

Serological Response to *Chlamydia pneumoniae* Infection

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The human serological response was analyzed by using sera from patients who were serologically positive but isolation negative for *Chlamydia pneumoniae* and from patients with proven *C. pneumoniae* infection based on serology and isolation. To assess whether seroreactivity to *C. pneumoniae* proteins had potential diagnostic value, the cross-reactivities of these sera to other *Chlamydia* species and of sera from patients infected with *C. trachomatis* and *C. psittaci* to *C. pneumoniae* proteins were determined. In all serum samples from patients with proven *C. pneumoniae* infections, reactivities were seen with 98-, 68-, 60-, 39.5-, and 30-kilodalton proteins. Similar patterns were seen in sera from patients who were serologically positive and isolation negative. The onset of seropositivity for *C. pneumoniae* was accompanied by reactivities against presumably shared chlamydial antigens and a *C. pneumoniae*-specific 98-kilodalton protein.

Chlamydia pneumoniae, formerly known as *Chlamydia* sp. strain TWAR (10), has been shown to be an important human respiratory pathogen that causes acute respiratory diseases and pneumonia (11-13, 15, 23). Infection with *C. pneumoniae* is worldwide, with 40 to 60% of individuals tested in each population having *C. pneumoniae* antibody (13, 26). *C. pneumoniae* has been associated with both epidemic and endemic occurrences of acute respiratory disease (11-13, 15, 23). Diagnosis of *C. pneumoniae* infection is based on isolation of the organism, by serological tests, or both. The serological tests used are the micro-immunofluorescence (micro-IF) test with *C. pneumoniae* elementary body antigen and the complement fixation test. The micro-IF test is specific for *C. pneumoniae*, while the complement fixation test measures genus-reactive antibodies that recognize the lipopolysaccharide. Recently, Ladany et al. (18) have developed an extracted antigen enzyme-linked immunosorbent assay for differentiating immunoglobulin G (IgG) antibodies that recognize *C. trachomatis* from those that recognize *C. pneumoniae*. Immunoblot analyses with anti-*C. pneumoniae* rabbit immune serum have demonstrated the presence of antibodies that recognize determinants that are shared among the *Chlamydia* spp. and apparent *C. pneumoniae*-specific determinants (4, 18). In order to investigate *C. pneumoniae* infection further, the humoral immune response was analyzed, and the results are described here.

MATERIALS AND METHODS

Strains. The *Chlamydia* strains used in this study included the following: *C. pneumoniae* TW-183, AR-39, AR-388, and LR-65 (11, 16); *C. trachomatis* B/TW-5/OT and L2/434/Bu (25); and *C. psittaci* 6BC (9) and meningopneumonitis (8). All *Chlamydia* strains were adapted to grow in HeLa 229 cells (17). The organisms were harvested and purified in a linear gradient of meglumine diatrizoate (Hypaque-76; Winthrop-Breon Laboratories, New York, N.Y.) (14). The final products usually contained 1.0×10^8 to 5.0×10^8 inclusion-forming units per ml of organisms.

Antisera. The human sera used in this study were selected from our serum bank based on micro-IF (26) and complement fixation test serologies. The criteria used for the

serological diagnosis of a current infection with *C. pneumoniae* in the micro-IF test were a fourfold antibody titer rise or, in a single serum sample, an IgM titer greater than 1:16 or an IgG titer greater than 1:512 (13, 26). An IgG titer greater than or equal to 1:16 and less than 1:512 is considered indicative of past infection (13, 26). IgG and IgM titers of $\leq 1:8$ are considered negative. The serum samples tested included those from patients who were (i) seropositive for *C. pneumoniae* and from whom the organism was isolated (three to five sequential serum samples following the course of infection were available from each patient [$n = 4$]; these isolates were designated AR-277, AR-231, AR-442, and AR-497); (ii) seropositive for *C. pneumoniae* but isolation negative ($n = 8$); (iii) seropositive for *C. trachomatis* only (included in group iii were sera from adults with low-titer *C. trachomatis* antibody [IgG titer, $< 1:32$; $n = 3$], babies with high-titer *C. trachomatis* antibody [IgG titer, $> 1:512$; $n = 2$], and patients with lymphogranuloma venereum [IgG titer, $\geq 1:512$; $n = 3$]); (iv) seropositive for *C. psittaci* and *C. pneumoniae* ($n = 1$); (v) complement fixation test positive but seronegative for *C. pneumoniae* and *C. trachomatis*, evidently *C. psittaci* ($n = 1$); and (vi) seronegative for all *Chlamydia* spp. ($n = 4$).

