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Virulence of *Campylobacter jejuni* for Chicken Embryos Is Associated with Decreased Bloodstream Clearance and Resistance to Phagocytosis

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The 11-day-old chicken embryo has been shown to be a useful animal model for comparing the virulence of human isolates of *Campylobacter jejuni*. Virulence in this system is associated with the ability to invade the chorioallantoic membrane and to survive and proliferate in vivo. In this study, the survival and multiplication of *C. jejuni* in the embryonic host was investigated. It was possible to enhance the virulence of a relatively avirulent *C. jejuni* strain by passaging it intravenously through the embryos. The resulting isogenic variants demonstrated enhanced abilities to survive in vivo but were still unable to invade when inoculated onto the chorioallantoic membrane. The bloodstream clearance of *C. jejuni* was studied, and virulent, but not avirulent, strains persisted and multiplied both in the bloodstream and in embryonic liver. Virulent strains also were cleared significantly more slowly from the bloodstream of adult BALB/c mice after intravenous challenge than were avirulent strains. *C. jejuni* strains which were cleared slowly in vivo were also ingested slowly in vitro by mouse peritoneal macrophages. Clearance studies in mice pretreated with cobra venom factor demonstrated that opsonization by serum complement was not a prerequisite for clearance of campylobacters from the murine bloodstream.

Campylobacter jejuni is recognized as an important cause of enterocolitis in man, and considerable progress has been made in recent years in understanding its pathogenesis (38). It now appears that the initial process by which *C. jejuni* colonizes the gastrointestinal tract may involve flagella (28, 31), lipopolysaccharide (28), or other surface structures. Colonization of the mucus gel may provide an alternative means of mucosal association (24). Like other enteric pathogens, *C. jejuni* appears to have an assortment of virulence factors for producing toxigenic and/or invasive disease. A choleralike enterotoxin (14, 19, 20, 26, 36) and various cytotoxins (16, 19, 20, 27, 39) have been identified. Although invasion is a well-documented phenomenon (38), the virulence determinants which confer the invasive phenotype and the extraintestinal survival of some strains are as yet unidentified.

Previous studies from this laboratory have demonstrated the usefulness of the chorioallantoically inoculated chicken embryo as an in vivo model for studying certain aspects of *C. jejuni* pathogenesis (9, 10). Strains varied greatly in their abilities to invade the chorioallantoic membrane (CAM) and kill the embryos, and both invasion and multiplication in the host are important determinants of virulence. Pathogenicity in this model is unrelated to carriage of plasmid DNA, siderophore production, bacterial motility, or cell envelope toxicity (10).

In this study, the survival and multiplication of *C. jejuni* in the embryonic host were investigated. Evidence is presented, by using both human isolates and passaged variants, that successful multiplication in vivo is associated with decreased bloodstream clearance and with the ability to resist engulfment by phagocytic cells.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. The *C. jejuni* strains used in this study have been described in previous publications (9, 10). Isogenic variants of *C. jejuni* LOPEZ were selected by in vivo passage, as described below. Brucella agar (BA) containing 4% defibrinated sheep blood (10) was used for routine culturing and for dilution plating. Broth cultures of *C. jejuni*, used for inoculation of chicken embryos and for determining in vitro growth rates, were grown in a Brucella broth (BB)-BA biphasic culture system (10). Incubation was at 37°C in a microaerobic growth environment (10).

Chicken embryo methodology. (i) Chicken embryos. Eleven-day-old inbred Hyline Variety SC chicken embryos (Hy-Line International, Dallas Center, Iowa) were inoculated chorioallantoically or intravenously as described previously (10).

(ii) Virulence enhancement of *C. jejuni* LOPEZ by intravenous passage in chicken embryos. *C. jejuni* LOPEZ was grown in biphasic culture medium to middle to late log phase (optical density at 625 nm, 0.5 to 1.0) and harvested by centrifugation. Cell pellets were resuspended and diluted in BB, and five embryos were inoculated with approximately 10^4 CFU per embryo. The actual number of viable CFU per milliliter in the suspension was determined by dilution plating. Forty-eight hours later, livers from the infected embryos were removed, homogenized in 1 ml of BB, diluted, and plated. Plates were examined after 48 h of incubation, and growth from the plate with the highest bacterial count was transferred once on BA plus 4% defibrinated sheep blood. After 24 h of incubation, this passaged strain, designated LOPEZ-1, was frozen in BB plus 15% glycerol. *C. jejuni* LOPEZ-1 was subsequently passaged in vivo two additional times as described above, with one exception. Livers from infected embryos were removed at 24 h rather than at 48 h postinfection. After each passage, the isolates (LOPEZ-2

and LOPEZ-3) were preserved for future study by freezing them in BB plus 15% glycerol.

(iii) Blood clearance and multiplication in vivo. Each chicken embryo was inoculated intravenously with 10^4 CFU of an individual *C. jejuni* strain. At various times postinoculation, blood samples from at least five embryos in each group were obtained by removing the shell at the air sac end and nicking the blood vessels with a sterile scalpel blade. Each 50-μl sample was initially collected into an equal volume of BB containing 10 U of sodium heparin per ml. Serial decimal dilutions were prepared in BB, and the number of *C. jejuni* organisms present per milliliter of blood was determined by plating. The embryos were then sacrificed, and the livers were excised, placed in 1 ml of BB, weighed, and homogenized. The mean number of CFU per 100 mg (wet weight) of tissue was determined by dilution plating.

