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Macrophage Activation During Experimental Murine Brucellosis: a Basis for Chronic Infection

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Evidence is presented that the chronicity of infection in CBA mice after injection of *Brucella abortus* 19 is related to a number of factors: (i) the relative resistance of *B. abortus* to macrophage killing, which allowed some bacteria to survive the peak of macrophage activity occurring at 14 days; (ii) the decline in macrophage activity thereafter (this decline was related in part to the presence of fewer bacteria to stimulate the bactericidal activity and also to specific, active suppressor mechanisms not identified in this study); and (iii) the insensitivity of the persistent *Brucella* organisms to activated macrophages. This was not due to a selection of genetically resistant bacteria, but possibly to their inaccessibility, either within "incompetent" macrophages or outside macrophages altogether.

Of those bacteria that grow mainly within macrophages, a number characteristically produce chronic infections, e.g., tuberculosis, leprosy, brucellosis, and salmonellosis. Recovery from or control of intracellular bacterial infections depends mainly on T lymphocytes producing lymphokines, which activate the bactericidal mechanisms of the macrophages (4, 20), attract more macrophages, in the form of their monocyte precursors, to the site of infection (21), and stimulate the proliferation of macrophages (20). Induction of this acquired immunity occurs relatively early in the disease, but in the above examples only a fraction of the bacteria are killed. A large population persists, often at a remarkably constant level, until the infection is finally resolved or the host dies (6, 8, 13, 28).

The present study concerns murine brucellosis, a useful model of the chronic type of intracellular bacterial infection. Although antibodies may play some role in protection against brucellosis (15, 27), the bactericidal phase in murine brucellosis coincides with the onset of cell-mediated immunity (13). Adoptive transfer of immunity is abolished by treatment of the cells with anti-Thy-1 serum and complement (unpublished data). Macrophage bactericidal activity is maximal at about 14 days postinfection, during the bactericidal phase (13), but wanes thereafter, despite persistence of as many as 10^6 bacteria per spleen.

The present work seeks to explore in vivo mechanisms that may limit the immune response despite the continued presence of the relatively high antigen load and to ask why the infection is chronic.

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MATERIALS AND METHODS

Mice. CBA/H mice were maintained by strict pedigreed brother-sister inbreeding in the Microbiology Animal Breeding Unit. Mice were infected between 8 and 10 weeks of age and were sex matched within experiments.

Bacteria. *Brucella abortus* 19, a smooth attenuated vaccine strain, was obtained from Commonwealth Serum Laboratories, Parkville, Victoria. *B. abortus* 45/20, a rough attenuated strain, was kindly supplied by G. Alton, Commonwealth Scientific and Industrial Research Organization, Division of Animal Health, Parkville. *Listeria monocytogenes* was a local human isolate with a 50% lethal dose of 3×10^3 for CBA mice. *Salmonella typhimurium* C_5 was the kind gift of I. Kotlarski, Department of Microbiology, University of Adelaide. All cultures were maintained by weekly subculture on horse blood agar and were renewed from lyophilized stock after fewer than 50 subcultures. No changes in colony type of *Brucella* or in virulence of any of the bacteria occurred.

Infection of mice. Bacteria were washed from 24-h nutrient agar plates with 1% horse serum in distilled water (serum water). The suspension was standardized turbidimetrically, and the dose was checked retrospectively by viable count (17). Mice were injected intravenously with 0.2 ml.

Chemotherapy. Mice were injected intramuscularly with 1 mg of streptomycin per day and were given approximately 3 mg of sulfadiazine per day in their drinking water.

Assay of bacterial numbers. Mice were killed by cervical dislocation. Spleens or livers were homogenized in 5 ml of serum water by using an Ultra-Turex homogenizer (Jarake and Kuntrel, Breisgau, West Germany). Dilutions were sampled onto nutrient agar. Colonies of *Listeria* were counted after 24 h, *Salmonella* after 48 h, and *Brucella* after 72 h. Each isolate

was recognizable by colony type. In most experiments with mixed infections the slow-growing *Brucella* was in excess, and only very rarely was difficulty encountered in obtaining counts.

Macrophage activation. Macrophage activity in vivo was measured by challenging infected mice with an unrelated organism and was expressed as log protection, i.e., the difference between log number of bacteria in normal mice and that in mice with activated macrophages (13).

