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Tear and Serum Antibody Response to *Chlamydia trachomatis* Antigens during Acute Chlamydial Conjunctivitis in Monkeys as Determined by Immunoblotting

HARLAN D. CALDWELL,[1]* SCOTT STEWART,[1] SHIRLEY JOHNSON,[2] AND HUGH TAYLOR[2]

Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840,[1] and Wilmer Institute, Johns Hopkins Medical Institute, Baltimore, Maryland 21205[2]

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In this study, we examined the temporal antibody response by immunoblotting analysis in tears and sera of three cynomolgus monkeys (*Macaca fasicularis*) with primary acute *Chlamydia trachomatis* serovar B conjunctivitis. The objective was to identify chlamydial antigens stimulating antibody during the host responses in the course of this self-limiting infection with the rationale that they may be protective antigens. The major outer membrane protein (MOMP), lipopolysaccharide (LPS), and polypeptides of 60 and 68 kilodaltons (kDa) were the predominant antigens recognized by immunoglobulin A (IgA) in monkey tears. Tear IgA antibody specific for the MOMP was first detected 14 days postinfection, whereas tear IgA reactive with LPS or the 68- and 60-kDa polypeptides was first detectable on day 21. Tear IgA antibodies specific for each of these antigens persisted in tears through day 56, 4 weeks after both peak clinical disease and recovery of the organism from the conjunctivae. In contrast, tear IgG antibodies peaked at approximately 28 days postinfection, the time of maximal inflammatory response. The IgG response in monkey sera was similar to that observed for tear antibodies, in that the MOMP, 60-, and 68-kDa polypeptides were the primary immunogens. The exception was that IgG antibody against these antigens was detected 1 week later than that observed for tear IgA antibodies. Of three monkeys that responded with tear IgA antibody against LPS, one did not have detectable serum IgG LPS antibody. The specificity of the tear IgA antibody response of monkeys was determined by immunoblotting nine other *C. trachomatis* serovars in addition to the homologous B serovar. The tear IgA response to the MOMP was predominantly B complex subspecies-specific (serovars B, Ba, D, and E), whereas the response to chlamydial LPS was found to be species-specific. The significance of these observations in relation to previous vaccine studies in nonhuman primates is discussed.

Trachoma is a blinding disease that results from repeated or recurrent infection of the conjunctival epithelium of humans with *Chlamydia trachomatis*. The etiologic agents of trachoma are the *C. trachomatis* serovars A, B, and C. In developing countries of the world, an estimated 500 million persons are afflicted by the disease, of whom approximately 7 million are blind (5). Preventive measures are clearly needed to control trachoma. Although chlamydiae are sensitive to antibiotics, mass systemic antibiotic therapy is not practical due to administrative problems associated with supervision of treatment of large numbers of people and to the cost of antibiotics and their possible adverse side effects (6). Immunoprophylaxis has not proven to be an effective means of preventing trachoma. In early vaccine trials in humans, elementary bodies produced only a short-lived protective immunity (7, 10) and induced a deleterious hypersensitivity to infection (9, 10). These results suggested that both a protective and a deleterious immune response were produced after immunization with whole chlamydiae. Thus, a successful vaccine for trachoma will require identification of the protective and deleterious antigens.

We have recently shown that the pathogenesis of chlamydial guinea pig inclusion conjunctivitis is mediated by a delayed-type hypersensitivity reaction, and the antigen that elicits this deleterious hypersensitivity is common to the genus *Chlamydia* (22). Similar observations have also been recently described in the cynomolgus monkey model for trachoma (20). The immune mechanisms that function in protective immunity are not known. Previous studies in monkeys indicate that protective immunity is specific for the infecting trachoma serovar (8, 21) and that tear immunoglobulins may play an important role in mediating this immune response (1, 15). In this report, we studied by immunoblotting the antibody response to chlamydial antigens with tears from monkeys with primary acute *C. trachomatis* conjunctivitis. Our goal was to identify antigens that induce tear antibodies specific for the infecting trachoma serovar and whose appearance in tears coincides with the resolution of infection. The hope is that these antigens may be useful molecules in the development of a subunit trachoma vaccine.

