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Systemic *Chlamydia trachomatis* Infection in Mice: a Comparison of Lymphogranuloma Venereum and Trachoma Biovars

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We developed a murine model of systemic infection with *Chlamydia trachomatis* biovar lymphogranuloma venereum (LGV). The pathological features of this infection resemble those of human LGV infection since both are characterized by granuloma formation. Mice developed resistance to reinfection with LGV, and this resistance was based on cellular immune mechanisms since it was transferable with immune spleen cells but not with immune serum. Resistance required viable organisms for induction. We compared LGV biovar infection with trachoma biovar infection. Trachoma biovar produced similar but less marked microbiological and pathological features. Cross-immunity was less apparent between serovars from trachoma and LGV biovars than it was between serovars within the same biovar. This model of systemic *C. trachomatis* infection will be useful in exploring virulence features of LGV.

Chlamydia trachomatis is the etiological agent for several distinctive chronic infectious diseases in humans (16). Two *C. trachomatis* biovars of human pathogens are recognized, each producing notably different diseases. Lymphogranuloma venereum (LGV) biovar produces systemic infection with prominent lymphatic involvement and granuloma formation (17). Trachoma biovar produces mucosal infection of the eye, respiratory tract, or genital tract. Mononuclear cell infiltration, germinal center formation, and late fibrosis characterize the pathology of trachoma (2). The factor(s) possessed by LGV and lacking in trachoma biovars that allows LGV to disseminate is unknown. LGV differs from trachoma biovars in a number of laboratory characteristics which may relate to its enhanced virulence. These characteristics include intracerebral lethality for mice, plaque formation in L cells, and the ability of effectively infect cell monolayers in the absence of centrifugation or host cell treatment with DEAE-dextran (9). The marked difference in clinical expression of disease is somewhat paradoxical given the high (greater than 95%) degree of genomic homology between LGV and trachoma biovars (5, 21).

We were interested in further understanding the "virulence" characteristics of LGV. Our approach was to examine the microbiological, immunological, and pathological features of LGV infection by using the murine model of systemic LGV infection initially described by Rake and Jones (13). We compared the microbiological and histopathological features of systemic LGV infection with those of systemic trachoma biovar infection and examined for evidence of cross-immunity.

MATERIALS AND METHODS

Organism. Serovars B/TW-5/OT, C/TW-3/OT, L_1/440/BU, L_2/434/BU, and L_3/404/BU were used. Serovars L_1, L_2, and L_3 are LGV biovars, and serovars B and C are trachoma biovars. Strains were grown in HeLa 229 cells (7). Organisms were partially purified by centrifugation at 500 × *g* for 10 min to pellet gross HeLa cell debris. Organisms were concentrated to 3×10^8 to 5×10^8 inclusion-forming units (IFU) per ml for trachoma and 1×10^9 to 4×10^9 IFU/ml for LGV from the above supernatant by centrifugation at 30,000 × *g* for 30 min and then were suspended in sucrose-phosphate-glutamate buffer (pH 7.4) (sucrose, 75 g/liter; KH_2PO_4, 0.52 g/liter; Na_2HPO_4, 1.22 g/liter; glutamic acid, 0.72 g/liter; distilled water to 1 liter) containing no antibiotics. Organisms were frozen at −70°C and thawed once before use. Dilutions were prepared in sucrose-phosphate-glutamate before inoculation.

Inoculation of mice. BALB/c female mice (Fred Hutchinson Cancer Research Center, Seattle, Wash.) 5 to 8 weeks of age were used. Organisms in 0.5 ml of sucrose-phosphate-glutamate buffer were injected intravenously (i.v.) via tail veins which had been dilated by immersion in warm water. To monitor the growth of *C. trachomatis*, groups of animals were sacrificed 1 h after inoculation and on days 1, 2, 3, 4, 7, 14, and 19 after infection. Quantitative cultures were performed on spleens and lungs. Animals were sacrificed by ether anesthesia and then exsanguinated.

For evaluation of resistance to reinfection, animals that were 19 days convalescent after primary infection were rechallenged i.v. with the same dilution of homologous organism, and quantitative growth was monitored in groups of animals 1 h after inoculation and on days 1, 2, and 3.

