Mycoplasma pneumoniae protein involved in the antibody response in human infection

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SUMMARY Antigen from purified *Mycoplasma pneumoniae* organisms treated with Tween-80-ether was used in a solid phase enzyme immunoassay and compared with the conventional lipid containing complement fixation antigen for measuring antibodies in sera from patients with aseptic or bacterial meningitis or with apparent *M pneumoniae* infection. In immunoblotting of the enzyme immunoassay antigen, enzyme immunoassay positive sera detected a polypeptide at $M_r = 180\,000\text{--}200\,000$, while enzyme immunoassay negative sera whether positive or negative in the complement fixation test did not. These results indicate that the enzyme immunoassay antigen containing the high molecular weight polypeptide can be used to measure *M pneumoniae* antibodies more specifically than the conventional lipid containing complement fixation antigen.

Considerable increases in the titres of complement fixing antibodies reactive with the glycolipid containing fraction of *Mycoplasma pneumoniae* have been reported in many kinds of infections and illnesses with or without other evidence of *M pneumoniae* infection.¹⁻³ Examples of such illnesses are the infections and disorders of the central nervous system.⁴⁻¹¹ Some of these antibody responses may be due to cross reacting antibodies stimulated by various conditions.¹² Such antibodies often develop during acute pancreatitis.^{13,14} On the other hand, antibodies against brain, lung, and liver tissue develop during *M pneumoniae* infections¹⁵ and injections of mycoplasmas other than *M pneumoniae* can cause central nervous system symptoms in animals.¹⁶⁻¹⁸

Our previous studies with enzyme immunoassay and a Tween-80-ether treated *M pneumoniae* antigen,¹⁴ instead of the conventional lipid containing complement fixation antigen, supported, in conjunction with clinical evidence, the view that some of the rises in complement fixation antibody titres may be due to false positive reactions against the glycolipid moieties of the antigen. It seemed possible that a more purified *M pneumoniae* protein antigen could eliminate these reactions and make the serological diagnosis of *M pneumoniae* infection more reliable.

To clarify the possible role of *M pneumoniae* in central nervous system infections, we examined

paired sera from patients with meningitis and meningoencephalitis with a fourfold or greater rise in complement fixation antibody titre to the *M pneumoniae* lipid antigen. Using the enzyme immunoassay with a protein containing antigen for *M pneumoniae*,¹⁴ sera from patients with bacterial meningitis did not show a serological response, whereas sera from patients with a *M pneumoniae* respiratory infection showed significant increases in antibody titre. The latter sera reacted in immunoblotting with a defined high molecular weight polypeptide separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and was transferable to nitrocellulose paper.

Material and methods

SERUM SPECIMENS

These included paired sera from 11 patients with aseptic meningitis and meningoencephalitis and four patients with bacterial meningitis, all with fourfold or greater increases in antibody titre in the *M pneumoniae* complement fixation test. Paired sera from five patients with pneumonia showing fourfold or greater rises in the concentration of antibody against *M pneumoniae* in the complement fixation test, and thus with a probable *M pneumoniae* infection, were used as positive controls. The negative control group consisted of paired sera from 12 patients with pneumonia due to causes other than *M*

pneumoniae and single sera from four one year old healthy children, all with low antibody titres in the *M pneumoniae* complement fixation test.

ANTIGENS

Mycoplasma pneumoniae enzyme immunoassay antigen was prepared and produced by Orion Diagnostica (Espoo, Finland) according to our specification.¹⁴ Standard *M pneumoniae* complement fixation antigen (Orion Diagnostica), which consisted of lipid components extracted with chloroform and methanol from cultivated purified *M pneumoniae* organisms,¹⁹ was used in the complement fixation test.

