Immunoblotting patterns with *Mycoplasma pneumoniae* of serum specimens from infected and non-infected subjects

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Summary. Two hundred and ninety-four serum specimens from 248 subjects, whose complement fixation (CF) titres to *Mycoplasma pneumoniae* were known, were further investigated by IgG immunoblotting. After analysis of *M. pneumoniae* proteins by SDS-PAGE, nine polypeptides (p) with mol. wts of 180–43 Kda were selected for immunoblotting studies. Antibodies to *M. pneumoniae* measured by immunoblotting appeared progressively with age; most subjects more than 19 years old gave positive results. For most of the polypeptides, there was an increase in the frequency of band detection when the CF titres were higher. Furthermore, paired serum specimens from 10 patients with *M. pneumoniae* infection, as demonstrated by a rise in CF antibody titre, were tested for IgG blotting patterns. Generally, p180 (the P1 adhesin of *M. pneumoniae*), p172 and p84 were shown to be the dominant targets of the immune response to this organism and may have diagnostic value.

Introduction

Mycoplasma pneumoniae causes a wide range of clinical manifestations, including primary atypical pneumonia. In most cases, the diagnosis of *M. pneumoniae* disease is confirmed by serology. The most widely used method for serodiagnosis is the complement fixation (CF) test with a *M. pneumoniae* glycolipid antigen.¹ Cross-antigenicities are known to occur between this glycolipid and similar antigens of other origins, resulting in non-specific diagnostic reactions.^{2–5}

The use of blotting methods provided a valuable tool for investigating the response to *M. pneumoniae* antigens.^{6–11} These studies pointed out the importance of antibodies to some proteins in the course of acute infection, especially those directed to a high-mol.-wt polypeptide (designated P1 and known to be the major adhesin of *M. pneumoniae*) of 160–190 Kda, according to different authors.^{12,13}

In the present study, we have investigated by immunoblotting the antibody response to *M. pneumoniae* antigens of subjects of all ages, with or without active infection as indicated by CF test results. The aims of this work were: (1) to provide epidemiological data on the distribution of antibodies in relation to age, by the immunoblot technique; (2) to evaluate the immune response to different polypeptides of *M. pneumoniae*; and (3) to define their putative value for improving the diagnosis of *M. pneumoniae* infection.

Materials and methods

Selection of serum samples

Two hundred and ninety-four serum specimens from 248 subjects (152 males, 96 females) were classified diagnostically by the CF test and then analysed by the IgG immunoblotting test. The investigated population comprised 14 hospitalised infants under 1 year old, 73 hospitalised children under 10 years old, 102 hospitalised patients over 10 years old and 59 healthy adult blood donors.

For an epidemiological study (see *Results*, section 2), serum specimens that gave negative results in the CF test (titre < 8) were examined from 191 of the 248 subjects described above. These fell into 10 age-groups (< 1, 1, 2–5, 6–9, 10–19, 20–29, 30–39, 40–49, 50–59 and > 59 years), each of about 20 subjects, and they comprised 59 healthy adults and 132 hospitalised patients; no clinical data were available for most of these patients. All these serum specimens were collected within the same 6-month period.

The remaining serum specimens belonged to selected patients with antibody titres to *M. pneumoniae* of ≥ 8 by CF test (range 8–1024). These included 10 patients with respiratory infection who exhibited a four-fold or more rise in titre by CF test on paired sera (mean time between the two samples: 20.5 SD 14.9 days).

M. pneumoniae strain and antigen preparation

The strain of *M. pneumoniae* (C200) was a gift from Dr S. Bossard (Faculté de Médecine Rockefeller,

Lyon). It was grown at 37°C for 3 days in bottles containing SP4 broth medium.¹⁴ This suspension, containing 10⁸ cfu/ml, was washed three times by centrifugation at 37 000 *g* at 4°C for 30 min with phosphate-buffered saline (PBS; 10 mM sodium phosphate in 13 mM sodium chloride solution), pH 7.2, and sonicated. The antigen was distributed in 0.5-ml volumes and these were stored at -80°C until use.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotting methods