For evaluation of cross-immunity, groups of mice were primed with serovar L_2 (10^6 IFU per mouse) or serovar B (10^8 IFU per mouse) and allowed to convalesce for 19 days. Then mice were rechallenged with the homologous or heterologous serovar (10^6 IFU of LGV per mouse and 10^8 IFU of trachoma per mouse), and quantitative growth in the spleen was determined 48 h later.

For evaluation of whether immunization with nonviable organisms would confer resistance, mice were immunized i.v. with 10^6 IFU of inactivated serovar L_2 per mouse and challenged on day 19 after immunization with 10^6 IFU of viable L_2 per mouse. The organisms used for priming were inactivated with 0.02% Formalin at 4°C for 3 days. Immunization with inactivated organism produced antibody titers of 1:64 against L_2 at day 4 after immunization.

Quantitative cultures. Quantitative cultures were performed on spleens and lungs (6). Preliminary experiments demonstrated that *C. trachomatis* could also be recovered from the liver. However, results from the liver were highly variable and cytotoxicity of the HeLa 229 cell sheet usually

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was produced. For these reasons, quantitative cultures were not routinely performed with livers.

Spleens and lungs were aseptically removed from mice which had been sacrificed and exsanguinated. Organs were weighed, and a 10% (wt/vol) dilution was prepared in sucrose-phosphate-glutamate buffer. Organs were homogenized with mortar and pestle to a homogenous slurry until no large pieces of tissue remained. Gross debris was removed by centrifugation at 300 × *g* for 10 min. The clarified supernatant was frozen at −70°C until it was assayed in cell culture.

Samples (0.1 ml) of the 10-fold dilutions were inoculated onto replicate HeLa cell monolayers pretreated with DEAE-dextran. After centrifugation at 900 × *g* for 1 h, the inoculum was removed and fresh culture medium was added. Cells were incubated for 2 days at 37°C for LGV biovar and for 3 days at 35°C for trachoma biovar. Cells were stained with May-Grunwald-Geimsa, and the number of inclusions was determined on primary isolation.

Serological studies. The indirect micro-immunofluorescent (micro-IF) assay, using multiple serovars of *C. trachomatis* elementary bodies, was used (20). Mouse sera were titrated to endpoint, and anti-mouse immunoglobulin (IgG, IgM, IgA) was used. Determination of IgM and IgG class antibody titer was not made.

Pathology. For evaluation of the histopathological response to primary infection and to reinfection, mice were sacrificed on selected days after challenge. Spleens, livers, and lungs were removed, fixed in 10% Formalin, sectioned, and stained with hematoxylin and eosin.

Passive transfer experiments. To determine whether resistance to reinfection was due to serum antibody or to immune spleen cells, passive transfer experiments were carried out. Sera from animals were obtained from a group of eight mice inoculated i.v. with serovar L_2 on three occasions at weekly intervals. These sera were pooled and had a micro-IF antibody titer to L_2 of 1:512. Normal sera from animals inoculated with uninfected HeLa cell debris without detectable micro-IF antibody to any *C. trachomatis* serovar were used as controls. Immune spleen cells were pooled from the spleens of eight mice 6 days convalescent from a primary infection with serovar L_2. Spleens were aseptically teased apart, and single-cell suspensions were prepared. Gross debris was removed by centrifugation at 100 × *g* for 10 min. Normal spleen cells from uninfected mice were prepared in an identical manner. Animals were given 0.5 ml of normal or immune sera i.v. or 2×10^8 normal or immune spleen cells in 0.5 ml of Hanks balanced salt solution 2 h before i.v. challenge with serovar L_2. Spleens were harvested 48 h after infection, and chlamydial growth was assayed. The degree of protection was assessed by determining the $\log_{10}$ reduction of IFU in spleens of animals that received control (nonimmune) preparations and immune preparations.

Statistical analysis. Two-tailed Student's *t* test was used for comparison of mean lung or spleen titers of organism. IFU were transformed to $\log_{10}$ for statistical comparison.

