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Protective Role of Magnesium in the Neutralization by Antibodies of *Chlamydia trachomatis* Infectivity

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Neutralization of the infectivity of *Chlamydia trachomatis* was assessed by using polyclonal antisera and monoclonal antibodies (MAbs). Polyclonal antisera and a species-reactive MAb as well as a subspecies-specific MAb, both of which were directed toward the major outer membrane protein of *C. trachomatis*, reduced the number of chlamydial inclusion-forming units in an in vitro assay. Neutralization was dependent on the presence of complement. The species-specific MAb reacted with all 15 serovars by a microimmunofluorescence assay and a dot blot enzyme-linked immunosorbent assay with heat-treated elementary bodies. On the other hand, this same MAb reacted with all serovars, except those in the C complex, by the dot blot enzyme-linked immunosorbent assay with viable organisms and neutralized in vitro all 10 serovars tested, except those in the C complex. When neutralization assays were performed in a solution containing Mg^{2+}, neutralization by both polyclonal antisera and MAbs was significantly reduced. A dose response to Mg^{2+} supplied as $MgSO_4$ revealed that all concentrations tested from 50 to 800 μM had some effect. Concentrations of greater than or equal to 400 μM $MgSO_4$ completely abolished neutralization at the lowest dilution of polyclonal antisera and species-reactive MAb tested. Although Mg^{2+} also blocked the neutralization effect of the subspecies-specific MAb, this neutralization was not as complete as that observed with the species-reactive MAb. Addition of Mg^{2+} to the assay over the initial 45 min of incubation of *C. trachomatis* with MAb and complement showed that the organisms could be rescued to some extent over the first 30 min of incubation, after which time neutralization of infectivity could not be reversed. *C. trachomatis* treated with Mg^{2+}, the species-reactive MAb, and complement were lethal to mice in an in vivo toxicity and infectivity assay, whereas mice injected with organisms incubated with the same MAb and complement without Mg^{2+} survived.

Chlamydia trachomatis is a human pathogen that is the proven etiologic agent of a variety of infections ranging from cervicitis to trachoma (5, 24). There are no known virulence factors for this organism, and little is known about how this organism escapes host defense systems. Polyclonal antisera and monoclonal antibodies (MAbs) have been used in efforts to dissect the components that contribute to the pathogenicity of this organism (2, 9, 16, 20, 30). From the results of those studies, it appears that the major outer membrane protein (MOMP) of *C. trachomatis* plays a key role since antibodies to the MOMP are able to neutralize infectivity. However, not all antibodies directed to this protein are inhibitory to the growth of the organism. Therefore, placement of epitopes in the outer membrane and, possibly, their interaction with other cell wall components are key factors that need to be defined. In an attempt to further elucidate the components involved in the neutralization of *C. trachomatis*, we used polyclonal antisera and MAbs to address this issue, as examined by in vitro and in vivo infectivity assays.

In this report we present evidence that Mg^{2+} in physiologic concentrations can effectively abolish the neutralizing ability of complement with polyclonal antisera and MAbs toward *C. trachomatis* infectivity. In a number of studies (8, 10–12, 21–23, 25, 27), antibody-complement killing of gram-negative rods has been examined. It has been reported that serum-resistant, gram-negative rods are susceptible to killing by serum and complement when tested in the absence of Mg^{2+} (21–23). Our finding, therefore, is similar to those reported with serum-resistant, gram-negative bacteria, in that Mg^{2+} also plays a key role in protecting *C. trachomatis* from the adverse effects of an antibody-complement membrane attack.

MATERIALS AND METHODS

Organisms. The chlamydial isolates used in this study were raised in HeLa 229 cells and frozen at −70°C in 0.2 M sucrose–0.02 M sodium phosphate (pH 7.2)–5 mM glutamic acid. The following *C. trachomatis* strains were used: L1 (440), L2 (434), L3 (404), A (G-17), B (HAR-36), Ba (Apache, 2), C (TW-3), D (ICCal-8), E (Bour), F (UW-6), G (UW-57), H (UW-4), I (UW-12), J (UW-36), and K (UW-31). *Chlamydia psittaci* Texas turkey strain was also used.

Rabbit polyclonal antisera. Rabbit antiserum to chlamydia was produced in a female New Zealand White rabbit (Simmonsen Laboratories, Gilroy, Calif.) by using Renografin-purified *C. trachomatis* serovar L3 (strain 404) elementary bodies and the injection schedule outlined by Caldwell et al. (3).

