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Effect of *Corynebacterium parvum* Treatment on the Growth of *Salmonella enteritidis* in Mice

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The growth of *Salmonella enteritidis* in mice pretreated with 700 μg of killed *Corynebacterium parvum* was less than that seen in normal CD-1 mice. In treated mice, there was an early increased inactivation of the blood, liver, and spleen bacterial populations, followed by a prolonged period of slow but continuous bacterial growth. The treated mice failed to develop significant delayed hypersensitivity and did not show the characteristic antibacterial immune response seen in untreated infected animals. Eventually sufficient resistance did develop in most of the treated animals to protect them against the lethal effects of the challenge infection. The peak *C. parvum* effect was seen when *S. enteritidis* was injected 7 to 14 days later. Injection of *C. parvum* 24 h after the bacterial challenge actually potentiated the *Salmonella* infection. There was no evidence of an increased specific humoral response by the *C. parvum*-treated mice, suggesting that the slower growth of the *S. enteritidis* was due to the continued enhanced killing of the bacterial population by the nonspecifically stimulated cells of the reticuloendothelial system, rather than to any specific augmentation of the host immune response.

Intravenous injection of killed *Corynebacterium parvum* into normal mice (13) results in an intense stimulation of the reticuloendothelial system (RES). There is an associated increase in resistance to some acute bacterial (1) and protozoan (18) infections and even to some tumor transplants (21). Part of this augmentation of the host's response after the introduction of *C. parvum* into the tissues may be explained by its adjuvant action (3, 14) and, since both humoral and cellular defenses are involved in acquired resistance to many facultative intracellular parasites (8, 16), any stimulus to the production of specific humoral factors could increase host resistance by promoting phagocytosis and the early inactivation of the challenge population. There is good evidence, however, that the elimination of intracellular microbial parasites often depends upon a cell-mediated immune response with a resulting state of delayed-type hypersensitivity (DTH) being developed against one or more microbial antigens (16, 17). There is an apparent contradiction between the overall protection afforded by *C. parvum* given before infection (1) and the depressive effect of this agent on T-cell reactivity (2, 19, 20). This paradox would be resolved if host resistance in the *C. parvum*-treated animal was found to depend upon nonspecific activation of the RES (12, 13).

The present study examines the effect of intravenously injected *C. parvum* on the development of DTH and acquired resistance to *Salmonella enteritidis*. DTH to the *Salmonella* test antigen was found to be severely impaired in the *C. parvum*-treated mice, which were, nonetheless, resistant to the challenge, showed increased rates of blood clearance, and had the capacity to inactivate an enlarged proportion of the challenge inoculum. These are all features that commonly result from stimulation of the RES. No doubt they are responsible for the survival advantage conferred on the mice by *C. parvum* treatment. However, the simultaneous depression of cell-mediated immunity in these animals was probably responsible for the slow eradication of organisms from the liver and spleen and so contributed to the protracted salmonella infections observed in some of the *C. parvum*-treated mice.

MATERIALS AND METHODS

Animals. Specific pathogen-free CD-1 mice (Charles River Farms, Wilmington, Mass.) were maintained, 10 to a cage, on sterile bedding and kept under Isocaps (Carworth Lab Cages, N.Y.) to prevent cross-infection (9). They were fed sterile pellets and water ad lib. Female mice weighing 18 to 24 g were used throughout.

Organisms. *S. enteritidis* NCTC 5694 and a strep-

tomycin-resistant variant (SM^R) were maintained under conditions described previously (5). Both strains were virulent for CD-1 mice (intravenous mean lethal dose (LD_{50}) = 5 to 10 $\times$ 10^3). The method for determining the LD_{50} values has been described elsewhere (9). Inocula were grown overnight at 37 C in tryptose soy broth (Difco), and then subcultured into 5 ml of fresh broth and incubated for 5 h at 37 C. The culture was harvested during the late logarithmic phase and diluted suitably in sterile saline immediately before injection into the mice. The number of viable organisms in the inoculum was checked by plating samples onto tryptose soy agar (TSA) and incubating overnight at 37 C.