(iv) Bactericidal assay. Fifty-microliter samples of blood from chicken embryos were collected into 25-μl portions of medium 199 with Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.4) (Sigma Chemical Co., St. Louis, Mo.) and 10 U of sodium heparin per ml (3). After samples were collected from 10 to 15 embryos, the diluted blood was pooled and held on crushed ice prior to use in the bactericidal assay. *C. jejuni* strains to be tested were grown in biphasic culture medium and harvested by centrifugation. Cell pellets were diluted in medium 199 to an approximate cell concentration of 10^5 CFU/ml. Each assay mixture contained 150 μl of either diluted blood or medium 199 and 50 μl of the bacterial suspension. Tubes were incubated for 1 h at 37°C. At time zero and 1 h later, 50-μl samples were removed, diluted, and plated in triplicate. Each *C. jejuni* strain was tested in triplicate.

Blood clearance in adult BALB/c mice. Six- to eight-week-old BALB/c mice (Research Biogenics, Inc., Bastrop, Tex.) were used to evaluate the bloodstream clearance of four strains of *C. jejuni*. Strains were grown in biphasic culture medium, harvested by centrifugation, and resuspended in BB to an approximate cell concentration of 10^7 CFU/ml. Groups of 5 to 10 mice were inoculated intravenously in the tail vein with 0.1 ml of the *Campylobacter* suspension. At various times postinoculation, mice were sacrificed by decapitation and blood was collected in sterile petri plates. Samples (100 μl) were immediately diluted (1:4) with BB containing 10 U of sodium heparin per ml. The number of CFU per milliliter of blood was determined by plating serial 10-fold dilutions onto selective medium (15). Each strain of *C. jejuni* was tested twice.

In vivo complement depletion. Complement depletion of adult BALB/c mice was achieved by intravenous injection of *Naja haje* cobra venom factor (CVF) (Diamedix Corp., Miami, Fla.). Groups of 5 to 10 mice were given 5 U of CVF intravenously in a volume of 0.1 ml at 0 and 48 h. This injection protocol has been shown to reduce the C3 activity in adult mice to less than 3% of normal (25).

At 72 h, the clearance of *C. jejuni* strains from the bloodstream of mice treated with CVF was compared with that of untreated control mice. Serum from uninfected mice was also collected in order to determine the effectiveness of the decomplementation. The C3 levels in complement-depleted mice relative to those in untreated mice were determined by double diffusion precipitation in agar (8) with specific goat anti-mouse C3 antiserum (Cooper Biomedical, Malvern, Pa.).

Serum bactericidal assay. Blood was obtained from 6- to 8-week old BALB/c mice and was allowed to clot for 30 min at room temperature. After being placed on ice for an additional 1 to 2 h, the blood was centrifuged and the serum was harvested. The serum was immediately frozen at −70°C for use the next day in the bactericidal assay. When necessary, complement activity in the serum was destroyed by heating at 56°C for 30 min.

The serum bactericidal assay was performed as described above for chicken embryo blood. Each assay mixture contained 125 μl of either serum or medium 199 and 125 μl of bacterial suspension. Each *C. jejuni* strain was tested in triplicate, and experiments were repeated three times.

In vitro phagocytosis assay. (i) Collection and cultivation of murine peritoneal macrophages. Peritoneal macrophages were induced and collected from 6-week-old BALB/c mice by the method of Conrad (7). Mice were injected intraperitoneally with 1 ml of Brewer's thioglycolate broth (Difco) to elicit the migration of macrophages into the peritoneum. Four days later, mice were sacrificed by carbon dioxide asphyxiation, and macrophages were harvested by peritoneal lavage with Hanks balanced salt solution (Sigma) containing 10 U of sodium heparin per ml. The cells were washed once with Hanks balanced salt solution, and cell viability was determined by trypan blue dye exclusion. Cells were resuspended to an approximate concentration of 4×10^6 cells per ml in RPMI 1640 medium (GIBCO) buffered with 20 mM HEPES and 25 mM sodium bicarbonate and supplemented with 20% heat-inactivated fetal calf serum (FCS; GIBCO). This medium, referred to below as RPMI-20% FCS medium, has been shown to optimize the survival of both macrophages and *C. jejuni* in an in vitro phagocytosis assay (23). Twenty-four-well tissue culture plates (no. 3047; Falcon Plastics, Oxnard, Calif.) were then seeded with 300 μl of the cell suspension to give a final cell density of approximately 2×10^5 to 4×10^5 macrophages per cm^2 (32). Peritoneal cells were allowed to adhere at 37°C with 5% humidified CO_2 for 2 h, and then nonadherent cells were removed by washing with RPMI 1640 medium. RPMI-20% FCS (500 μl) was then added to each well. After an additional incubation period of 16 h, the adherent cells were washed again and used in the phagocytosis assay (described below). The viability of adherent cells was determined to be >95% by trypan blue dye exclusion. The cell population was demonstrated to contain an average of 96% macrophages by nonspecific esterase staining (30). In order to establish the ratio of *C. jejuni* cells to macrophages in the phagocytosis assay, the actual number of adherent macrophages per well was determined by counting representative cell populations with an inverted-phase-contrast microscope (1).

(ii) Phagocytosis assay. The phagocytosis assay used in these studies was adapted from the procedure described by Kiehlbauch et al. (23). *C. jejuni* strains were grown in biphasic medium and harvested by centrifugation. The cell pellets were diluted in RPMI-20% FCS to an approximate cell concentration of 1.0×10^7 CFU/ml. Adherent macrophages were incubated for 1 h with fresh RPMI-20% FCS prior to use in the assay. At the end of the incubation period, the medium was removed from the cells and 30 μl of BALB/c normal mouse serum (NMS) was added to each well. This was followed immediately by the addition of 270 μl of the cell suspension.

Multiple control wells containing only NMS and the *Campylobacter* suspension were also prepared for each strain tested. These wells were sampled at time zero in order to establish the number of *C. jejuni* cells initially present in

TABLE 1. Virulence of *C. jejuni* strains for chicken embryos inoculated chorioallantoically and intravenously

C. jejuni strain	LD_{50} at 72 h[a]	
	Chorioallantoic inoculation[b]	Intravenous inoculation
6324	$>3.3 \times 10^8$	7.4×10^4
LOPEZ	$>1.0 \times 10^8$	1.0×10^5
289504	3.1×10^2	2.5×10^{0}[b]
303955	1.4×10^2	5.8×10^{-1}

[a] All values represent the pooled results of two replicate experiments, each with five embryos inoculated per dose of bacteria.
[b] Data reported previously in reference 10.

the assay. Additional samples were subsequently removed at 0.5 and 1 h in order to monitor the viability of the *C. jejuni* cells in the absence of phagocytes.

