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Differences in Susceptibilities of the Lymphogranuloma Venereum and Trachoma Biovars of *Chlamydia trachomatis* to Neutralization by Immune Sera

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Sera from seven patients from whom a *C. trachomatis* serovar L2 strain was isolated were tested in vitro for their ability to neutralize the infectivity of this organism. In one patient an inguinal lymph node was culture positive, whereas the remaining six patients had positive rectal biopsies. Sera from four of the patients, including the patient with the lymph node isolate, failed to neutralize serovar L2(434). In addition, the homologous strain recovered from the inguinal lymph node was available and was resistant to neutralization by the homologous sera. However, the same sera effectively neutralized a trachoma serovar, E(Bour). All four sera had inclusion immunofluorescent-antibody titers to *C. trachomatis* serovar L2 of 2,048 to 16,384 and microimmunofluorescent-antibody titers to the lymphogranuloma venereum biovar were equal or higher in all cases than to the 12 serovars of the trachoma biovar. The three remaining sera, while neutralizing the infectivity of the L2 strains tested, neutralized serovar E to a greater extent. These sera had the same inclusion immunofluorescent antibody titers as the sera that failed to neutralize serovar L2. To see whether this difference in the sensitivity of the biovars toward neutralization could be characterized, sera were obtained from mice immunized with different doses of both serovars L2 and E. Sera obtained from mice immunized with serovar E were able to effectively neutralize the homologous strain. In contrast, neutralization of the immunizing strain, L2(UCI-20), was not seen with sera obtained on days 7, 14, and 21 after immunization from animals receiving 8×10^5 and 8×10^4 inclusion-forming units of L2(UCI-20); however, these same sera neutralized serovar E. However, with a higher immunizing dose of L2 (10^7 IFUs), both E and L2 were neutralized with sera obtained 7 and 14 days after immunization. Therefore, the relative resistance to neutralization by serovar L2 compared with that of serovar E in the mouse model was inoculum dependent.

The species *Chlamydia trachomatis* is composed of 15 established serovars which are divided into two biovars, the lymphogranuloma venereum (LGV) biovar and the trachoma biovar. Members of the LGV biovar, in general, cause more systemic illness and are more invasive (8). In animal models they are more lethal and in cell cultures they are easier to propagate than the trachoma biovar strains, probably due to their invasive properties (2, 8). These two biovars also differ in their DNA structure as shown by restriction endonuclease analysis (3, 4) and their outer membrane proteins with regard to charge (60 and 12 kilodaltons) and molecular mass (12 kilodaltons) (1).

In this report we present evidence that failure to be neutralized by homologous immune sera is a property possessed by some strains of the LGV biovar and is not evident with strains in the trachoma biovar. Microorganisms able to evade the bactericidal activity of normal sera or specific antibody and complement have been shown in human infections and with animal models to be more invasive than serum-susceptible strains (9, 10). Therefore, this relative resistance exhibited by strains of the LGV biovar may confer a selective advantage that contributes to their more invasive properties compared with those of strains of the trachoma biovar, which are predominately localized to mucous membranes.

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MATERIALS AND METHODS

Chlamydia strains. The *C. trachomatis* strains used in this study were from isolates obtained from the American Type Culture Collection (Rockville, Md.), S.-P. Wang, C.-C. Kuo, J. Schachter, and S. Darougar as well as from clinical isolates raised in our laboratory (UCI strain). Stock isolates used were 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). All stock strains were raised in either HeLa 229 cells or McCoy cells and frozen in a sucrose-phosphate glutamic acid buffer (2-SPG) at −70°C (6).

Clinical isolates were collected, placed in 2-SPG, and cultured on McCoy or HeLa 229 cells. To obtain high titers, strains were passed at least four times on HeLa 229 or McCoy cells before being frozen in 2-SPG at −70°C. Clinical isolates were serotyped by microimmunofluorescence (MIF) with monoclonal antibodies (11).

