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Neutralization of *Chlamydia trachomatis*: Kinetics and Stoichiometry

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Monoclonal antibodies to the major outer membrane protein of *Chlamydia trachomatis* were used to neutralize *C. trachomatis* infectivity in HeLa 229 cells and to determine the kinetics and stoichiometry of the reaction. In vitro neutralization of *C. trachomatis* infectivity proceeded as a first-order reaction and required an activation energy of approximately 20 kcal/mol (ca. 84 kJ/mol). The rate of neutralization was linear with respect to antibody concentration and reaction temperature. The efficiency of neutralization decreased exponentially as the ratio of noninfective to infective chlamydiae increased in the antigen preparation. The neutralization assay was also significantly affected by reaction parameters such as the reaction volume and the duration of incubation. Stoichiometric calculations showed that an average ratio of 10^3 and 10^4 immunoglobulin molecules per chlamydial particle was required to yield 50% neutralization by monoclonal antibodies specifying serovar-specific and species-specific epitopes, respectively. The implications of these findings for vaccine design and for the role of the major outer membrane protein in the pathogenesis of chlamydial infections are discussed.

Seroepidemiologic data (6) and controlled vaccine trials (26) suggest that *Chlamydia trachomatis* induces immunity. Humoral immunity appears to be important, since antibody produced by experimentally infected animals or from naturally infected humans can neutralize *C. trachomatis* infectivity in vitro and in vivo (12, 18, 19, 23, 27). In vitro neutralization of infectivity is potentially an important and versatile assay that can be used to characterize host humoral immunity, to evaluate protective antigens in vaccine design, and, with monoclonal antibodies (MAbs), to delineate structure-function relationships for target antigens. Early studies of neutralization showed that antibodies from natural *C. trachomatis* infections, as measured either by an in vitro assay or by protection in the mouse toxicity prevention test, correlated with microimmunofluorescent (micro-IF) but not with complement fixation antibodies (2, 4, 22). Neutralization was enhanced by the addition of complement and anti-globulin (5, 7, 12, 18). More recent studies using MAbs and monospecific polyclonal antibodies showed that the major outer membrane protein (MOMP) is a target for neutralizing antibodies. These studies showed that neutralization is dependent on bivalent immunoglobulin G (IgG) binding and depending on the host cell culture conditions, its mechanism of action appears to be due to the prevention of attachment of elementary bodies (EBs) to the host cell or inhibition of porin function on MOMP after the antibody-coated chlamydiae are internalized (7, 20, 21, 24). Despite these advances in our understanding of chlamydial infectivity, the neutralization assay has proved difficult to replicate in different laboratories, largely because of a lack of standardization and an understanding of the basic stoichiometry of the reaction.

We were therefore interested in determining the kinetics and stoichiometry for the neutralization of *C. trachomatis* infectivity for HeLa cells by using MAbs to MOMP. To further characterize the reaction, we evaluated factors which may significantly affect neutralization, such as antibody concentration, heat inactivation of *C. trachomatis* EBs, reaction volume, and incubation time and temperature. Our results suggest that neutralization of *C. trachomatis* with MAbs to MOMP resembles neutralization of viruses in that it proceeds with time as a first-order reaction and that its rate is proportional to antibody concentration and incubation temperature. Unlike viral neutralization, a larger amount of antibody and a higher activation energy are required for the chlamydial reaction to occur. Neutralization was dramatically affected by the proportion of noninfectious EBs in the antigen preparation. These results may explain some of the discrepant results regarding *C. trachomatis* in vitro neutralization.

MATERIALS AND METHODS

Organisms. *C. trachomatis* serovars D (UW3/Cx) and L_2 (434/Bu) were grown in HeLa 229 cells as previously described (20). EBs were harvested from 90 to 100% infected HeLa cell cultures after 48 to 72 h and were purified through a 35% Renografin cushion and then on a discontinuous 40 to 52% Renografin gradient (7). Purified EBs were aliquoted and stored at −70°C in sucrose phosphate glutamate buffer (SPG; 200 mM sucrose, 10 mM sodium phosphate, 5 mM L-glutamate, pH 7.2) until being used. Purified EBs were diluted in SPG to give a final concentration of between 3×10^3 and 3×10^4 inclusion-forming units (IFU) per HeLa cell monolayer culture on 12-mm glass coverslips in all neutralization assays.