The extent of phagocytosis over time was determined by comparing the number of intracellular CFU with the total number of intracellular plus extracellular CFU present at 0.5 and 1 h as follows. A 100-μl portion of the *Campylobacter* suspension was removed, diluted, and plated in triplicate to determine the number of extracellular bacteria present. The remainder of the bacterial suspension was then aspirated from the well, and the cell monolayer was washed five times with RPMI 1640 medium. After the last wash, 300 μl of fresh medium was added to the well, and the macrophages were lysed by the addition of 300 μl of 0.2% Triton X-100 in RPMI 1640 medium. A 100-μl portion of the resulting suspension, which contained the intracellular bacteria released from the macrophages, was then removed and plated. Individual *C. jejuni* strains used in these assays were tested in duplicate, and experiments were repeated three times.

LD_{50} determinations. Fifty percent lethal dose (LD_{50}) values were calculated by the method of Reed and Muench (34).

Statistics. Statistical significance was determined by the Student *t* test.

RESULTS

Virulence of *C. jejuni* strains for chicken embryos inoculated chorioallantoically and intravenously. As described previously, 11-day-old Hy-Line Variety SC chicken embryos inoculated chorioallantoically are useful for comparing the virulence of human isolates of *C. jejuni* and *Campylobacter coli* (10). Four isolates of *C. jejuni* that were used in the previous investigation were selected for use in the present study. Table 1 summarizes the LD_{50}s calculated at 72 h when graded doses of these isolates were administered chorioallantoically and intravenously to chicken embryos.

Two of the *C. jejuni* strains, 6324 and LOPEZ, were relatively avirulent after chorioallantoic inoculation, with LD_{50}s in excess of 10^8. In contrast, *C. jejuni* 289504 and 303955 were highly virulent when introduced by this route (LD_{50}s of 3.1×10^2 and 1.4×10^2, respectively). When *C. jejuni* 6324 and LOPEZ were administered intravenously to chicken embryos, the LD_{50}s decreased by approximately 3.5 logs, suggesting that these strains were avirulent because they were relatively noninvasive. The LD_{50}s of the highly virulent strains 289504 and 303955, however, also decreased 2 logs after intravenous inoculation. This suggested that the latter were not only invasive but that they also possessed enhanced abilities to survive and proliferate in the embryonic bloodstream.

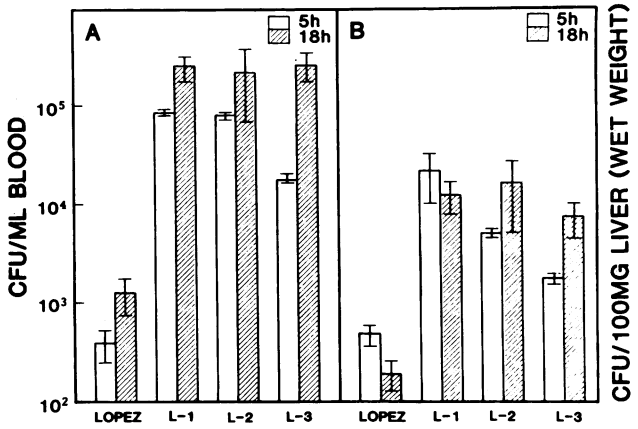

FIG. 1. Relative virulence of *C. jejuni* LOPEZ, LOPEZ-1 (L-1), LOPEZ-2 (L-2), and LOPEZ-3 (L-3) for 11-day-old Hy-Line Variety SC chicken embryos. Embryos were inoculated intravenously with 10^4 CFU; at 5 and 18 h postinfection, the mean number of CFU per milliliter of blood (A) and the mean number of CFU per 100 mg (wet weight) of liver (B) were determined. Each value shown represents the mean ± the standard error of the mean for five embryos.

Virulence enhancement of *C. jejuni* LOPEZ by intravenous passage in chicken embryos. Experiments were initiated to determine whether the virulence of an avirulent strain could be enhanced by serial intravenous passage in 11-day-old chicken embryos. *C. jejuni* LOPEZ was passaged three times through the embryos, and the virulence of each of the resulting variants, designated LOPEZ-1, LOPEZ-2, and LOPEZ-3, was compared with that of the parent strain (Fig. 1).

Intravenous passage in chicken embryos appeared to greatly enhance the ability of strain LOPEZ to survive in vivo. Significantly higher numbers of bacteria were recovered from the blood and the livers of embryos infected with the passaged variants LOPEZ-1, LOPEZ-2, and LOPEZ-3 than were recovered from those infected with strain LOPEZ ($P < 0.001$). This relationship was true at both 5 and 18 h. There was no indication that multiple passages in vivo increased the survival of *C. jejuni* LOPEZ-2 or LOPEZ-3 relative to that of LOPEZ-1. Therefore, additional intravenous passages were not carried out.

Relative virulence of *C. jejuni* LOPEZ and LOPEZ-3 for chicken embryos inoculated intravenously and chorioallantoically. To establish whether in vivo passage selected not only for increased survival in vivo but also for enhanced invasive ability, graded doses of *C. jejuni* LOPEZ and LOPEZ-3 were inoculated intravenously and chorioallantoically into 11-day-old chicken embryos. Table 2 summarizes the LD_{50}s calculated at 24, 48, and 72 h postinoculation. Whereas there was a 4.5-log difference in the virulence of the two isolates 72 h after intravenous inoculation, no differences were seen after chorioallantoic inoculation. These results clearly demonstrated that passaging strain LOPEZ intravenously did not enhance its ability to invade across the CAM.