Chlamydia serology. Sera obtained from patients were assayed by two different indirect immunofluorescent assays. The MIF assay used elementary bodies of the 15 serovars of *C. trachomatis* as the antigens as previously described (5). In the other method, which will be referred to as the inclusion indirect immunofluorescence assay (IFA), acetone-fixed McCoy cells infected with serovar L2(434) were used (5). Dilutions of sera in phosphate-buffered saline (0.01 M, pH 7.4) were placed on the antigen-containing slides and allowed to incubate at 37°C for 30 min, followed by washing in phosphate-buffered saline and water. Subsequently, a

TABLE 1. MIF titer of human serum samples from patients with a *C. trachomatis* serovar L2 infection

Patient no.	MIF titer of serovar:														
	A	B	Ba	C	D	E	F	G	H	I	J	K	L1	L2	L3
1	2,048	4,096	4,096	256	4,096	4,096	2,048	1,024	1,024	256	512	2,048	2,048	8,192	1,024
2	512	512	512	512	512	512	512	256	128	256	512	128	1,024	1,024	1,024
3	1,024	1,024	1,024	256	2,048	1,024	1,024	1,024	256	512	512	256	2,048	2,048	512
4	1,024	2,048	2,048	512	2,048	2,048	1,024	1,024	512	256	1,024	1,024	2,048	2,048	2,048
5	1,024	512	512	256	1,024	1,024	512	1,024	1,024	256	1,024	1,024	1,024	1,024	1,024
6	512	512	1,024	256	512	512	256	256	256	256	512	256	1,024	1,024	512
7	512	256	512	256	512	512	512	1,024	256	256	256	512	512	1,024	1,024

fluorescein isothiocyanate-conjugated second antibody reactive with the primary antibody was added, incubated, and washed as before. Slides were read with an Olympus microscope equipped with epi-illumination and a 100-W mercury vapor light source.

In vitro neutralization. In vitro neutralization assays were performed as previously described (6). Serum samples were diluted in phosphate-buffered saline. Dilutions were performed in 96-well microdilution plates. Lyophilized guinea pig serum (M. A. Bioproducts, Whittaker, Md.) was reconstituted in Hanks balanced salts solution, 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]) were added to the appropriate antibody dilutions and control mixtures that contained no antibody. All reactions contained 5% guinea pig serum. A comparison of controls containing 5% guinea pig serum with and without 10% normal human serum consistently gave the same neutralization results; therefore controls used in this study contained 5% guinea pig serum without added human serum. 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 phosphate-buffered saline immediately before infection. The chlamydial inoculum was adjusted so that 20 to 30% of the control monolayers were infected, i.e., 40 IFU per ×400 field. Cells were infected by centrifuging them at $1,000 \times g$ 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), and cycloheximide (1 μg/ml) was added. 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 previously described mouse monoclonal antibody, E4 (6), that recognizes all 15 serovars of *C. trachomatis*. Monolayers were then washed, a goat anti-mouse 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 cover slip in the test and control assays were read, an average was taken, and the results were expressed as a percentage reduction of IFU from control monolayers. All determinations were performed at least twice on different days, except where noted due to a limiting amount of serum. A ≥50% reduction from control IFU in infectivity was defined as neutralization.

Mouse immunizations. Thirty-eight female BALB/c mice (Simmonsen Laboratories, Gilroy, Calif.) 4 to 6 weeks of age were divided into eight groups; mice received a single intraperitoneal injection of various concentrations of IFU (in phosphate-buffered saline) of either L2(UCI-20) or E(Bour) raised in McCoy cells. Sera obtained by periorbital bleeding from each group of mice receiving the same dose were collected and pooled before injection and 7, 14, and 21 days after immunization. Blood was allowed to clot on ice, and serum was separated and frozen at −70°C. Serum samples were assayed by inclusion IFA and in vitro neutralization.

Data analysis. The two-tailed Student *t* test was used to analyze the data.

RESULTS

Neutralization studies with sera from patients infected with serovar L2. An isolate of *C. trachomatis*, L2(UCI-20), was cultured from an inguinal lymph node from a patient (no. 4) with LGV. This isolate was typed as serovar L2 by using monoclonal antibodies raised in our laboratory. Acute- and convalescent-phase sera obtained from this patient had MIF titers to L2 of 1,024 and 2,048, respectively, and inclusion IFA titers of 4,096 and 8,192, respectively. MIF titers to the other *C. trachomatis* serovars were equal to or less than the titer to L2 (Table 1).