Killed *C. parvum* suspension was kindly provided by C. Adlam, Wellcome Laboratories, Beckenham, Kent, U.K. The suspension contained 7 mg (dry weight) of cells per ml. Mice were usually injected intravenously with 0.1 ml of suspension 7 to 10 days prior to challenge. Heat-killed *Mycobacterium tuberculosis* $H_{37}R_v$ (11) was homogenized in sterile saline to give a suspension containing 7 mg (dry weight) of cells per ml, and mice were injected intravenously with 0.1 ml of suspension and tested 7 days later. Heat-killed *S. enteritidis* (60 C for 60 min) was homogenized in sterile saline (7 mg/ml), and 0.1 ml was injected intravenously into CD-1 mice. The mice initially showed some endotoxic reaction, but only two of the 50 mice died.

Enumeration of bacteria in vivo. The livers and spleens were removed aseptically from groups of five randomly selected mice and homogenized separately in cold sterile saline. The homogenates were diluted suitably and plated on TSA (6). Heart blood (0.1 ml) was plated onto TSA. In challenge experiments, the homogenates were plated on TSA, with or without 20 μg of streptomycin per ml of agar. Separate counts of the drug-resistant challenge and drug-sensitive residual vaccinating populations were recorded.

DTH determinations. Mice were injected in one hind footpad with 2.0 μg of protein test antigen diluted in 0.03 ml of sterile saline. The antigen was obtained from a 72-h culture filtrate of *S. enteritidis* as described earlier (10). The foot swelling, with reference to the contralateral foot, was measured at 3 and 24 h with dial gauge calipers (Schnelltäster, Kroplin). An increase of 1.8 U or more (0.18 mm) was significant at the 1% level.

Serology. Mice were bled by heart puncture, and the serum was separated and heated at 56 C for 30 min before storage at -20 C. Hemagglutinin titers were measured by using alkali-treated *S. enteritidis* lipopolysaccharide (Difco) absorbed onto washed sheep red blood cells (4). The reciprocals of the serum titers were expressed as $\log_2$ values ($\pm$ standard error).

RESULTS

Growth of S. enteritidis in C. parvum-treated mice. Normal mice were injected intravenously with 700 μg of *C. parvum* in 0.1 ml of saline or with 0.1 ml of saline alone, and 7 days later the two groups were infected intravenously with about 10^4 viable *S. enteritidis* 5694. The challenge organism was rapidly cleared from the blood of the *C. parvum* mice and taken up in the liver and spleen in approximately equal numbers (Fig. 1). Both the liver and spleen bacterial counts declined substantially over the first 150 min of the challenge period so that by this time the survivors represented only 1% of the original inoculum. This early, extensive inactivation of the challenge infection contrasts with the incomplete blood clearance and slower rate in decline shown by the liver and spleen populations in the control animals (Fig. 1). As a result, up to 20% of the inoculum survived the initial inactivation period, and this 10-fold difference in the size of the 24-h challenge populations in the two groups of mice was sufficient to account for the higher survival of the *C. parvum*-treated animals (Fig. 2). Thus, it can be said that *C. parvum* treatment, by markedly stimulating both blood clearance and bacterial inactivation (clearly the effect of a nonspecific increase in phagocytic function),

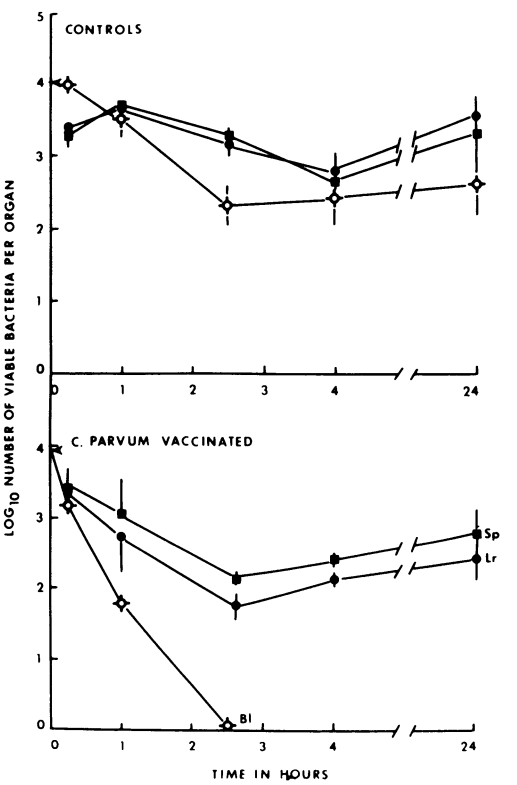

FIG. 1. *Viable counts of S. enteritidis in the liver (Lr), spleen (Sp) and blood (Bl) of C. parvum-treated mice (bottom) or in normal controls (top) over the first 24 h of the infection.*