Separation of chlamydial antigens by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins of purified chlamydial elementary bodies were separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (19) with 12.5% polyacrylamide gels. Approximately 20 μ g of protein, as determined by the method of Lowry et al. (20), was added to the gels. Protein bands were visualized by staining the gels with Coomassie brilliant blue. Molecular weights were determined by using the protein standards (Pharmacia Fine Chemicals, Piscataway, N.J.) phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100) and α -lactalbumin (14,400).

Immunoblot analysis. Western blots (immunoblots) were performed by the method of Towbin et al. (24). Following transfer, blots were blocked for 1 h with Tris-buffered saline (TBS)-3% bovine serum albumin, incubated overnight with human immune serum (diluted 1:20) in TBS-1% bovine serum albumin-0.05% Tween 20, washed with TBS-0.05% Tween 20 (six times for 5 min each time), incubated with goat anti-human immunoglobulin (diluted 1:1,000) conju-

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TABLE 1. Serological responses by micro-IF and immunoblot analyses in sequential serum samples from patients with *C. pneumoniae* infection proven by isolation

Patient isolate	Time (days) sera obtained after onset ^a	Micro-IF titer ^b		Recognition of the following <i>Chlamydia</i> proteins by immunoblot analysis ^c :															
		IgM	IgG	98-kDa			68-kDa			60-kDa			MOMP			30-kDa			
				Pn	Tr	Ps	Pn	Tr	Ps	Pn	Tr	Ps	Pn	Tr	Ps	Pn	Tr	Ps	
AR-277	4	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	21	128	16	+++	-	-	++	+++	++	+++	++	++	++	+	+	+	+	+	(+)
	49	512	128	+++	-	-	+	+++	++	+++	++	++	+	+	+	+	+	+	(+)
	140	0	64	++	-	-	+	++	++	++	++	++	+	+	+	+	(+)	+	(+)
	320	0	32	++	-	-	(+)	+	++	+++	++	++	-	-	-	-	-	(+)	-
	580	0	16	-	-	-	(+)	(+)	++	+	-	-	-	-	-	-	(+)	-	-
AR-231	16	8	0	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	42	16	32	+	-	-	++	++	++	+	-	-	+	+	++	+	-	-	(+)
	408	0	512	++	-	-	(+)	(+)	(+)	+	-	-	+	(+)	(+)	-	-	-	-
AR-442	3	8	0	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-
	17	256	32	+	-	-	++	+	++	+	+++	++	(+)	-	-	(+)	-	-	++
	47	64	512	++	-	-	++	+	++	+++	++	-	+	+	+	(+)	+	-	-
	103	0	128	++	-	-	++	-	+	++	++	-	+	++	+	(+)	-	-	-
	632	0	16	-	-	-	(+)	-	-	-	+	-	-	-	-	-	-	-	-
AR-497	25	512	32	+++	-	-	+	+	+	+	-	+	+	+	+	-	+	-	-
	40	1,024	256	+++	-	-	+	+	+	+	-	+	+	+	+	-	+	-	-
	53	256	256	+++	-	-	+	+	+	+	-	+	+	+	+	-	+	-	-
	88	16	128	+++	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-
	606	0	16	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+

^a Time after onset of symptoms.

^b Titer is the reciprocal of serum dilutions.

^c Symbols: -, no reaction; (+), weak reaction; +, ++, and +++, successively stronger reactions. Abbreviations: Pn, *C. pneumoniae*; Tr, *C. trachomatis*; Ps, *C. psittaci*.

gated to horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo.) for 1 h, and washed six times with TBS-0.05% Tween 20 followed by two washes with TBS. Color development was observed on the addition of 4-chloro-1-naphthol (Sigma). Protein transfer was monitored by staining the blots with India ink, and molecular weight markers were visualized by using a gold stain (Aurodyne; Janssen Life Science Products, Piscataway, N.J.).

RESULTS

Immunoblotting with human serum. The reactivities of sera obtained from patients with proven *C. pneumoniae* infection are summarized in Table 1. Representative immunoblots of sera with *C. pneumoniae*, *C. trachomatis*, and *C. psittaci* are presented in Fig. 1. Because of the various responses of sera from different individuals, only the reactivities that were common in the sera from all or the majority of patients are emphasized. As shown in Fig. 1a, when the acute-phase serum sample was compared with the convalescent-phase serum sample from a patient with proven *C. pneumoniae* infection, the emergence of antibodies to several chlamydial proteins was observed simultaneously with seroconversion. A strong reaction was observed with 98-, 68-, and 60-kilodalton (kDa) proteins. This pattern of reactivity was observed in all seropositive sera tested from the four individuals with proven *C. pneumoniae* infection (Table 1). All but one serum sample recognized proteins of 30 and 49 kDa; and all serum samples had very weak reactivities with the *C. pneumoniae* (39.5 kDa), *C. psittaci*, and usually, *C. trachomatis* major outer membrane proteins (MOMPs). Of the predominant reactivities that were observed, all but the 98-kDa protein appeared to be shared among the *Chla-*

mydia spp. This reactivity persisted through the course of seropositivity.