Antibodies. MAbs UM-1, UM-3, and UM-4 were prepared and characterized as previously described (16). UM-1 and UM-3, both isotype IgG1, are MAbs with subspecies specificity for the MOMP of B complex serovars of *C. trachomatis* by immunoblot and micro-IF. UM-4, isotype IgG2b, is a MAb which recognizes a serovar-specific epitope on the MOMP of serovar L_2. MAb C11, isotype IgG2b, was kindly provided by J. B. Mahony, McMaster University, Hamilton, Canada, and MAb 597 was provided by ADI Diagnostics,

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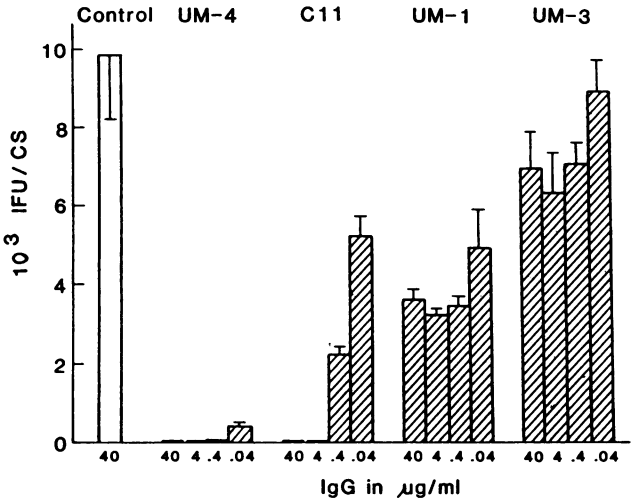

FIG. 1. Neutralization of infectivity of *C. trachomatis* serovar L_2 EBs for HeLa 229 cells. Purified serovar L_2 EBs were incubated with IgG from normal mouse serum and MAbs UM-1, UM-3, UM-4, and C11 at 37°C for 30 min. Residual infectivity was determined in HeLa cells by counting IFU/cs. Data shown are the means and standard deviations of triplicate samples.

Inc., Mississauga, Canada. Both of these MAbs recognize species-specific epitopes on MOMP by immunoblot and Micro-IF reactions. Purified IgG was prepared from hybridoma culture supernatant or ascites fluid by protein A-Sepharose CL-4B chromatography and dialyzed against 0.01 M phosphate-buffered saline (PBS), pH 7.0. Concentrations of purified IgG were determined from spectrophotometric readings by using the extinction coefficient $E^{1\%}_{280\ nm} = 13.5$.

Neutralization assay. The standard neutralization assay combined 180 μl of *C. trachomatis* EB in SPG with 180 μl of antibody diluted in 0.01 M PBS, pH 7.0, containing 100 μg of normal mouse IgG (NMIgG) per ml. NMIgG at 100 μg/ml was used as a protein carrier in all experiments except when otherwise stated. The reaction mixture was incubated at 37°C for 30 min with continuous shaking. Residual infectivity was determined by inoculation of the reaction mixture onto confluent 24-h-old coverslip cultures of HeLa 229 cells without centrifugation. Cells infected with serovar D were pretreated with 30 μg of DEAE-dextran per ml for 20 min. The inoculum was allowed to adsorb for 2 h at room temperature and then rinsed off with Hanks balanced salt solution. Cultures were incubated at 35 to 37°C for 48 to 72 h in medium containing 1 μg of cycloheximide per ml. *C. trachomatis* inclusions were stained with fluorescein-conjugated MAbs (Syva Co., Palo Alto, Calif.) and quantitated by counting 30 high-power (×400) fields per coverslip (10). Experiments were performed in triplicate. Figure 1 illustrates the residual inclusion counts per coverslip (IFU/cs) obtained with serovar L_2 and four MAbs (UM-1, C11, UM-3, and UM-4), using standard assay conditions. A MAb is considered neutralizing when it reduces the infectivity of the antigen by 50% compared with that of controls. Thus, by this criterion, UM-1, UM-4, and C11 were neutralizing MAbs for serovar L_2. UM-4 was incorrectly reported as a nonneutralizing MAb in a previous publication (16).

Thermal stability of EBs. Serovar L_2 and D EBs, diluted in SPG to yield a final concentration of between 3×10^3 and 10^4 IFU/cs, were combined with an equal volume of PBS alone, PBS containing 1 mg of bovine serum albumin (BSA) per ml, or NMIgG at concentrations of 0.1 and 0.01 mg/ml in PBS. Mixtures were incubated at 37°C, and aliquots were assayed for inclusion formation at 0, 15, 30, and 60 min.

To evaluate the effect of noninfectious EBs in the inocula on neutralization, UV-inactivated serovar L_2 EBs were added to a control preparation of L_2 EBs from the same pool at control/inactivated-EB ratios of 1:1, 1:2, 1:5, and 1:10 (infectivity ratio). EBs were checked for residual viability in cell culture after UV treatment. The reaction volume was kept constant, and the reactions were incubated at 37°C for 30 min. Separate controls were used for each reaction to take into account decreased uptake and endocytosis of the control inocula when UV-inactivated EBs were added in large numbers. Residual infectivity was determined as described above. Percent survival was calculated as (residual IFU/total IFU) × 100. Log_e percent survival was plotted against the infectivity ratio to derive a mathematical relationship between neutralization efficiency and the proportion of control versus inactivated EBs in the antigen preparation used for a neutralization reaction.

