Med Microbiol Immunol (1989) 178:113–120

Medical Microbiology and Immunology

Monoclonal antibody neutralization of unmanipulated *Chlamydia trachomatis* serovar A infection of human epithelioid cells (A-431)

Ibrahim S. Barsoum, Thomas A. Goodman, Lisa K. Hardin, and Daniel G. Colley

Departments of Microbiology and Medicine, Vanderbilt University School of Medicine and Veterans Administration Medical Center, Nashville, TN 37212, USA

Abstract. A human epithelioid cell line (A-431) was tested in parallel with McCoy fibroblast cells for the growth of trachoma-related serovar A *Chlamydia trachomatis* without centrifugation or cycloheximide addition. A-431 cells were 4–7 times more susceptible to infection with serovar A than McCoy cells in such unmanipulated cultures. Murine monoclonal antibodies (MAbs) developed against serovar A were then evaluated for their ability to inhibit unmanipulated serovar A infectivity of A-431 cells. Two of seven MAbs tested neutralized infectivity by more than 50%. An IgG2a MAb (2C8) that is specific for serovar A, and another IgG2a MAb (4E3) that reacts equally with serovars A and L2 neutralized infectivity of serovar A by $72.2 \pm 3.7\%$ and $56.0 \pm 5.8\%$ (mean ± SEM of 7 experiments) respectively. Mouse immune serum (MIS) raised against serovar A elementary bodies (EB) neutralized infectivity of serovar A by $76.0 \pm 4.9\%$ (mean ± SEM of 7 experiments). Immunoblot detection of serovar A EB polypeptides separated by SDS-PAGE indicated that 2C8 reacted with a 16 kD and 4E3 reacted with a 12 kD polypeptide while MIS reacted with several polypeptides including the major outer membrane protein (MOMP). These studies show that the human epithelioid cell line A-431 is a more susceptible host than McCoy cells in unmanipulated cultures, and that 2 MAbs neutralize serovar A infectivity of A-431 cells. Identification of antigenic moieties of importance in unmanipulated chlamydial infections may help in the development of potential vaccines against trachoma.

Introduction

Chlamydia trachomatis is usually grown in vitro in McCoy [15, 17] or HeLa [8] cells. While the L2 serovar readily infects these cells, most other serovars,

Offprint requests to: I. S. Barsoum

especially the ocular serovars A, B and C, require artificial manipulations to enhance the infection of the host cells. Such manipulations include centrifugation of the chlamydial inoculum over the host cells, pretreatment of the host cells with DEAE-dextran [9, 16] and the addition of cycloheximide to the culture medium [15]. A-431, a human epithelioid cell line originally derived from an epidermoid carcinoma [4], was tested in parallel with McCoy mouse fibroblast cells for the growth of trachoma-related serovar A without centrifugation and without the addition of cycloheximide. Moreover, we have used A-431 cells to test the neutralization of unmanipulated infectivity of serovar A with murine monoclonal antibodies (MAbs) prepared against *C. trachomatis* serovar A. We describe two MAbs that neutralized the infectivity of serovar A in A-431 cells by more than 50%.

Material and methods

Growth of cell lines and C. trachomatis stocks. McCoy cells (originally obtained from Dr. Julius Schachter, Department of Laboratory Medicine, University of California, San Francisco) were grown in Eagle's MEM (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum, 290 μg/ml glutamine, 10 μg/ml gentamicin, 10 units/ml mycostatin, 1 μg/ml fungizone, 3 μM/ml glucose and adjusted to pH 7.2 with 7.5% Na bicarbonate, later referred to as complete medium/McCoy (CM/M). Cells were passaged every 4–5 days. A-431 cells (obtained from Drs. C. Stoscheck and R. Gates, Dept. of Biochemistry, Vanderbilt University and the VA Medical Center) were grown in Dulbecco's Modified Eagle's medium and Ham's nutrient mixture F-12 (Sigma Chemical Co., St. Louis, MO) supplemented with 5% heat-inactivated fetal calf serum, 50 μg/ml gentamycin, 1 μg/ml fungizone and 10 units/ml mycostatin (complete medium/A-431; CM/A). Cells were passaged once every week. *C. trachomatis* serovar A was initially grown in McCoy cells in tissue culture flasks and partially purified stocks of elementary bodies (EB) were prepared as described previously [2].