RESULTS

Macrophage activation during course of infection of CBA mice with *B. abortus* 19. CBA mice were injected intravenously with 5.5 $\times$ 10^5 viable *B. abortus* 19, and groups of five mice were killed at intervals to determine the bacterial count in spleen and liver. Figure 1 shows that initially there were 10 times as many *B. abortus* organisms in the liver as in the spleen, but numbers in the liver soon fell to approximately 10^4, persisting there for some weeks. In the spleen, bacterial numbers increased to a peak of about 5 $\times$ 10^7, which persisted from day 7 to 14 and was followed by a sharp drop in which 90% of the bacteria were killed during 3 days. Remaining bacteria in the spleen persisted at a remarkably constant level until 6 weeks postinfection, when they began gradually to decrease. Occasional organisms were isolated as long as 3 months postinfection.

Macrophage activation during the various phases of brucellosis was examined by secondarily infecting the mice with *L. monocytogenes* and following the growth of that strain over the succeeding 3 days (Fig. 2). Maximum activity was seen at 2 weeks after *Brucella* infection, during the early bactericidal phase (log protection in spleen = 8, liver = 6). During the steady state of *Brucella* infection, anti-*Listeria* macrophage activity gradually diminished. When the *Brucella* numbers were waning 6 weeks after infection, macrophage activity against listeriae was markedly reduced, especially in the spleen (log protection in spleen = 4, liver = 5).

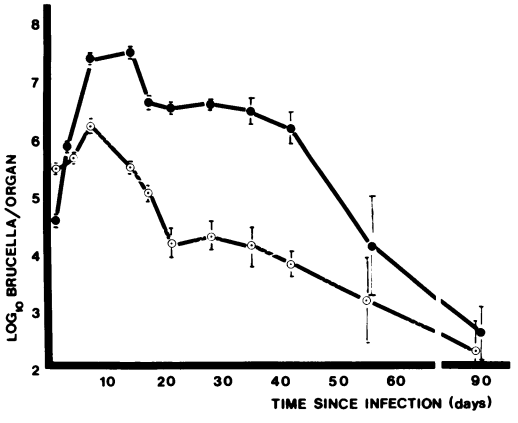

FIG. 1. *Growth of 5 $\times$ 10^5 B. abortus 19 in spleens (●——●) and livers (⊙-·-·-⊙) of CBA mice. Counts were performed on groups of five mice. Vertical bars indicate one standard error.*

Early activation of macrophages induced by *Brucella* organisms. Various doses of *Brucella* strain 19 given to normal CBA mice were followed at intervals by intravenous challenge with 10^4 listeriae. Bacterial counts were performed 3 days later. Table 1 shows a very rapid onset of macrophage activation after *Brucella* infection, with a higher *Brucella* dose giving higher macrophage activity. When listeriae were given at the same time as brucellae, the results were variable. Exacerbation of both infections when listeriae were given together with high doses of brucellae was seen in some experiments.

Live or alcohol-killed smooth *Brucella* strain 19 organisms were compared with live or killed rough *Brucella* strain 45/20 for their ability to activate macrophages. Figure 3 shows that the live smooth and rough *B. abortus* strains produced a comparable infection of the spleen and liver of CBA mice. When 6 $\times$ 10^5 live organisms of either the smooth or rough strain were injected, early and persistent macrophage activation resulted (Fig. 4). On the other hand, 10^9 alcohol-killed bacteria produced only a transient early activation of the macrophages.

Effect of reducing *Brucella* numbers during steady state. The role of numbers of brucellae in maintaining macrophage activation was investigated by reducing those numbers with antibiotics. Mice infected with brucellae 21 days earlier were treated by combined streptomycin/sulfanilamide chemotherapy for 8 days, and bacterial numbers were followed over the succeeding 2.5 weeks (Table 2). The numbers of brucellae slowly returned to the level seen in untreated infection. Regrowth was not as rapid as it was early in infection (cf. Fig. 1). In a second experiment, when *Brucella*-infected mice were given chemotherapy and, 2 days after withdrawal of antibiotics, were challenged with 10^4 listeriae, it was shown (Table 3) that anti-*Listeria* protection had diminished, implying that the level of bacteria plays a role in maintaining macrophage activation.

Effect of superinfecting mice with *B. abortus* during different stages of brucellosis. Mice infected with *B. abortus* 19 for different times were superinfected with 2 $\times$ 10^8 *B. abortus* 19 organisms. Figure 5 shows the counts of brucellae in spleen and liver at intervals after superinfection and in the absence of superinfection. The mice were able to reduce the number of secondary brucellae by 1 to 3 logs over a week.