Neutralization kinetics and activation energy. Antibody concentration, reaction volume, and incubation temperature all affect the rate of the neutralization reaction. To examine the effect of antibody concentration on the kinetics of *C. trachomatis* neutralization, serovar L_2 EBs were incubated with three different concentrations of MAb UM-4 IgG or NMIgG (3.2, 16, and 32 μg/ml) at 37°C. Reaction mixtures were monitored for residual infectivity at various time intervals over 60 min. The $\log_{10}$ survival fraction (residual IFUs/total IFUs in the control) was plotted as a function of time to give kinetic curves for the reaction at each antibody concentration. Regression analyses were calculated for the linear portion of the plot, and reaction rates were plotted against antibody concentration.

The effect of reaction volume on the efficiency of neutralization was determined by setting up duplicate assays using serovar L_2 EBs and MAbs UM-4 and C11. Just prior to incubation, the volume of one set of reaction mixtures was increased from 360 μl to 1 ml by using a 1:1 mixture of SPG and PBS containing 10 μg of NMIgG per ml. The reactions were incubated at 37°C for 30 min, and residual infectivity was assayed in HeLa 229 cells.

To determine the effect of temperature on neutralization kinetics, triplicate reaction mixtures of serovar L_2 EBs and MAb UM-4 IgG or NMIgG (32 μg/ml) were incubated at 4, 22, and 37°C. Aliquots of each reaction mixture were taken at various times and placed on ice before inoculation onto HeLa cell monolayers, to determine residual infectivity. The percent survival and survival fraction were calculated for each temperature, and the rate of the neutralization reaction was plotted against the reciprocal of the absolute temperature for each reaction. The Arrhenius activation energy for the neutralization reaction was derived from the reaction rates and temperatures.

Since serovar D is more heat labile than serovar L_2, the optimal temperature and duration of incubation were tested with serovar D and MAb 597 under standard assay conditions as described with the following variations for incubation: 37°C for 30 min, 37°C for 60 min, 22°C for 60 min, and 4°C overnight.

Stoichiometry. Particle counts for each of the neutralization reactions were made by electron microscopy to assess the ratio of EB particles to IFU (11). The number of IgG molecules in each reaction mixture was calculated from the spectrophotometric quantitation of pure MAb IgG as described under "Antibodies," with $M_r = 155{,}000$. The ratio of

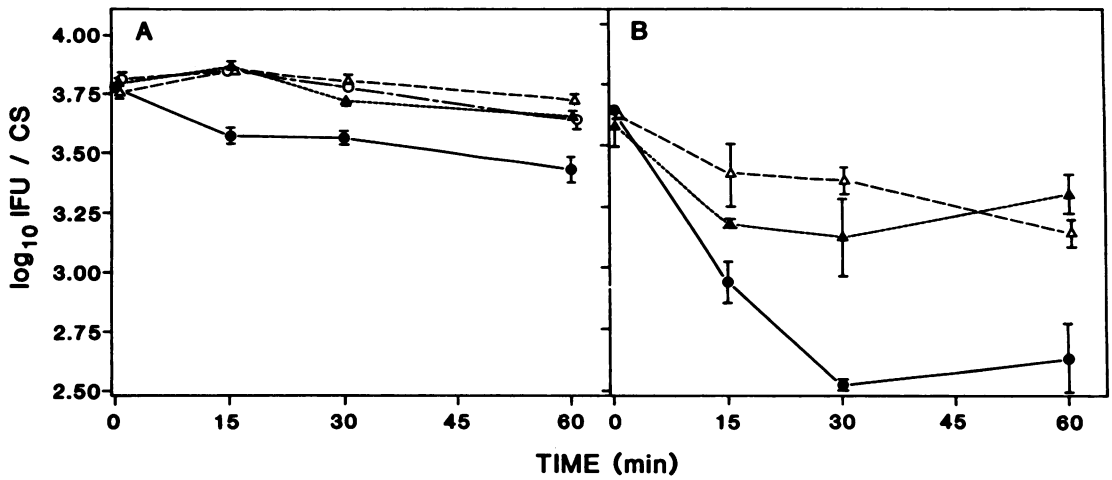

FIG. 2. Heat inactivation of *C. trachomatis* infectivity. Purified EBs of serovars L_2 (A) and D (B) were incubated at 37°C in PBS (●), bovine serum albumin at 1 mg/ml (▲), and normal mouse IgG at 10 (○) and 100 (△) μg/ml. At various times as shown, aliquots were taken to determine residual infectivity for HeLa 229 cells. Data shown are the means and standard deviations from triplicate experiments.