Both acute- and convalescent-phase sera were used for in vitro neutralization assays with the homologous isolate, L2(UCI-20). No significant neutralization was observed with two different sources of complement, guinea pig and human sera, at concentrations of 5 and 10%. In Table 2 are the neutralization data from the high-titer patient no. 4 convalescent-phase sera against various serotypes and strains of

TABLE 2. Neutralization of *C. trachomatis* strains by serum[a] from patient no. 4

Biovar	Serovar	Strain	No. of assays	% Reduction ± SD with the following serum dilution:		
				10^{-1}	10^{-2}	10^{-3}
LGV	L2	UCI-20	6	9 ± 17	9 ± 9	0 ± 12
	L2	ATCC-434	4	9 ± 11	1 ± 14	1 ± 0
	L2	UW-297	1	13	31	14
	L2	UW-329	2	62 ± 19	19 ± 27	18 ± 6
	L1	ATCC-440	3	45 ± 10	1 ± 3	0 ± 0
	L1	IOL-1962	2	19 ± 6	4 ± 4	1 ± 1
	L1	EA-1	1	15	15	0
	L3	EA-3	2	31 ± 17	35 ± 45	0 ± 3
	L3	EA-4	2	0 ± 0	0 ± 1	0 ± 0
Trachoma	E	Bour	2	100 ± 5	95 ± 1	77 ± 11
	E	MI-2659	2	83 ± 11	55 ± 24	30 ± 4
	E	MI-2225	2	88 ± 2	61 ± 21	2 ± 3
	D	MI-1555	2	79 ± 3	59 ± 17	23 ± 6
	G	MI-2130	2	87 ± 5	65 ± 14	14 ± 2
	F	MI-8	1	69	47	33
	C	TW-3	1	84	50	53
	J	MI-2341	1	74	38	0

[a] Serum was from patient no. 4, who was infected with L2(UCI-20), and had an L2 MIF titer of 2,048 and an inclusion IFA titer of 8,192.

TABLE 3. Results of in vitro neutralization assays with homologous sera and *C. trachomatis* isolates[a]

Strain	Serovar	Inclusion IFA antibody titer	% Reduction ± SD with the following serum dilution:		
			10^{-1}	10^{-2}	10^{-3}
MI-2659	E	<32	11 ± 16	4 ± 6	0 ± 0
MI-8	F	64	0 ± 0	2 ± 3	3 ± 5
MI-1555	D	64	22 ± 11	5 ± 8	7 ± 11
MI-2341	J	128	91 ± 12	69 ± 35	0 ± 0
MI-2225	E	256	79	25	6
MI-2130	G	1,024	98 ± 3	51 ± 42	23 ± 6

[a] Both the isolate and serum were collected on the same day from the patient. All assays were performed twice on different days, with the exception of those for strain MI-2225.

C. trachomatis. If we consider a ≥50% reduction in infectivity at a 10^{-1} dilution of serum as neutralization, then only one of the nine strains of the LGV biovar were neutralized compared with all eight strains of the trachoma biovar ($P < 0.001$). Six trachoma biovar strains were neutralized at a 10^{-2} dilution of serum compared with none of the strains neutralized in the LGV biovar.

Homologous human serum collected the same day as the positive culture was available for six of the non-LGV isolates neutralized by the heterologous human serum from the L2-infected patient (no. 4). Table 3 lists results of in vitro neutralizations for these six serum samples as well as the corresponding inclusion IFA titers of the homologous sera. Sera with an inclusion IFA titer of <64 did not neutralize the homologous strain. However, the higher-titered sera were able to neutralize the homologous strain. The fact that some of the strains were not neutralized, most likely due to the low level of specific antibody in the sera, supports the fact that the neutralization of these strains (Tables 2 and 3) seen with the heterologous L2 (patient no. 4) serum was antibody specific.

Sera from six patients from whom rectal *C. trachomatis* L2 isolates were cultured were also tested in neutralization assays against *C. trachomatis* L2(UCI-20), L2(434), and E(Bour). MIF titers of these sera against the 15 serovars of *C. trachomatis* can be seen in Table 1. In addition, the homologous L2(25667R) isolate was available from one of the six patients (no. 2) and tested against the homologous serum. Sera from three of the patients (no. 1, 6, and 7) gave results similar to those of the serum obtained from patient no. 4 in that they did not neutralize the two L2 strains tested, yet at a 10^{-1} serum dilution they neutralized strain E(Bour) (Fig. 1). The antibody dilutions that gave a 50% reduction in serovar E infectivity for these sera were $<1 \times 10^{-3}$ for patient no. 4, 1×10^{-2} for patient no. 7, 2×10^{-3} for patient no. 1, 1.6×10^{-2} for patient no. 6.