Chorioallantoic passage of *C. jejuni* LOPEZ-3. *C. jejuni* LOPEZ-3 was also passaged chorioallantoically to determine if its invasive ability could be enhanced. Chicken embryos were inoculated on the CAM with 10^7 CFU of *C. jejuni* LOPEZ-3 per embryo. Forty-eight hours later, campylobacters were reisolated from the embryonic livers essentially as described for the in vivo passage of *C. jejuni* LOPEZ. After four more such passages, the virulence of the

TABLE 2. Virulence of *C. jejuni* LOPEZ and its passaged variants for chicken embryos inoculated intravenously and chorioallantoically

Strain	Time (h) postinoculation	LD_{50}[a]	
		Intravenous inoculation	Chorioallantoic inoculation
LOPEZ	24	$>1.3 \times 10^6$	$>5.3 \times 10^7$
	48	5.5×10^5	5.3×10^7
	72	1.6×10^5	2.1×10^7
LOPEZ-3[b]	24	1.9×10^4	$>7.4 \times 10^7$
	48	7.1×10^1	2.3×10^7
	72	7.1×10^1	1.1×10^7
LOPEZ-8[c]	24	1.2×10^3	$>1.8 \times 10^7$
	48	2.3×10^1	4.7×10^7
	72	1.8×10^1	7.9×10^6

[a] All values represent the pooled results of two replicate experiments, each with five embryos inoculated per dose of bacteria.
[b] *C. jejuni* LOPEZ reisolated from embryonic livers after three serial intravenous passages through the embryos.
[c] *C. jejuni* LOPEZ-3 reisolated from embryonic livers after five serial chorioallantoic passages through the embryos.

resulting variant, LOPEZ-8, was compared with that of LOPEZ-3 by intravenous and chorioallantoic inoculation (Table 2).

Although *C. jejuni* LOPEZ-8 appeared to be somewhat more virulent than LOPEZ-3 by intravenous challenge, there was again no evidence of increased invasiveness following chorioallantoic inoculation. Attempts were also made to directly passage strain LOPEZ chorioallantoically. After three serial passages on the CAM, the resulting isolate was no more invasive than its parental strain (data not shown). Thus, it was concluded from these experiments that it was not possible to select an invasive derivative of *C. jejuni* LOPEZ by serial passage in vivo.

Bactericidal activity of chicken embryo blood for *C. jejuni* LOPEZ, LOPEZ-3, 6324, 289504, and 303955. Experiments were initiated to determine the mechanisms responsible for the differences in virulence of these strains (and passaged variants) for 11-day-old chicken embryos. Differences in virulence could not be related to possible differences in the susceptibility of the isolates to the bactericidal effects of chicken embryo blood. All five isolates retained their viability for at least 60 min when incubated in medium 199 or in chicken embryo blood diluted 1:2 in medium 199 (data not shown).

Clearance and multiplication of *C. jejuni* isolates in the blood and liver of chicken embryos. Some insight into the mechanisms that may be responsible for the observed differences in virulence was provided by studies on the clearance and multiplication of campylobacters in the bloodstreams and livers of infected embryos. Groups of embryos were inoculated intravenously with approximately 10^4 CFU of *C. jejuni* LOPEZ, 6324, 289504, or 303955 per embryo. At various times postinoculation, blood samples from individual embryos were obtained, and the numbers of *C. jejuni* present per milliliter of blood were determined (Fig. 2). Embryos were then sacrificed, livers were excised, and the mean numbers of CFU per 100 mg (wet weight) of tissue were determined (Fig. 3).

The data shown in Fig. 2 clearly demonstrate that the avirulent strains, LOPEZ and 6324, were being cleared from the embryonic bloodstream during the first 9 h after infection. Significant numbers of these two strains did not reappear in the circulation until 24 h postinoculation. In contrast, strains 289504 and 303955 not only persisted in the circulation but multiplied extensively, reaching levels of 2×10^6 and 7×10^6 CFU per ml of blood, respectively, 18 h after inoculation. By 24 h postinoculation, 4 of 10 embryos infected with strain 289504 and 9 of 10 embryos infected with strain 303955 had succumbed to the infection.

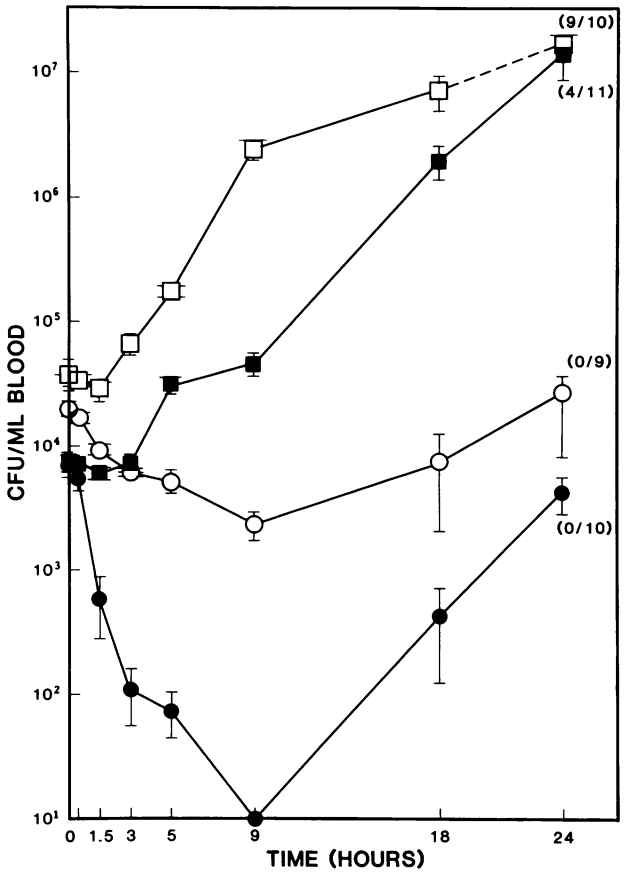

FIG. 2. Clearance and multiplication of *C. jejuni* LOPEZ (●), 6324 (○), 289504 (■), and 303955 (□) in the blood of 11-day-old Hy-Line Variety SC chicken embryos. Groups of embryos were inoculated intravenously with approximately 10^4 CFU per embryo, and the mean number of CFU per milliliter of blood was followed with time. Each point represents the mean ± the standard error of the mean of two separate experiments in which five embryos were used per group. Each fraction in parentheses is the number of dead embryos over the total number inoculated for that time.