IgG molecules to EBs in the neutralization of serovars L_2 and D by two species-specific MAbs, C11 and 597, and in the neutralization of serovar L_2 by the serovar-specific MAb UM-4 were plotted against the percent neutralization attained for each reaction.

Statistical analyses. Determinations of infectivity (IFU/cs) were converted to log values to stabilize variance. All data were analyzed by randomized blocked analyses of variance, and linear regression parameters were calculated by the method of least squares.

RESULTS

Heat inactivation. Serovar L_2 and D EBs incubated in PBS at 37°C lose infectivity for HeLa cells (Fig. 2A and B). Infectivity after 15, 30, and 60 min at 37°C was significantly lower than the infectivity at time zero for both serovars ($P < 0.0001$). Serovar L_2 EBs were less heat labile than serovar D EBs at all time points after zero ($P < 0.0001$). Addition of a protein carrier reduced thermal inactivation. When serovar L_2 EBs were suspended in 10 or 100 μg of NMIgG per ml or 1 mg of BSA per ml, infectivity remained unchanged after 30 min at 37°C; for serovar D EBs, the concentrations of NMIgG and BSA needed to be increased 10-fold to preserve infectivity. Despite the presence of a protein carrier, the infectivity of serovar D EBs was approximately 20 to 30% of time zero values after an incubation period of 60 min at 37°C.

Effect of the infectivity ratio on neutralization efficiency. To evaluate the potential effect of noninfectious EBs on neutralization, we used antigen preparations that contained increasing proportions of UV-inactivated serovar L_2 EBs in the standard neutralization assay. MAb UM-4 neutralized 99% of the infectivity in the control preparation (mean survival = 0.78%). In the presence of UV-inactivated EBs, the mean survival increased from 1.7 to 86% for infectivity ratios of 1:1 and 1:10, respectively. Figure 3 shows the linear relationship that exists between the percent survival for each reaction and the infectivity ratio. This relationship can be described mathematically as follows: percent survival = $0.91e^{(0.47r)}$, where r is the infectivity ratio. This logarithmic relationship implies that neutralization decreases exponentially with increasing proportions of inactivated EBs in the antigen preparation used for the neutralization reaction.

Effect of antibody concentration on the kinetics of neutralization. We compared the rate of neutralization of serovar L_2 infectivity with three concentrations of UM-4 IgG. Figure 4A shows that neutralization, inversely expressed by the survival fraction, is more effective with increasing concentrations of antibody in the reaction mixture. The reaction proceeded with time as a first-order reaction until saturation. The rate of neutralization at each antibody concentration was determined by a regression line fitted to the linear portion of the kinetic curve (Fig. 4B). When the reaction rates were plotted against the antibody concentration, a

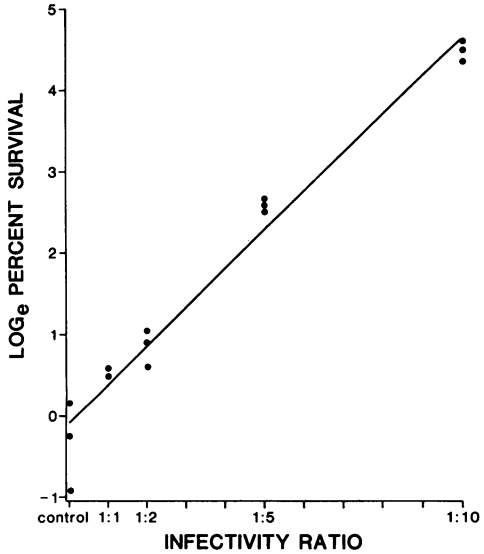

FIG. 3. Relationship between neutralization efficiency and the proportion of inactivated EBs in the neutralization reaction. Purified serovar L_2 EBs were UV inactivated and added in various proportions to a control EB preparation from the same pool. These mixtures were used as antigens and incubated with MAb UM-4 at 37°C for 30 min. Residual infectivity of each mixture was determined in HeLa 229 cells. Percent survival is calculated as (residual IFUs/total IFUs in controls) × 100. Each datum point (●) represents the result of a separate experiment.