M. pneumoniae proteins were separated on polyacrylamide 7.5% gels at 30 mA for 5 h by the discontinuous system of Laemmli.¹⁵ Samples were diluted 1 in 4 in 0.06 M Tris-HCl buffer, pH 6.8, containing SDS 2% w/v, 2-mercapto-ethanol 5% v/v, glycerol 10% v/v and bromophenol blue 0.01% w/v, and heated at 95°C for 4 min before electrophoresis. The protein was transferred from polyacrylamide gel to polyvinylidene difluoride membrane (Immobilon®, Millipore) electrically¹⁶ at 0.8 mA/cm² of gel/h, by use of a semi-dry Novablot system (LKB-Pharmacia Fine Chemicals). The transfer buffer consisted of 38 mM glycine, 48 mM Tris-HCl, SDS 0.0375% w/v and methanol 20% v/v. After transfer, the membrane sheets were cut into 3–4-mm wide strips, immersed for a few seconds in undiluted methanol and pre-incubated for 1 h at 37°C with 10 mM Tris-saline buffer containing skimmed milk 5% w/v to block non-specific protein binding. The sera, diluted 100-fold in the same buffer, were placed in contact with the strips for 3 h at 37°C with gentle shaking. The blot preparations were then washed six times with 10 mM Tris-saline and incubated for 1 h at 37°C with a peroxidase-conjugated rabbit anti-human IgG (Dakopatts, France), diluted 500-fold in 10 mM Tris-saline containing skimmed milk 0.5% w/v. After a further washing as described, the peroxidase substrate (4-chloro-1-naphthol 0.3% w/v, H₂O₂ 0.003% v/v in 10 mM Tris-saline) was added for a few minutes to develop the coloured reaction. Non-immunological staining of the blots with amido-black 0.1% w/v was performed to control the efficacy of the transfer step.

Complement fixation (CF) test

This test was performed according to the micro-method of Sever.¹⁷ Two HD₅₀ units of complement and 1 unit of *M. pneumoniae* complement-fixing antigen (Behring Institute, Germany) were used in the test. All the sera were incubated at 56°C for 20 min and the fixation time at 4°C was 18 h.

Results

Immunoblotting analysis of M. pneumoniae proteins

The proteins of *M. pneumoniae* were analysed by SDS-PAGE and blotting methods (fig. 1, part A, lane

3). The uninoculated culture medium was treated and analysed in the same conditions used for the *M. pneumoniae* antigen and no bands were detected by SDS-PAGE analysis (date not shown). Human serum specimens eliciting various immunological patterns with *M. pneumoniae* by the CF test (absence of antibodies, high antibody titres, seroconversions) were used to define the major proteins recognised by the immune response. From more than 30 different bands recognised by some samples, nine polypeptides (p) of 180, 172, 120, 115, 92, 84, 66, 55 and 43 Kda, respectively, were selected on the following criteria: (1) these proteins shared mol. wts close to those previously described by other authors;^{7–9, 11} (2) they were not detected in serum specimens from infants of 12–24 months without evidence of *M. pneumoniae* infection; (3) they were recognised with a higher frequency (see results below) in some other specimens. Some typical patterns are illustrated in fig. 1, part B.

Epidemiological data from CF-negative subjects according to age

One hundred and ninety-one serum specimens with CF titres <8 were tested by immunoblotting, to investigate the age-distribution of specific anti-*M. pneumoniae* antibodies in non-infected subjects (fig. 2). In infants <1 year, a few reactive bands, probably corresponding to maternal antibodies, were observed. As expected, the number of bands detected increased between the ages of 2 and 19 years, the usual period for *M. pneumoniae* primary infection. Most of the subjects older than 19 years exhibited antibodies to *M. pneumoniae*, more especially those against proteins of high mol. wt; antibodies to p180 appeared to be the most interesting marker of seropositivity, being present in 80–90% of subjects >19 years old.

Frequency of antibodies to M. pneumoniae proteins in relation to CF-test status

All 294 serum specimens from the 248 subjects were examined for anti-*M. pneumoniae* IgG antibodies by immunoblotting. The sera were divided into three groups according to their CF titres: <8 (negative), 8–32 (weak positive) and >32 (strong positive), respectively. Generally, the higher the CF titre of a serum, the more polypeptide bands were detected (table I). However, in subjects with high CF titres (>32), only three of the nine marker proteins elicited specific antibodies at a frequency >80%—p180, p172 and p84 (table I).