Significant differences were seen not only when virulent and avirulent strains were compared but also when each of the *C. jejuni* strains was compared with the other three. With four exceptions, all values obtained from 30 min to 3 h postinoculation were highly significant ($P < 0.001$). When strain 6324 was compared with either 289504 or 303955 at 18 h postinoculation, the values were significant ($P < 0.01$). There were no significant differences when strains 6324 and 289504 were compared at 3 h postinoculation or when strains 289504 and 303955 were compared at 18 h postinoculation.

Of the four strains tested, *C. jejuni* 303955 was clearly the most successful at multiplying in vivo. Between 1.5 and 9 h postinoculation, the number of bacteria detected in the bloodstream doubled once every 72 min. It is likely that the

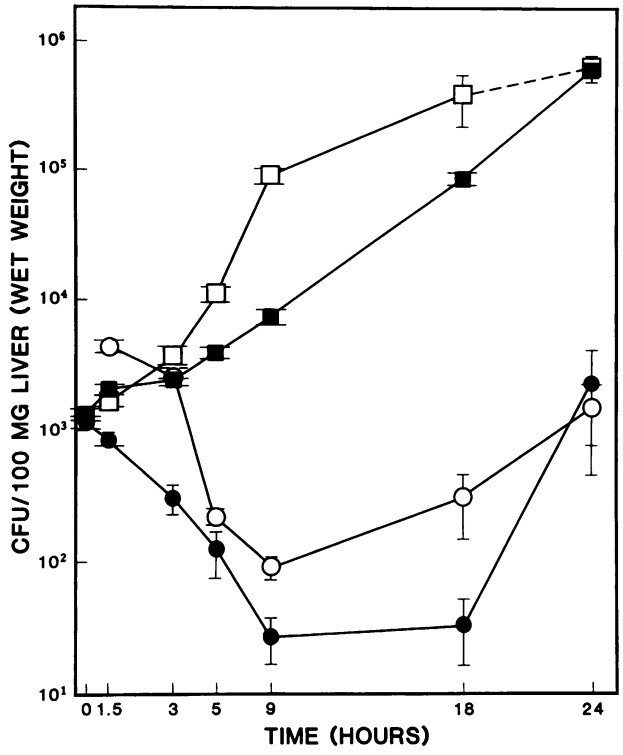

FIG. 3. Multiplication of *C. jejuni* LOPEZ (●), 6324 (○), 289504 (■), and 303955 (□) in the livers of 11-day-old Hy-Line Variety SC chicken embryos. Groups of embryos were inoculated intravenously with approximately 10^4 CFU per embryo, and the mean number of CFU present in embryonic livers was followed with time. Each point represents the mean ± the standard error of the mean of two separate experiments in which five embryos were used per group.

rapid growth rate of this strain enabled it to reach a high enough cell density to kill 90% of the embryos by 24 h postinoculation.

Similar results were obtained when the mean number of campylobacters recovered from embryonic livers was plotted against time (Fig. 3). The numbers of highly virulent strains 289504 and 303955 increased steadily in the livers of the embryos, reaching levels of 8×10^4 and 4×10^5 per 100 mg (wet weight) of tissue, respectively, by 18 h postinoculation. In contrast, decreasing numbers of strains LOPEZ and 6324 were recovered from liver tissues between 1.5 and 9 h after inoculation. A resurgence in the growth of these two strains did not occur until 18 to 24 h postinoculation. There was no statistical difference when strains LOPEZ and 6324 were compared at 3 and 24 h postinoculation. All other values, however, were highly significant ($P < 0.01$ for strains 289504 and 303955 compared at 90 min and for strains 6324 and 303955 compared at 3 and 18 h; $P < 0.001$ for all other values).

By using the same experimental method described above, the in vivo survival and multiplication of the passaged variant LOPEZ-3 was then compared with those of its parental strain, LOPEZ (Fig. 4). *C. jejuni* LOPEZ-3 exhibited a reduced initial clearance and an increased ability to multiply in the embryo relative to strain LOPEZ. *C. jejuni* LOPEZ-3 was recovered in significantly higher numbers ($P < 0.001$) than strain LOPEZ from the bloodstream and from embryonic livers, which were sampled at each time point.

Thus, in vivo passage of strain LOPEZ clearly selected for a variant with an increased ability to survive in vivo. However, unlike infection with the highly virulent strains 289504 and 303955, embryos infected with *C. jejuni* LOPEZ-3 did not succumb to infection within 24 h of intravenous inoculation of 10^4 campylobacters. Assuming that there is a critical number of bacteria required for embryonic death, it is likely that *C. jejuni* LOPEZ-3 was unable to multiply to this threshold within 24 h. This interpretation was substan-