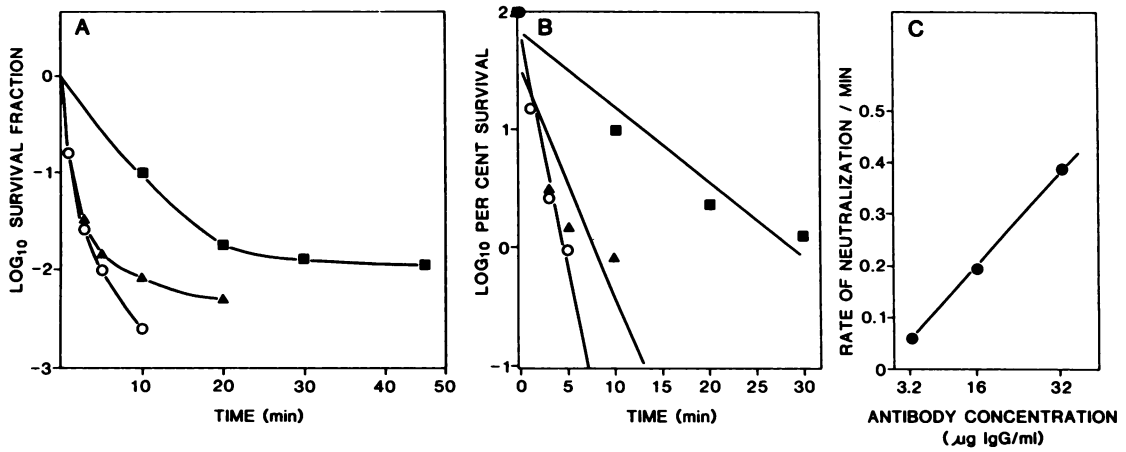

FIG. 4. Kinetics of neutralization of serovar L_2 infectivity by MAb UM-4. Purified EBs were incubated with MAb UM-4 IgG at 32 (○), 16 (▲) and 3.2 (■) μg/ml and NMIgG as a control at 37°C. (A) At various times as indicated, aliquots of the reaction mixtures were taken and placed on ice until inoculated onto HeLa 229 cells for determination of residual infectivity. Survival fraction = residual IFU/total IFUs in controls. (B) Regression line fitted to the linear portions of the kinetic curve in panel A. (C) Relationship between the rate of the neutralization reaction and antibody concentration.

straight line was obtained, indicating that the rate of neutralization was directly proportional to the antibody concentration (Fig. 4C).

From the above results, we surmise that reaction volume can affect the efficiency of the neutralization reaction by altering the final concentration of the reactants. To directly test this hypothesis, we compared a reaction volume of 360 μl used in our standard assay with a 1-ml volume, which has been used in other neutralization assays (8, 15). Figure 5 shows the mean percent survival of serovar L_2 infectivity after neutralization with MAbs UM-4 and C11, using four antibody concentrations and the two different volumes. Neutralization was more effective at the smaller volume for

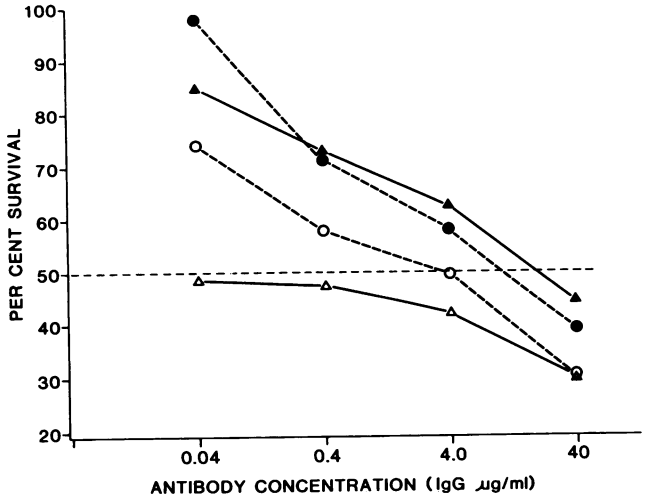

FIG. 5. Effect of reaction volume on the neutralization of serovar L_2 infectivity by MAbs UM-4 (▲, 1 ml; △, 360 μl) and C11 (●, 1 ml; ○, 360 μl). Reaction mixtures of EBs and UM-4 IgG (40, 4, 0.4, and 0.04 μg/ml) were made in a total reaction volume of 360 μl in duplicate. Just prior to incubation at 37°C for 30 min, the reaction volume of one set of reaction mixtures was brought up to 1 ml with a 50:50 mixture of antigen and antibody diluents (SPG and NMIgG in PBS). Residual infectivity was determined in HeLa 229 cells as described in the text.

both antibodies at all concentrations tested. The difference in mean percent survival between the two volumes was significant for MAb UM-4 at all concentrations ($P < 0.0001$), whereas for MAb C11, the difference became significant at the lower antibody concentrations of 0.4 and 0.04 μg of IgG per ml ($P < 0.0034$ and 0.0001, respectively). If 50% neutralization is to be used as a cutoff point for identifying an antibody as neutralizing, then it can be seen that the reaction volume became critical for reactions in which the antibody concentrations were low.