Three serum samples (patients no. 2, 3, and 5) neutralized all isolates tested, yet in all cases the greatest amount of neutralization was seen with serovar E. Of the homologous isolate-serum pair tested from patient no. 2, there was 50% neutralization at a serum dilution of 6.5×10^{-2}. This was similar to the results for the other L2(434) isolate tested against this serum. However, with this serum there was a 1-log-unit difference in the dilution giving 50% infectivity reduction with serovar E compared with that with serovar L2.

Antibody titers measured by inclusion IFA did not correlate with neutralization titers for the L2 serovars (Fig. 1). However, there was a correlation of inclusion IFA antibody titer with degree of neutralization of serovar E.

Mouse model of serovar L2 resistance to neutralization by specific L2 antisera. In an attempt to determine whether this difference in susceptibility to neutralization seen in humans between the LGV and trachoma biovars could be reproduced in an animal model, BALB/c mice were injected with either L2(UCI-20) or E(Bour). Preimmunization MIF and inclusion IFA titers from the animals did not reveal any antibody to *C. trachomatis*.

Two different sets of immunizations were performed. In the first set of immunizations six groups of three mice each were injected with concentrations ranging from 8×10^7 to 8×10^5 IFU for L2(UCI-20) and 8×10^9 to 8×10^7 IFU for E(Bour). Another set of animals were injected with L2(UCI-20) with concentrations from 8×10^6 to 8×10^4 IFU, which overlapped those previously used, and concentrations of E(Bour) from 8×10^6 to 8×10^5 IFU. The results shown in Fig. 2 represent six of the groups that were immunized.

Serum samples taken before immunization as well as 7, 14, and 21 days after the single intraperitoneal injection were tested in vitro against both L2(UCI-20) and E(Bour) for their ability to neutralize the infectivity of these strains. Preimmunization sera did not reduce the infectivity of either strain tested. Sera from animals immunized with the highest dose of L2(UCI-20), 8×10^7 IFU, effectively neutralized both the homologous strain, L2(UCI-20), and serovar E. Neutralization was seen 7 and 14 days after immunization at a 10^{-1} serum dilution; however, neutralization was not seen despite a higher inclusion IFA titer with sera obtained at day 21 after immunization. Sera obtained by using a lower inoculum of L2(UCI-20), 8×10^5 to 8×10^4 IFU, although able to neutralize the infectivity of E(Bour), were not able to effectively neutralize the immunizing L2(UCI-20) strain. These differences in neutralization of L2 and E were significant at days 14 ($P < 0.001$) and 21 ($P < 0.05$) for an immunizing dose of 8×10^5 IFU of L2 and at days 7, 14, and 21 ($P < 0.05$) for 8×10^4 IFU of L2 inoculum.

Sera obtained from animals immunized with the higher doses of E(Bour), 8×10^9 or 8×10^8 IFU, neutralized both E and L2(UCI-20) (Table 4 and Fig. 2). Sera from all other groups of mice immunized with E neutralized serovar E but failed to neutralize L2(UCI-20).

Inclusion IFA titers obtained with all of the mouse sera can be seen in Table 4. As with the human sera, there was a correlation between inclusion IFA titer and the ability of sera to neutralize infectivity of serovar E. The only exception to this was serum obtained on day 21 after the injection of 8×10^7 and 8×10^6 IFU of serovar L2. In contrast to the results with serovar E, however, there was no clear correlation of inclusion IFA titer and ability to neutralize L2(UCI-20). From the inclusion titer IFA alone one could not predict whether strain L2(UCI-20) would be neutralized and, if neutralized, to what degree.

DISCUSSION

Although no specific virulence factors for *C. trachomatis* have yet been described, it is clear from clinical manifestations that serovars belonging to the LGV biovar that cause systemic infections are more invasive than the trachoma strains, which cause mainly localized genital or ocular infections. Not only are the LGV strains isolated from more severe systemic infections in humans, but they have also been shown in animal models to be more virulent (2, 13). In this report we propose that resistance to neutralization of infectivity by immune sera as demonstrated by some LGV strains may be a virulence property possessed by some of these invasive strains of *C. trachomatis*.