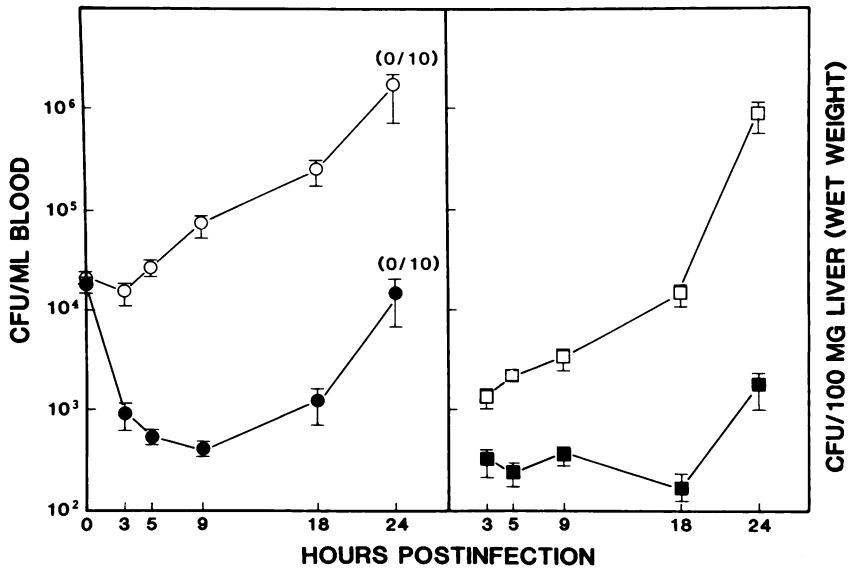

FIG. 4. Clearance and multiplication of *C. jejuni* LOPEZ (closed symbols) and LOPEZ-3 (open symbols) in the blood and livers of 11-day-old Hy-Line Variety SC chicken embryos. Groups of embryos were inoculated with approximately 10^4 CFU per embryo, and the mean number of CFU present in the blood and livers was followed with time. Each point represents the mean ± the standard error of the mean of two separate experiments in which five embryos were used per group. Each fraction in parentheses is the number of dead embryos over the total number inoculated for that time.

tiated by the observation that 7- to 10-fold higher numbers of campylobacters were recovered from the livers and blood of embryos infected with strains 289504 and 303955 24 h postinoculation than from those of embryos infected with *C. jejuni* LOPEZ-3.

The increased survival and multiplication within the host of the more virulent strains was not the result of a more rapid growth rate. A comparison of growth rates in biphasic culture medium indicated that the doubling times for the four *C. jejuni* strains (LOPEZ, 6324, 289504, and 303955) were similar, ranging from 2.2 to 2.6 h during exponential growth. The two most virulent strains, 289504 and 303955, exhibited longer lag periods and reached a lower final density than did the avirulent strains. Similarly, the two passaged variants (LOPEZ-3 and LOPEZ-8) grew more slowly in vitro than the parental strain, LOPEZ, and their final cell densities were only half that of LOPEZ (11).

It is evident from the data just presented that virulent *C. jejuni* strains are able to resist being cleared from the embryonic bloodstream after intravenous challenge. Although 11-day-old chicken embryos are incapable of mounting an immune response, they do possess phagocytic capabilities (22, 29). Thus, to ensure survival in vivo, campylobacters must be able to avoid the phagocytic cells of the reticuloendothelial system. Therefore, it was of interest to determine whether virulent *C. jejuni* strains were less susceptible to phagocytosis by macrophages in vitro.

Thioglycolate-elicited BALB/c mouse peritoneal macrophages were chosen for these studies since *C. jejuni* has been shown to be readily phagocytized in vitro by this mononuclear cell type (23). Before performing in vitro assays, however, the clearance of avirulent and virulent *C. jejuni* strains from the bloodstreams of adult BALB/c mice was investigated.

Clearance of *C. jejuni* strains from the bloodstreams of adult BALB/c mice after intravenous inoculation. Groups of 6- to 8-week old BALB/c mice were injected intravenously with approximately 10^6 CFU of *C. jejuni* LOPEZ, 6324, 289504, or 303955 per mouse (Fig. 5). Within 5 min after inoculation, there was a 1.5-log difference in the number of avirulent (LOPEZ and 6324) and virulent (289504 and 303955) campylobacters circulating in the murine bloodstream ($P < 0.001$). From 30 to 120 min postinoculation, significant differences were seen not only when virulent and avirulent strains were compared but also when each strain was compared with the other three. With three exceptions, all values at 30, 90, and 120 min were highly significant ($P < 0.001$). The exceptions were the virulent strains, 289504 and 303955, compared at 90 and 120 min ($P < 0.01$) and the avirulent strains, LOPEZ and 6324, compared at 120 min (not significant).

The most virulent strain in the chicken embryo system, 303955, was also cleared most slowly from the murine bloodstream. The clearance rate of *C. jejuni* 289504, also a virulent strain in the chicken embryo, appeared to parallel that of strain 303955. The two strains which were avirulent in chicken embryos (LOPEZ and 6324) were also cleared rapidly from the murine circulation. However, contrary to the results obtained with chicken embryos, strain 6324 was cleared more rapidly from the murine bloodstream than was strain LOPEZ.

While the clearance data obtained with mice generally substantiated those obtained with chicken embryos, there were two notable differences between the avian and murine model systems. First, both virulent and avirulent *C. jejuni* strains were eventually cleared from the murine bloodstream. Blood samples obtained from mice 24 h after intravenous inoculation with any of the four *C. jejuni* strains were invariably culture negative for campylobacters. Second, although the bloodstream clearance of *C. jejuni* LOPEZ-3 was retarded compared with that of its the avirulent parental strain, it did not resist clearance to the same extent as the virulent strains (data not shown). This suggests that additional factors in the mouse affect clearance of these passaged variants.