Effect of temperature on the kinetics of neutralization. The rates of neutralization at three temperatures (4, 22, and 37°C) were compared by using serovar L_2 and MAb UM-4 IgG (32 μg/ml). Neutralization was significantly affected by temperature. The effect was biphasic (Fig. 6A). The plot of log percent survival against time of incubation consists of an initial linear portion, with an exponential decrease in residual infectivity with time, and a plateau portion during which the rate of reaction did not change appreciably over time. Regression analyses on the linear portions of the graph yielded three slopes that represent the rates of neutralization at those temperatures (Fig. 6B). Neutralization proceeded 90-fold faster at 37°C than that at 4°C. The plot of the rate of neutralization against the reciprocal of absolute temperature ($1/T$) yielded a straight line, suggesting that increased temperature favors the formation of activated complexes of antigen and antibody (Fig. 6C). The reaction can thus be described by the Arrhenius equation (9): $2.3 \log k_1/k_2 = E_a/R(1/T_2 - 1/T_1)$, where E_a = activation energy, R = gas constant, T_1 and T_2 = reaction temperatures in degrees Kelvin, and k_1 and k_2 = reaction rates at T_1 and T_2.

The Arrhenius activation energy for the chlamydial neutralization using MAb UM-4 was determined to be 17.6 kcal/mol (ca. 73.6 kJ/mol) from the reactions at 4 and 22°C and 22.4 kcal/mol (ca. 93.7 kJ/mol) from the reactions at 22 and 37°C.

Because serovar D EBs are more heat labile than serovar L_2 EBs, we determined the optimal temperature and duration of incubation for serovar D and MAb 597. A reaction temperature of 37°C for 30 min was significantly more effective than incubation at 37°C for 60 min, 22°C for 60 min, and 4°C overnight ($P < 0.05$ and 0.001 for MAb IgG

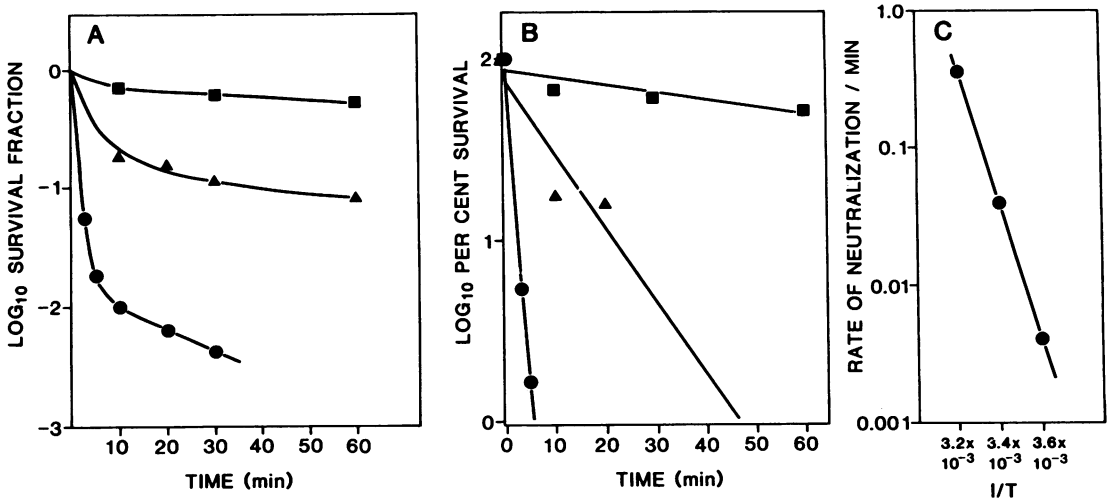

FIG. 6. Kinetics of neutralization of serovar L_2 infectivity at different temperatures of incubation. Purified EBs were incubated with 28.6 μg of UM-4 IgG or NMIgG per ml at 4°C (■), 22°C (▲), and 37°C (●). (A) At various times as indicated, aliquots of each reaction were taken and placed on ice until inoculated onto HeLa cells for determination of residual infectivity. (B) Regression slopes for the linear portions of the kinetic curve in panel A. (C) Relationship between the rate of the neutralization reaction and the incubation temperature.

concentrations of 4 and 0.4 μg/ml, respectively). For reactions incubated at 4°C overnight and at 37°C for 60 min, the infectivities of the control preparations were only 16 and 33% of duplicate controls incubated at 22°C for 60 min or at 37°C for 30 min, respectively.

Stoichiometry. We obtained particle counts by electron microscopy of EBs in some of the reaction mixtures described above and calculated the number of IgG molecules in the mixture that produced a range of neutralization results, assuming full functionality of the IgG preparations. The ratio of total EB particles to IFU for various experiments varied from 39:1 to 83:1, with a mean of 67:1 for serovar L_2. Serovar D, similar to most other non-lymphogranuloma venereum serovars with which we have worked, had a greater range of EB/IFU ratios, varying between 100:1 and 1,678:1 with a mean of 1,619:1. Figure 7 shows that when the IgG/EB ratios for serovars L_2 and D were plotted against percent neutralization attained by MAbs C11 and 597, respectively, 50% neutralization of serovar L_2 and D infectivity can be obtained at a ratio of 10^4 IgG molecules per EB in the reaction mixture, while the corresponding ratio for the serovar-specific MAb UM-4 was 10^3 IgG molecules per EB.