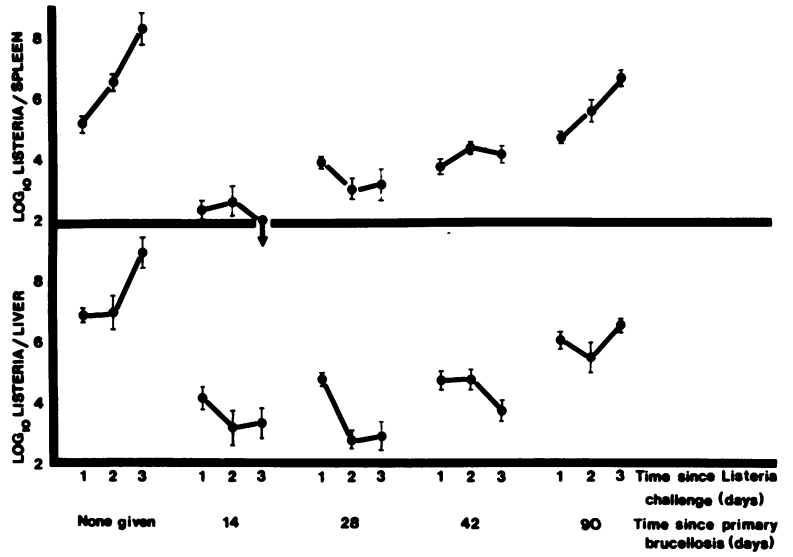

FIG. 2. *In vivo protection against 5 × 10³ L. monocytogenes at different times after infection of CBA mice with 5 × 10⁵ B. abortus 19. Counts were performed on groups of five mice 1, 2, or 3 days after Listeria challenge. Vertical bars indicate one standard error.*

TABLE 1. *Macrophage activation after different doses of Brucella strain 19*[a]

Dose of brucella	Time of *Listeria* challenge[b]		
	Day 0	Day 4	Day 7
3×10^5	0.67	2.50	3.95
1.6×10^6	0	2.91	3.85
8×10^6	1.05	3.50	4.33
4×10^7	0	4.70	5.24
2×10^8	—[c]	4.13	5.24

[a] Mice were injected with different doses of *B. abortus* 19 and at intervals were challenged with 1.1×10^4 listeriae. Bacterial counts on the spleens of groups of five mice were performed 3 days later.

[b] Log protection, i.e., the difference between viable listeria numbers in *Brucella*-injected mice and in control mice not receiving brucellae.

[c] The infection was exacerbated in these mice, and all died by day 3.

In the spleen, the preexisting infection at 2, 4, and 6 weeks interfered with the assessment of final removal of the second dose. Without means to distinguish them, it was not possible to say what proportion of the resulting spleen populations represented primary and secondary infecting organisms.

To see whether the massive increase in bacterial numbers after superinfection increased macrophage activation, the mice carrying a primary infection were given 5×10^8 *Brucella* strain 19, and these plus control mice were challenged 0, 3, or 7 days later with 5×10^3 listeriae. Figure 6 shows the results of bacterial counts 2 days after challenge. As before (Fig. 2), clearance of listeriae was maximal 14 days after primary *Brucella* infection and waned thereafter. Macrophage activation rapidly increased after 5×10^8 brucellae were injected into normal mice or mice that had been infected with brucellae 42 or 90 days earlier. However, superinfection with 5×10^8 brucellae at days 14 or 28 failed to boost macrophage activity.

Boosting macrophage activity during chronic brucellosis with an unrelated infection. To elicit a secondary response to *L. monocytogenes* during chronic brucellosis, mice were infected with 10^3 listeriae and, 2 weeks later,

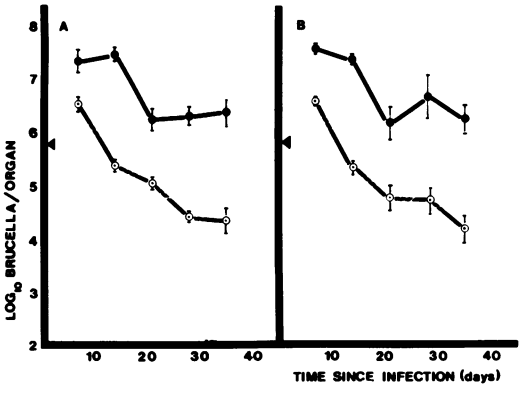

FIG. 3. *Growth of 5 × 10⁵ B. abortus strains 19 (A) and 45/20 (B) in the spleens (●——●) and livers (⊙- - - -⊙) of CBA mice. Counts were performed on groups of five mice. Vertical bars indicate one standard error.*

