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Detection of Antibody to *Chlamydia psittaci*: Comparison of Antibody Titers in Human Sera Assayed by Enzyme-linked Immunosorbent Assay (ELISA) and Microplate Immunofluorescence Antibody Technique (MFA)

Hiroko BESSHO and Akira MATSUMOTO
Department of Microbiology, Kawasaki Medical School, Kurashiki 701-01, Japan
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ABSTRACT. Antibodies to *Chlamydia psittaci* in sera of healthy human adults, immunized and nonimmunized animals, rabbits and mice, were assayed by the enzyme-linked immunosorbent assay (ELISA) and then the titers were compared with those assayed by the microplate immunofluorescence antibody technique (MFA). The titers of rabbit and mouse sera determined by these two different methods were virtually identical to each other in both specificity and sensitivity. However, many of the human sera showed nonspecific, false positive reaction in the ELISA test. The results indicated that the MFA was more reliable than the ELISA and that the titers obtained by the ELISA had to be examined by other titration method to confirm the specificity. Such disadvantage was not seen in the MFA for the titration of antichlamydial human sera.

Key words : MFA — ELISA — Antibody — *Chlamydia psittaci* — Nonspecific reaction

We established a simple titration method, microplate immunofluorescence antibody technique (MFA), to detect the antibody against *Chlamydia psittaci* and reported that the MFA was more sensitive, specific and simple than the complement fixation test (CF) which is commonly used for serodiagnosis of human chlamydial infection.[1] The anti-chlamydial antibody titers of immunized rabbit sera and sera collected from a psittacosis patient determined by the MFA were constantly 8 to 16 times higher than those determined by the CF, and were almost equivalent with those assayed by the ELISA. In the present study, we assayed the human, rabbit and mouse sera by the ELISA and MFA, and compared the titers to one another to confirm the usefulness of the MFA for serodiagnosis of human chlamydial infection.

MATERIALS AND METHODS

Propagation of *Chlamydia* strain : *Chlamydia psittaci* Izawa-1 strain, which was isolated from a budgerigar kept by a severe psittacosis patient,[2] was used throughout this study. The organisms were propagated in suspension L cell cultures and elementary bodies (EBs) were purified from the infected L cells at 72 hours postinoculation by the method of Tamura and Higashi.[3] The biological properties of the organisms were reported previously.[2]

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The purified EBs were inactivated by UV irradiation and disrupted by sonic treatment with an ultrasonic generator BH-200 P (Tomy Seiko Co., LTD. Japan) (90w) for 30 min at 4°C to use as an antigen in the ELISA test.

Preparation of sera : Human sera were collected from blood samples which were kindly supplied from 26 healthy workers in this medical school, and stored at -20°C until use. From some sera IgG was partially purified by repeated precipitation with 50% and 33% ammonium sulfate solutions. Non-immunized New Zealand rabbit sera were obtained before immunization and hyperimmune sera were obtained after 4 times intramuscular injections of the UV-inactivated EBs with Freund's complete adjuvant. Immunized mouse sera were obtained from 10 ICR mice after an intraperitoneal injection. Nonimmunized mouse sera were collected from 10 normal mice. All sera were stored under sterile condition at -20°C until use.

MFA : The MFA was carried out as described previously.[1] Briefly, the infected monolayered L cells at 20 to 24 hours postinoculation in an ordinary glass culture vessel were dispersed with a trypsin-EDTA solution and 10 μl of the suspension (about 10^6 cells/ml) was put into each well in a Terasaki plate (Flow Lab. Inc., USA) and allowed to form monolayer at 37°C in a humidified atmosphere composed of 5% CO_2 and 95% air for 2 to 6 hours. After twice washings with PBS, the cells were fixed with ethanol for 30 min at room temperature, and then washed with PBS. Ten μl of the serum serially diluted was put into the wells and the plate was incubated for 45 min at 37°C. After several washings, the cells in the wells were overlaid with antihuman IgG FITC labeled goat serum solution (10 μl of 10 times diluted solution/well, Medical and Biological Lab., LTD., Japan), incubated for 45 min at 37°C, and then examined under a fluorescent microscope. The antiserum titer was determined at highest dilution of serum showing the definite fluorescence of inclusions.