DISCUSSION

Neutralization of *C. trachomatis* infectivity with MAbs to MOMP proceeds with first-order kinetics, having a large activation energy of approximately 20 kcal/mol (ca. 84 kJ/mol) and requiring nearly equal stoichiometric amounts of antibody molecules to MOMP molecules. Neutralization is highly sensitive to multiple factors in the assay.

The most important factor in the neutralization process is the heat lability of EBs. This lability has been noted in several earlier studies in which as much as 50 to 70% of the antigen could be inactivated after incubation at 37°C for 1 h (2, 12, 23). This is of critical importance because the efficiency of the neutralization reaction decreases exponentially with an increasing proportion of inactivated EBs in the antigen preparation, suggesting that the quality of the antigen is a very important factor for the assay. This is especially critical for the non-lymphogranuloma venereum serovars since these serovars have a high EB particle/IFU ratio. Thus, the use of antigen preparations which have been partially inactivated through improper purification, prolonged storage, or repeated freezing and thawing may also substantially affect the detection of neutralization.

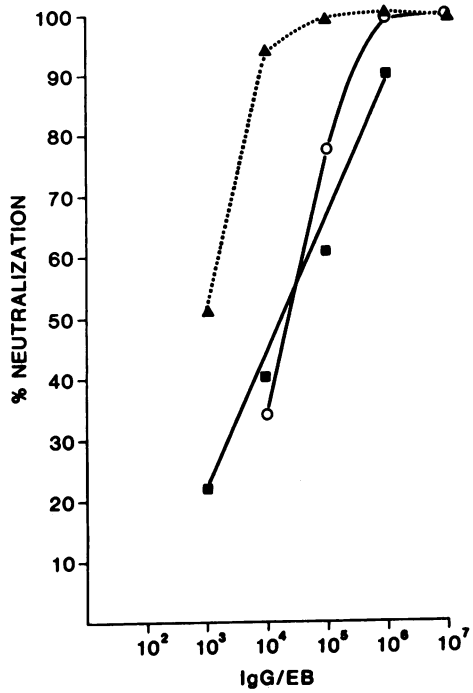

FIG. 7. Correlation between the IgG molecule/EB ratio and neutralization. Percent neutralization is calculated as [(IFU for the control − IFU for the test MAb)/IFU for the control] × 100. Serovar D and L_2 EBs were reacted with different concentrations of MAbs 597 and C11, respectively, and serovar L_2 EBs were reacted with MAb UM-4 by using standard neutralization assay conditions. EB particle counts were obtained by electron microscopy for each reaction mixture. The IgG molecule/EB ratio was plotted against the percent neutralization for each reaction.

The addition of BSA or normal mouse IgG as an antibody diluent also serves to reduce thermal inactivation of EBs during the incubation process, but its effectiveness depends on the concentration of the protein carrier and the serovar. Heat-inactivated EBs bind antibody but do not form inclusions. Thus, addition of an adequate amount of protein carrier to the reaction mixture ensures that thermal inactivation of *C. trachomatis* EBs is minimized as a confounding factor for neutralization. The use of normal serum as a control and 1% normal serum as a diluent for reactions in which serum is the source of antibody not only minimizes heat inactivation of EBs but also takes into account the nonspecific inactivation of EBs by normal sera (3, 14).

To further characterize the neutralization reaction, we monitored the kinetics of the reaction with various antibody concentrations and temperatures of incubation. Neutralization is favored by high antibody concentration and a reaction temperature of 37°C. Both kinetic curves showed marked biphasic rates of neutralization, with the initial exponential rate gradually declining to a plateau. This may reflect the decline in availability of antibody binding sites as the reaction proceeds and the increase of steric constraints on binding as more sites on the same EB have been bound. Two conclusions pertaining to the duration of incubation and reaction volume in the neutralization assay can be drawn from these data. In the present study and that reported by Su et al. (24), the neutralization reaction was essentially completed within 10 min at 37°C. Since the use of an incubation period at 37°C longer than 30 min leads to excessive thermal inactivation of EB infectivity, short incubation periods are clearly preferred. The reaction volume experiment illustrates the importance of using a small reaction volume, especially when an unknown serum or secretion is being tested or when the antibody concentration may be low or limiting.