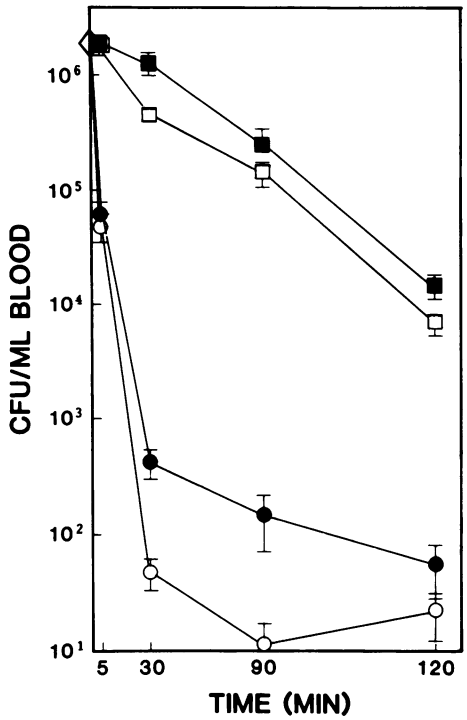

FIG. 5. Clearance of *C. jejuni* LOPEZ (●), 6324 (○), 289504 (□), and 303955 (■) from the blood of adult BALB/c mice. Groups of mice were inoculated intravenously with approximately 10^6 CFU per mouse, and the mean number of CFU per milliliter of blood was followed with time. Each point represents the mean ± the standard error of the mean of two separate experiments in which five mice were inoculated for that time.

Bactericidal activity of NMS against *C. jejuni* strains. The possibility that the apparent differences in clearance seen in BALB/c mice might be the result of differences in the susceptibility of the strains to the bactericidal effects of NMS was investigated. All four strains demonstrated some serum sensitivity (15 to 73% survival after 1 h). However, there was little or no correlation between clearance from the murine bloodstream and the serum susceptibility of these strains. Bactericidal activity was completely eliminated by heating the serum to 56°C for 30 min, suggesting that complement mediated the killing effect (11).

Phagocytosis of *C. jejuni* strains in vitro by BALB/c mouse peritoneal macrophages. An in vitro phagocytosis assay was used to determine whether clearance was correlated with differences in susceptibility to phagocytosis. Cell suspensions of *C. jejuni* LOPEZ, 6324, 289504, and 303955 were combined with adherent BALB/c mouse peritoneal macrophages at a ratio of 400:1. The extent of phagocytosis over time was determined by comparing the number of intracellular CFU with the total number of intracellular plus extracellular CFU present at each time period (Table 3). The four

TABLE 3. Phagocytosis of *C. jejuni* LOPEZ, 6324, 289504, and 303955 by BALB/c mouse peritoneal macrophages

Time (h)	% Phagocytosis of strain[a]:			
	LOPEZ	6324	289504	303955
0.5	9.7 ± 0.8	5.6 ± 0.2	3.1 ± 0.1	1.4 ± 0.1
1.0	18.0 ± 0.7	13.2 ± 0.05	7.5 ± 0.4	3.2 ± 0.3

[a] Data are expressed as means ± standard errors of the mean of duplicate samples determined as follows: [number of intracellular CFU/(number of intracellular CFU + number of extracellular CFU)] × 100.

strains were phagocytized in vitro at rates which were directly proportional to their observed clearance rates in vivo. Similar results were obtained when the assay was performed without 10% NMS (data not shown), suggesting that opsonization by serum complement was not a significant factor in the phagocytosis of *C. jejuni* strains. This was confirmed by determining clearance in vivo in complement-depleted mice.

Clearance of *C. jejuni* in complement-depleted mice. The clearance studies in adult BALB/c mice were repeated, this time with two groups of mice for each *C. jejuni* strain. One group of mice was pretreated with CVF, which causes depletion of alternative complement pathway components, including C3 (6, 33), while the second group received no pretreatment. The efficiency of depletion was determined by double diffusion precipitation in agar with specific goat anti-mouse C3 antiserum. By using this technique, C3 was undetectable in the undiluted sera of CVF-treated mice. In the untreated mice, however, C3 could be detected through a 1:32 dilution. This suggests that the C3 level in CVF-treated mice was reduced by at least 97%. Both groups were injected intravenously with 10^6 CFU of *C. jejuni* LOPEZ, 6324, 289504, or 303955 per mouse, and the mean number of CFU per milliliter of blood was determined at various times postinoculation (Fig. 6).

With a single exception, none of the four strains was cleared more slowly from the blood of CVF-treated mice than from the blood of mice which had received no pretreatment. The exception was the avirulent strain LOPEZ, which at a single time (15 min postinoculation) was cleared significantly more slowly from the bloodstream of CVF-treated mice ($P < 0.001$). This result was verified by testing *C. jejuni* LOPEZ two additional times, suggesting that complement may play some role in the clearance of this strain from the murine bloodstream. However, since complement depletion did not appear to delay the rapid clearance of the avirulent strain 6324 or to retard the clearance of the highly virulent strains 289504 and 303955, the phagocytosis of these strains in vivo probably occurs by a mechanism which is independent of complement opsonization.

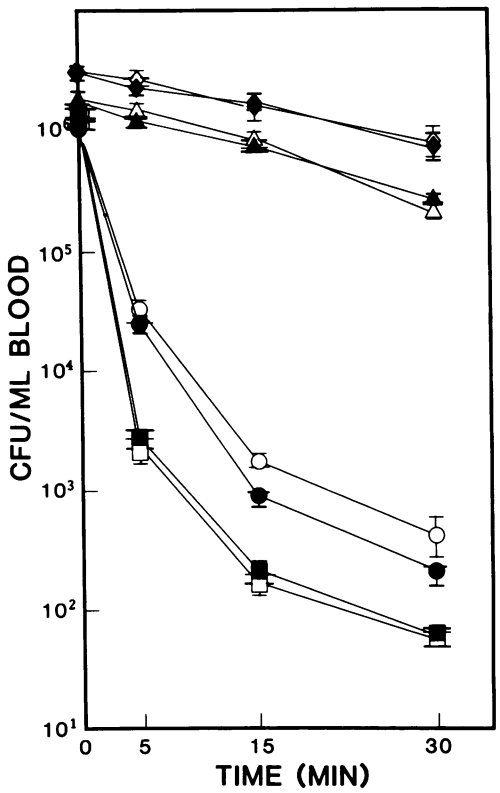

FIG. 6. Clearance of *C. jejuni* LOPEZ (●), 6324 (■), 289504 (▲), and 303955 (◆) from the blood of untreated (closed symbols) and CVF-treated (open symbols) BALB/c mice. Groups of mice were inoculated intravenously with approximately 10^6 CFU per mouse, and the mean number of CFU per milliliter of blood was followed with time. Each point represents the mean ± the standard error of the mean of two separate experiments in which five mice were inoculated for that time.