MAb production. Four-week-old female BALB/c mice (Simonsen) were immunized by an intraperitoneal injection of 50 μg of purified elementary bodies of serovar C, E, or L1 dispersed in complete Freund adjuvant, followed by intravenous injections of 50 μg of purified elementary bodies on days 7 and 14. The spleen of the immunized mouse was removed for fusion on day 17. Mouse spleen cells were fused with the mouse myeloma cell line S194/5XXO.Bu.1. A modification of the method described by Kohler and Milstein (13) was used to fuse, select, and propagate MAb-producing hybridomas. Tissue culture fluid was screened for MAb to *C. trachomatis* by microimmunofluorescence (MIF) and dot blot enzyme-linked immunosorbent assay (ELISA). Hybridomas were selected and cloned by limiting dilution. Hybridomas were grown, and 10^6 cells were injected intraperitone-

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ally into female mice (age, 4 to 8 weeks) that had been injected intraperitoneally 2 weeks previously with Pristane (Sigma Chemical Co., St. Louis, Mo.). Ascitic fluids were collected, centrifuged (10,000 × *g* for 30 min at 4°C), and frozen at −70°C.

MIF. MIF was performed by the procedure of Wang and Grayston (29, 30) with the following modifications. With some MAb and polyclonal antisera, the assay was done with and without Mg^{2+} (800 μM $MgSO_4$) added to the phosphate-buffered saline (PBS) diluent and wash. Testing was performed with elementary bodies in 0.01 M PBS (pH 7.4) containing 0.02% Formalin, as well as with elementary bodies suspended in PBS without Formalin.

Dot blot ELISA. A modification of the procedure described by Zhang et al. (31) was employed for dot blot ELISA. Nitrocellulose paper (pore size, 0.2 μm) was soaked in PBS (0.01 M, pH 7.4) for 5 min and positioned in a dot blot block (Bio-Rad Laboratories, Richmond, Calif.). A solution of 20 μg of protein per ml of elementary bodies pelleted through 40% Renografin-76 (E. R. Squibb & Sons, Princeton, N.J.) in 0.01 M HEPES buffer (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4) containing 0.15 M NaCl was applied (0.05 ml per dot) and was allowed to filter by gravity for 30 min, and then a vacuum was applied. In some cases heat-treated (56°C, 30 min) elementary bodies were applied. Wells were washed three times with PBS–0.05% Tween 20 (PBS-T). BLOTTO buffer (0.2 ml) (4) was added to each well, and the blot was incubated at 42°C for 30 min. This was followed by three washes with PBS-T and the addition of 0.1 ml of the MAb dilution in PBS, further incubation for 30 min at 37°C, and three washes with PBS-T. Goat anti-mouse immunoglobulin G (IgG) and IgM horseradish peroxidase-tagged sera (0.1 ml; Organon Teknika, Philadelphia, Pa.) were added to each well. Blots were again incubated (37°C, 30 min) and washed three times with PBS-T. Positive reactions were visualized by color development with 0.1% hydrogen peroxide and 4-chloro-1-naphthol (0.5 mg/ml; Sigma) in PBS.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (14). A total of 5 μg of chlamydia protein, as measured by the method of Lowry et al. (15), was applied to each lane of the gel. Gels were silver stained by a modification of the method of Oakley et al. (18), by using 10% ethanol in the ammoniacal silver and developer solution, or were transferred to nitrocellulose paper (pore size, 0.2 μm) by a modification of the blotting procedure of Towbin et al. (28). The Western blots were prepared as follows. (i) Gels were transferred to 0.2-μm-pore-size nitrocellulose paper overnight at 4°C in Tris–glycine–20% ethanol in a transfer cell (Bio-Rad) at 40 V. (ii) The nitrocellulose blots were then blocked with BLOTTO at 42°C for 30 min and rinsed three times for 10 min each with PBS, incubated with mouse ascites fluid (1:1,000) for 1 h at room temperature, rinsed as with PBS as described above, incubated for 30 min with a peroxidase-conjugated goat anti-mouse antibody (Organon Teknika), diluted in PBS, rinsed with PBS as described above, and developed with 0.1% hydrogen peroxide–4-chloro-1-naphthol (0.5 mg/ml) in PBS for 5 to 15 min.