MATERIALS AND METHODS

Organisms. The *C. trachomatis* serovars/strains used were A/Har-13, Ba/Apache-2, C/Har-32, E/Bour, F/IC-Cal-3, purchased from the American Type Culture Collection, Rockville, Md., and strains B/TW-5/OT, D/UW-3/Cx, G/UW-57/Cx, H/UW-4/Cx, and I/UW-12/Ur, which were originally obtained from C.-C. Kuo, University of Washington, Seattle. Chlamydiae were grown in HeLa 229 cells, and elementary bodies were purified by discontinuous density gradient centrifugation in Renografin (E. R. Squibb & Sons, Princeton, N.J.) as previously described (3).

* Corresponding author.

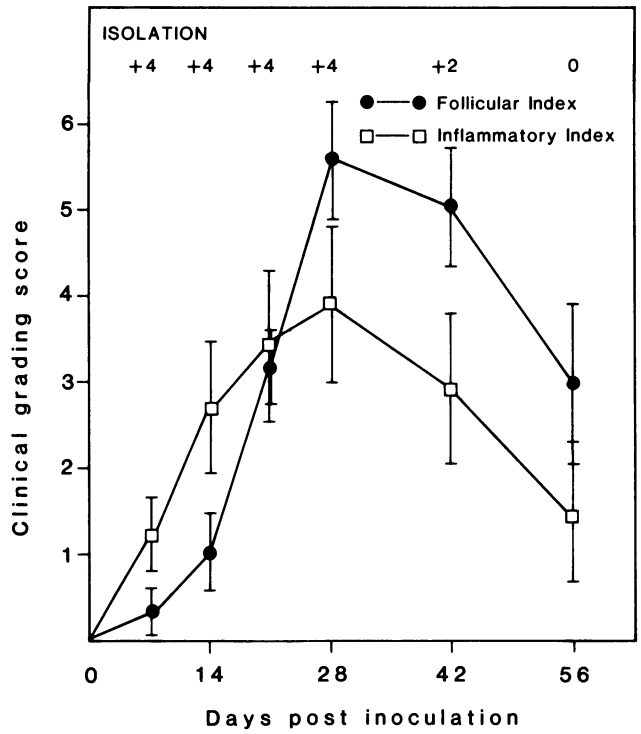

FIG. 1. Clinical and microbiological evaluation of cynomolgus monkeys with experimental eye infections with *C. trachomatis* serovar B. The graph shows the mean aggregate inflammatory and follicular grading scores for the right and left eyes of three monkeys and the isolation results during the course of experimental infection. Inflammatory and follicular scores at each point are the mean values from the three animals plus or minus the standard error of the mean. The isolation results at each time point are expressed as the number of inclusions observed by immunofluorescence staining of McCoy cell monolayers inoculated with clinical specimens 48 h before staining. 0, negative; 1, 1 to 9 inclusions at ×100 magnification; 2, 10 to 20 inclusions per microscopic field at ×100 magnification; 3, 1 to 9 inclusions at ×600 magnification; 4, greater than 10 inclusions per microscopic field at ×600 magnification.

Animals. Three adult male cynomolgus monkeys (*Macaca fasicularis*), designated Z-1, Z-2, and Z-3, were inoculated with 2,000 50% infective doses of *C. trachomatis* serovar B in the conjunctival sac of each eye on day 0. The clinical course of infection was monitored by using an inflammatory and follicular scoring system (19). The inflammatory response was the aggregate score for the severity of papillae, bulbar conjunctival injection, chemosis, and ocular discharge. The response was ascertained separately for each eye of each animal, and the mean score was determined for each monkey. The follicular response, the aggregate score of the grading of tarsal, bulbar, and limbal follicles, was also a mean score of the response of both eyes of each animal.The collection of blood and tears and the isolation of chlamydiae were also done as previously described (19). In this study, only primary isolation data were included in the results.

Immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting were essentially done as previously described (2). Preparatory sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on whole-cell lysates of the B serovar with a 12.5% gel. After electrophoresis, polypeptides were electrophoretically transferred to nitrocellulose paper (NCP) (HAHY; Millipore Corp., Bedford, Mass.), and the entire NCP sheet was incubated with BLOTTO (11) for 30 min at room temperature. The NCP was then cut into 0.5-cm-wide strips, and individual strips were incubated with samples containing tear antibody or sera. Tear antibody was eluted from cellulose sponges (0.5 by 0.5 cm; Weck-Gel Surgical Spears, Edward Weck & Co., Research Triangle Park, N.C.) in 1 ml of BLOTTO by incubation overnight at 4°C. The sample was further diluted (1:5) in BLOTTO, and 5 ml of the dilution was used for immunoblotting. Sera were diluted 1:50 in BLOTTO, and 5 ml of the serum dilution was used for immunoblotting. Tears and sera collected from individual monkeys at specified times pre- and postinfection were incubated with NCP strips containing chlamydial antigens overnight at room temperature. NCP strips were washed with 25 mM sodium phosphate–0.15 M sodium chloride, pH 7.2 (phosphate-buffered saline [PBS]) and probed to detect immunoglobulin G (IgG) or IgA. For IgG detection, NCP was incubated with 5 ml of ^{125}I-protein A (5×10^4 cpm/ml) in PBS for 2 h at room temperature. The NCP strips were then washed in PBS, air dried, and subjected to autoradiography with Kodak X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) and a Lightning-Plus intensifying screen (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) overnight at −70°C. For IgA detection, NCP strips were incubated overnight with the diluted specimen, washed in PBS, and incubated with a 1:200 dilution of rabbit anti-monkey IgA (Fc specific) (Nordic Immunological Laboratories, El Toro, Calif.) for 2 h at room temperature. The NCP was washed in PBS, probed with ^{125}I-protein A, and processed for autoradiography as described above.

RESULTS

Clinical observations. The clinical course and isolation of chlamydiae during experimental *C. trachomatis* infection of the three cynomolgus monkeys used in this study are shown in Fig. 1. The clinical disease has been described in detail elsewhere (19). Briefly, 1 week after infection (day 7), all monkeys had acute chlamydial follicular conjunctivitis. Maximal clinical disease occurred at day 28 postinfection. Maximal recovery of chlamydiae from the upper conjunctivae of monkeys was obtained on days 7 through 28 postinoculation. The number of chlamydiae recovered from the conjunctivae decreased substantially after day 28, and all three monkeys were primary culture negative on day 56. Chlamydiae were recovered from one monkey (Z-1) on day 56 on the second cell culture passage.

Immunoblotting analysis of tear antibodies. Tear specimens collected from monkeys before and during experimental infection were analyzed by immunoblotting with the homologous B serovar as test antigen (Fig. 2). Immunoblots were probed to detect tear IgG (Fig. 2A) and IgA (Fig. 2B) antibodies. No preexisting tear antibodies reactive with chlamydial antigens were found in animals Z-2 and Z-3 before infection (day −7). Animal Z-1 had preexisting tear IgA which reacted with the 45-kilodalton (kDa) protein. All three monkeys had detectable IgG antibody specific for the major outer membrane protein (MOMP) on day 21 postinfection (Fig. 2A). As judged by the intensity of the autoradiographic signal, this response peaked on day 28 in tears of two of the three monkeys (Z-2 and Z-3) and was poorly to not detectable by day 56. Tear IgG from monkeys Z-2 and Z-3 recognized additional high-molecular-mass components, 60 and 68 kDa, respectively. Only monkey Z-2 had detectable IgG antibodies specific for chlamydial lipopolysaccharide (LPS), and this response was observed only on day 28 postinfection.