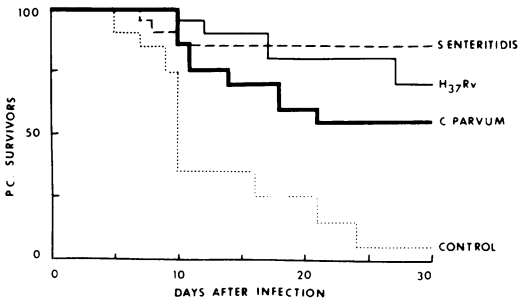

FIG. 2. *Survival of mice treated with heat-killed bacterial suspensions 7 days prior to challenge with 10^4 viable S. enteritidis.*

conferred a clear-cut survival advantage on treated animals. However, once this initial inactivation was complete, the surviving *S. enteritidis* in the *C. parvum*-treated mice began to multiply in the liver and spleen, though at a slower rate than in untreated controls (Fig. 3).

The typical immune response developed by untreated mice was characterized by a sharp change in growth rate of the *Salmonellae* about day 6, and this was associated with the development of high levels of DTH with little evidence of Arthus reactivity (Fig. 3). This has been taken as evidence of a cell-mediated immune response against the infecting organism (5, 7). On the other hand, the *C. parvum*-treated mice developed considerable Arthus (3 h) footpad sensitivity after the *Salmonella* challenge, but most of this foot swelling had disappeared by 24 h (Fig. 3), suggesting that the development of DTH had been impaired by the *C. parvum* treatment. This apparent absence of DTH correlated with the inability of the treated animals to eradicate the challenge organisms from the tissues.

Reinfection of the *S. enteritidis*-infected mice on day 12 with 10^4 viable *S. enteritidis* SM^R was associated with an immediate inactivation of the drug-resistant population in both liver and spleen (Fig. 3). However, the rate of decline of viable SM^R organisms in the *C. parvum*-treated mice was considerably slower than in the corresponding controls; 7 days were required to inactivate 99% of the challenge inoculum in the treated group compared with 2 days for a similar kill in the controls. Much reduced levels of DTH were also observed in the *C. parvum*-treated mice after reinfection. This again points to an impairment of cell-mediated immunity in these animals.

A curious feature of the *Salmonella* growth curves observed in all of the *C. parvum*-treated mice was a reversal of the initial distribution of the challenge population between the liver and spleen (Fig. 1, 3). The relative proportions of both the liver and spleen bacterial populations frequently remained inverted until the infection reached near lethal proportions. The reason for this shift and its significance concerning the evolution of the infection in the treated animal is still unknown.

The early inactivation of an intravenous inoculum of *S. enteritidis* and its subsequently slower rate of growth in the *C. parvum*-treated mice could theoretically have been due to an enhanced production of specific opsonins against the somatic *Salmonella* antigens. However, passive hemagglutination tests using purified *S. enteritidis* lipopolysaccharide against sera taken from *C. parvum*-treated mice failed to reveal detectable levels of such antibodies (Table 1). Even when these mice were challenged with *S. enteritidis*, the hemagglutinin titers found in sera harvested on day 12 of the infection were still significantly lower ($P < 0.01$) than they were for the *Salmonella*-infected controls. This depressed humoral response by the *C. parvum*-treated animal was ascribed to the lowered bacterial population and, thus, to a smaller antigenic stimulus. For that matter, the reduced growth by *S. enteritidis* could also be thought to account for the apparent absence of DTH in treated mice. If this were so, it should be possible to induce a normal cell-mediated

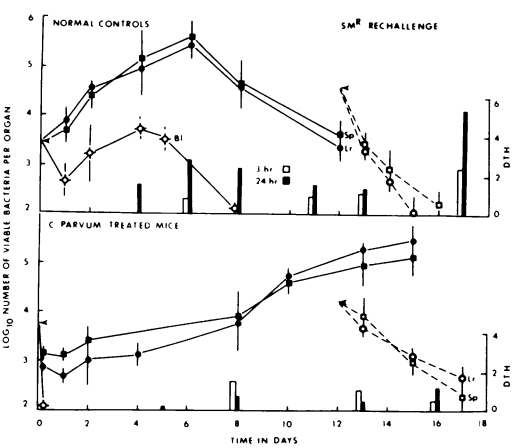

FIG. 3. *Growth of S. enteritidis in normal (top) or C. parvum-treated mice (bottom) after intravenous challenge; Sp, spleen; Lr, liver; Bl, blood. Broken lines represent a streptomycin-resistant rechallenge population injected on day 12. Open histograms represent the average 3-h foot swelling (average of five determinations), and the solid bars represent the 24-h increases in average foot thickness following injection of 2 μg of Salmonella test antigen.*