Another set of five serum samples from a seropositive, isolation-positive individual (AR-442) provided a unique opportunity to follow the appearance and disappearance of antibody to *C. pneumoniae* proteins. These serum samples were collected over an approximately 2-year period and consisted of acute-phase sera with an antibody titer considered to be negative, followed by samples containing various titers and by a final serum sample that had an extremely low titer. This is a usual finding because *C. pneumoniae* antibody persists for long periods of time (26). No reactivity to the 98-kDa protein was observed in the first serum sample, a strong reactivity was observed in the next three serum samples, and no reactivity was observed with the 98-kDa protein when the titer decreased to a very low level (Table 1). There were no proteins with similar molecular weights recognized by these serum samples in either *C. trachomatis* or *C. psittaci*.

All serum samples from *C. pneumoniae*-seropositive, isolation-negative patients (IgM titer, $\geq 1:125$) that were examined recognized a 98-kDa *C. pneumoniae* protein (Fig. 1b). Again, this reactivity was *C. pneumoniae* specific because no recognition of analogous *C. trachomatis* or *C. psittaci* proteins was observed. The spectrum of protein recognition by serum samples from seropositive, isolation-negative patients was similar to that by serum samples from isolation-positive patients. Antibodies to the 98-, 68-, 60-, and 49-kDa proteins were consistently present; and most reacted with a 30-kDa protein. A very weak reaction was observed with the chlamydial MOMPs.

In all sera tested from individuals with *C. trachomatis*

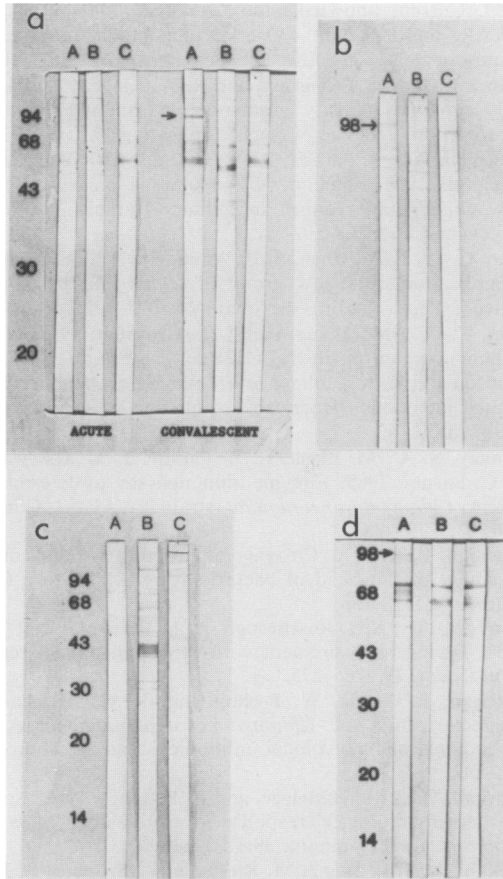


FIG. 1. Immunoblot analysis of whole-cell lysates of *Chlamydia* elementary bodies with human sera. Whole-cell lysates of the different *Chlamydia* spp. were electrophoresed through a 13% sodium dodecyl sulfate-polyacrylamide gel, electrophoretically transferred to nitrocellulose, and incubated with sera from a patient (AR-277) with proven *C. pneumoniae* infection (a), with sera from an individual who was seropositive for *C. pneumoniae*, but from whom the organism was not isolated (b), with sera from an individual with antibody against *C. trachomatis* only (c), and with sera from an individual with a *C. psittaci* infection who was also seropositive for *C. pneumoniae* (d). For each panel, the lanes are as follows: A, *C. pneumoniae*; B, *C. trachomatis*, and C, *C. psittaci*.

infection, no reactivity was observed with the 98-kDa protein of *C. pneumoniae* (Fig. 1c). Unlike the weak recognition of sera from patients infected with *C. pneumoniae* for the homologous MOMP, a strong reactivity with the *C. trachomatis* MOMP was observed with sera containing *C. trachomatis* antibody. In the serum sample from the patient that was seropositive for both *C. psittaci* and *C. pneumoniae*, reactivity with the 98-kDa *C. pneumoniae* protein was observed (Fig. 1d).