The immunoblot profiles shown in Fig. 2B represent both tear IgG and IgA antibody responses to chlamydial antigens. Differences in the autoradiographic signals of immunoreactive antigens or the appearance of immunoreactive antigens not observed in Fig. 2A was interpreted as indicating chlamydia-specific IgA antibodies. Tear IgA antibodies reactive with the MOMP were first detected on day 14 postinfection in all three monkeys. The MOMP IgA antibodies increased dramatically by day 21 and were present in tears through day 56. Tear IgA antibodies reactive with the 60- and 68-kDa proteins and LPS were first detected on day 21 postinfection in animals Z-2 and Z-3 and on day 28 in animal Z-1 and were present through day 56.

Immunoblotting analysis of serum antibodies. The serum IgG antibody responses of monkeys Z-1, Z-2, and Z-3 are shown in Fig. 3. The MOMP was the primary antigen recognized by all three monkeys, with antibody first detected at day 21 postinfection. In addition to the MOMP, each animal also recognized the 60-kDa polypeptide. Animals Z-2 and Z-3 had detectable IgG against the 60-kDa protein on day 21, whereas animal Z-3 first recognized this protein on day 28. Antibodies to the MOMP and the 60-kDa protein were detectable through day 56. No preexisting IgG antibodies were observed for either the MOMP or the 60-kDa protein. A weak but detectable antibody response was observed in sera of animals Z-1 and Z-2 to the 68-kDa protein on day 28, whereas monkey Z-3 had preexisting antibodies to this protein. Antibodies reactive with a 45- and a 32-kDa protein were found in sera of monkeys Z-2 and Z-3, respectively; however, antibodies specific for these antigens were also present before chlamydial infection. Monkeys varied in their serum IgG response to chlamydial LPS. Monkey Z-2 had serum IgG antibody against LPS that was first detected on day 21 postinfection and remained detectable through day 56. Monkey Z-3 had a weak serum antibody response to LPS on days 14 through 56, and no LPS response was observed with serum from monkey Z-1.

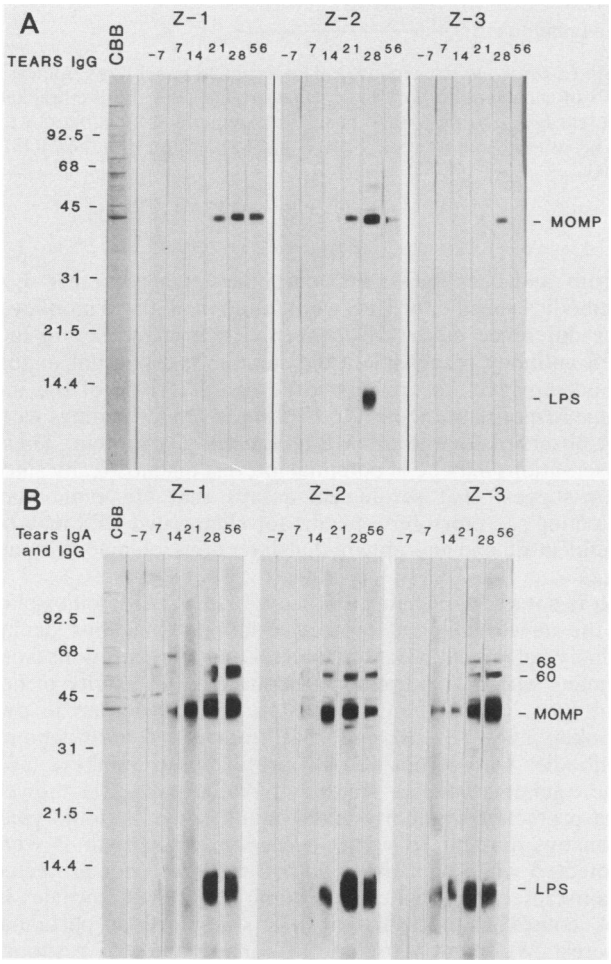

FIG. 2. Immunoblotting analyses of the temporal tear antibody response of cynomolgus monkeys with experimental *C. trachomatis* conjunctivitis. (A) Immunoblots of monkeys Z-1, Z-2, and Z-3 were probed for tear IgG antibodies; (B) immunoblots of tears from the same monkeys probed for IgA and IgG antibodies. Tears were collected from animals at 7 days before infection (−7) and at weekly intervals postinfection (7, 14, 21 days, etc.). CBB, Coomassie brilliant blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis polypeptide profile of the infecting *C. trachomatis* serovar B. The immunoreactive polypeptides are identified on the right side of the figure.