TABLE 1. *Hemagglutination titers for mouse sera after killed C. parvum or S. enteritidis pretreatment followed 7 days later by infection with living S. enteritidis*

Pretreatment	Days after infection		
	1	6	12
C. parvum	<2.0[a]	<2.0[b]	3.1 ± 0.25[b]
S. enteritidis	10.0[a]	8.8 ± 1.10	-[c]
Saline	<2.0[a]	3.5 ± 0.45	5.6 ± 0.77
C. parvum without challenge	<2.0[a]	-[c]	-[c]

[a] Log_2 of the inverse hemagglutinin titer for pooled sera.
[b] Mean (± standard error) for five determinations.
[c] Not tested.

immune response merely by increasing the size of the challenge dose 100-fold. Mice were therefore injected intravenously with *C. parvum* 7 days prior to their challenge with 5×10^5 viable *S. enteritidis* (100 LD_{50}'s). The growth rate of the bacterial challenge of the liver and spleen was still markedly reduced compared with the controls (Fig. 4), but the larger bacterial population induced an antibacterial response as judged by the inhibition of further growth by the organisms in the liver and spleen from day 6 onwards. Despite this evidence for a cell-mediated response to the further growth of the massive challenge infection, the *C. parvum*-treated mice developed barely significant levels of DTH (0.15 ± 0.05 mm).

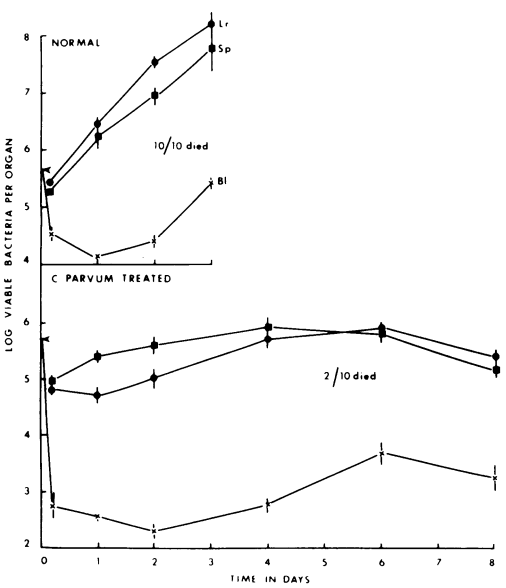

FIG. 4. *Growth of S. enteritidis in C. parvum-treated and control mice (top) after the introduction of 100 LD_{50} dose of virulent organisms by the intravenous route.*

Effect of heat-killed M. tuberculosis or S. enteritidis on anti-Salmonella immunity. There was a possibility that the slowed *Salmonella* growth in the *C. parvum*-treated mice was due to antigenic competition as a result of the massive inoculum of dead corynebacteria. To investigate this possibility, mice were injected with equivalent amounts of two other bacterial suspensions. The first was *M. tuberculosis* $H_{37}R_v$, selected as an unrelated organism unlikely to induce a cross-reacting humoral response, and the second was heat-killed *S. enteritidis*, known to induce high levels of specific antibodies in intravenously vaccinated mice (7). Groups of mice were injected with 1,400, 700, and 350 μg of heat-killed *C. parvum* or *M. tuberculosis* ($H_{37}R_v$) or with either 700 or 350 μg of heat-killed *S. enteritidis* (the 1,400 μg dose proved to be too toxic for the mice). Seven days later, the pretreated mice, together with a group of normal controls, were infected with increasing numbers of viable *S. enteritidis*, and the LD_{50} values for each group were determined (Table 2). Injection of 350 μg of heat-killed *S. enteritidis* or *M. tuberculosis* $H_{37}R_v$ increased the size of the median lethal dose of viable *Salmonellae* approximately five-fold. This should be compared with the 10-fold increase observed in the *C. parvum*-treated animals (Table 2). Increasing the dosage of *C. parvum* or $H_{37}R_v$ to 1,400 μg seemed to have little further protective effect.