Immunoprecipitation. Immunoprecipitation was performed with MAbs that were negative by the Western blot procedure. The procedure of Giugmi et al. (6) was adapted for our immunoprecipitation experiments.

In vitro neutralization. Rabbit polyclonal antisera raised in our laboratory to *C. trachomatis* serovar L3 was heat inactivated at 56°C for 30 min before use. Ascites fluid was run through an affinity column (CM Affi-gel blue; Bio-Rad) and subsequently dialyzed against three changes of PBS and stored at −70°C until use. In addition, heat-inactivated ascites fluid was run in parallel in MIF and neutralization assays with column-treated ascites fluid to ensure that there was no loss of activity due to column treatment. No inactivation was noticed; only a slight dilution was observed which could be accounted for by the dilution of the material run through the column.

Antibody was diluted in PBS; Hanks balanced salt solution (HBSS; Irvine Scientific, Irvine, Calif.); or PBS with added Ca^{2+}, Mg^{2+}, or glucose, depending on the experiment. Dilutions were performed in 96-well microtiter plates. Lyophilized guinea pig serum (GPS; M. A. Bioproducts, Whittaker, Md.) was reconstituted in HBSS, divided into fractions, and stored at −70°C until it was used as a source of complement. Chlamydia (4×10^4 inclusion-forming units [IFU]) was added to the appropriate antibody dilutions and control mixtures which contained no antibody. All reactions contained 5% GPS. Reactions were incubated at 37°C for 45 min. The mixtures were then used to infect HeLa 229 cell monolayers contained in glass vials (15 by 45 mm) which were washed twice with PBS immediately before infection. The chlamydial inoculum was adjusted so that 20 to 30% of the control monolayers were infected, i.e., 40 IFU/×400 field. Cells were infected by centrifuging them at 1,000 × *g* for 1 h at room temperature, followed by stationary incubation at 37°C for 1 h. Afterward, 1 ml of Eagle minimal essential medium with Earle salts–5% fetal bovine serum–gentamicin (50 μg/ml), with or without cycloheximide (1 μg/ml), was added to all monolayers. Cells were incubated at 37°C for 48 h, at which time they were fixed in methanol followed by incubation at 37°C for 30 min with a rabbit polyclonal anti-chlamydial serum. Monolayers were then washed, and then a goat anti-rabbit horseradish-peroxidase-tagged antiserum (Organon Teknika) was added and the monolayers were incubated at 37°C for 30 min. Hydrogen peroxide (0.1%) and 4-chloro-1-naphthol (0.5 mg/ml) were used for color development. Ten × 400 fields per each cover slip in the test and control assays were read, an average was taken, and the results were expressed as a percentage of the IFU in control monolayers. Each experiment was repeated at least three times on separate days.

Mg^{2+} in vitro rescue experiments. In vitro neutralization assays were performed as outlined above, with the following modifications. All components used, including microtiter plates, were prewarmed to 37°C before use. All test reaction mixtures contained the same concentration of elementary bodies, dilutions (10^{-2}) of MAb in PBS, and GPS. Controls were the same, except that MAb was not included. $MgSO_4$ (800 μM) was added at time zero and at 1, 2, 5, 15, and 30 min of incubation to control and test mixtures. One set of mixtures received no additional Mg^{2+} through the 45 min of incubation. For each time point, test reactions were compared with control monolayers that received Mg^{2+} at the same time for the percentage of IFU. All determinations were done in duplicate. In addition, the experiment was repeated three times on separate days.

In vivo toxicity and infectivity neutralization. *C. trachomatis* serovar L2 was raised in 24-well microtiter plates. At 48 h after infection, HeLa 229 cell monolayers, which were 100% infected, were rinsed twice with PBS. Cells were scraped into PBS, sonicated by a 30-s burst (low setting, 2) by using a needle probe on an ultrasonic homogenizer (Braun-Sonic 2,000; B. Braun Instruments, Burlingame,