ELISA: The ELISA was performed in a polystylene (Flow Lab. Inc., USA, Falcon Becton Dikinson & Co.,USA, and Sumitomobakurite Co., Japan) and polyvinyl (Flow Lab. Inc., USA and Sumitomobakurite Co., Japan) plates having 96 round wells by the modified method of Engvall and Perlmann.[4] The EB-antigen suspension (5 μg protein/ml) was prepared with 0.05M carbonate buffer pH 9.6 and 0.1 ml of the suspension was put into each well to sensitize the well surface. The plate was incubated at 4°C overnight and then washed 3 times with PBS containing 0.01% Tween 20 (Wako Purechemical Industries LTD., Japan). One tenth ml of the serially diluted serum was added to each well and incubated at room temperature for 2 hours. After three time washings, 0.1 ml of a 1 : 2,000 diluted goat antiserum to human, mouse or rabbit IgG labeled with alkaline phosphatase (Sigma Chemical Co., USA) was added. After 2 hour incubation at room temperature, the plate was washed and 0.1 ml of the substrate solution containing *p*-nitrophenyl phosphate (1 mg/ml in diethanolamine buffer, pH 9.8) was added, followed by incubation at 37°C for 30 min. The reaction was terminated by the addition of 3N NaOH (50 μ1) and assayed with a micro ELISA autoreader MR 580 (Dynatech Lab. Inc, USA) at 405 nm of wave length. Readings above 0.2 were regarded as positive reactions.

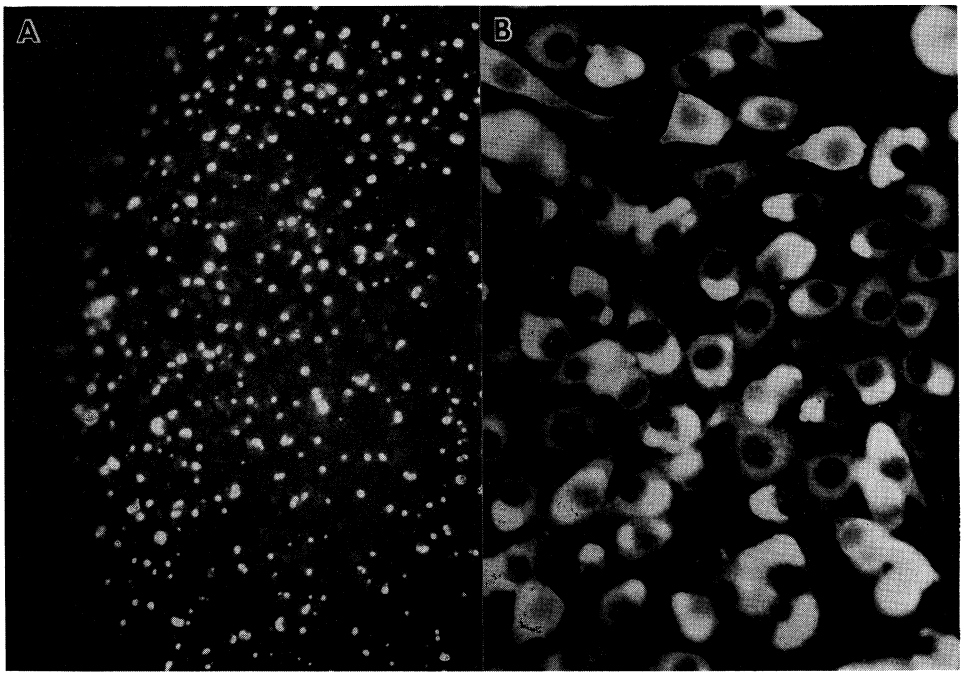

Fig. 1. Positive reaction in MFA. A number of intracytoplasmic inclusions showing positive reaction are seen. A: A part of a well. Inclusions are recognized as scattering bright spots all over the well surface at low magnification (10×10). B: Cells showing positive reaction at higher magnification. Inclusions in host cells are specifically stained and readily distinguished. Round dark area in each cell is nucleus.