Growth studies carried out in *Salmonella*-infected mice pretreated with 700 μg of either heat-killed $H_{37}R_v$ or *S. enteritidis* indicated that both groups of animals had an increased ability to kill the challenge organism in liver and spleen over the first 24 h, but that this was followed by a nearly normal growth rate until peak counts were observed about day 6 (Fig. 5).

TABLE 2. *Effect of pretreatment of mice with suspensions of various killed bacteria 7 days prior to determination of LD_{50} values for intravenous S. enteritidis 5694*

Pretreatment	Median lethal dose[a]			
	1,400 μg[b]	700 μg	350 μg	0 μg
C. parvum	5.2[a]	4.8	4.75	3.65
M. tuberculosis	4.65	4.6	4.2	3.70
S. enteritidis		4.25	3.95	3.40

[a] Log_{10} of the median lethal dose of viable organisms required to kill 50% of the mice within 28 days of challenge (5, 8).
[b] Intravenous *C. parvum* dosage.

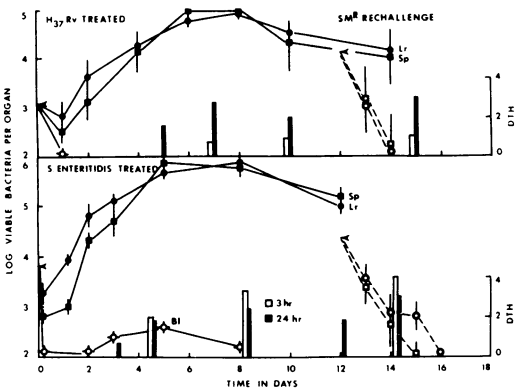

FIG. 5. *Growth of an S. enteritidis challenge in mice treated with 700 μg of heat-killed M. tuberculosis $H_{37}R_v$ (top) or S. enteritidis 5694 (bottom) 7 days previously. Both groups of mice were then reinfected intravenously with 10^4 S. enteritidis SM^R on day 12 (broken lines). Histograms represent footpad reactivity to the Salmonella test antigen.*

Survival curves shown in Fig. 2 indicate that killed $H_{37}R_v$ and *S. enteritidis* both induced substantial protection against the *Salmonella* challenge. The observed differences in percentage of survival in these two groups was probably not significantly different from that obtained with *C. parvum;* however, the growth curves were strikingly different, exhibiting both a normal immune response and high levels of DTH (Fig. 5). Strong Arthus (3 h) reactivity developed in the *S. enteritidis*-treated animals, making the 24-h reactions more difficult to interpret. As expected, the serum antibody levels in these mice were also greatly elevated (Table 1), and blood clearance of the *S. enteritidis* challenge was both rapid and complete (Fig. 5). The response to reinfection with *S. enteritidis* SM^R showed that both the $H_{37}R_v$- and *S. enteritidis*-treated mice were capable of developing high levels of cell-mediated immunity despite the massive antigenic load of killed bacilli introduced into the RES prior to challenge. These results suggest, therefore, that the immune response was impaired only by the *C. parvum*.

Time course of the host response to C. parvum. Groups of mice were injected intravenously with 700 μg of *C. parvum* at various times with respect to the *S. enteritidis* challenge. When *C. parvum* was injected 24 hr after challenge, the infection progressed more rapidly (Fig. 6); when given at the same time, it had little or no effect. When the challenge was delayed for 7 or 14 days after the *C. parvum*, the growth of *S. enteritidis* in both the liver and spleen was depressed. However, if the time interval between treatment and challenge was further increased to 28 days, the growth curves for *S. enteritidis* returned substantially towards normal, and DTH was again a feature of the host response (Fig. 6).

DISCUSSION

In terms of host survival (1), *C. parvum* has a protective effect if given before infection with *S. enteritidis*, but not after (Fig. 6). Because *C. parvum* clearly has a marked stimulatory effect on the RES (13), it is probably this factor that was responsible for the increase in survival shown in Fig. 2, but *C. parvum* has also been reported to act as an immunological adjuvant for a variety of unrelated antigens (3, 14). Thus, the presence of *C. parvum* could have stimulated an accelerated humoral response to the surface antigens of *S. enteritidis*. This possibility seems unlikely, however, in view of our inability to detect hemagglutinins in the serum of *C. parvum*-treated mice at the time of challenge or even quite late in the *Salmonella* infection (Table 1). Substantial antibody titers were detected in mice given 700 μg of heat-killed *S. enteritidis*, but the growth patterns of the challenge *Salmonellae* remained essentially normal (8), whereas those for animals treated