TABLE 1. In vitro neutralization of *C. trachomatis* infectivity

Antibody[a]	Immunoglobulin class	Western blot or immunoprecipitation specificity	Serovar specificity[b]	Serovar assayed for neutralization[c]	50% infectivity reduction antibody dilution
Rabbit polyclonal antisera	Polyclonal antisera	Polyclonal antisera	Genus	E (32,000)	1.7 ± 10^{-3}
MAb E4	IgG2b	MOMP	Species	E (51,200)	7.0 ± 10^{-3}
MAb C1.1	IgG1	MOMP	Subspecies (L3, A, C, H, J)	C (204,800)	4.3 ± 10^{-7}
MAb L1.1	IgG2b	MOMP	Subspecies (L1, L2, L3, B, Ba, D, E, K)	L1 (12,000)	$<1 \pm 10^{-1}$[d]
MAb E7	IgG1	Unreactive	Genus	E (6,400)	$<1 \pm 10^{-1}$[d]

[a] Immunizing antigens were polyclonal antisera, L3; MAb E4, E; MAb C1.1, C; MAb L1.1, L1; MAb E7, E.
[b] Determined by MIF.
[c] Neutralization data shown are for the homologous serovar, except for the polyclonal antisera. Values in parentheses are reciprocal dilutions.
[d] No neutralization was observed at the lowest dilution (1×10^{-1}) tested.

Calif.), and centrifuged at 500 $\times$ *g* for 10 min. The supernatant was removed and diluted in PBS to give 5×10^9 IFU/ml. A 1:10 dilution of MAb E4 fluid was made in the serovar L2 suspension containing 5% GPS. In addition, $MgSO_4$ (800 μM) was added to the Mg^{2+} treatment group. The control mixture consisted of L2 treated with 5% GPS and PBS alone or serovar L2 treated with 5% GPS and MAb E7, which failed to neutralize *C. trachomatis* infectivity in vitro. Mixtures were incubated for 45 min at 37°C, followed by a 0.2-ml injection into the tail vein of female BALB/c mice (age, 4 to 6 weeks; Simonsen). Results of preliminary experiments established the lethal dose of untreated serovar L2 under these conditions to be 10^9 IFU. The time of death of the animals was recorded at 24-h intervals.

Complement fixation. A standard complement fixation assay was performed with and without added $MgSO_4$ (800 μM) by the procedure outlined in the *Manual of Clinical Laboratory Immunology* (19). The only modification was that PBS containing Mg^{2+} was substituted for the test antibody dilution.

RESULTS

In vitro neutralization of *C. trachomatis* infectivity. Rabbit polyclonal antisera, as well as the MAbs in Table 1, were tested for their ability to neutralize chlamydial infectivity in HeLa 229 cells. Polyclonal antisera and MAbs E4 and C1 · 1, which are to the MOMP of *C. trachomatis*, as determined by Western blot analysis and immunoprecipitation, respectively, neutralized infectivity (Table 1). Complement, which was supplied in GPS, was necessary for this inhibition by both polyclonal and monoclonal antisera. GPS was tested at concentrations of 1, 5, and 10%. Neutralization at 1% was appreciably lower than that at 5%; neutralization at 10% lowered the control IFU and at 5% achieved optimal neutralization, while it did not affect control infectivity. Therefore, 5% GPS was used in all subsequent assays. Cycloheximide and centrifugation did not have any effect on the neutralization assays, since the same degree of neutralization was achieved with and without either or both of these treatments. MAb L1 · 1, although it was also against MOMP and had MIF titers comparable to those of MAb E4, failed to neutralize L1, one of the serovars for which it showed specificity. MAb E7, which appeared by MIF to react with all serovars of *C. trachomatis* as well as *C. psittaci*, and thus was genus reactive, was unreactive by Western blot and also failed to neutralize infectivity when tested with serovar E.

MAb C1 · 1 reacted only with members of the C- complex and C-related complex when examined by MIF. MAb E4, however, reacted with all serovars when tested by Western blot and MIF. No detectable differences were noted in the reactivities between MAb E4 and the different serovars when tested by Western blot. MIF titers were 6,400 for serovar C; 25,600 for serovars I and K, and 51,200 for all other serovars. Dot blot ELISA with heat-treated chlamydia resulted in the reactivity of MAb E4 with all serovars. When non-heat-treated viable organisms were used, however, all serovars except those belonging to the C- complex reacted with MAb E4. An example of this can be seen in Fig. 1 with representative serovars of the B, C, and C-related complexes. The neutralization activity of MAb E4 against members of the B, C, and C-related complexes is shown in Fig. 2. It is interesting that while by Western blot and MIF this MAb reacted similarly with all serovars, it failed to neutralize the infectivity of serovars in the C complex, while it quite effectively neutralized B complex and C-related complex members. The difference seen in neutralization may be explained by the failure of the C complex to react with MAb E4 by dot blot ELISA with viable organisms (Fig. 1). Without heat treatment or acetone fixation to expose this conserved region in C complex members, MAb E4 does not bind because of the surface inaccessibility of this epitope. In addition, this MAb, as expected, since it did not react with *C. psittaci* by either MIF or Western blot, was not effective in neutralizing the infectivity of a *C. psittaci* strain.