RESULTS

Figure. 1 illustrates one of the wells showing positive reaction by the MFA. All inclusions in the host cells are readily recognized for their morphology and highly specific staining. The antibody titers in the sera determined by the ELISA and MFA are summarized in Figure. 2. With one exception (serum showing 1 : 4 by MFA), the normal rabbit sera, which were negative by the MFA, were also found to be negative by the ELISA. All immunized rabbit sera, which were highly positive by the ELISA, showed positive reaction at approximately identical levels. The results from mouse sera are divided into two groups ; one is negative by the ELISA and MFA. These negative sera were collected from nonimmunized mice, and the other is composed of immunized, positive group in both methods. These results indicate that the antibodies in the animal sera are detectable in a good correlation between the two different methods. However, such a correlation was not clearly seen in the human sera. Only 6 of 26 sera were positive in both methods, but other 20 sera which were negative by the MFA, were undoubtedly positive by the ELISA. Two of 6 showed considerably high titers (1 : 256 and 1 : 1024) in the ELISA, although their titers were only 1 : 4 by the MFA. From these results, it is very likely that the false positive reaction is frequently occurred in human sera as assayed by the ELISA. This

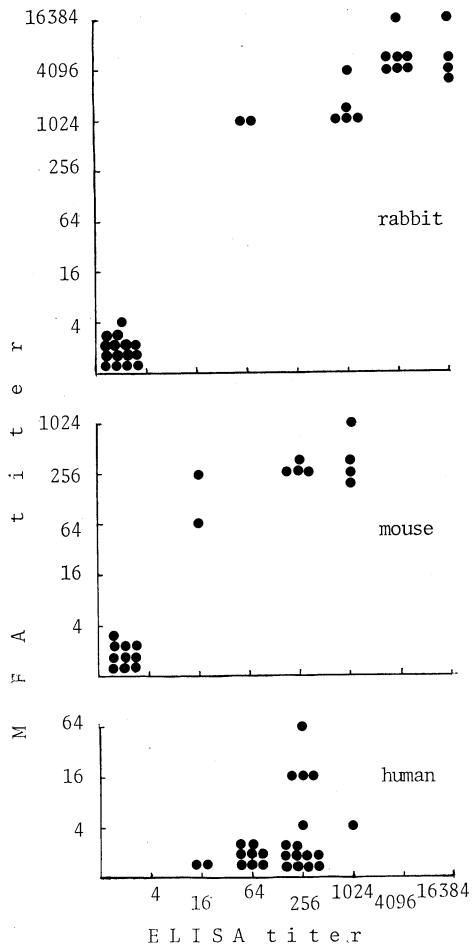

Fig. 2. Comparison of antibody titiers to *C. psittaci* by MFA and ELISA. Each dot indicates a serum obtained from an individual animal or man. The rabbit and mouse sera are clearly divided into two, nonimmunized and immunized, groups, indicating a good correlation between the titers assayed by ELISA and MFA. Such a correlation is not seen in human sera.

nonspecific reaction was observed in all plates purchased from the different makers, and was not prevented by the treatment of the sensitized wells with 1% bovine serum albumin or human serum albumin solution before the addition of the antiserum tested. It is, therefore, concluded that the ELISA does not reliably distinguish the difference between real- and false-positive human antisera. There are some possibilities for the nonspecific, false-positive reaction in the ELISA; (i) nonspecific association or adhesion of the human antibody to the well surface, (ii) nonspecific association or adhesion of antihuman enzyme conjugated goat serum to the well, and (iii) specific or nonspecific association of the enzyme conjugated goat serum to the antigen covering the well surface.