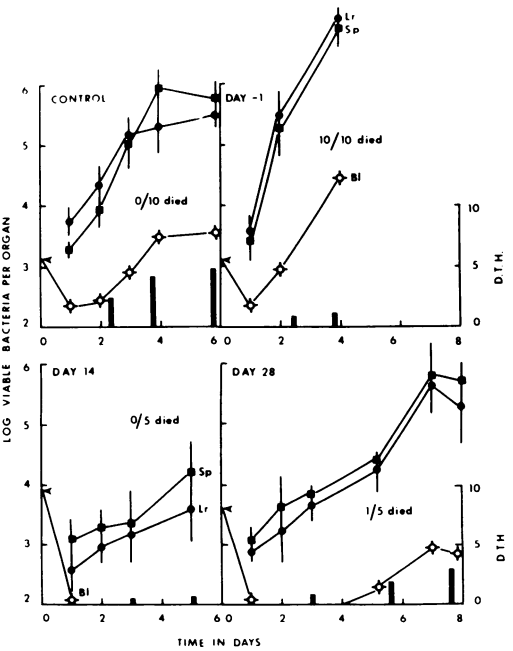

FIG. 6. *Growth of S. enteritidis in mice receiving 700 μg of heat-killed C. parvum one day after the Salmonella challenge (top right) or 14 or 28 days prior to infection with 10^4 viable S. enteritidis (bottom). Histograms represent the DTH reactions.*

with *C. parvum* were not. Whereas minute traces of specific or cross-reacting opsonin may be produced in *C. parvum*-treated mice (thus accounting for the increased blood clearance and inactivation of organisms during the early hours of infection), such factors can hardly explain the continued slower growth of the *Salmonellae* over the next 14 days. On the other hand, the presence of large numbers of nonspecifically activated macrophages in the grossly enlarged livers and spleens of the *C. parvum*-treated animals could limit bacterial growth in vivo for as long as the stimulatory effect of the *C. parvum* persisted. It is known that *C. parvum*-stimulated macrophages do have an increased bactericidal capacity (12). It thus seems reasonable to postulate that the restrained growth of the *Salmonellae* in *C. parvum*-treated mice is due primarily to the nonspecific effects of RES activation. However, there is no question that *C. parvum* treatment also reduced the level of DTH in the *Salmonella*-infected mice. Furthermore, a challenge population of drug-resistant *S. enteritidis* was inactivated more slowly in these mice than in control animals. This suggests that an important component of the immune response is not operating normally in the *C. parvum*-treated mouse. This accords with accumulating evidence that *C. parvum* tends to inhibit T-cell function. The response to phytohemagglutinin, the mixed lymphocyte reaction, the graft-versus-host reaction (19), and the development of contact sensitivity (2) have all been reported to be depressed in mice treated with *C. parvum*.

Regardless of the mechanism involved, the *C. parvum* effect reached a maximum about day 7, but when the challenge and the *C. parvum* were injected simultaneously or even 24 h apart, no obvious change in the *Salmonella* growth curve could be seen (Fig. 6). The time lag before the treated mice displayed a depressed ability to develop DTH presumably represents the time required for activated macrophages to develop in response to the *C. parvum* stimulus (15). This view is consistent with the idea that the activated macrophages in the liver and spleen not only directly restrain the growth of the *Salmonellae* in vivo, but are also responsible for the treated animal's inability to develop an effective antibacterial resistance to the residual challenge infection. However, it is interesting to note that depression of T-cell function by *C. parvum*-activated macrophages has been described to occur in vitro (20). The picture of an overall increased resistance to a lethal challenge infection in the face of an apparently reduced capacity to mount a cellular immune response is also similar to that already described for *C. parvum*-treated mice receiving murine tumor transplants (21). It seems reasonable that the *C. parvum*-activated macrophages within the RES could be the primary effector in both systems.

It is interesting to note that bacterial growth was enhanced when *C. parvum* was given 24 h after infection (Fig. 6). This would seem to rule out the use of this reagent for therapeutic purposes. However, the combination of nonspecific antibacterial activity and a possible specific depression of T-cell-mediated immunity (recently extended to include allograft reactions; 15) would seem to raise the possibility of using *C. parvum* to suppress the rejection mechanism while achieving a desirable increase in the level of nonspecific antimicrobial resistance. Such a situation would be an improvement upon the usual consequence of immunosuppressive drug therapy.

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