDG reactions with CF titers.** With few exceptions, positive DDG reactions were obtained with postinfection sera that had chlamydial CF titers ranging from 8 to 8256 (Table 1). Most of the postinoculation sera tested had CF titers of ≥32. There appeared to be no correlation between the intensity of the DDG reaction and the CF titer.
When 21 sera representing consecutive bleedings of a calf injected with small doses of live epizootic bovine abortion chlamydiae were tested, only sera having a CF titer of $\geq 32$ were DDG positive (Table 2). Other discrepancies between CF and DDG positiveness appeared in tests of fractions of chlamydial antisera separated by sucrose gradient separation (see below).

**DDG reactions of serum fractions.** Results of DDG tests of chlamydial antisera produced in two turkeys, a pigeon, calf, rabbit, and sheep and fractionated by sucrose gradient centrifugation during previous experimentation (5) are compared with CF results in Table 4. Fractions three through five represented immunoglobulin G antibodies, and fractions six through 10 represented immunoglobulin M antibodies. Positive DDG reactions corresponded with CF titers of $\geq 8$, with the exception of fractions three through five of turkey 756 serum. These fractions were CF negative,
but positive DDG reactions were obtained. A similar discrepancy occurred in fraction three of sheep 205 serum.

Multiple lines were formed by several turkey antisera, as described above, and sucrose gradient fractions of turkey 756 antiserum formed multiple precipitin lines. Multiple lines were also obtained with fractions four and five from rabbit 2 but not with whole serum.

**Characterization of deoxycholate extract.**
Immunoelectrophoresis of the deoxycholate extract of strain SA-2 indicated that two antigens were present (Fig. 2). These antigens had similar electrophoretic mobilities.

Boiling the extract for 30 min as described previously (1) did not affect the formation of the two lines observed by immunoelectrophoresis. However, treatment of the extract with periodate using the procedure of Barron and Collins (1) destroyed the activity of both antigens.

**DISCUSSION**

DDG, as used in this study, appears to detect chlamydial antibodies satisfactorily in the sera of a variety of animal species. There was good agreement between development of gross lesions in infected animals and the results by DDG. As expected, an antibody response was not detected in animals inoculated with a chlamydial agent which was not infectious for that species. All of the animals tested were infected experimentally in the laboratory and the results by DDG encourage extension of the use of this procedure into field studies in cases of natural infections.

The method did not seem to be as sensitive as the CF test, and when DDG was positive the CF titer was at least 8. The method of DDG inherently does not have remarkable sensitivity for antibody detection, but the procedure is rather simple and this moderate insensitivity actually may be of some advantage in field work to eliminate minor reactors.

The deoxycholate extract antigen detected antibodies to the chlamydial group antigen(s) because it was prepared from the SA-2 strain of *C. trachomatis*, and sera tested were collected from animals infected with *C. psittaci* strains. The results obtained by immunoelectrophoresis suggested that the SA-2 extract contained more than one antigen. At the present time, the number of group antigens in chlamydiae has not been fully established. Recently, Kuo et al., (3a) reported three group-specific antigens, two of which were demonstrable in deoxycholate extracts and one in cell wall preparations.

The majority of sera produced one line of reaction when tested by DDG. However, mul-

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**Table 2. Double diffusion in gel (DDG) and complement fixation (CF) results from consecutive bleedings of a calf inoculated with the EBA strain of *C. psittaci***

| Post inoculation (day)* | DDG* | CF  |
|-------------------------|------|-----|
| PI                     | Neg  | Neg |
| 2                      | Neg  | Neg |
| 4                      | Neg  | Neg |
| 6                      | Neg  | Neg |
| 8                      | Neg  | Neg |
| 10                     | Neg  | Neg |
| 12                     | Neg  | Neg |
| 14                     | Neg  | 6   |
| 16                     | Neg  | 32  |
| 18                     | Neg  | 8   |
| 21                     | Neg  | 12  |
| 28                     | Neg  | 4   |
| 35                     | Neg  | 4   |
| 39                     | Neg  | 4   |
| 41                     | Neg  | 6   |
| 43                     | Neg  | 32  |
| 45                     | W    | 128 |
| 50                     | W    | 32  |
| 64                     | Neg  | 16  |
| 68                     | W    | 32  |
| 69                     | W    | 32  |
| 72                     | W    | 40  |

* Calf 5,309 was inoculated with <10 ELD₃₀ chlamydiae on days 0, 37, and 66.
* W = weak.
* PI = preinoculation.

**Table 3. Titration of antigen and sera by double diffusion in gel***

| Animal | Antigen dilution 1: | Antiserum dilution 1: | CF* |
|--------|---------------------|----------------------|-----|
| Calf 5309 | 1 W W | 16 32 | 32 |
| Rabbit 258 | 1 S M W | 16 32 | ND* |
| Sheep 205 | 1 S S S S M | 16 32 | 192 |
| Turkey 919 | 1 S M | 16 32 | 8 |

* CF = complement fixation; indirect CF was used for turkey serum.
* ND = not done.
Table 4. Comparison of double diffusion in agar gel (DDG) with complement fixation (CF) tests on fractions of sera collected from sucrose gradients

| Animal | Animal no. | Test | Sucrose fraction | Whole serum |
|--------|------------|------|-----------------|-------------|
|        |            |      | IgG             | IgM         |
|        |            |      | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  |
| Pigeon | 732        | CF*  | 0   | 0   | 2   | 32  | 32  | 8   | 0   | 0   | 0   | 0   | 0   |
|        |            | DDG  |     |     |     |     |     |     |     |     |     |     |     |
| Turkey | 154        | CF   | 0   | 0   | 4   | 32  | 32  | 8   | 0   | 0   | 0   | 0   | 0   |
|        |            | DDG  |     |     |     |     |     |     |     |     |     |     |     |
| Calf   | 756        | CF   | 0   | 0   | 0   | 0   | 2   | 8   | 16  | 16  | 0   | 0   | 64  |
|        |            | DDG  |     |     |     |     |     |     |     |     |     |     |     |
| Rabbit | 2          | CF   | 0   | 0   | 8   | 28  | 28  | 24  | 24  | 8   | 20  | 6   | 0   |
|        |            | DDG  |     |     |     |     |     |     |     |     |     |     |     |
| Sheep  | 205        | CF   | 0   | 0   | 0   | 4   | 8   | 24  | 48  | 64  | 48  | 8   | 0   |
|        |            | DDG  |     |     |     |     |     |     |     |     |     |     |     |

* CF data. Reprinted from Page et al. (5). Indirect CF test was used for turkey serum and fractions.
  * S = strong, M = moderate reaction, W = weak.
  * Multiple lines of reaction.

Fig. 2. Immunoelectrophoresis of deoxycholate extract antigen. Well contains deoxycholate extract of C. trachomatis strain SA-2. Trough contains rabbit antiserum 226 to C. psittaci strain psittacosis (6BC).

Multiple lines were observed with some turkey sera and sucrose fractions collected from turkey sera and a rabbit serum. The fact that the antigen was prepared as a deoxycholate extract might account for difficulties in resolution of lines. A possible explanation for positive DDG results in the absence of CF activity in sucrose fractions of turkey serum containing slow-sedimenting antibodies is that CF antigen detected antibodies to only one of the group antigens present in chlamydiae.

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