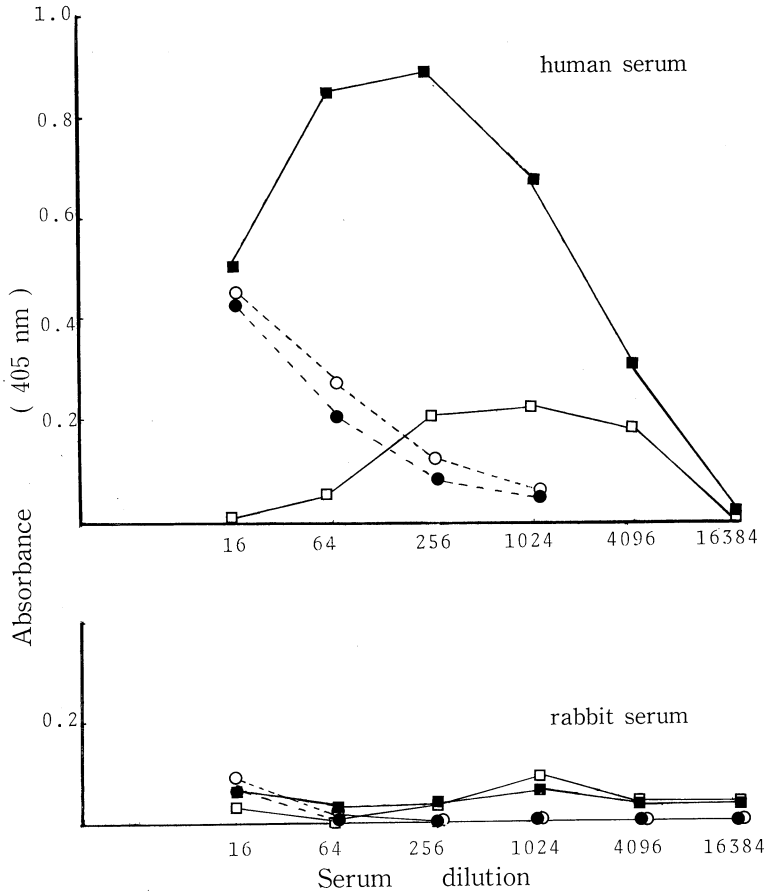

Fig. 3. Effects of chromic acid treatment of plate and heat-inactivation of sera on the titers by ELISA.
○---○ : native serum assayed in untreated plate
●---● : inactivated serum assayed in treated plate
□—□ : native serum assayed in treated plate
■—■ : inactivated serum assayed in treated plate
When the treated plate was used for the inactivated human serum, the absorbance was extremely increased, although no effect was detected in the rabbit sera.

Preliminary experiments indicated no reaction between the antigen (EBs of Izawa strain) and the enzyme conjugated goat serum. Therefore, the third possibility was excluded. When the wells were treated with chromic acid mixture, washed and then used in the ELISA test, nonspecific reaction of the human sera, negative by the MFA, was extremely enhanced (Fig. 3). When untreated plate was used for inactivated and native sera, the titers of both sera were identical to each other. In contrast, a significant difference in titer between the native and inactivated sera was seen when the treated plates were used. The native serum showed only about 0.2 in absorbance at 256 to 1024-fold dilution, but the same serum after heat-inactivation at 56°C for 30 min showed extremely high level of the absorbance, and consequently a titer more than 1 : 4096 was