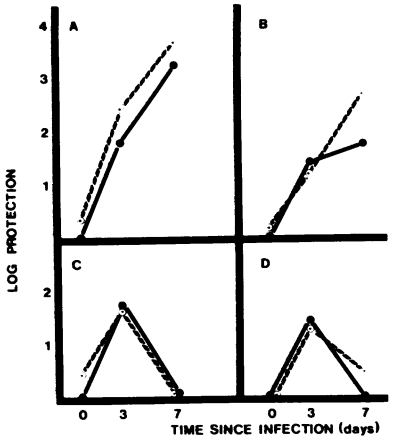

FIG. 4. *Protection conferred against 5 × 10^3 L. monocytogenes by B. abortus strains 19 (●——●) or 45/20 (⊙- - -⊙). Live B. abortus, protection measured in (A) spleen or (B) liver; Alcohol-killed B. abortus, protection measured in (C) spleen or (D) liver. Groups of five mice.*

TABLE 2. *Effect of transient chemotherapy on bacterial numbers in chronic brucellosis*[a]

Days since infection	Days since withdrawal of antibiotics	Antibiotic-treated mice	Untreated mice	Ratio[b]
29	3	5.21 ± 0.11[c]	6.53 ± 0.18[c]	1:20
36	10	5.53 ± 0.39	6.35 ± 0.34	1:6.6
43	17	5.85 ± 0.22	6.01 ± 0.14	1:1.5

[a] Mice were infected intravenously with 5 × 10^5 *B. abortus* 19. Chemotherapy of half the mice was begun 21 days later and continued for 5 days. Bacterial counts were performed on groups of five mice at intervals thereafter.

[b] Ratio of bacterial numbers in antibiotic-treated to untreated mice.

[c] Geometric means of bacterial numbers in the spleens of five mice ± standard error.

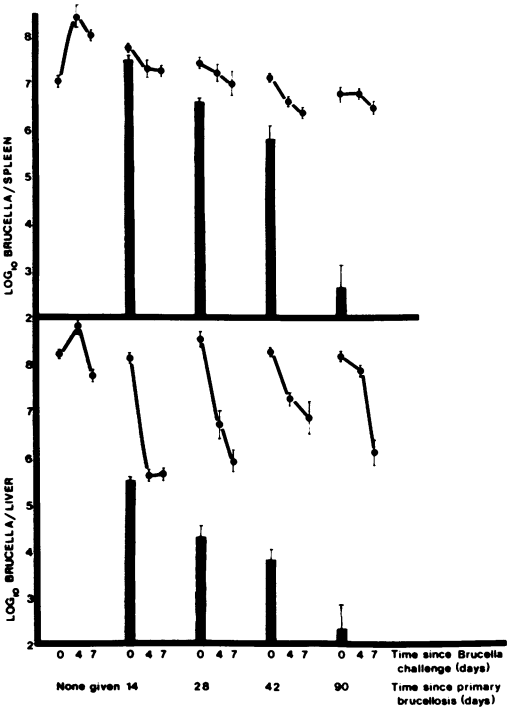

FIG. 5. *Superinfection with 5 × 10^8 B. abortus 19 of CBA mice already infected with 5 × 10^5 brucellae at different times before. Histograms represent the number of brucellae in groups of five control mice not superinfected. Graphs represent number of brucellae in groups of five mice at intervals after superinfection. Vertical bars represent one standard error.*

when the resulting macrophage activation had waned, were given 5 × 10^5 *Brucella* strain 19. A second infection with 10^3 listerae was given 4 weeks later. Numbers of brucellae and listeriae were examined in these mice and appropriate controls 3 and 7 days later. Table 4 shows that

TABLE 3. *Effect of chemotherapy of chronic brucellosis on macrophage activity against listeriae*

Treatment[a]	Log_{10} listeriae per organ[b]		Log_{10} brucellae per organ[b]	
	Spleen	Liver	Spleen	Liver
No brucellosis	7.54 ± 0.13	7.85 ± 0.20		
Untreated brucellosis	4.77 ± 0.44 (3.77)	3.95 ± 0.24 (3.90)	5.22 ± 0.25	3.29 ± 0.46
Treated brucellosis	6.32 ± 0.12 (1.22)	5.17 ± 0.06 (2.68)	3.15 ± 0.52	2.18 ± 0.18

[a] Mice infected 21 days earlier with 5 × 10^5 *Brucella* strain 19 were either treated for 8 days with antibiotics or left untreated. After 2 days to allow the antibiotics to be excreted, the *Brucella*-infected mice and uninfected controls were challenged with 10^4 listeriae. Viable bacterial counts were performed on their spleens and livers 3 days later.