DISCUSSION

Human isolates of *C. jejuni* show striking differences in virulence when inoculated chorioallantoically into 11-day-old chicken embryos (10). By using this method of inoculation, it was possible to screen a large number of isolates for virulence and to identify both highly virulent and relatively avirulent *C. jejuni* strains. By inoculating embryos intravenously with these strains, it became apparent that at least two separate factors were contributing to virulence in this model system. Virulent strains were more invasive than avirulent strains, and they had an enhanced ability to survive and proliferate in vivo.

The results presented here demonstrate that the chicken embryo is useful not only for comparing the pathogenicity of *Campylobacter* isolates but also for manipulating the virulence of individual *C. jejuni* strains. By using this system, it was possible to enhance the virulence of a relatively avirulent *C. jejuni* strain by serially passaging it intravenously and chorioallantoically through the embryos. Although the resulting isogenic variants did not gain the ability to invade across the chorioallantoic membrane, they did acquire an enhanced ability to survive and multiply in vivo.

Insight into the mechanisms responsible for the observed differences in virulence was provided by studies on the clearance and multiplication of campylobacters in the blood and livers of embryos after intravenous challenge. Highly virulent *C. jejuni* strains not only persisted in the embryonic circulation but multiplied extensively in vivo, reaching levels which were high enough to kill 40 to 90% of the embryos by 24 h postinoculation. In contrast, avirulent strains were cleared from the embryonic circulation early after inoculation, and significant numbers of these strains did not reappear in the bloodstream until 18 to 24 h later. It is likely that the resurgence of these strains during this time was the result of the survival and multiplication of a small number of virulent cells which were present in the original population.

Alternatively, a mutation may have occurred in vivo which resulted in a new cell population with enhanced virulence.

Similar results were obtained when the bloodstream clearance of *C. jejuni* LOPEZ and its passaged variant, LOPEZ-3, was studied. When embryos were inoculated intravenously with equivalent numbers of *C. jejuni* LOPEZ and LOPEZ-3, the variant persisted in the embryonic bloodstream and demonstrated an enhanced ability to multiply in vivo relative to the parental strain.

Reduced in vivo survival of avirulent *C. jejuni* in the chicken embryo was not due to the bactericidal effects of chicken embryo blood. This was not surprising, since the complement system is not developed in embryos at this stage (4). Thus, it was concluded that the differences in clearance must involve differences in the interaction of these strains with the phagocytic cells of the reticuloendothelial system, which are present and functional in 11-day-old chicken embryos (22, 29, 35). Not only were avirulent strains cleared from the embryonic bloodstream, but decreasing numbers were also recovered from embryonic livers. This suggested that avirulent campylobacters were ingested and killed by phagocytic cells. In contrast, the persistence of virulent strains (and variants) in the bloodstream after intravenous inoculation necessarily indicated that they were able to avoid the phagocytic defenses of the embryos.

Clearance studies of adult mice provided additional evidence that virulent *C. jejuni* strains were less readily phagocytized in vivo. Virulent strains were cleared significantly more slowly from the murine circulation after intravenous challenge than were avirulent strains.

There was a positive correlation between the in vivo clearance data for adult mice and the phagocytosis of *C. jejuni* strains by mouse peritoneal macrophages in vitro. Strains which were cleared rapidly in vivo were also ingested more quickly by phagocytes in vitro. It is possible that the apparent differences in ingestion could be due to differences in intracellular killing by macrophages or to bacterial replication rather than to differences in ingestion rates. The latter possibility seems unlikely considering the relatively short incubation period used in these studies. Likewise, on the basis of the published studies by Kiehlbauch and coworkers (23) in which *C. jejuni* was shown to survive intracellularly in BALB/c macrophages for up to 6 to 7 days, the former alternative also seems unlikely.

In the nonimmune host, opsonization by serum complement has been demonstrated to be important in the clearance of some microorganisms from the circulation (21). In particular, fixation of the third component of complement (C3b) is considered to be a critical prerequisite for phagocytosis (12, 18, 21). In vivo experimental evidence for the phagocytosis-promoting activity of C3 has been obtained by using animals which have been depleted of complement by prior injection with CVF (2, 5, 25, 37). Clearance studies of adult BALB/c mice pretreated with CVF did not indicate that opsonization by serum complement was a prerequisite for the clearance of *C. jejuni* from the murine bloodstream. Only the clearance of *C. jejuni* LOPEZ appeared to be affected by depletion of serum C3. Recent evidence suggests that microorganisms can interact with phagocytic cells independently of opsonic factors. Some bacteria can directly bind to complement receptors without C3 opsonization (17). Other reports suggest that binding is mediated by lectinlike molecules which are present either on the bacterial cell surface or in the plasma membrane of the phagocyte (13, 17). Additional nonspecific factors which have been shown to influence phagocytosis include the charge, the hydrophobicity, and the chemical composition of the particle surface (18). It remains to be determined which cell surface characteristics potentially influence the phagocytosis of *C. jejuni*.

In summary, these studies suggest that virulent *C. jejuni* strains share the ability to invade the CAM of chicken embryos and to survive and multiply in vivo. Additional evidence that virulence is associated with resistance to phagocytosis was obtained from clearance studies of adult mice and in vitro studies using mouse peritoneal macrophages.

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