RESULTS

Optimal dose of *C. trachomatis*. Optimal challenge dose was determined by inoculation of 10-fold dilutions of organism. Spleens and lungs were assayed for the infectivity titer at 48 h after inoculation. Serovar L_2 was lethal at 10^8 IFU per mouse; death occurred within 24 h (Table 1). No mice receiving 10^7 IFU or less died. With serovar B, the mortality was one of eight mice inoculated with 10^8 IFU and none with 10^7 IFU and less. The titers in spleens and lungs were proportional to the inoculum given, except with serovar L_2, which showed paradoxically greater growth in the spleens of mice inoculated with 10^6 IFU than with 10^7 IFU. Mice inoculated with 10^7 IFU looked sicker than those receiving 10^6 IFU. Therefore, this effect may have been due to greater toxicity to the susceptible host cell at the higher dose.

TABLE 1. Infective doses of *C. trachomatis* for mice after i.v. inoculation

Serovar	Inoculum (IFU/mouse)	Mortality[a]	Mean $\log_{10}$ IFU of organisms at 48 h postinfection per[b]:	
			Spleen	Lung
L_2	10^8	9/9		
	10^7	0/9	4.80 (6)	4.97 (7)
	10^6	0/9	5.55 (5)	3.96 (7)
	10^5	0/3	5.15 (3)	2.10 (3)
B	10^8	1/8	4.08 (7)	3.65 (7)
	10^7	0/7	2.38 (5)	2.80 (7)
	10^6	0/7	2.18 (7)	1.94 (7)

[a] Number of mice that died/number inoculated; all deaths occurred within 24 h.

[b] Numbers in parentheses indicate the number of mice studied at each dose.

Based on these results, we chose an inoculum of 10^6 IFU per mouse for serovar L_2 and 10^8 IFU per mouse for serovar B in experiments examining the kinetics of chlamydial growth after i.v. inoculation.

Growth of *C. trachomatis* in spleens and lungs. Both serovar L_2 and B strains successfully grew in spleens and lungs of mice infected i.v. with the organism. Isolation of the organism from spleens and lungs 1 h after injection was taken as the base-line level of infection. At 24 h after infection with 10^8 IFU of serovar B, no infectious chlamydiae were detectable in either spleens or lungs (Fig. 1). A single peak of infectivity was detectable at 48 h, after which infectivity rapidly decayed so that by day 7 both organs were apparently free of infection. After infection with 10^6 IFU of serovar L_2, multiplication proceeded from 1 h postinfection to a plateau level over days 1, 2, 3, and 4 without an eclipse phase and then decayed over the succeeding days; both organs were free of infection by day 14.

On rechallenge with the homologous serovar and dose of organism, animals 19 days convalescent from an initial infection were resistant to reinfection (Fig. 1). This was evidenced by a lower titer of peak multiplication and a more rapid rate of clearance. Resistance appeared more marked in spleens than in lungs. Similar degrees of resistance were noted for serovar B and serovar L_2.

Cross-immunity between serovars of *C. trachomatis*. We next examined the serovar specificity of this resistance, using animals 19 days convalescent from an initial infection and rechallenging them with a heterologous strain (Table 2). Spleens were harvested from groups of mice 48 h postchallenge, and infectivity assays were performed. Mice that were primed with uninfected HeLa cell material and then challenged with either serovar B or L_2 did not display resistance (data not shown). Mice convalescent from an initial infection with serovar B or L_2 were resistant to the homologous serovar to the greatest extent. Mice were also resistant to challenge with heterologous serovars of the same biovar, i.e., serovar B versus C and L_2 versus L_1 and L_2. However, serovar B-primed mice demonstrated no resistance to challenge with serovar L_2. On the other hand, L_2-primed mice demonstrated resistance to challenge with