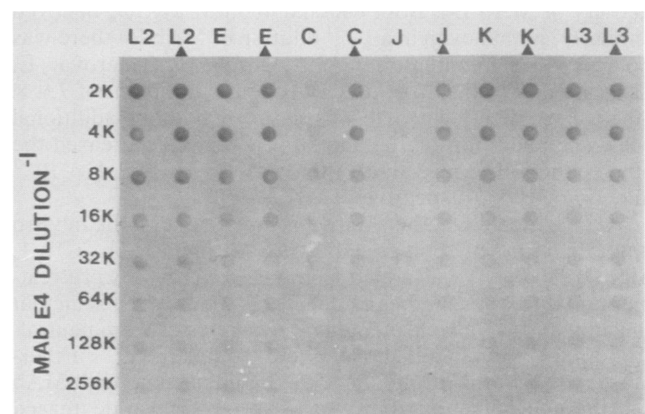

FIG. 1. Dot blot with the indicated *C. trachomatis* serovars. Each dot represents 1 μg of purified elementary bodies. Both nontreated and heat treated (▲) (56°C, 30 min) preparations were tested against the dilutions of MAb E4 listed to the left of the blot (K indicates thousands).

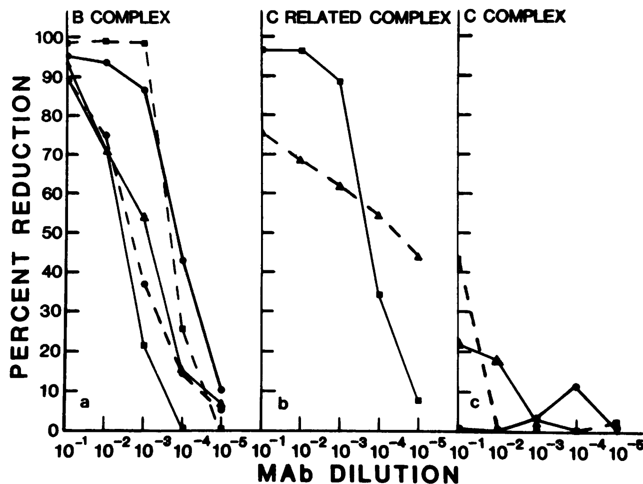

FIG. 2. In vitro neutralization of *C. trachomatis* infectivity by MAb E4 and complement. (a) The infectivity reduction curves were divided into the serovars belonging to the B complex consisting of serovars B (●- -●), D (■- -■), E (●—●), L1 (■—■), and L2 (▲—▲). (b) Serovars K (▲) and L3 (■), which are in the C-related complex. (c) C complex serovars C (▲), J (●), and H (- - - -). Each point is the average of at least three separate experiments run on different days.

TABLE 2. Effect of Mg^{2+} on in vitro neutralization of *C. trachomatis* infectivity

Antibody	Mg^{2+} [a]	Serovar	% Control ± at the following antibody dilutions[b]:		
			10^{-1}	10^{-2}	10^{-3}
Polyclonal antisera	−	E	9 ± 4	8 ± 4	41 ± 33
Polyclonal antisera	+	E	82 ± 15	117 ± 10	118 ± 29
E4	−	E	5 ± 1	7 ± 3	13 ± 4
E4	+	E	95 ± 1	105 ± 4	93 ± 1
C1.1	−	C	18 ± 13	9 ± 4	12 ± 4
C1.1	+	C	59 ± 28	68 ± 12	64 ± 4

[a] A concentration of 800 μM $MgSO_4$ was used.
[b] All reactions contained 5% GPS in PBS (0.01 M, pH 7.4).