ENZYME IMMUNOASSAY

The antigen was diluted in a sodium carbonate-sodium bicarbonate buffer, pH 9.6. Serum and conjugate dilutions were made in phosphate buffered saline (PBS) supplemented with 0.2% Tween 20 and 10% sheep or newborn calf serum. Heavy chain specific, alkaline phosphatase conjugated antibodies against human IgG and IgM were used as conjugates (Orion Diagnostica). The solid phase enzyme immunoassay procedure was as described elsewhere.¹⁴ All sera were diluted 1/100 for the assay.

SDS PAGE AND IMMUNOBLOTTING

For immunoblotting both enzyme immunoassay and complement fixation antigens of *M pneumoniae* were used. The proteins were separated by SDS 10% polyacrylamide slab gel electrophoresis prepared according to Laemmli.²⁰ After separation, the proteins were transferred electrophoretically to nitrocellulose sheets and immunoblotted according to the procedure by Towbin *et al*.²¹ modified as described elsewhere.²² The transfer was checked by the reversible heparin toluidin blue staining procedure. For immunological detection of the transferred proteins, the sheets were sequentially exposed to the patient sera, diluted 1/50 in PBS supplemented with 0.2% Tween 20 and 2% bovine serum albumin, then to ¹²⁵I-labelled staphylococcal protein A, and finally to autoradiography.

Results

CORRELATION BETWEEN ENZYME IMMUNOASSAY AND COMPLEMENT FIXATION TITRES

The results of the enzyme immunoassay were first obtained as absorbance values (A_{405}). A moderately positive convalescent phase serum of the positive control group was chosen as the positive reference serum. The mean of the negative control group results was calculated in both the IgG and IgM test

Fig. 1 IgM antibody response against the Tween-ether treated antigen in the enzyme immunoassay test in various groups of patients with diagnostic rises or high titres of complement fixation antibodies against *M pneumoniae*. Healthy controls include patients with pneumonia with low complement fixation antibody titres against *M pneumoniae*. Values for the acute (1) and convalescent phase (2) are shown.

and used as the negative reference. Using these reference values the results were expressed as enzyme immunoassay units (EIU).

$$x = \frac{S-M1}{M2-M1} \times 100 \text{ EIU}$$

where x = result of the test, S = absorbance value of the sample, M1 = mean absorbance of the negative control group, and M2 = absorbance of the positive reference serum.

None of the sera from the four patients with bacterial meningitis (three caused by *Haemophilus influenzae* and one by *Neisseria meningitidis*) showed pronounced increases in either the IgM or the IgG antibody concentrations in enzyme immunoassay despite significant increases in complement fixation antibody titres (Figs. 1 and 2). Most patients with aseptic meningitis and encephalitis also had low concentrations of antibodies against the enzyme immunoassay antigen, again despite the fourfold or greater rise in the complement fixation test. Two patients with encephalitis like neurological symp-

Fig. 2 IgG antibody response against the enzyme immunoassay antigen in the patient groups of Fig. 1.

toms had significant concentrations of antibodies in enzyme immunoassay as well. Both patients had respiratory infections associated with neurological symptoms: one had an acute respiratory infection, negative cold agglutinin titres, and a lower IgG concentration against the enzyme immunoassay antigen in the convalescent phase serum than in the acute phase sample; the other had a rise in antibody concentration in the enzyme immunoassay and an increase in the cold agglutinin titres and developed pneumonia after the central nervous system symptoms (Figs. 1 and 2). Sera from all the patients with a typical pneumonia and positive complement fixation titres were clearly positive in the *M pneumoniae* enzyme immunoassay test as well (Figs. 1 and 2). These patients also had raised cold agglutinin titres in their convalescent phase sera. Sera from healthy one year old children and from patients with pneumonia with no increase in complement fixation antibody titre did not respond in the enzyme immunoassay test to the Tween 80-ether treated *M pneumoniae* antigen.