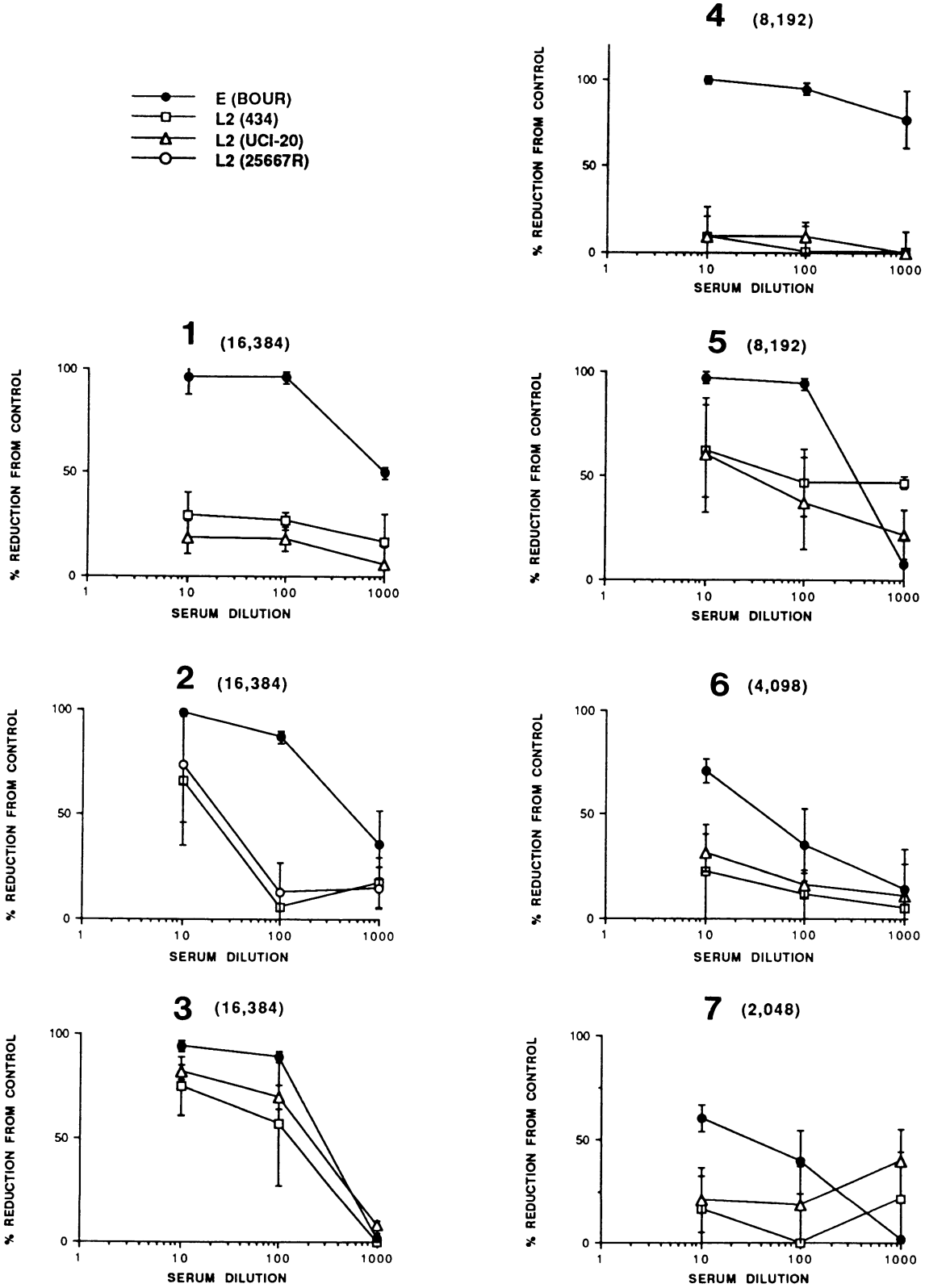

FIG. 1. In vitro neutralization of *C. trachomatis* strains by human sera from patients infected with serovar L2. Results are expressed as percentage reduction of IFU from that of the control, and each point is the mean from at least two determinations. Vertical bars represent the standard deviations (±) for each point. Inclusion IFA titers for each sera are in parenthesis after the patient number.