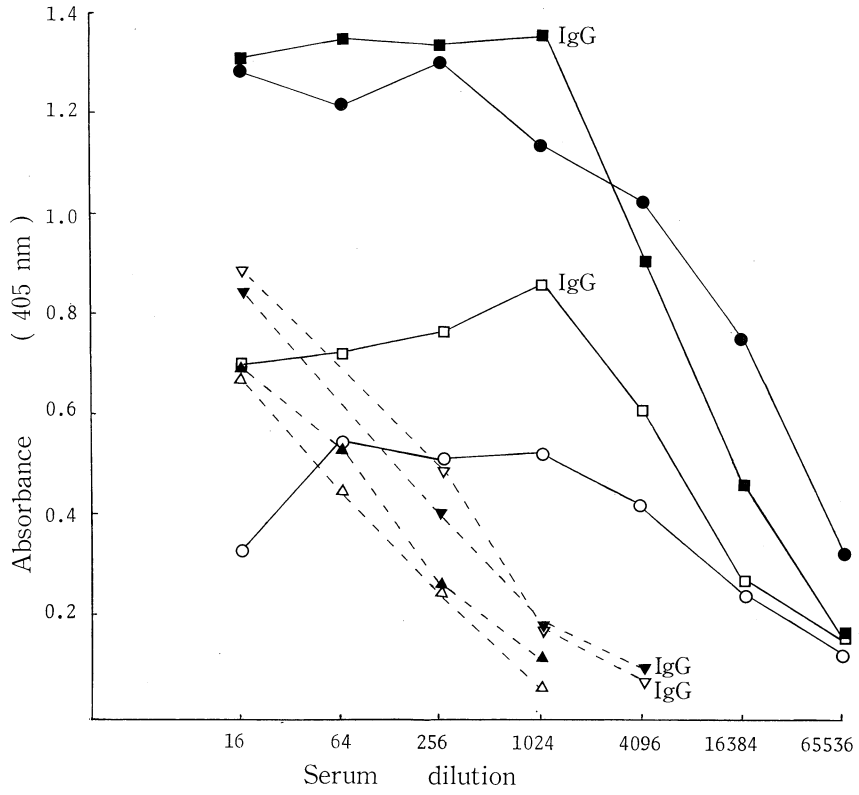

Fig. 4. Effects of chromic acid treatment of plate and heat-inactivation of sera on ELISA titers of human sera and crude IgG.
○——○ : native serum assayed in treated plate
●——● : inactivated serum assayed in treated plate
□——□ : crude IgG assayed in treated plate
■——■ : inactivated crude IgG assayed in treated plate
△---△ : native serum assayed in untreated plate
▲---▲ : inactivated serum assayed in untreated plate
▽---▽ : crude IgG assayed in untreated plate
▼---▼ : inactivated crude IgG assayed in untreated plate
All sera and IgG solutions were prepared from a serum at a 1 : 64 by the MFA.

obtained. This peculiar enhancement was never seen in the rabbit sera even if the treated plates were used for the inactivated serum. Identical phenomena were observed when the crude human IgG solutions were assayed by the ELISA using the treated plates. As shown in Figure. 4, no significant difference in titer between the native and inactivated sera was observed (1 : 256) by the use of the untreated plates. Similarly, the crude IgG solutions before and after inactivation showed an identical titer (1 : 1024) in the untreated plates. However, if the treated plates were used, the reaction was remarkably enhanced, and such an enhancement was much more significant in the sample after than before inactivation. Consequently, the titers of native serum, inactivated serum and IgG solution before inactivation resulted in a 1 : 16384, and a titer more than 1 : 16384, probably 1 : 65536, was obtained for the inactivated IgG solution. As shown in Figures. 3 and 4, the absorbance was decreased and disappeared in

proportion to the serum dilution. Besides, an equal quantity of the enzyme conjugated goat serum was put into each well in the ELISA system. Therefore, these facts may exclude the second possibility above mentioned, and may indicate that the nonspecific reaction is resulted from the nonspecific association or adhesion of the human antibodies to the well surface.

DISCUSSION

We established a simple method, MFA, for the titration of antibody against chlamydiae.[1] In the MFA, the antibody titer is determined by the following two points ; (i) confirmation of the presence of specific-stained areas in the host cells, and (ii) identification of the stained areas as the intracytoplasmic inclusions containing chlamydiae. These two points can be examined simultaneously under a fluorescent microscope. These are the reasons why the MFA is highly specific.

The ELISA is considered as a sensitive and highly specific method for antibody detection and used widely for screening of monoclonal antibody producing cells. In the present study, it was, however, demonstrated that human sera, negative and relatively low titer by the MFA, showed frequently high, nonspecific reaction which might be resulted from the nonspecific adhesion of the IgG to the well surface of the plate used in the ELISA system. The nonspecific reaction was extremely enhanced when the sera were inactivated and assayed in the plate treated with chromic acid mixture. Such a curious phenomena was never seen in the rabbit and mouse sera even if the sera were inactivated and assayed in the treated plate. Therefore, it seems that the anti-chlamydial human antibody titer determined by the ELISA should be reexamined by the other methods, such as the MFA and microimmunofluorescent technique (MIF).[5] Levy *et al.* reported anti-chlamydial antibodies in patient sera by the ELISA, in which they determined the titers based on an absorbance level standardized by the MIF and regarded absorbance more than 0.225 as positive reaction.[6] According to their manner, the serum titer less than 1 : 16 may not be determined for the nonspecific reaction. Actually, in many cases, the sera tested by the ELISA were diluted 100-fold and more before titration depending on antibody titer and antigen.[7–10] This might be due to the false positive reaction in the human serum at higher concentration. From the results obtained in the present study, it is confirmed again that the MFA is, so far as human chlamydial antibody at relatively low titer is assayed, more specific and convenient than the ELISA test.

Acknowledgment

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