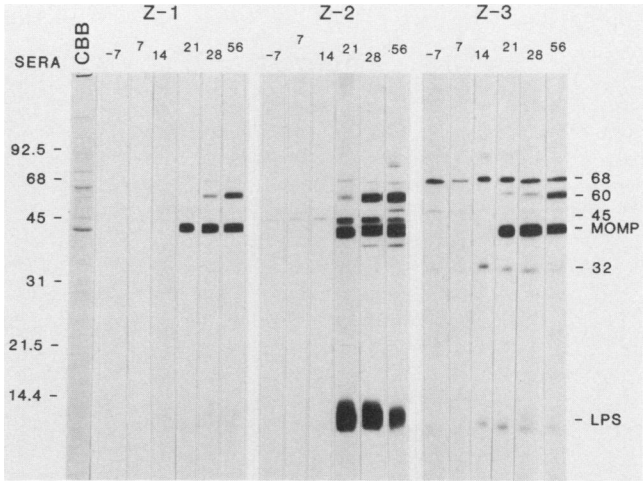

FIG. 3. Immunoblotting analyses of the temporal serum antibody response of cynomolgus monkeys with experimental *C. trachomatis* conjunctivitis. Immunoblots of monkeys Z-1, Z-2, and Z-3 were probed for IgG antibodies. The results did not change when the same sera were probed to detect IgA antibodies (data not shown). CBB, Coomassie brilliant blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis polypeptide profile of the infecting *C. trachomatis* serovar B. Numbers at top indicate days postinfection. Numbers at either side indicate molecular mass (in kilodaltons).

Immunoblot serovar specificity of tear antibody response. The *C. trachomatis* specificity of the monkey tear antibody response is shown in Fig. 4. Figure 4A shows a Coomassie blue-stained gel of the 10 different *C. trachomatis* serovars tested. The MOMPs are illustrated in the figure and, as previously described, vary in their subunit molecular mass with respect to serovar (4, 17). Fig. 4B and C show the accompanying immunoblots probed for tear IgG and IgA antibodies, respectively. The immunoblots shown were probed with a pooled tear preparation consisting of tear specimens from all three animals collected on days 21 and 28 postinfection. Pooling specimens was necessary to obtain sufficient volumes of tear antibody required for incubation with NCP containing multiple serovar polypeptide profiles. Both the IgG and IgA anti-MOMP responses were specific for the MOMPs of the B serogroup (serovars B, Ba, D, and E). No immunoreactivity was found with the MOMPs of the G or F serovar or with the C serogroup serovars C, H, and I; however, weak immunoreactivity was observed with the