[b] Geometric mean of groups of five mice ± standard error. Parentheses indicate log protection, i.e., the difference in bacterial counts between the groups given primary burcellosis and those controls receiving no *Brucella* infection.

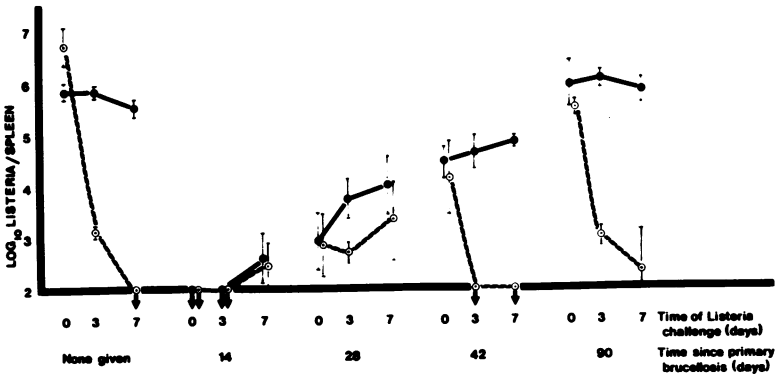

FIG. 6. *Development of anti-Listeria protection in mice superinfected with 5 × 10^8 B. abortus 19 at different intervals after a primary infection with 5 × 10^5 B. abortus. The superinfected mice (⊙- - -⊙) and control, non-superinfected mice (●——●) were challenged 0, 3, and 7 days after superinfection. Counts of listeriae in groups of five mice were performed 2 days later. Vertical bars represent one standard error.*

TABLE 4. *Effect of macrophage activation by secondary and primary listeriosis on chronic and acute brucellosis*

Group	Log_{10} brucella per spleen[a]		Log_{10} listeriae per spleen[a]	
	3 days	7 days	3 days	7 days
A. Chronic brucellosis + secondary listeriosis[b]	6.17 ± 0.22	6.27 ± 0.14	2.57 ± 0.23	<2.00
B. Chronic brucellosis + primary listeriosis	6.29 ± 0.18	6.12 ± 0.15	5.06 ± 0.35	<2.00
C. Chronic brucellosis alone	6.15 ± 0.15	6.37 ± 0.28		
D. Acute brucellosis + secondary listeriosis	6.04 ± 0.09	6.93 ± 0.09	2.98 ± 0.09	<2.00
E. Acute brucellosis + primary listeriosis	6.12 ± 0.13	6.86 ± 0.11	7.49 ± 0.35	3.36 ± 0.21
F. Acute brucellosis alone	6.57 ± 0.06	7.92 ± 0.04		
G. Secondary listeriosis			2.58 ± 0.49	<2.00
H. Primary listeriosis			7.26 ± 0.22	<2.00

[a] Geometric mean of groups of five mice ± standard deviation.

[b] Mice in the test group were given their first dose of 10^3 listeriae and 2 weeks later 5 × 10^5 *Brucella* strain 19. Twenty-eight days after the *Brucella* injection they were boosted with a second dose of 10^3 listeriae, and viable bacterial counts were performed on their spleens 3 and 7 days later. Appropriate controls were included as explained in the text.

a secondary response to *Listeria* could be demonstrated in the presence of underlying brucellosis (group A versus group B). However, despite the increased activity of the macrophages towards listeriae, no clearing of the chronic *Brucella* infection was seen (group A versus group C). This was in contrast to the suppression of *Brucella* organisms given at the same time as either a primary or secondary infection with listeriae (groups D and E versus group F), showing that brucellae were at least potentially susceptible to *Listeria*-activated macrophages.

Changes in susceptibility of brucellae to activated macrophages during infection. To test for a possible genetic change in the resistance of brucellae to activated macrophages, *Brucella* organisms were recovered from 28-day-infected mice and subcultured once on horse blood agar to provide sufficient numbers of bacteria. The growth in the liver and spleen of these "recovered brucellae" was similar to that of in vitro-cultured *Brucella* strain 19 (Fig. 7).