Infectivity of McCoy and A-431 cells by C. trachomatis serovar A. Cell lines grown in tissue culture flasks were trypsinized and 2×10^5 cells in 1 ml of their respective CM were grown on coverslips in shell vials for 18–24 h. The vials were washed with Dulbecco PBS, 0.9 ml CM/M or CM/A added and 0.1 ml of stock chlamydia EB diluted in sucrose-phosphate-glutamate (SPG) buffer (10^4–10^6 IFU/ml) was added in each of 2 vials. Two uninoculated vials were used as controls. The cultures were incubated for 48 h at 37°C in 5% CO_2. In another set of experiments, 0.1 ml of inoculum was added directly to the cells, left to be absorbed for 1 h at room temperature, the inoculum removed by rinsing the cells with PBS, and incubated as above. At the termination of culture cells were washed with PBS, fixed in absolute methanol and stained with Giemsa stain diluted 1:25 with phosphate-buffered water pH 7.0 for 1 h. Coverslips were destained briefly in absolute ethanol, mounted on clean slides with Permount (Fisher). Each coverslip was examined microscopically with 100× oil immersion lens and the number of infected cells (containing inclusion bodies) in 300–600 cells were counted. Percentage infectivity was calculated as follows: number of infected cells/total cells counted × 100.

Monoclonal antibodies preparation and purification. Balb/c mice were immunized with irradiated (100 KiloRad) EB of serovar A in complete Freund's adjuvant with two subsequent reexposures. Sensitized spleen cells, 3 days after the last immunization, were fused with murine myeloma cells 8653 [7] using polyethylene glycol. After HAT selection, hybridomas were screened for antibodies against EB of serovar A and L2 by ELISA in microtiter plates [2]. Positive hybridomas were transferred to 24 well plates, grown to confluency, reassayed by ELISA and subcloned. Subclones were retested and injected into pristane-treated Balb/c mice for the production of antibody containing ascites. Purified monoclonal IgG was obtained by passage of the ascitic fluid through a protein A-sepharose CL-4B column (Pharmacia, Uppsala, Sweden) as previously described [5]. Specificity of the purified MAb was tested by ELISA against serovars A and L2 [2] and by micro-immunofluorescent test as described by Wang [19].

Neutralization assay. The method described by Lucero and Kuo [11] was used, with some modifications. Briefly, 0.1 ml of purified MAb diluted in Hank's balanced salt solution (HBSS) was mixed with 0.1 ml of fresh guinea pig complement (GPC, 5% final concentration) (GIBCO), 0.7 ml HBSS and 0.1 ml of *C. trachomatis* EB diluted in SPG (approximately 10×10^6 IFU/ml). Control tubes containing only GPC and diluted *C. trachomatis* EB were included in each experiment. Tubes were incubated for 1 h at 37°C with periodic shaking and 0.1 ml of the mixture was added to 0.9 ml CM in each of 2 vials containing 2×10^5 A-431 cells grown on coverslips for 18 h. The vials were incubated at 37°C in 5% CO_2 without further manipulations. At 48 h cells were washed with PBS, fixed, stained with Giemsa, and the number of infected cells was counted as before. Percent neutralization was calculated as follows: number of infected cells in the absence of MAb–number of infected cells in the presence of MAb/Number of infected cells in the absence of MAb $\times$ 100. Parallel experiments were done to test the effect of the presence or absence of GPC on the neutralization of chlamydia with MAb.

Immunoblotting. Immunoblotting was done according to the procedure described by Towbin et al. [18]. Briefly, purified EB of *C. trachomatis* were electrophoresed in 12% acrylamide gels in a minigel system (Bio-Rad, Richmond, Ca) and blotted onto nitrocellulose paper (NCP) (Schleicher & Schuell, Keene, NH) overnight at 4°C. The paper was cut into strips and diluted MAb, MIS, normal mouse serum (NMS) and OKT3 (an IgG2a class-matched control) (ATCC, Rockville, MD) were added to the NCP strips in Accutran disposable incubation trays (Schleicher & Schuell). Bound antibodies were detected by the addition of peroxidase labelled goat anti-mouse IgG (Tago, Ca). Color reaction was developed by the addition of 4-chloro-l-naphtol (Sigma) and 0.3% H_2O_2.