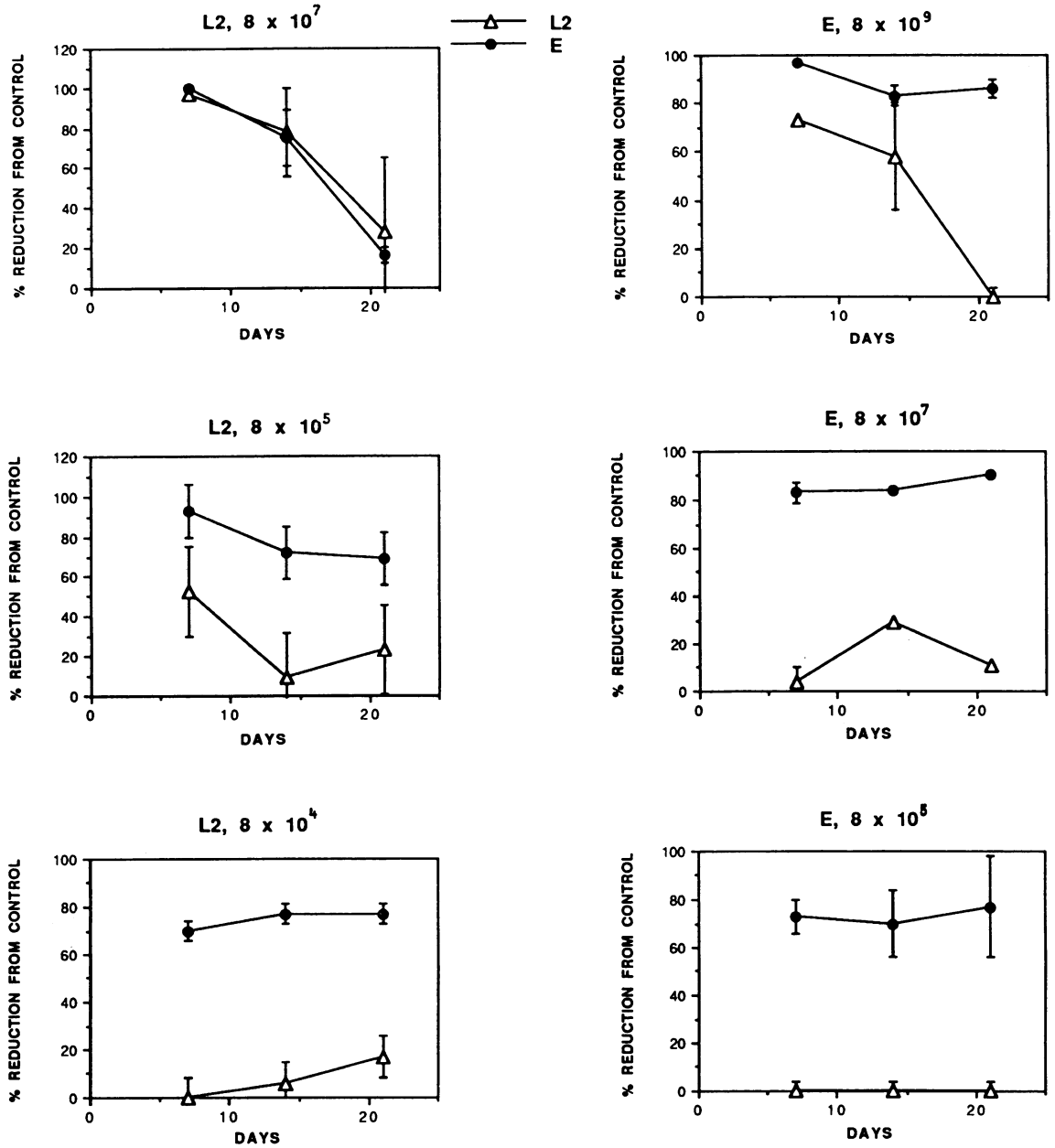

FIG. 2. In vitro neutralization data from sera obtained from mice immunized with either L2(UCI-20) or E(Bour). The serovar and innoculating dose (IFU) used for each group are at the top of each graph. Results are for the 10^{-1} dilution of serum for each time point. Each point represents the mean of at least duplicate experiments for pooled sera from at least three mice. Vertical bars represent the standard deviations (±) for each time point determination.