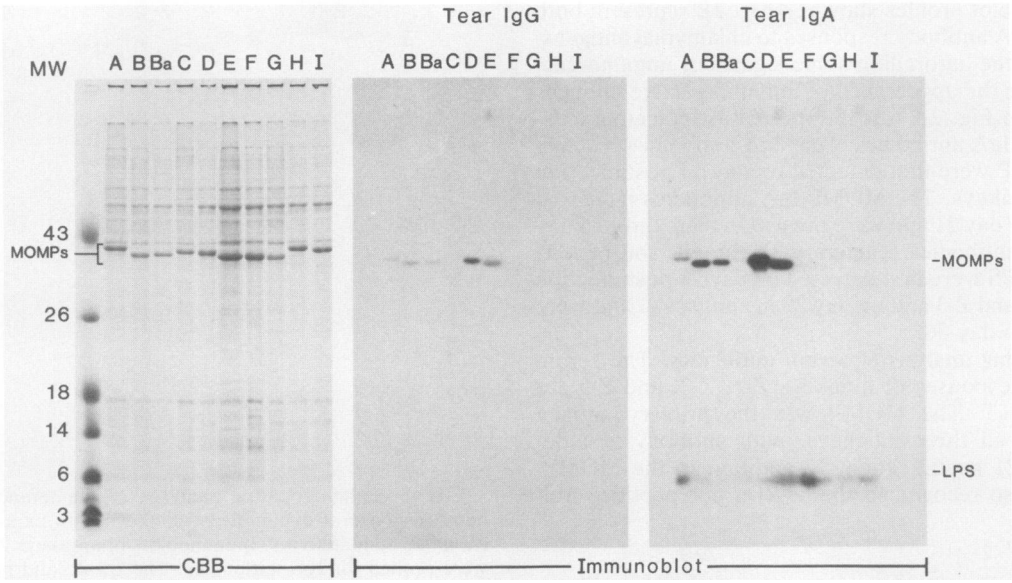

FIG. 4. Specificity of the tear antibody response of monkeys infected with *C. trachomatis* serovar B. The left-hand panel is a Coomassie brilliant blue-stained gel of the *C. trachomatis* serovars tested. The MOMPs of each serovar vary in M_rs and are indicated by the bracket. The middle panel is an immunoblot probed only with ^{125}I-protein A to detect tear IgG. The right-hand panel is an immunoblot incubated with rabbit anti-monkey IgA followed by iodinated protein A to detect both tear IgG and IgA antibodies. Notice that IgA response to the MOMP is predominantly B serogroup specific. MW, Molecular mass (in kilodaltons).

MOMP of the A serovar. Dilution of the tear specimens did not change the specificity of reactions shown in Fig. 4, indicating that the predominant tear IgG and IgA response against the MOMP that is detected by immunoblotting is predominantly B complex specific. Tear IgA LPS antibodies reacted with a species specificity in that they recognized this antigen in lysates of each of the *C. trachomatis* serovars tested. IgA antibodies reactive with the 68- and 60-kDa polypeptides were not observed in immunoblots probed with the pooled and diluted tear specimens. The reason for not detecting these antibodies is most likely that they were present in lower concentrations than anti-MOMP or -LPS antibodies and their immunoreactivity was lost after dilution of the tear samples.

DISCUSSION

In this report we studied by immunoblotting the antibody response in tears and sera of cynomolgus monkeys with acute *C. trachomatis* conjunctivitis. Our findings show that the MOMP, 60-kDa polypeptide, and LPS are the primary immunogens recognized by tear antibodies during this self-limiting *C. trachomatis* infection of the conjunctival epithelium. Tear IgA specific for the MOMP was first detected 14 days postinfection, was B complex specific, and remained detectable 28 days after peak recovery of chlamydiae. Tear IgA antibodies reactive with the 60-kDa protein and LPS were first detectable 21 days postinfection, were reactive with all *C. trachomatis* serovars tested, and also were detectable 28 days after peak recovery of chlamydiae from the conjunctivae. In contrast, tear IgG specific for these antigens was more transient, with maximal responses coinciding with the peak inflammatory period of the infection, suggesting that tear IgG antibody may in part be due to transudation. This conclusion is supported by the observation that monkey Z-2 was the only animal with detectable serum and tear IgG LPS antibodies, whereas tear IgA antibodies specific for LPS were found in all three monkeys. The difference observed between tear IgA and serum IgG LPS antibody responses in the same monkey is not understood; however, it appears to be characteristic of the immune response of the host to LPS since similar findings were not observed for the MOMP or the 60-kDa protein. These observations may be of potential interest diagnostically since they suggest that serological assays that are capable of detecting secretory IgA specific for chlamydial LPS may be useful in diagnosing chlamydial infections of mucosal surfaces.