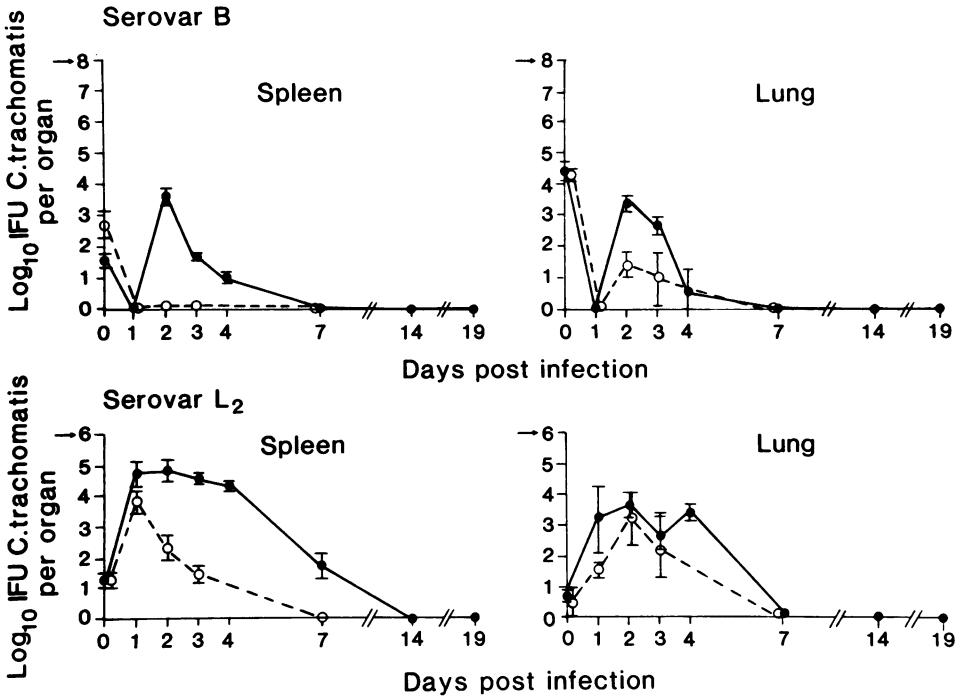

FIG. 1. Growth of *C. trachomatis* in spleens and lungs of mice infected i.v. Arrows indicate the inocula of organism injected. Time zero values represent infectivity recovered 1 h postinfection. Solid circles and lines indicate the growth of *C. trachomatis* in mice primarily infected; open circles and dashed lines indicate mice that were reinfected with homologous organism at the same dose. Values are means from three to four mice per time point, and vertical lines indicate 1 standard deviation of the mean.

the trachoma biovars, serovars B and C, although the resistance was weaker against trachoma than against LGV biovars. Mice immunized with Formalin-killed L_2 were not protected from challenge with L_2.

Passive transfer of resistance with immune serum or spleen cells. For a determination of whether serum antibody or immune spleen cells contributed to the observed resistance, passive transfer experiments were performed with serovar L_2. Results showed that immune sera exerted no significant reduction on the growth of serovar L_2 (Table 3). The residual micro-IF antibody titers to L_2 at the time of spleen assay 2 days after passive immunization were 1:64 to 1:256. Immune spleen cells, on the other hand, were more effective in reducing the growth of the same organism. This finding was consistent with lower (10^6 IFU) and higher (10^8 IFU) inoculum concentration. In this experiment, none of the mice that received 10^8 IFU of L_2 serovar died.

Host response to *C. trachomatis* infection. Spleen weights and serum micro-IF antibody were monitored as parameters of the host response infection. Splenomegaly has been reported to follow murine *Chlamydia psittaci* infection (1). Splenomegaly also accompanied *C. trachomatis* infection (Table 4). With primary infection (10^8 IFU of serovar B or

TABLE 2. Resistance to i.v. challenge with homologous and heterologous serovars of *C. trachomatis*

Priming[a] (serovar/strain)	Challenge[b] (serovar/strain)	Log_{10} reduction of IFU in spleen[c]	*P*
B/TW-5	B/TW-5	2.23	<0.01
	C/TW-3	2.02	<0.01
	L_2/434	−0.02	NS[d]
L_2/434	L_2/434	2.70	<0.01
	L_1/440	1.52	<0.01
	L_3/404	1.08	<0.01
	B/TW-5	0.74	<0.01
	C/TW-3	0.54	<0.05
Formalin-killed L_2/434	L_2/434	0.01	NS

[a] B/TW-5 was given i.v. at 10^8 IFU per mouse; L_2/434 was given i.v. at 10^6 IFU per mouse; controls were given HeLa material.

[b] B/TW-5 and C/TW-3 were given i.v. at 10^8 IFU per mouse; L_1/440, L_2/434, and L_3/404 were given i.v. at 10^6 IFU per mouse on day 19.

[c] At 48 h postchallenge, each group contained five to eight mice; titers represent nonimmunized minus immunized values.