Next, macrophages were activated by infecting mice with 10^3 listeriae, and 3 days later the *Listeria*-infected and uninfected mice were challenged with 6.4 × 10^5 in vitro-cultured *Brucella* strain 19 or with the recovered brucellae. Viable

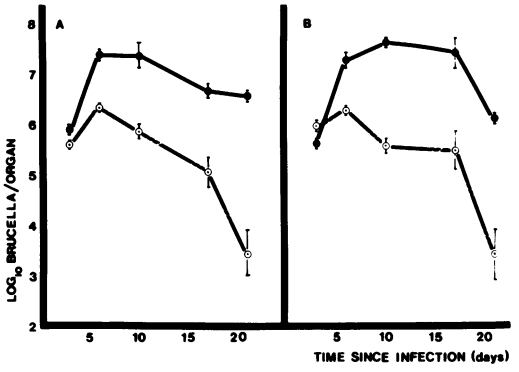

FIG. 7. *Growth of in vitro-cultured B. abortus 19 (A) or of B. abortus recovered from 28-day-infected mice (B) in spleens (●——●) and livers (⊙-·-·-⊙) of CBA mice. Counts were performed on groups of five mice. Vertical bars represent one standard error.*

counts on the spleens and livers of the mice 7 days later showed no differences between in vitro-cultured *Brucella* strain 19 and brucellae recovered in vivo (Table 5).

Relative susceptibility of brucellae and listeriae to activated macrophages. Comparison of Fig. 2 and 5 suggests that listeriae are more susceptible than brucellae to activated macrophages. To investigate this more clearly, mice were infected intravenously with a "third-party" strain, *S. typhimurium* (10^3). At 28 days postinfection, surviving healthy mice were given a secondary infection with 10^4 listeriae or brucellae. Counts on the two organisms 3 days later are shown in Table 6. Despite the slower growth of *Brucella,* it is less susceptible to macrophage activation than is *Listeria,* which was significantly suppressed in the *Salmonella*-infected mice. Because of the high numbers of salmonellae still in the liver, no estimate of brucellae or listeriae in that organ was possible.

Macrophage activation by brucellae or listeriae. Mice were infected with 5×10^5 brucellae or 10^3 listeriae 14 or 5 days, respectively, before being challenged with 10^4 salmonellae. The times chosen were the time of maximum macrophage activation in the two infections. Three days later, counts on the number of salmonellae in spleen and liver of previously infected mice were compared with those in mice given only salmonellae (Table 7). Both *Brucella*- and *Listeria*-infected mice suppressed salmonellae, with the *Brucella*-infected mice showing some superiority.

DISCUSSION

Infection of CBA mice with *B. abortus* 19 could be divided into four phases: (i) an early phase of active growth in both the liver and spleen, when bacterial numbers reached a peak in the spleen of 10^7 to 10^8 and about 10^6 in the liver; (ii) a bactericidal phase, which occurred in the liver 7 to 21 days postinfection and in the spleen 14 to 17 days postinfection and which removed 90 to 99% of the bacteria; (iii) a plateau, when bacteria remained at a constant level between 10^6 and 10^7 in the spleen and 10^4 in the liver; and (iv) a final recovery phase, beginning about 42 days postinfection, when the bacterial numbers began gradually to fall, so that after 3 months only occasional bacteria were recovered. A remarkable feature of the plateau phase was the consistency of the numbers. Not only were the numbers at 42 days after infection not significantly different from those at 21 days, but within any one group of five mice the highest and the lowest counts varied less than fivefold. Similar periods of very constant levels of infection have been described by others for brucellosis (8, 13, 23), tuberculosis (5, 6), salmonellosis (11, 28), and pertussis (7).

TABLE 5. *Susceptibility of in vitro-cultured Brucella strain 19 or of brucellae recovered from 28-day-infected mice to macrophages activated by Listeria infection*[a]

Source of brucellae	Listeriae	Log_{10} brucellae per organ[b]	
		Spleen	Liver
In vitro	−	7.49 ± 0.10	6.65 ± 0.27
In vitro	+	6.27 ± 0.15	5.15 ± 0.07
In vivo	−	7.75 ± 0.04	7.00 ± 0.06
In vivo	+	6.35 ± 0.12	5.16 ± 0.10

[a] Three days after mice were infected with 10^3 listeriae, they and uninfected controls were challenged with 6.4×10^5 *Brucella* strain 19 either from in vitro culture or recovered from mice infected 28 days earlier. Bacterial counts were performed on the spleens and livers of the mice 7 days after *Brucella* infection.