IDENTIFICATION OF POLYPEPTIDE ANTIGEN
SDS-PAGE showed over 10 protein bands in the concentrated Tween 80-ether treated *M*

pneumoniae antigen. Electrophoresis of the complement fixation antigen also gave several polypeptide bands. When patient sera, previously tested with both the enzyme immunoassay and the complement fixation test, were used in immunoblotting to identify the antigenic polypeptides separated by SDS-PAGE, one zone at $M_r = 180\,000\text{--}200\,000$ was repeatedly detected by enzyme immunoassay positive sera (Fig. 3). No such band could be found in the complement fixation antigen with sera positive both in the enzyme immunoassay and the complement fixation test. Also, enzyme immunoassay negative sera, whether negative or positive in the complement fixation test, failed to detect this band in either type of antigen.

The acute phase patient sera which had a low response in the *M pneumoniae* enzyme immunoassay test often failed to show the $M_r = 180\,000\text{--}200\,000$ antigen or gave a faint reaction; convalescent phase sera were able to detect the zone clearly.

Fig. 3 Immunoblotting of *M pneumoniae* polypeptides separated by SDS-PAGE and transferred to nitrocellulose sheets, with a known enzyme immunoassay positive convalescent phase serum of a patient with pneumonia (1 and 2) and a serum of a healthy one year old child with no enzyme immunoassay or complement fixation antibodies against *M pneumoniae* (3 and 4). Both sera were diluted 1/50 and 1/100. Arrow indicates the $M_r = 180\,000\text{--}200\,000$ position.

Discussion

The enzyme immunoassay test using a protein containing antigen treated with Tween 80-ether seems to be more specific for *M pneumoniae* infection than the conventional complement fixation test, which is based on a lipid containing antigen. In enzyme immunoassay, the sera from patients with bacterial meningitis and most sera from patients with aseptic meningoencephalitis showed no significant antibody titres or diagnostic rises in antibody concentration. The small increase seen in some of the convalescent phase sera may have been due to minor cross reactive common bacterial components present in the enzyme immunoassay antigen. In addition to its specificity, compared with the complement fixation test, enzyme immunoassay is a simple procedure and it is possible to measure both IgM and IgG antibody concentrations separately, which gives some information of the phase of the immune response.

In some cases of aseptic meningitis and encephalitis, *M pneumoniae* may be the actual cause of infection, but definite conclusions on the association between *M pneumoniae* and the central nervous system symptoms cannot yet be made. The two patients with encephalitis as the clinical diagnosis who had high antibody concentrations in both the complement fixation and enzyme immunoassay test also had respiratory symptoms during the course of their illnesses, and thus may have experienced two diseases simultaneously: a respiratory *M pneumoniae* infection and a widespread central nervous system infection. Moreover, one of these two patients had a lowered enzyme immunoassay IgG antibody value in the convalescent phase serum. Isolation of *M pneumoniae* from the respiratory tract would have been most informative, but could not be done in this retrospective study.

Immunoblotting has previously proved useful for detecting immunologically active components from otherwise unpurified material.²¹⁻²³ In the present study we used a Tween 80-ether treated fraction of *M pneumoniae* organisms as antigen in the enzyme immunoassay test. This antigen preparation was used in immunoblotting to define antigenic polypeptides. Sera positive in the enzyme immunoassay and complement fixation test detected one major polypeptide at $M_r = 180-200-000$, corresponding to the mycoplasma surface protein P1 concerned in the attachment of the organisms to respiratory epithelium described by Hu *et al.*^{24,25} We did not detect this polypeptide when either complement fixation positive/enzyme immunoassay negative or complement fixation negative/enzyme immunoassay negative sera were used. These results also agree well with those made with whole *M pneumoniae*

organisms and monoclonal antibodies.^{26,27} In these studies the main antigenic entity of the *M pneumoniae* organism is a high molecular weight protein.

The enzyme immunoassay antigen containing this antigenic polypeptide appears to be reliable in the diagnosis of *M pneumoniae* infection.

We thank Dr Antti Pönkä for some of the patient sera, Viivi Perttunen and Pirjo Sarjakivi for technical assistance, and Anne Brisk for secretarial assistance. This work was supported by grants from the Finnish Medical Society Duodecim and the Finnish Medical Research Council.

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