Immunoblotting patterns of subjects according to M. pneumoniae immune status

In the light of the results (fig. 2) defining the presence of anti-*M. pneumoniae* antibodies in relation to age, we considered the possibility that, in subjects

Fig. 1. Blotting analysis of *M. pneumoniae* proteins, as indicated by amido-black staining (A, lane 3) and by immunological staining, on human serum specimens with various reactivities to *M. pneumoniae*: negative (B, lane 4), low positive (B, lanes 5 and 6), high positive (B, lanes 7, 8 and 9). Mol. wt standards (BioRad) are shown in A, lanes 1 and 2. (a, photographs of original gel and membrane; b, diagrammatic representation.)

under 19 years old, CF titres > 32 might be related to primary infection whereas, beyond 19 years, CF titres > 32 were more likely to reflect re-infections. In our own experience¹⁸ and that of others,^{19,20} CF titres of ≥ 64 are considered to be presumptive evidence of recent infection. Therefore, we divided the population into four groups according to age and serum-CF titres (table II), to investigate whether antibodies to certain polypeptides could help distinguish primary infections

from re-infections. In group II (age 1–19; CF titre > 32), presumed to include mainly primary infections, all the polypeptides were detected markedly more often than in group I (age 1–19; CF titre < 8). On the contrary, in group IV (age > 19; CF titre > 32), presumed to represent mainly re-infections, a single protein (p172) elicited a significantly increased immune response in comparison with group III (age > 19; CF titre < 8) (table II).

Fig. 2. Immunoblotting study of antibodies to nine *M. pneumoniae* marker proteins (p180: ●, graph a; p172: ○, graph a; p120: ▲, graph a; p115: ●, graph b; p92: ○, graph b; p84: ▲, graph b; p66: ●, graph c; p55: ○, graph c; p43: ▲, graph c) in 191 CF-test negative subjects according to age.

Immunoblotting analysis of paired sera with a four-fold or more CF antibody-titre rise

Particular attention was given to the blotting patterns of paired serum specimens from 10 patients who exhibited a four-fold or greater rise of antibody titre by the CF test (table III). In all but two (nos. 8 and 10, table III) of the 10 patients, a CF antibody rise was correlated with an increase in the number of bands detected by immunoblotting. Six of the 10 patients (nos. 1–6, table III) presented very few bands (0–2) in the early-phase serum specimen, whereas, for

the other four patients (nos. 7–10), the first serum specimen already gave positive reactions with several of the *M. pneumoniae* proteins. The only antigens for which antibodies were detected in the convalescent-phase specimens of all 10 patients were p180 and p84.

Discussion

The epidemiological data demonstrated that the frequency of protein-band detection was correlated

Table I. Frequency of IgG antibodies to *M. pneumoniae* proteins by immunoblotting in relation to CF-test results

CF titre	Number of sera	Percentage of sera with polypeptide (Kda) band in IgG blot								
		p180	p172	p120	p115	p92	p84	p66	p55	p43
< 8	204	55	39	36	41	28	47	37	44	22
8–32	33	88	79	42	58	24	70	58	76	64
> 32	57	100	95	53	63	49	83	70	70	61

Table II. Comparison of IgG antibodies to *M. pneumoniae* proteins by immunoblotting between presumed uninfected and infected subjects (indicated by CF titre) of younger and older age-groups

Group	Age (years)	CF titre	Number of subjects	Number (%) of subjects with polypeptide band (Kda) in IgG immunoblot								
				p180	p172	p120	p115	p92	p84	p66	p55	p43
I	1-19	<8	78	15(19)	11(14)	10(13)	16(21)	12(15)	23(29)	24(31)	22(28)	10(13)
II	1-19	>32	26	26(100)*	25(96)*	12(46)*	15(58)*	10(38)†	18(69)*	15(58)†	18(69)*	17(65)*
III	>19	<8	113	88(78)	67(59)	62(55)	67(59)	45(40)	68(60)	45(40)	62(55)	32(28)
IV	>19	>32	15	15(100)	13(87)‡	8(53)	10(67)	8(44)	12(80)	11(73)	9(60)	7(47)

*p < 0.001 by Yates' χ^2 test, compared with group I.†p < 0.05 by Yates' χ^2 test, compared with group I.‡p < 0.05 by Yates' χ^2 test, compared with group III.**Table III.** Immunoblotting results for 10 patients exhibiting a significant rise of *M. pneumoniae* CF-antibody titres in paired serum specimens