Several serum samples from individuals who were seronegative for all *Chlamydia* spp. were tested. No serum sample recognized the 98-kDa protein, while one serum sample recognized the 60-kDa protein that was cross-reactive among the *Chlamydia* spp.

DISCUSSION

Analysis of the human humoral response to *C. pneumoniae* infection showed a similar spectrum of antigen recog-

nition by sera from patients infected with *C. pneumoniae* as that by anti-*C. pneumoniae* rabbit immune sera (4). Both anti-*C. pneumoniae* rabbit immune sera and human sera recognized immunoreactive proteins of 98, 68, 60, and 30 kDa and the 39.5-kDa MOMP. Unlike sera from *C. trachomatis*- and *C. psittaci*-infected patients, which exhibited strong reactivities to the homologous MOMPs, sera from *C. pneumoniae*-infected patients demonstrated weak reactivities with the *C. pneumoniae* MOMP. Generally, these reactivities also extended to the analogous *C. psittaci* and *C. trachomatis* MOMPs, suggesting that the recognition was a genus reactivity. Analogous results were observed with rabbit immune sera against the different *Chlamydia* spp. (4). The similar weak recognition of human sera for the *C. pneumoniae* MOMP and the cross-reactivities with the MOMPs of the other *Chlamydia* spp. give further support to our previous suggestion that the *C. pneumoniae* MOMP is less immunogenic and antigenically complex than are the MOMPs of the other *Chlamydia* spp.

Analysis of antigens recognized during human *C. trachomatis* (3, 22, 27) or guinea pig *C. psittaci* (2) infections has demonstrated that most have antibodies against 68- and 60-kDa proteins and the MOMP (2, 3, 22, 27) and that both the MOMP and the 60-kDa proteins contain genus-reactive determinants (1, 6, 7, 21, 22). Moreover, a 75-kDa protein that contains genus-reactive determinants is recognized during both *C. trachomatis* (21) and *C. pneumoniae* (5) infections. Ladany et al. (18) have also reported cross-reactive antigens of *C. trachomatis* and *C. pneumoniae* by Western blot (immunoblot) analysis by using rabbit hyperimmune sera. Thus, in addition to the genus-reactive determinant found in the lipopolysaccharide, there appear to be several antigenic reactivities that have been previously shown to be shared among *C. trachomatis* and *C. psittaci* that also extend to *C. pneumoniae*.

The onset of seropositivity indicative of *C. pneumoniae* infection was accompanied by reactivities that were shared among the species and by a 98-kDa protein that appeared to be *C. pneumoniae* specific. In immunoblot experiments with rabbit immune sera against *C. trachomatis*, *C. psittaci*, and *C. pneumoniae*, only the recognition of the 98-kDa protein was *C. pneumoniae* specific (4). An immunoreactive protein with a similar molecular weight containing a genus-reactive determinant has been reported for *C. trachomatis* (21). However, none of the sera tested from *C. pneumoniae*-infected patients that reacted with the 98-kDa protein recognized the *C. trachomatis* protein, suggesting that the reactive epitope is *C. pneumoniae* specific.

Proteins with other molecular weights have been suggested to have antigenic reactivities that are specific for *C. pneumoniae*. Immunoblot analysis of human serum samples that were reactive with *C. pneumoniae* or *C. trachomatis* by enzyme-linked immunosorbent-assay demonstrated *C. pneumoniae*-specific protein bands at a molecular mass range of 50 to 60 kDa (18). On the other hand, 30- and 80-kDa *C. pneumoniae* proteins were recognized by anti-*C. pneumoniae* rabbit immune serum but not by anti-*C. trachomatis* rabbit immune serum (18). Although we observed reactivities of human sera from patients with *C. pneumoniae* infections with protein bands in the same apparent molecular mass range (50 to 60 kDa), the recognition extended to *C. psittaci*, *C. trachomatis*, or both.

In an analysis of both rabbit and human serum samples, while a plethora of reactivities were observed, only recognition of the 98-kDa protein band was observed in all samples tested to be specific for *C. pneumoniae*. Our finding

that seropositivity for *C. pneumoniae* is associated with the appearance of antibody against a protein that appears to have a species-reactive determinant represents a potential diagnostic tool for the recognition of *C. pneumoniae* infections.

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