Mg^{2+} effect on in vitro neutralization. In performing preliminary in vitro neutralization assays, it was noted that the neutralization effect on Mab E4 was blocked when assays were performed in HBSS rather than in PBS. Therefore, the components of HBSS were added separately to PBS and the neutralization assay was performed. When added alone to PBS, glucose, bicarbonate, or Ca^{2+} had no effect on the ability of MAb E4 to neutralize chlamydial infectivity. However, on the addition of Mg^{2+} to PBS, the neutralization effect of MAb E4 was abolished. The addition of Ca^{2+} to the solution of Mg^{2+} containing PBS did not reverse the antineutralization effect of the added Mg^{2+}. Both $MgCl_2$ and $MgSO_4$ were used as a source of Mg^{2+}, and the results obtained were the same; therefore, all subsequent determinations were done with $MgSO_4$. There was a dose effect seen with different concentrations of Mg^{2+} on the neutralization activity of MAb E4. Both 800 and 400 μM $MgSO_4$ behaved similarly, in that even at a 10^{-1} dilution of MAb E4 there was no reduction in infectivity of *C. trachomatis* serovar E. Compared with the 50% infectivity reduction point of 7.0×10^{-3} of MAb E4 when it was assayed without additional Mg^{2+}, the addition of 100 and 50 μM $MgSO_4$ increased the 50% reduction in infectivity titer of MAb E4 to 2.4×10^{-1} and 1.6×10^{-2}, respectively.

To investigate whether Mg^{2+} had any effect on the in vitro neutralization of polyclonal antisera and MAb C1 · 1, $MgSO_4$ (800 μM) was added to the *C. trachomatis* pretreatment mixtures. From the results (Table 2), it can be seen that the added Mg^{2+} also protected *C. trachomatis* from neutralization by polyclonal antisera and complement. Furthermore, while not as pronounced as the effect seen with MAb E4, the neutralization ability of MAb C1 · 1 also decreased when 800 μM $MgSO_4$ was present.

Experiments were also performed in which $MgSO_4$ (800 μM) was added at different times during the initial MAb E4 complement treatment of serovar E. With a 10^{-2} MAb E4 dilution, the ability of Mg^{2+} to rescue chlamydia decreased progressively over the first 30 min, at which time it was unable to rescue the elementary bodies (Fig. 3).

To determine whether $MgSO_4$ (800 μM) by itself inhibits the binding of antibody to elementary bodies, MIF titers were obtained by diluting polyclonal antisera and MAb in PBS with and without Mg^{2+}, as well as by washing slides in PBS with and without added Mg^{2+}. In every case, the titers were the same, regardless of whether Mg^{2+} was present.

$MgSO_4$ (800 μM) was also tested for anticomplement activity in a standard complement fixation assay. No anticomplementary activity of Mg^{2+} was noted, in that there was no decrease in hemolytic activity of complement toward sensitized erythrocytes.

In vivo *C. trachomatis* neutralization. To test MAb E4 for its ability to neutralize chlamydial infectivity or toxicity in vivo and to determine whether Mg^{2+} had any effect, *C. trachomatis* serovar L2 was pretreated with MAb E4–5% GPS with and without 800 μM $MgSO_4$, PBS–5% GPS, and a nonneutralizing MAb E7–5% GPS. Serovar L2 was pretreated with the combinations given above for 45 min. Subsequently, 0.2 ml containing 10^9 IFU was injected into the tail vein of mice, and deaths were recorded (Fig. 4). All mice that received L2 pretreated with PBS (n = 14) and MAb E7 (n = 5) died within the first 48 h. Those animals that received L2 pretreated with MAb E4 and Mg^{2+} (n = 11) all died

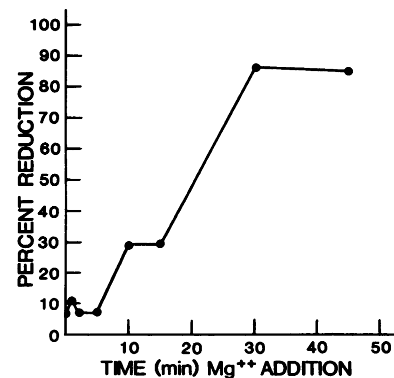

FIG. 3. Rescue by Mg^{2+} of *C. trachomatis* serovar E from MAb E4 and complement as measured by in vitro neutralization. At the indicated times, $MgSO_4$ (800 μM) was added to a suspension of elementary bodies in PBS containing MAb E4 (at a 10^{-2} dilution) and 5% GPS. Controls for each time point did not contain antibody but contained the same concentration of elementary bodies and GPS, and Mg^{2+} was added at the same times as the test mixtures.