Results

Infectivity of *C. trachomatis* serovar A added to cells without centrifugation or cycloheximide addition was significantly greater in A-431 cells than in McCoy cells. The percentage infectivity was 4–7 times more in A-431 human epithelioid

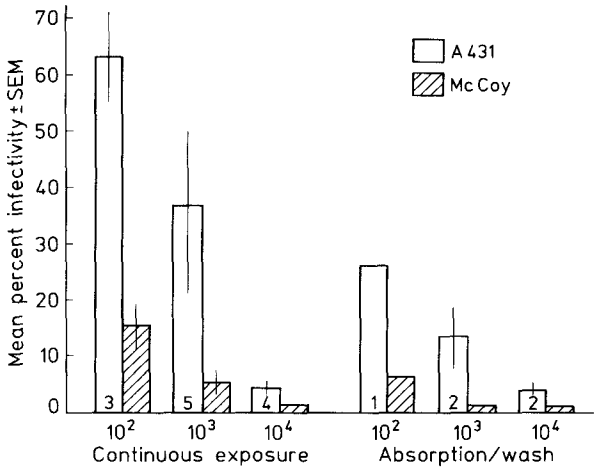

Fig. 1. Mean percentage of infectivity ± SEM of 1–5 experiments (n value given in open bar of each set) of *C. trachomatis* serovar A added to A-431 and McCoy cells for 48 h (continuous exposure) or absorbed for 1 h, washed and replaced with complete medium (absorption/wash) without centrifugation or addition of cycloheximide. The reciprocals of the dilutions of *C. trachomatis* stocks used are given below each set of bars

cells than in McCoy mouse fibroblast cells in repeated experiments using 10^2–10^4 dilutions of serovar A EB stock (Fig. 1). Infectivity of serovar A EB incubated for 1 h and washed was less in both cell lines than when EB were cultured continuously. Nonetheless, even in these absorption/wash studies A-431 showed higher infectivity rates than McCoy cells (Fig. 1). Subsequently, in 6 continuous exposure and 4 absorption/wash experiments using a single dilution (10^3) of serovar A and A-431 cells the mean percent infectivities (±Standard error of the mean, SEM) were 42.5 ± 8.1 and 25.3 ± 6.2 respectively.

Neutralization. Two of seven MAbs raised against *C. trachomatis* serovar A neutralized unmanipulated infectivity in A-431 cells by more than 50%. The IgG2a MAb, 2C8, and another IgG2a MAb, 4E3, neutralized this infectivity by 72.2 ± 3.7% and 56.0 ± 5.8% (mean ± SEM), respectively (Fig. 2). Mouse immune serum (MIS) raised against serovar A EB showed 76.0 ± 4.9% (mean ± SEM) neutralization while a class-matched (IgG2a) control MAb (OKT3) and normal ascitic fluid showed only 25.3 ± 7.6% and 25.7 ± 0.6% neutralization respectively (Fig. 2). Effective neutralization occurred only in the presence of guinea pig complement (GPC). In the absence of GPC, low neutralization rates were obtained. Neutralization obtained with MAb 2C8 in the presence and the absence of GPC respectively was 49.7 ± 6.3% and 24.5 ± 5.2%. That obtained with MIS was 60.0 ± 2.5% and 40.0 ± 2.5% (mean ± SEM of 3 experiments), respectively. The specificities of these MAbs to *C. trachomatis* serovars, as determined by Micro-IF and ELISA, are shown in Table 1. MAb 2C8 was specific for serovar A in Micro-IF and ELISA at a low titer, while 4E3 showed cross reactivity with other serovars in the Micro-IF and to L2 by ELISA.

