Enzyme-Linked Immunosorbent Assay for Detection of Mycoplasma pneumoniae Antibodies

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The enzyme-linked immunosorbent assay (ELISA) described by Engvall and Perlmann has been used for the detection of antibodies against Mycoplasma pneumoniae. These organisms, grown in flat-bottom wells of microtiter plates, were used as antigen. Preliminary results suggest that ELISA is specific and that its sensitivity is somewhere in between those of the metabolic inhibition and radioimmunoprecipitation methods. Unlike other serological methods, such as metabolic inhibition and mycoplasmacidal tests, in which the presence of antibiotics in sera may give false results, ELISA provided results that were not influenced by antibiotics. Furthermore, ELISA offers the possibility of measuring not only immunoglobulin M, but also complement-independent antibodies, especially those of the immunoglobulin A class. Theoretical aspects concerning the different sensitivities of some serological reactions are discussed. In the field of specific immunological techniques, complement fixation, metabolic-inhibition (MI), and mycoplasmacidal tests have been widely used for the serological diagnosis of diseases caused by Mycoplasma pneumoniae. A large amount of literature exists concerning their specificity, sensitivity, and reproducibility (2–4, 12, 19; F. Busolo, M. Zorzenon, and I. Piacentini, Boll. Ist. Sieroter. Milan., in press). Radioimmunoprecipitation has recently been proposed, which, due to its elevated sensitivity, offers great advantages over other methods, though posing serious problems concerning worker safety and elimination of the radioisotopes. In other sectors of diagnostic serology, a new method, enzyme-linked immunosorbent assay (ELISA), has been proposed which overcomes the above-mentioned problems. It is based on the work of Engvall and Perlmann (6) on specific antibodies against "solid-phase" adsorbed antigens using an enzyme-conjugated anti-gammaglobulin. The adaptation of micromethods makes this technique extremely versatile and practical, as can be seen in its wide application in serological diagnosis of bacterial (5, 8, 11, 15), mycoplasmal (9), viral (18, 19), and parasitic (17, 20) disease.

In this brief communication we report the results obtained from 50 subjects with suspected primary atypical pneumonia examined with the immunoenzymatic method applied for the first time to the diagnosis of human diseases caused by mycoplasmas.

MATERIALS AND METHODS

Mycoplasma and culture conditions. The strain of M. pneumoniae was kindly provided by the National Collection of Type Culture, Colindale, London. Culture media consisted of tryptic soy broth (Difco) containing: 15% unheated horse serum, 0.25% yeast extract, 1% glucose, 0.002% cresol red, and 1,000 U of penicillin G per ml. The pH was adjusted to 7.4 with 1 N NaOH.

Human sera. Human sera were obtained from pediatric outpatients of the Pediatric Clinic of the University of Padua.

MI test. The MI test method proposed by Taylor-Robinson et al. (16) was used, with the addition of a monodispersed cell suspension of M. pneumoniae. Antigen preparation. Elisa type microtiter plates with flat-bottomed wells (Cooke Engineering Co.) were used according to the following techniques.

(i) M. pneumoniae cultures in Roux flasks (surface area, 150 cm²) yielded, after washing, about 4 mg of protein per flask as determined by the Lowry method (13). Mycoplasmas, mechanically removed and suspended in phosphate-buffered saline (pH 7.2), were conserved at −80°C. A 40-µl sample of a suitable dilution in carbonate buffer at pH 9.8 was put in each well. For passive sensitization, the plates were left overnight at 4°C.

(ii) A 100-µl volume of culture in logarithmic growth phase was inoculated into each well (10⁴ to 10⁶ color change units per well). After 4 days of incubation, the pH had diminished about 0.8 unit. After removal of the liquid, the mycoplasmas that had adhered to the bottom of the well were washed with phosphate-buffered saline containing 0.05% Tween 20 and then fixed in 10% Formalin for 10 min at 4°C. After three washings, the plates were hermetically sealed in a plastic bag and kept at −30°C until used.

Preparation of conjugated antisera. Goat anti-human immunoglobulins G, M, and A combined (IgG-M-A), IgM, and IgA antisera (Hyland, Costa Mesa, Calif.) were conjugated with alkaline phosphatase essentially as described by Engvall and Perlmann (6). The enzyme preparation used was alkaline phosphatase.
tase, Sigma type VII (Sigma Chemical Co., St. Louis, Mo.; specific activity, 1,000 U/mg of protein). The conjugated antisera, diluted in tris(hydroxymethyl)aminomethane-hydrochloride (pH 8) containing 0.001 M MgCl₂ and 0.02% NaN₃, were kept at −80°C.

ELISA. Before testing, the antigen fixed to the bottom of the wells was washed with phosphate-buffered saline containing 0.05% Tween 20, which prevented nonspecific adsorption of the antibody to the solid phase. Samples of 50 µl of different serum dilutions (in phosphate-buffered saline + 0.05% Tween 20) were placed in the wells. The plates were incubated for 2 h at 37°C. The liquid was removed, and the wells were washed twice with phosphate-buffered saline + Tween. A 50-µl sample of conjugated antiserum diluted in tris(hydroxymethyl)aminomethane-hydrochloride buffer was added to each well, and the plates were incubated again for 2 h at 37°C. After two washings, 250 µl of 3-phenylphenol phosphate (1 mg/ml) solution in carbonate buffer (pH 9.8) was added, and plates were incubated for 1 h at 37°C. The reaction was then stopped with the addition of 50 µl of 3 N NaOH. Absorbance was read at 400 nm in a Perkin-Elmer 124 double-ray spectrophotometer using microcuvettes.