[b] Geometric mean of groups of five mice ± standard error.

TABLE 6. *Relative susceptibility of brucellae and listeriae to activated macrophages*[a]

Pretreatment	Log_{10} bacteria per spleen[b]	
	Brucella	*Listeria*
None	4.23 ± 0.06	7.28 ± 0.27
Salmonella	3.91 ± 0.05	4.74 ± 0.40
Log protection[c]	0.32	2.54

[a] Mice were infected with 10^3 salmonellae, and 28 days later survivors, or previously uninfected mice, were challenged with 10^4 brucellae or listeriae. Bacterial counts were performed 3 days later.

[b] Geometric mean of groups of five mice ± standard error.

[c] Log protection, i.e., the difference between counts in untreated and treated mice.

TABLE 7. *Macrophage activation by Brucella or Listeria organisms*[a]

Pretreatment	Log_{10} salmonellae per organ[b]	
	Spleen	Liver
None	3.84 ± 0.25	4.43 ± 0.28
Brucella	1.40 ± 0.24 (2.40)	2.09 ± 0.19 (2.34)
Listeria	2.63 ± 0.27 (1.21)	3.09 ± 0.07 (1.34)

[a] Mice were infected with 5×10^5 brucellae or 10^3 listeriae 14 or 5 days, respectively, before challenge with 10^4 salmonellae. Bacterial counts were done on the spleen and liver 3 days later.

[b] Geometric mean of groups of five mice ± standard error. Parentheses indicate log protection, i.e., the difference between counts in untreated and treated mice.

One basic reason for *B. abortus* surviving the bactericidal phase to establish a chronic infection must be its relative resistance to activated macrophages, compared with *L. monocytogenes*, which causes an acute infection. Thus when macrophage activation was induced by *S. typhimurium* infection, growth of listeriae was suppressed 300-fold, brucellae scarcely at all (Table 6). On the other hand, *Brucella*-infected mice were at least as active against salmonellae as were *Listeria*-infected mice (Table 7).

The mice remained able to deal with high numbers of superinfecting brucellae at all times (Fig. 5). The superinfecting bacteria were reduced up to 1,000-fold in the liver within 1 week and 20- to 50-fold in the spleen. The contribution of antibody to this removal is unknown. Others (23, 24) using genetic markers to distinguish their strains showed that when mice were given a second dose of brucellae about 1 month after the first, the primary and secondary organisms were present in comparable numbers 1 to 3 weeks later. A similar equilibrium was seen in murine tuberculosis (6), but in salmonellosis the superinfecting strain was in the minority (28).

Macrophage activity in the spleens and livers of *Brucella*-infected mice was tested by challenging these mice with *L. monocytogenes* and measuring nonspecific protection against that strain. This approach was first used by Mackaness (13), who showed that in vivo protection against listeriae correlated wtih in vitro macrophage activity. We were able to confirm in CBA mice his findings that protection against listeriae peaked at a time corresponding with the onset of bactericidal activity against brucellae, and to demonstrate this in the liver as well as the spleen. Significant protection was measured in mice challenged with listeriae 3 days after *Brucella* infection (Table 1). The degree of protection was greater the higher the dose of brucellae. Protection occurred whether the injected brucellae were smooth (strain 19) or rough (45/20), dead (alcohol killed) or alive. This early activation may be a non-immunological effect of the injection of particles on the bactericidal mechanisms of the spleen. Alternatively, it may be due to lipopolysaccharide-activating macrophages, although whether strain 45/20 has an endotoxin with this property is a matter of controversy (1, 8, 12). Protection was not seen 7 days after injection of killed organisms, despite the high dose (10^9), but persisted when living bacteria were given. Whether this results from a special property of live bacteria in stimulating cell-mediated immunity or is merely a reflection of the continued presence of antigen is an old question (2) which is not answered by the present study.

As did Mackaness (13), we found that after the peak of anti-*Listeria* activity 14 days after *Brucella* infection, the total protective activity of the spleen declined, despite the continued presence of relatively high levels of *Brucella* antigen (approximately 10^6 bacteria) which might be expected to continue to stimulate the immune system. The level of bacterial antigen apparently does play a role in the degree of macrophage activity in the spleen. Thus reduction of the numbers of brucellae during the plateau phase by chemotherapy reduced the protection against listeriae (Table 3). Withdrawal of chemotherapy allowed the numbers of brucellae to increase, although at a rate slower than the initial growth phase of the organism (Table 2).