Immunoblotting. Immunoblot analysis of the reactivities of the MAbs against serovar A EB components separated first by SDS-Page showed that 2C8 reacted with a 16 kD and 4E3 reacted with a 12 kD polypeptide component. MIS showed reactivity with several polypeptides including the major outer memebrane protein (MOMP) band at 39 kD also with 29, 55, 72 and 85 kD polypeptide bands (Fig. 3).

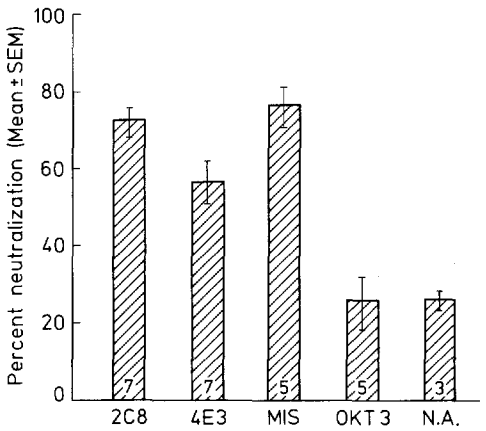

Fig. 2. Neutralization of *C. trachomatis* serovar A infectivity in A-431 epithelioid cells with monoclonal antibodies 2C8 and 4E3, anti-mouse immune serum (MIS), an unrelated monoclonal antibody with IgG2a isotype (OKT3) and normal ascitic fluid (N.A.) diluted 1:100 in Hank's balanced salt solution. The number of duplicate experiments is given under each bar

Table 1. Specificity of Monoclonal Antibodies to *C. trachomatis* serovars

MAb	Micro-IF[a]									ELISA[b]	
	A	B	CJ	DE	FG	H	I	L1L2	KL3	A	L2
2C8	8[c]	–	–	–	–	–	–	–	–	0.204	0.05
4E3	640	160	160	160	160	–	–	160	–	0.605	0.478

[a] Microimmunofluorescent assay using acetone fixed EB. The final dilution giving positive results is given

[b] ELISA using 20 μg/ml protein of serovars A and L2 irradiated EB, using 1:4 dilution of MAb and read at 490 nm wave length

[c] Reciprocal of the titer

Discussion

We have shown that A-431, a human epithelioid cell line, is more susceptible to infection with serovar A *Chlamydia trachomatis* than McCoy cells when infections are done in the absence of centrifugation or cycloheximide. In a previous study of the interaction between *C. trachomatis* serovar A and McCoy cells in the absence of centrifugation, Lee [10] reported that vials used for the infection of McCoy cells monolayer with the aid of centrifugation were unsuitable for the unaided infection with *C. trachomatis* and *C. psittaci.* In another study by Moorman et al. [13], *C. trachomatis* inocula producing 50–80% infections in McCoy cells with centrifugation resulted in infection rates of 15–30% and 5–10% of primary cultures of human endometrial and ectocervical epithelial cells. In the present study human epithelioid A-431 cells were 4–7 times more susceptible to infection with *C. trachomatis* serovar A than McCoy cells without manipulation of the cells (Fig. 1). We have also successfully grown serovar C, another endemic trachoma-related serovar, in A-431 cells and have obtained partially purified EB from these cultures (unpublished observation). The use of A-431 provides an in vitro model that may