**Titration of the antigen.** Determination of the optimal antigen concentrations was done using varied dilutions of antigen (1.5 to 48 µg of proteins per well), a positive reference serum diluted 1:16, and conjugated antiserum diluted 1:200.

**Determination of a working dilution of conjugate antiserum.** Five dilutions were run (from 1:100 to 1:500) in the presence of a predetermined quantity of antigens and against suitable dilutions of anti-*M. pneumoniae* reference serum. This was included in all the following tests as a check against the stability of the conjugate.

**RESULTS AND DISCUSSION**

**Titration of the antigen.** The maximum absorbance values were reached at 24 to 48 µg of protein per well (Fig. 1), corresponding to the optimal concentration of *M. pneumoniae* to be used in the ELISA. These absorbance values were also reached with *M. pneumoniae* cultures grown on the bottoms of the wells. At the working level, then, it is possible to follow both methods, either adding samples obtained from *M. pneumoniae* cultures of 24 µg of protein to each well or cultivating the mycoplasmas directly in the wells and fixing them after 4 days or whenever the pH falls from 7.4 to 6.6.

In any case, the fixing of the mycoplasmas was done using 10% Formalin as reported by other authors working with various antigens (11). As has been demonstrated in a series of preliminary experiments conducted using “fixed” and “non-fixed” antigens, the Formalin treatment has no significant effect on determinant antigenic sites of *M. pneumoniae*. The choice of fixed antigens was also motivated by the fact that under equal experimental conditions, fixed antigens demonstrated absorbance values spread over a narrower range than those of nonfixed antigens.

**Titration of the conjugate antiserum.** Suitable dilutions of the antiserum were assayed with 4-day-old cultures grown in the wells. The absorbance values of the positive reference serum with respect to the five concentrations of anti-human IgG-M-A (from 1:100 to 1:500) tested are determined. As expected, all dilutions of the patient’s serum showed elevated absorbance values and growing nonspecific absorption at the higher conjugate concentrations. Theoretically, all conjugate dilutions could have been chosen as suitable even if those of 1:100, 1:200, and 1:300 ensured a wide absorbance range (0.10 to 0.90). A dilution of 1:200 was selected on the basis of the data presented in Fig. 2. At conjugate dilutions of 1:500 and 1:100, absorbance values were obtained of 0.40 and 0.91, respectively, with a plateau at the 1:200 dilution. For economic reasons and because of the above-mentioned equal absorbance ranges, we chose the 1:200 dilutions. As reported in the literature, storage at 4°C for 8 months does not modify the conjugate’s activity.

**ELISA.** In Fig. 3 the results obtained with the M1 method are compared to those obtained with the ELISA. The critical part of the tests was the endpoint determination. The endpoint in the M1 test corresponded to the highest dilution of patient serum that still prevented a color change in the culture medium. The reading was taken against a simultaneous color change in the con-

![Fig. 1. Antigen titration on solid phase. For passive sensitization of microplates, suitable dilutions of *M. pneumoniae* in carbonate buffer at pH 9.8 (40 µl) were put in each well and left overnight at 4°C. Assay was performed using a positive reference serum diluted 1:16 and the conjugated antiserum diluted 1:200.](image-url)
ELISA FOR M. PNEUMONIAE ANTIBODIES

The statistical analysis of the results in Fig. 3 shows that the values obtained by the ELISA method correlate well with those obtained by the MI test ($P < 0.01$). Of 50 sera examined, 19 had a titer below 1:16 with both methods used. In the majority of the remaining sera, the titers obtained with ELISA were slightly higher than those obtained with MI. In considering this difference, the class of immunoglobulins involved in these reactions should be noted.

In Fig. 4 and 5 two positive sera have been tested with polyvalent conjugated antiserum anti-IgG-M-A, monospecific anti-IgM, and anti-IgA. In Fig. 4, the ELISA reveals antibodies mostly belonging to the IgM class with a low level of serum IgA. The elevated titer of IgM with respect to total immunoglobulin may be due to the higher background of its negative controls. There may also be better conjugation of the enzyme to the antibody. Nonspecific absorption to the solid phase is always greater for IgM (Fig. 6). In Fig. 5, IgA also appears.

The persistence of IgM antibodies for long periods was examined in only four patients. Three of them showed low titers of IgM 1 year after the onset of disease. These results agreed with those of Fernald et al. (7) and Biberfeld and Sterner (1). Both MI and ELISA tests were applied to study paired sera of eight patients. The antibody titers from 0 to 64-fold when the convalescent sera were collected at 20 days after the onset of disease.

To make a serological diagnosis of M. pneumoniae infections, it is necessary to demonstrate the so-called seroconversion phenomenon. In...
Although and antigens where the method, obtained with sera IgA

4-day-old microplate diluted x (zg polyvalent and monospecific conjugated antibody Serum diluted (reciprocal)

In testing reproducibility we found that in three separate tests the titers obtained with the ELISA never varied more than twofold.

The ELISA method, which we have applied for the first time to detect anti- M. pneumoniae antibodies, can also be employed with patients placed under antibiotic therapy at the time the blood sample is drawn. Unlike the MI and mycoplasmalidial techniques, there is no danger of false-positives due to the presence of antibiotics in the serum.

In summary, the low cost, simplicity, speed, and sensitivity of the ELISA test make it a promising serological technique in the diagnosis of infections caused by M. pneumoniae.

LITERATURE CITED