It is not known what role, if any, tear IgA antibodies play in the development of protective immunity against ocular chlamydial infection. It is of interest that eye secretions from humans with active trachoma neutralized infectivity of homologous but not heterologous trachoma serovars in owl monkey eyes (1, 15) and that transfer of antitrachoma antibodies with serum was not protective in monkeys (16). The trachoma vaccine studies of Wang et al. (21) showed that protective immunity in the monkey eye is homotypic. Monkeys immunized with *C. trachomatis* serovar B were protected when challenged with B but were not protected against infectious challenge with the trachoma C serovar. In this context, the results of this study are of particular interest. We found here that the tear IgA antibody response was directed against determinants located on the MOMP of the infecting B serovars with no detectable antibody reactive with the MOMP of the C serovar.

We are aware of one other study in which immunoblotting has been used to analyze the serological response of persons with culture-proven *C. trachomatis* infection. Newhall et al. (13) studied the serological response of serum IgG from a single serum specimen of women with cervicitis who were culture-positive contacts of men with nongonococcal urethritis. They found that a 60- to 62-kDa protein was the

predominant immunogen recognized by serum IgG antibodies. Antibodies to the MOMP were described as being observed frequently but were weak in their immunoreactivity. Since similar reactions against the MOMP were observed in some control sera, these investigators considered the reactions to be nonspecific. Additionally, they did not report detecting antibodies against chlamydial LPS. Since the observations of these investigators differ substantially from those reported here, some discussion concerning the possible reasons for these discrepancies is appropriate. We considered the possibility that the discrepancies in findings resulted from the fact that two different chlamydial diseases in different host species were studied. This explanation is not entirely satisfactory, since both species were primates with diseases caused by *C. trachomatis* infections at mucosal surfaces. We feel that there are more probable alternative explanations to explain the differences in these studies. The MOMP of *C. trachomatis* is known to be an antigenically complex molecule. Polyclonal monospecific MOMP antisera (3, 4) and monoclonal antibodies reactive with the MOMP (12, 14, 18) have defined antigenic determinants that confer type, subspecies, and species serospecificities to the protein. Furthermore, we have recently observed heat-sensitive serotyping antigenic determinants located on the MOMP that are destroyed by the immunoblotting procedure (unpublished observations). As we showed here by immunoblotting, the antibody response to the MOMP during experimental infection of monkeys was largely subspecies specific, suggesting that the more antigenically unique portions of the protein are immunodominant. The chlamydiae isolated from the culture-positive women in the studies of Newhall et al. (13) were not serotyped, and a single serovar (L_2) was used as test antigen. It is conceivable that the reason these investigators failed to see an appreciable antibody response to the MOMP in the sera of some women was because they were infected with a *C. trachomatis* serovar whose MOMP was antigenically distinct from that of the L_2 serovar. Serovars G, F, H, I, and J, all of which are capable of causing cervicitis, would be examples of serovars that may not induce appreciable amounts of cross-reacting antibody against the MOMP of the L_2 serovar. Alternatively, the antibody response in humans may be preferentially directed at the heat-sensitive MOMP serotyping determinants which would have been denatured by the immunoblotting procedure. In the same study by Newhall et al. (13), antibodies reactive with chlamydial LPS were not described. Although we saw variable results in the sera of monkeys in their responses to LPS, tear IgA LPS antibodies were consistently detected in all three animals (Fig. 2B). We have previously reported that the use of Tween 20 or other nonionic detergents reduces the signal obtainable against LPS by immunoblotting (2). In this study, we did not use detergents in the immunoblotting assay. In contrast, in the study by Newhall et al. (13), Tween 20 was used. This rather minor (although perhaps very important) difference in methods may be the reason for the differences in detecting LPS antibodies in their studies and those reported here.

We believe that the results described here adequately reflect the serological response to chlamydial antigens as determined by immunoblotting analysis. Our argument is based on the following points: (i) we have studied the temporal antibody response in both tears and sera in what is a relevant animal model for *C. trachomatis* infections of mucosal surfaces, (ii) in addition to studying the antibody response to the homologous infecting *C. trachomatis* serovar, we have also addressed serospecificity of the response to other *C. trachomatis* serovars, and (iii) the immunoblotting procedures used in this study detect both protein and nonprotein chlamydial antigens.

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