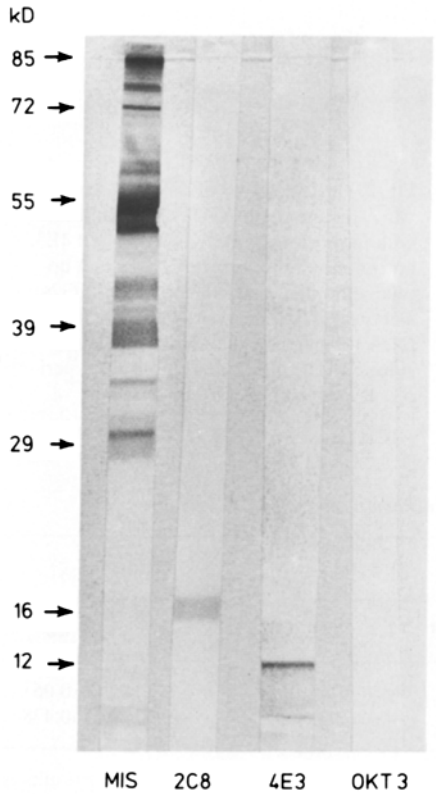

Fig. 3. Immunoblot analysis of the reactivities of mouse immune serum (MIS), neutralising MAbs 2C8 and 4E3 and a class-matched unrelated MAb OKT3 with *C. trachomatis* serovar EBs electrophoresed in 12% acrylamide gel and blotted onto nitrocellulose paper. Arrows indicate the molecular densities (kD) of polypeptide bands that reacted with MIS or MAbs.

more closely approximate the natural host-cell interaction that results in chlamydial infections, in that artificial manipulations are not required.

Previous neutralization studies have used polyclonal rabbit antisera against MOMP to neutralize infectivity of L2 serovar in HeLa cells [1] or MAb to MOMP to neutralize the infectivity of serovars B, H, I, K and L2 in the presence of complement [11]. Peeling et al. [14] described an in vitro neutralization of *C. trachomatis* serovar L2 and I with MAb that was complement independent but was dependent on optimal concentrations of both chlamydia and MAb. In a recent study by Zhang et al. [22] a panel of MAbs against serovar B which were species specific did not neutralize infectivity in HeLa cells in vitro but showed in vivo neutralization of infectivity of the organism for monkey eyes and protected mice from toxic death after i.v. injection of serovar B EB [22]. In the present study, 2 MAbs neutralized the in vitro infectivity of serovar A in A-431 cells, and this neutralization was done in the presence of complement. Optimum neutralization was obtained at 10^1–10^2 dilutions of MAb. In the present study successful in vitro neutralization occurred only in the presence of complement. Lower infectivity and lower neutralization rates were obtained in the absence of complement. Previous studies have also shown that complement enhances antibody-mediated neutralization while it had no effect by itself on chlamydial inactivation [3, 11, 12].

Neither 2C8 or 4E3 reacted by immunoblotting with the MOMP moiety which is approximately 39 kD [14, 22]. Parallel studies with MIS indicate the transfer to NCP, and reactivity of a 39 kD component suggesting that MOMP transferred and retained some immunoreactive epitopes in our system. Previous studies have shown reactivity of MAb to the MOMP using EB of serovars B [22], I and L2 [14], but serovar A has not been previously tested. A recent study, by Ward et al. [20] showed that sera from trachoma patients were characterized by a weak immune response to MOMP despite the presence of an important serotype specific determinant on this protein. These authors speculated that this lack of antibody to MOMP could explain the susceptibility of children in hyperendemic areas to recurrent infection.

Recent studies have shown that EB of serovars J and L2 possess proteins of 31 and 18 kD that bind to HeLa cells [21]. Antisera raised against these proteins inhibited chlamydial-host cell binding and were thus neutralizing. These data suggest that these chlamydial adhesins may play key roles in the early steps of chlamydia-host cell interaction, and that antibodies directed against them may be protective. A similar study identified 2 serovar L2 components of 32 kD and 18 kD that bound to HeLa cell surface membrane moieties [6].

In this study, we were able to neutralize in vitro, unmanipulated infectivity of trachoma-related serovar A in human epithelioid cells A-431. The use of A-431 provides an alternative to the widely used, but manipulation-requiring, McCoy and HeLa cells. The development of these two neutralizing MAbs may assist in further definition of the infectivity process of *C. trachomatis* and ultimately in vaccine development to prevent this process.

Acknowledgements. We gratefully acknowledge the secretarial and administrative assistance of Judith O'Connell. This work was supported by a grant from the Edna McConnell Clark Foundation (284–0059) and in part by the Veterans Administration.