A number of attempts have been made to boost macrophage activation during brucellosis by giving either live superinfection (25) or killed organisms (8, 13). In the present experiments, mice infected with approximately 5×10^5 brucellae were superinfected at various times with 5×10^8 brucellae, and macrophage activity was tested by challenging with *Listeria* organisms (Fig. 6). When listeriae were given at the same time as 5×10^8 brucellae, there was no cross protection. However, when listeriae were given 3 or 7 days after the high *Brucella* dose, considerable protection was seen in mice not given a primary infection of brucellae or in mice infected with brucellae 90 days earlier. This protection was somewhat accelerated in mice infected with brucellae 42 days before superinfection. In this group protection against listeriae given 3 days after brucellae was absolute. However, in mice infected 14 or 28 days before superinfection, macrophage reactivation was suppressed. Thus, although the anti-*Listeria* activity in these mice due to the primary *Brucella* infection was still quite high, the protection against listeriae given

7 days after the superinfecting dose was not as high as that in any of the other boosted groups. This was not due to more rapid removal of the superinfecting brucellae because, as in Fig. 6, the *Brucella* organisms were removed at approximately the same rate in all groups. These results suggest some control mechanism preventing further macrophage activation. Ralston and Elberg (25) made a possibly similar observation in guinea pigs. Recall of macrophage activation was less efficient 8 weeks after primary infection than it was at 12 or 32 weeks, although the primary infection was cleared between 8 and 12 weeks.

Any proposed control mechanism would have to be specific since a secondary response to *Listeria* could be demonstrated 28 days after *Brucella* infection (Table 4, group A versus group B). This secondary response is unlikely to be due to antibody, since repeated studies have shown that antibody does not protect against *Listeria* (16, 22). Interestingly, macrophage activation induced by either primary or secondary listeriosis did not affect the plateau numbers of brucellae. On the other hand, mice given brucellae and listeriae together showed 1/10 the number of brucellae seen in controls after one week (Table 4, groups D and E versus group F), showing that *Brucella* organisms were potentially susceptible to macrophage activation by listeriae. Thus brucellae during the plateau phase appear to be resistant or inaccessible to the macrophage activation induced by listeriae.

The concept of selection of genetically resistant brucellae during the course of infection was tested by taking organisms from 28-day-infected mice, subculturing once, and injecting them into mice with *Listeria*-activated macrophages. They behaved no differently from organisms cultured in the normal way, nor was their growth curve in normal mice different from that of in vitro-grown *B. abortus* 19 (Table 5, Fig. 7).

It would be simplistic to assume that the degree of protection in *Brucella*-infected mice reflected the activity of each and every macrophage. First, macrophages are not a uniform population (10), and it is possible that the brucellae reside during the plateau period in macrophages which cannot be activated either by the initial anti-*Brucella* bactericidal phase or by primary or secondary listeriae. (They may not even reside in macrophages—microscopic study of 10^6 bacteria per spleen is very difficult). Second, macrophages are a constantly turning over population. Dannenberg et al. (3) showed that the half-life of the cells in a tubercle in a rabbit lung was 7 to 14 days. Although granulomas are not seen in the spleen of *Brucella*-infected mice, turnover of cells undoubtedly occurs. Third, there is variation in the total numbers of macrophages in the spleen during brucellosis, and probably also in the liver. Thus the waning of total anti-*Listeria* protective activity in the spleens between 21 and 42 days may partly represent loss from the spleen of the macrophages that gathered there in large numbers from days 14 to 28 (Riglar and Cheers, in preparation). Nevertheless, Mackaness (13) found that individual in vitro macrophage activity against listeriae also waned at this time. It is intriguing that macrophage activity is waning at the time when the infection is about to be finally resolved (42 days). Possibly the final resolution is brought about not by macrophages, but by antibody, which could contact brucellae as they pass from one transient macrophage to another.

There is suggestion of specific feedback control of macrophage reactivation between days 21 and 35, although the results are difficult to interpret. A number of mechanisms have been proposed for such controls, and some have actually been demonstrated to occur in other infections. They include immune complexes blocking antigen receptors on T lymphocytes (14), suppressor T cells (9, 11), and activated macrophages themselves acting as a feedback mechanism (18, 26). We do not believe that suppressor macrophages are the mediators of suppression in murine brucellosis, although we have been able to demonstrate them in the spleens of *Brucella*-infected mice (Riglar and Cheers, in preparation). Other possible suppressor mechanisms are being studied.

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