Patients			Acute serum specimen		Late serum specimen	
No.	Age (years)	Sex	CF titre	Number of polypeptide bands (Kda)	CF titre	Number of polypeptide bands (Kda)
1	3	M	<8	1 (66)	32	3 (180, 84, 66)
2	4	M	<8	2 (84, 55)	128	8 (180, 172, 120, 115, 84, 66, 55, 43)
3	4	M	<8	0	32	8 (180, 172, 120, 115, 84, 66, 55, 43)
4	6	M	<8	0	64	6 (180, 172, 92, 84, 66, 43)
5	19	M	<8	2 (55, 43)	1024	7 (180, 172, 115, 92, 84, 55, 43)
6	19	F	8	1 (115)	1024	5 (180, 172, 115, 84, 55)
7	12	M	<8	4 (180, 172, 84, 66)	64	9 (180, 172, 120, 115, 92, 84, 66, 55, 43)
8	26	F	<8	8 (180, 172, 120, 115, 84, 66, 55, 43)	64	9 (180, 172, 120, 115, 92, 84, 66, 55, 43)
9	26	M	32	4 (180, 66, 55, 43)	128	9 (180, 172, 120, 115, 92, 84, 66, 55, 43)
10	46	M	8	5 (180, 172, 115, 84, 55)	64	5 (180, 172, 115, 84, 55)

with the age of subjects over the range 2-19 years (fig. 2); the presence of specific antibodies in some of the young children agrees with the study of Sakurai *et al.*,²¹ who isolated *M. pneumoniae* with high frequency in children with respiratory disease up to the age of 3 years, suggesting that the pathological role of this organism may be more important in the 3-6 years age-group than previously suspected.

We tried to evaluate whether antibodies to any of the polypeptides would enable distinction between primary infections and re-infections (table II). However, no clear difference was noted between the IgG immunoblotting patterns of sera from patients believed to fall within these two categories. Even at a presumed distance in time from active infection in the case of older group-III subjects with low CF-antibody titres (table II), antibodies to all the marker proteins, with the exception of p172, were present at a high frequency. The latter antigen was detected significantly more frequently in group IV subjects, although the diagnostic value of this finding seems limited.

All 10 patients with indications of recent infection (paired sera showing CF antibody rise) showed increasing numbers of reactive bands. Six of these could be considered as primary infections, since little or no antibody was detected in early-phase serum by immunoblotting, but, in the four remaining cases, it was not evident whether acute-phase reactions repre-

sented a later stage of primary infection or a re-infection (table III).

As a whole, these results tend to confirm the role of protein P1 (p180 in our hands) as the dominant antigen of *M. pneumoniae*, as demonstrated by the following data: (1) detection of antibodies to P1 was age-related, being infrequently present before 5 years of age, but present in >80% of the subjects beyond 10 years; (2) subjects with high CF titres were also constantly positive for anti-p180 antibody by immunoblotting; (3) all paired-serum specimens exhibiting a significant rise in *M. pneumoniae* CF antibodies were positive for anti-p180 antibodies. The presence of p180 antibodies in 19% of the younger CF-negative subjects (group 1 in table II) is probably a reflection of previous *M. pneumoniae* infection and not of non-specificity of these antibodies. All these data provide encouragement to develop tests based upon protein P1 for diagnostic purposes. Preliminary tests with immunoenzymatic assays, with either the whole P1 protein^{10,22,23} or synthetic peptides corresponding to immunodominant epitopes of P1,²⁴ have already given interesting results.

The antigen p84 is another interesting target of the immune response. As with p180, antibodies to p84 were present in the convalescent-phase serum specimens of the 10 selected patients with CF antibody rises. The p84 protein, with p180 and p172, was one

of three recognised with a frequency of >80% in subjects with high CF titres (table I) and its frequency increased significantly in parallel with rising CF antibody titres (table II).

The results of these IgG blotting patterns with a large number of serum specimens suggest a potential diagnostic value for some polypeptides during the

course of *M. pneumoniae* infection, especially p180, p172 and p84. Should IgM and IgA affinity for these three proteins also be demonstrated, the immunoblotting technique may provide a particularly valuable tool for the diagnostic confirmation of current infection.

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