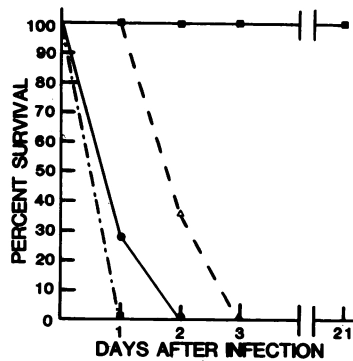

FIG. 4. Survival curve of BALB/c mice injected with 10^9 IFU of *C. trachomatis* serovar L2 treated with PBS–5% GPS (n = 14; ●); MAb E7 (10^{-2} dilution) in PBS–5% GPS (n = 5; —·—·); MAb E4 (10^{-2} dilution) in PBS–5% GPS with Mg^{2+} (800 μM) added (n = 11; △); or MAb E4 (10^{-2} dilution) in PBS–5% GPS (n = 14; ■).

within 72 h, while all animals injected with elementary bodies that were pretreated with MAb E4 without Mg^{2+} (n = 14) survived beyond 3 weeks, at which time observations were terminated.

DISCUSSION

Neutralization of *C. trachomatis* infectivity by polyclonal antisera and MAbs was investigated by using an in vitro assay and an in vivo mouse model. In this regard, we, as others, (16, 20, 31) have found that in addition to polyclonal antisera, MAbs to some MOMP epitopes neutralize *C. trachomatis* infectivity. Lucero and Kuo (16) reported that neutralizing activity resides mainly in serovar-specific antibodies. In this study, however, we found that MAb E4, while it reacted with all 15 serovars of *C. trachomatis* by MIF and thus was species reactive, neutralized members of the B complex and C-related complex but failed to neutralize members of the C complex. This observation is an indication that while all serovars possess the same epitope, the position of this epitope in the outer membrane influences the ability of an antibody-complement system to attack this organism. This finding was corroborated by MIF with acetone fixation and dot blot ELISA with heat-treated organisms in which all 15 serovars reacted similarly to MAb E4. When non-heat-treated, nonfixed organisms were used, however, all serovars except those of the C complex reacted. Zhang et al. (31) have reported a species-specific *C. trachomatis* MAb that reacted by MIF with all 15 serovars, but when it was tested with nontreated organisms by dot blot it reacted with only two serovars. They postulated that, in general, conserved, i.e., species-specific, epitopes are not readily accessible to antibody unless they are exposed by acetone fixation. While we found that this was also true of the C complex members with the conserved epitope recognized by MAb E4, in the majority of serovars, i.e., those in the B complex and the C-related complex, this region was surface exposed.

One of our most interesting findings was the protective role that Mg^{2+} appears to have in blocking the neutralization of *C. trachomatis* by antibody and complement. From MIF data with and without Mg^{2+}, it does not appear that Mg^{2+} directly influences antibody binding; and from results of studies with sensitized sheep erythrocytes, Mg^{2+} does not seem to affect the ability of complement to lyse erythrocytes. Furthermore, from our findings we know that the presence or absence of Mg^{2+} at the concentrations we tested did not impair the infectivity and, thus, the attachment or internalization of elementary bodies. Control elementary bodies with and without additional Mg^{2+} did not differ in the number of IFU. This observation is supported by the work of Sneddon and Wenman (26), who found that removal of Mg^{2+} from the initial infection process did not affect the amount of radiolabeled chlamydia ingested.

It should also be emphasized that Mg^{2+} reversed neutralization at concentrations that were half and also equal to those found under physiologic conditions. Reversal of neutralization was found with both polyclonal antisera and MAb. With the two neutralizing MAb we investigated, it appeared that Mg^{2+} was able to fully reverse neutralization with the species-specific MAb E4, as it did with polyclonal antisera; but it only partially reversed neutralization with MAb C1 · 1, which was a subspecies-specific MAb. Whether the position of the epitope in the membrane or other parameters are responsible for this differential effect remains to be answered.