On the basis of the reaction rates at different temperatures, the activation energy for the neutralization of serovar L_2 by MAb UM-4 was determined to be approximately 20 kcal/mol (ca. 84 kJ/mol). The Arrhenius activation energy of a reaction is a measure of the energy required to activate the reactants to a reactive state, which, in this case, is to form an antigen-antibody complex. The major factors involved are the diffusion coefficients of the reactants and the energy required to induce a topological fit. E_as for most virus neutralization reactions are in the range of 6 to 9 kcal/mol (ca. 25 to 38 kJ/mol) (9, 17). The relatively high value for *C. trachomatis* neutralization is consistent with the neutralization reaction requiring both high temperature and high antibody concentration to drive the reaction. The requirement for high concentrations of antibody for *C. trachomatis* neutralization was initially reported by Philip et al. (22), who observed that neutralization was correlated with micro-IF titers of 1,024 or greater. The difference between the chlamydial and viral energy requirements for neutralization is likely multifactorial. A major difference relates to the size of chlamydiae and viruses used in the neutralization kinetics experiments. The diameter of an EB is over 10 times that of a poliovirus, implying a >2,000-fold difference in volume and hence a corresponding difference in mass. Thus, EBs require more energy of diffusion to bring antigen and antibody together. Another reason may relate to the mechanism of neutralization. If neutralization requires cross-linking of MOMP molecules, then additional rotational energy is required for the bivalent binding of IgG on MOMP after the initial antigen-antibody contact. The cross-linking hypothesis of *C. trachomatis* neutralization is supported by several lines of evidence. Caldwell and Perry (7) showed that intact Ig was required for neutralization. The enhancement of neutralization by the addition of anti-globulin also suggests that Ig cross-linking may be a mechanism of neutralization (5). We previously reported that in antibody excess, *C. trachomatis* neutralization exhibited a prozone phenomenon and that MAbs to MOMP inhibited porin function (20, 21). These observations are also consistent with the cross-linking hypothesis.

Stoichiometric calculations of IgG-mediated neutralization showed that ratios of approximately 10^4 IgG molecules per EB are required for 50% neutralization of serovars D and L_2 by using the species-specific MAbs for MOMP. Su et al. (24) estimated that there are 2.86×10^4 MOMP epitopes on the surface of each EB. Our data obtained with the species-specific MAbs are consistent with the binding of most MOMP epitopes for an EB to be rendered noninfectious. Although the corresponding ratio for the serovar-specific MAb UM-4 is considerably less (10^3 IgG molecules per EB), these ratios are nevertheless generally higher than that required for viral neutralization, in which a few antibody molecules may be sufficient for neutralization.

Early studies of poliovirus neutralization suggested a single-hit model of virus inactivation. Later studies with MAbs led to a hypothesis consistent with neutralization resulting from a locking together of virion structure by antibody cross-linking (1). A simple theoretical model that shows first-order kinetics as well as provides for a multi-hit phenomenon was initially proposed for the neutralization of animal viruses by Tyrrell and Horsfall (25). In that model, neutralization proceeds in a stepwise fashion, in which each antibody which binds a virion decreases the infectivity of the virion by $1/n$, where n represents the number of binding sites on the virion, if all binding sites are critical for infectivity. For most viruses, however, a number less than n, say x, may be the threshold required to render the virus noninfective (neutralized). The values for n and x are 30 and 4, respectively, for polioviruses (13). For chlamydial neutralization, n may equal 2.86×10^4 and x may be between 10^3 and 10^4. Further studies with a wider panel of monoclonal antibodies specifying different epitopes on MOMP are required to delineate this important relationship.

Several aspects of these in vitro neutralization studies using antibodies to MOMP may have implications for humoral immunity in natural infections and for vaccine design. The high EB particle/IFU ratio, especially for non-lymphogranuloma venereum serovars, may mean that a substantial proportion of antibodies developed in response to an infection or a vaccine are expended in binding noninfective EBs, regardless of the relative neutralizing efficiencies of the different types of antibody produced. This may have evolved as a mechanism for chlamydiae to evade host defenses. Secondly, the differing in vitro neutralization efficiencies of MAbs specifying different epitopes on MOMP may mean that in polyclonal sera these antibodies are competitive and that although a MOMP vaccine with species specificity is desirable, it may evoke antibodies that are relatively inefficient at neutralizing the infectivity of EBs compared with serovar-specific antibodies. The higher efficiency of the serovar-specific MAb to MOMP in neutralizing the infectivity of EBs may be reflected in the predominantly serovar-specific protective immune response seen in natural human infections and experimental infections in primates. Competition studies with more MAbs with different specificities are required to define these relationships and how they may affect antibody-mediated protection in vivo. Thirdly, if the

serovar-specific antibodies can neutralize chlamydial infectivity more effectively than species-specific antibodies in natural infections, then, in the long term, the serovar-specific regions of MOMP must be susceptible to immune selection pressure and this selection may be the underlying mechanism for the evolution of serovars and new variants. The species-specific regions of MOMP may be conserved for important functions suggested for MOMP such as porin formation and adhesion. Further neutralization studies using antibodies with different specificities to MOMP against different serovars and new variants will be important for a better understanding of the role of MOMP in the pathogenesis of chlamydial infections and for vaccine design.

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