Serum from the patient with an L2 isolate from an inguinal lymph node tested in this study had a high chlamydial antibody titer; therefore, overall lack of antibody is an unlikely explanation for the resistance to neutralization seen with the homologous L2(UCI-20) strain when tested with this serum. The possibility exists that this patient may have had previous exposure to the trachoma biovars, and this could serve as an explanation for the greater neutralization of the trachoma biovar strains; however, MIF titers to the other biovars did not demonstrate a higher titer than that obtained with L2. In addition, data from the animal experiments in which mice were used that had no evidence of previous exposure to *C. trachomatis* supported neutralization results obtained with the human sera. Similarly, sera obtained from three of the six patients with rectal L2 isolates were also unable to effectively neutralize the L2 isolates tested. However, the same serum samples neutralized serovar E. Although they showed some neutralizing ability toward the L2 isolates tested, the remaining three sera in all cases neutralized serovar E to a greater extent.

Schoolnik et al. (9) have reported similar findings when examining *Neisseria gonorrhoeae* isolates and sera obtained from patients with disseminated gonococcal infection. Here they found six patients with disseminated gonococcal infections that acute and convalescent sera failed to neutralize or kill the homologous infecting strain. However, these same

TABLE 4. Inclusion IFA titers of mice immunized with L2(UCI-20) or E(Bour)

Immunogen	IFU injected	IgM titer[a]	IgG[b] titer (L2 neutralization/E neutralization) on day:		
			7	14	21
L2(UCI-20)	8×10^7	64	256 (+/+)	512 (+/+)	1,024 (−/−)
	8×10^6	32	256 (+/+)	512 (+/+)	1,024 (−/−)
	8×10^5	32	128 (−/+)	256 (−/+)	512 (−/+)
	8×10^4	<32	64 (−/+)	256 (−/+)	256 (−/+)
E(Bour)	8×10^9	32	128 (+/+)	512 (+/+)	1,024 (−/+)
	8×10^8	<32	32 (−/+)	128 (−/+)	256 (−/+)
	8×10^7	<32	32 (−/+)	128 (−/+)	256 (−/+)
	8×10^6	<32	64 (−/+)	128 (−/+)	128 (−/+)
	8×10^5	<32	32 (−/+)	64 (−/+)	64 (−/+)

[a] Immunoglobulin M (IgM) titers shown were from sera obtained on day 7; on both days 14 and 21 sera had titers of <32 for immunoglobulin M.
[b] IgG, Immunoglobulin G.

sera, which did not possess bactericidal antibodies to the homologous strain, effectively killed *N. gonorrhoeae* strains isolated from patients with uncomplicated urethritis. Sera from the other two patients with disseminated gonococcal infections had a low level of bactericidal antibodies toward the homologous strain and higher bactericidal levels to strains from patients with localized infections. Therefore, it seems that the more invasive strains of *C. trachomatis* and *N. gonorrhoeae* may share a common property that contributes to their pathogenicity.

In the mouse model presented here, it appears that this relative resistance to neutralization by the LGV strains is dependent on the immunizing dose. In a study of a model of systemic *C. trachomatis* infection in mice, Brunham et al. (2) described what they referred to as a paradoxic finding in that there was greater growth of *C. trachomatis* in spleens of mice inoculated with 10^6 IFU of L2 than in mice inoculated with 10^7 IFU of L2. Based on our data, it is interesting to speculate whether this finding by Brunham et al. (2) is due to sera from the mice immunized with 10^6 IFU not being as effective in neutralizing serovar L2 in the initial stages of infection compared with sera from animals receiving a higher inoculum. These authors also reported that passive transfer of sera obtained from mice immunized with serovar L2 failed to protect the animals from a subsequent L2 challenge. We obtained similar results with serovar L1 as the antigenic stimulus (13). It would be interesting to see whether this finding is true of homologous serum that neutralizes the homologous strain in vitro. Yong et al. (12) have described a differential effect of human mononuclear phagocytes against the LGV and trachoma biovars. They reported that the lack of neutralization of macrophage infectivity by the LGV biovar by homologous antibody may be a unique observation among strains of the LGV biovar not shared by strains of the trachoma biovar. Our data on the relative resistance to neutralization by immune sera among the biovars substantiate this finding.

In summary, the greater degree of resistance to neutralization by immune sera exhibited by members of the LGV biovar over the trachoma biovar strains may give the LGV strains an advantage during critical stages of the infection process and thus be a factor in their frequent association with more invasive infections. This finding is most likely a multifactorial property, as it has been found with other microorganisms (7, 10). Determining the mechanisms responsible for this property will have many implications for the future development of effective broadly reacting chlamydial vaccines.

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