The effect of Mg^{2+} that we observed might help to explain some of the conflicting data that have appeared in the literature concerning in vitro neutralization of *C. trachomatis* infectivity. Peeling et al. (20) described a MAb, AE11, that was species reactive by MIF and neutralized in vitro, independent of complement, serovars L2 and I. In their in vitro neutralization assay (20), they used PBS without an additional source of Mg^{2+}. Lucero and Kuo (16), however, using the same MAb and 5% GPS as a source of complement, were not able to demonstrate neutralization. However, their in vitro assays (16) were performed in HBSS which we found blocked neutralization because of the Mg^{2+} that was present. Using HBSS, they found that subspecies- and type-specific MAbs neutralized infectivity, but that no species-specific MAbs were capable of neutralization. Their finding is similar to that obtained in our assays run with additional Mg^{2+}. In this case, MAb E4, which is species reactive, did not cause neutralization, and subspecies-reactive MAb C1 · 1 neutralized up to 40% of the inoculum. Howard (9), using polyclonal antisera raised to two different serovars, A and B, in an in vitro neutralization assay with a Mg^{2+}-containing diluent, found that while the antisera raised to HAR-13 (serovar A) and that to HAR-36 (serovar B) showed broad cross-reactivities by MIF, neutralization was strain specific. In that study, if Mg^{2+} showed preferential protection of conserved, i.e., species-specific, epitopes, then this may account for the fact that the in vitro system failed to show in vitro cross-neutralization. Zhang et al. (31), in investigating the neutralization of *C. trachomatis* by MAb, found no in vitro neutralization, yet they found in vivo neutralization for some of the bispecies and subspecies MAbs they tested. This finding, along with our in vitro results with magnesium as well as results of our time course experiment, which showed that the incubation time is important for optimizing neutralization, emphasize that the neutralizing abilities of antibody and complement depend on the environmental conditions of the test system.

Our in vivo experiments correlated with our in vitro data with MAb E4. Here PBS- and MAb E7-treated elementary bodies killed mice within the first day of infection. However, the neutralizing antibody MAb E4 with complement completely destroyed the ability of a lethal dose of serovar L2 to kill the mice. Additionally, magnesium added to MAb E4 complement-treated elementary bodies enabled 60% of the mice to survice until 48 h after infection and allowed the remaining 40% to survive up to 3 days after infection. It is therefore interesting to speculate whether *C. trachomatis*,

during a natural infection, has evolved as a protective mechanism a structural component in which Mg^{2+} is instrumental in protecting the organism against antibody-complement attack.

The next question that remains is how Mg^{2+} interferes with MAb- and complement-mediated neutralization of *C. trachomatis*. The inhibitory effect of physiologic levels of Mg^{2+} on membrane antibody-complement attack has been reported for several gram-negative bacteria (21–23). These organisms are considered to be serum resistant under conditions in which physiologic levels of Mg^{2+} are present and are usually gram-negative bacteria with a smooth phenotype, containing lipopolysaccharide molecules with long polysaccharide side chains (7, 8, 21–23). It has been reported by Reynolds and colleagues (21–23) that *Salmonella typhimurium* C5 is resistant to antibody and complement; however, when these organisms were suspended in physiologic saline without Mg^{2+}, they became sensitive to attack by antibody and complement. It was first suggested that these observations reflected the fact that removal of Mg^{2+} might alter the structure of the lipopolysaccharide and, thus, weaken its attachment to the bacteria, thereby exposing the underlying membrane and making the organism susceptible to antibody-complement attack (23). More specifically, further work with serum-resistant and -sensitive strains of *Escherichia coli*, *Salmonella* spp., and *Pseudomonas aeruginosa* indicates that in both serum-resistant and -sensitive strains that complement attaches and is activated; however, the C5b-9 complexes that are formed on the serum-resistant organisms are not stably inserted into the bacterial membrane (7, 8, 10–12, 25, 27). Therefore, Mg^{2+} might affect the lipopolysaccharide conformation and, thus, alter the binding of activated C5 complexes. Since chlamydia appears to be more closely related to rough lipopolysaccharide-containing gram-negative organisms than to those with long O side chains (1, 17), it will be of interest to determine whether C5b-9 complexes insert stably into *C. trachomatis*.

In summary, we have described a species-reactive, i.e., *C. trachomatis*, MAb that appears to be limited in neutralization activity to serovars in the B complex and the C-related complex, those in which this epitope is surface accessible. In addition, we have described the inhibitory effect of physiologic levels of Mg^{2+} on polyclonal antisera-complement and MAb-complement systems, as assessed by in vitro neutralization and in vivo toxicity and infectivity prevention by elementary bodies. The exact mechanisms of this interaction and its in vivo implications remain to be answered.

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