References

1. Caldwell HD, Perry LJ (1982) Neutralization of *Chlamydia trachomatis* infectivity with antibodies to the major outer membrane protein. Infect Immun 38:745–754
2. Colley DG, Goodman TA, Barsoum IS (1986) Ocular sensitization of mice by live (but not irradiated) *Chlamydia trachomatis.* Infect Immun 54:9–12
3. Fedorko DP, Clark RB, Nachamkin I, Dalton HP (1987) Complement dependent in vitro neutralization of *Chlamydia trachomatis* by subspecies-specific monoclonal antibody. Med Microbiol Immunol 176:225–228
4. Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H, Parks WP (1973) In vitro cultivation of human tumors: Establishment of cell lines derived from a series of solid tumors. J Nat Cancer Inst 51:1417–1423
5. Goding SW (1976) Conjugation of antibody with fluorochromes: modification of the standard methods. J Immunol Meth 13:315–226
6. Hackstadt T (1986) Identification and properties of chlamydial polypeptides that bind eucaryotic cell surface components. J Bacteriol 165:13–20
7. Kearny JF, Radbruch A, Liesgang B, Rajewski K (1979) New mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. J Immunol 123:1548
8. Kuo CC, Grayston JT (1976) Interaction of *Chlamydia trachomatis* organisms and HeLa 229 cells. Infect Immun 13:1103–1109

9. Kuo CC, Wang SP, Grayston JT (1973) Effect of polycations, polyanions and neuraminidase on the infectivity of trachoma-inclusion conjunctivitis and lymphogranuloma venereum organisms in HeLa cells: sialic acid residues as possible receptors for trachoma-inclusion conjunctivitis. Infect Immun 8:74–79
10 Lee CK (1981) Interaction between a trachoma strain of *Chlamydia trachomatis* and mouse fibroblasts (McCoy cells) in the absence of centrifugation. Infect Immun 31:584–591
11. Lucero ME, Kuo CC (1985) Neutralization of *Chlamydia trachomatis* cell culture infection by serovar-specific monoclonal antibodies. Infect Immun 50:595–597
12. Mergan DW, Stiver G, Peeling R, Maclean IW, Brunham RC (1988) Complement enhancement of neutralizing antibody to the structural protein of *Chlamydia trachomatis.* J Infect Dis 158:661–663
13. Moorman DR, Sixbey JW, Wyrick PB (1986) Interaction of *Chlamydia trachomatis* with human genital epithelium in culture. J Gen Microbiol 132:1055–1067
14. Peeling R, Maclean IA, Brunham RC (1984) In vitro neutralization of *Chlamydia trachomatis* with monoclonal antibody to an epitope on the major outer membrane protein. Infect Immun 46:484–488
15. Ripa KT, Mardh PA (1977) Cultivation of *Chlamydia trachomatis* in cycloheximide treated McCoy cells. J Clin Microbiol 6:328–331
16. Rota TR, Nichols RL (1973) *Chlamydia trachomatis* in cell culture. I. Comparison of efficencies of infection in several chemically defined media, at various pH and temperature values, and after exposure to diethylaminoethyldextran. Appl Microbiol 26:560–565
17. Thomas BJ, Evans RT, Hutchinson GR Taylor-Robinson D (1977) Early detection of chlamydial inclusions combining the use of cycloheximide-treated McCoy cells and immunofluorescence staining. J Clin Microbiol 6:285–292
18. Towbin H, Stachelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. PNAS 76:4350–4354
19. Wang SP (1971) A micro-immunofluorescence method. Study of antibody response to TRIC organisms in mice. In trachoma and related disorders caused by chlamydial agents. Nichols RL (ed) Excerpta Medica, Amsterdam, pp 273–288
20. Wards ME, Treharne JD, Murray A (1986) Antigenic specificity of human antibody to chlamydia in trachoma and lymphogranuloma venereum. J Gen Microbiol 132:1599–1610
21. Wenman WM, Meuser RU (1986) *Chlamydia trachomatis* elementary bodies possess proteins which bind to eucaryotic cell membranes. J Bacteriol 165:602–607
22. Zhang Y-X, Stewart S, Joseph T, Taylor HR, Caldwell HD (1987) Protecitve monoclonal antibodies recognize epitopes located on the major outer membrane protein of chlamydia trachomatis. J Immunol 138:575–581

Received September 27, 1988