[d] NS, Not statistically significant.

TABLE 3. Passive transfer of resistance with immune serum or spleen cells with L_2 serovar of *C. trachomatis*

Component transferred[a]	Expt	Challenge dose (IFU per mouse)	Log_{10} reduction of IFU in spleen[b] (n)	*P*
Serum	1	10^6	0.1 (3)	
	2	10^8	0.2 (4)	
Spleen cells	1	10^6	0.6 (4)	<0.01
	2	10^8	0.9 (4)	<0.01

[a] Mice were given 0.5 ml of normal or hyperimmune (1:512) serum or 2 × 10^8 normal or immune (6 days postinoculation) spleen cells in 0.5 ml of Hanks balanced salt solution i.v. 2 h before challenge inoculation.

[b] Titer difference between mice receiving normal serum or cells and those receiving immune serum or cells. A residual titer in mice that received immune spleen cells but were not challenged was detected in the spleen of only one of four mice tested. This titer was 1.30 log_{10} IFU; the average titer for the four mice was 0.58 log_{10} IFU.

TABLE 4. Splenomegaly response among mice primarily infected and rechallenged with *C. trachomatis*

Serovar	Inoculation[a]	Mean spleen wt (mg) ± SD at day postinfection[b]							
		0	1	2	3	4	7	14	19
B	Primary	117 ± 5	100 ± 10	167 ± 12	200 ± 20	260 ± 46	183 ± 21	183 ± 21	160 ± 10
	Rechallenge	150 ± 0	186 ± 21	250 ± 20	253 ± 36	ND[c]	230 ± 30	ND	ND
L_2	Primary	110 ± 14	128 ± 17	168 ± 10	210 ± 8	233 ± 17	168 ± 25	158 ± 5	145 ± 6
	Rechallenge	130 ± 27	185 ± 10	183 ± 15	198 ± 5	ND	142 ± 15	ND	ND

[a] The i.v. inoculum dose per mouse was 10^8 IFU of serovar B and 10^6 IFU of L_2.
[b] Three mice were used per time point to calculate spleen weights in serovar B-infected mice; four mice per time point were used for serovar L_2 infection.
[c] ND, Not determined.

10^6 IFU of serovar L_2), maximum spleen weights were observed at day 4 when spleens were ca. twofold heavier than they were immediately postinfection. Responses were similar for both serovar B and L_2. On rechallenge, splenomegaly appeared sooner than during primary infection. Significant increases in spleen weight were detectable by 24 h postrechallenge, whereas on primary infection, significant increases were not detectable until 48 h postinfection.

After primary infection with 10^8 IFU of serovar B and 10^6 IFU of serovar L_2, serum antibody was detectable by day 3, peaked between days 4 and 7 (peak titer, 1:256 with L_2 and 1:64 with serovar B), and persisted until at least day 19. After reinfection, significant increases in serum micro-IF antibody were also apparent.

Histopathological findings. After primary inoculation with serovar L_2 (10^6 IFU), the histological findings in spleens were hyperplasia of white pulp with a peak reaction on days 2 and 3, with mild polymorphonuclear infiltration that gradually subsided and returned to normal by day 7. The most notable findings were in the liver, where granuloma formation was evident. This reaction peaked on day 4 with the appearance of mononuclear cells. The granuloma formation was decreased but still observable on day 19. The findings in the lungs included mild patchy interstitial infiltration with predominantly polymorphonuclear leukocytes on days 2 and 3 which gradually returned to normal by day 7.

On rechallenge inoculation with serovar L_2, in the liver, many foci of polymorphonuclear leukocyte infiltration were seen on day 1, with subsequent infiltration with mononuclear cells apparent on days 2 and 3. Histological changes in the lungs were generally not remarkable, except for mild patchy interstitial pneumonia on days 2 and 3.

Histological findings in mice inoculated with serovar B were similar to but less marked than those described for serovar L_2.

DISCUSSION

We have developed a model of systemic *C. trachomatis* infection in mice. This model appears to be particularly relevant to LGV infection in humans since both are characterized by granuloma formation (17). This model, however, does not fully mimic human LGV disease in which cutaneous or mucosal inoculation is followed by regional lymphatic infection. Bacteremic spread, however, is a feature of human LGV infection and thus this model partially mimics the human disease. This model may also have some application to the study of some forms of trachoma biovar infection since case reports of endocarditis (19), myocarditis (4), and meningoencephalitis (10) due to systemic infection with trachoma biovar have been reported. This model of systemic chlamydial infection may have its greatest usefulness in determining virulence characteristics of LGV.

Mice have a high degree of native resistance in i.v. infection with either LGV or trachoma biovar of *C. trachomatis*. The vast bulk of organisms are killed within 1 h of inoculation. The basis for this intrinsic resistance was not pursued in our studies but may result from uptake of organism by nonpermissive host cells, agglutination by serum immunoglobulins, or bacteriolysis (e.g., by complement) during the intravascular phase of distribution of the organism. Using infectivity assayed 1 h after inoculation as the base line of infection, we documented that replication of the organism did occur in both spleens and lungs. By 2 to 3 days after infection, a ca. 1,000-fold increase occurred with serovar L_2 and a 100-fold increase occurred with serovar B.

Clearance of organism from lungs and spleens was fairly rapid: it was complete by day 7 with serovar B and by day 14 with serovar L_2. Animals convalescent from an initial infection were resistant to rechallenge with the homologous strain as demonstrated by lower peak titer of multiplication of organism and more rapid clearance.

Different growth curves were seen after serovar L_2 and serovar B infection. During serovar B infection, an eclipse phase occurred at day 1 during which no infectious organisms were recoverable. This was not seen with L_2 infection, in which peak titers of infectivity were reached at day 1 and remained relatively stable over the subsequent 3 days. These in vivo growth curves somewhat reproduce the growth curves seen with trachoma biovars in cell culture (3) and LGV biovars in chicken embryo yolk sac culture (12). Presumably these different growth curves reflect a more rapid multiplication rate by LGV than by trachoma biovars.

The growth curve in the lung after serovar B i.v. inoculation was kinetically similar to, although less efficient than, that previously found after intranasal inoculation (6). With similar inocula, peak titers of infectivity were ca. 1,000-fold less in lung tissue after i.v. inoculation than after intranasal inoculation. After inoculation by either route, peak titers were reached on days 2 to 3 and infectivity persisted for 1 to 2 weeks.

An unexpected finding of this study was that resistance to reinfection, although nonspecific, was somewhat biovar restricted. Mice primed with trachoma biovar were resistant to challenge with antigenically dissimilar trachoma biovars but were completely susceptible to heterologous challenge with LGV. On the other hand, LGV-primed animals were most resistant to challenge with the same or other LGV serovars and least resistant to challenge with trachoma biovar. LGV-primed mice in general appeared more resistant than did trachoma-primed animals. These results suggest that important antigenic differences exist between LGV and trachoma biovars that are involved in limiting infection.

Passive transfer experiments suggested that resistance to reinfection for serovar L_2 *C. trachomatis* was due to cellular

immune mechanisms since it was transferrable with immune spleen cells. Several other findings were also compatible with resistance to LGV being dependent on cellular immunity. These are lack of serovar specificity of immunity and the need for live organisms for the establishment of active infection to induce cellular immunity (8).

Transferred spleen cells likely contained B lymphocytes, T lymphocytes, and macrophages. The experimental procedure did not allow determination of the transferred effector cell type. Resistance due to transferred B lymphocytes producing antichlamydial antibody was not considered likely since mice uniformly lacked antichlamydial antibody to *C. trachomatis* 2 days after transfusion of immune spleen cells. We favor a T-lymphocyte-mediated mechanism of resistance and speculate that gamma (γ) interferon released by T lymphocyte may be the active mediator. Chlamydiae generally are susceptible to the inhibitory action of interferon (4, 15) and are able to induce its production. Gamma interferon has recently been demonstrated in lymphokines which activate nonphagocytic somatic cells against intracellular infection (11, 18, 23). Additional studies with purified spleen cell populations, lymphokines produced by these cells, and purified gamma interferon in this model system may shed light on the immune effector systems involved in control of chlamydial infection. Williams et al. (22) have shown that resistance against mouse pneumonitis agent (a mouse biovar of *C. trachomatis*) in nude mice is also T-cell dependent.

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