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Serum-mediated Immune Cellular Responses to *Brucella melitensis*

I. Role of a Macrophage-stimulating Factor in Promoting Ingestion of *Brucella* by Streptomycin-protected Cells

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Injection of rabbits with living *Brucella melitensis* Rev I induced the appearance of a macrophage-stimulating-factor (MSF) in the sera of these animals. MSF was involved in ingestion of bacilli, hastening the formation of protected loci as measured by the addition of lethal amounts of dihydrostreptomycin. When sufficient time had been allowed for effective ingestion, streptomycin had little effect. This in turn allowed for multiplication of bacilli intracellularly in the presence of 5 to 250 μg of drug per ml. MSF mediated more effective ingestion by both immune and normal macrophages. Under such conditions, there was little, if any, intracellular growth restriction by macrophages from immune animals. The activity appeared within the first 5 days after injection with 10^9 organisms and was present for several months. Three weeks after injection, the activity of serum was partially heat-labile. All activity was removed by absorption with heat-killed or living Rev I cells, suggesting that a specific globulin is concerned.

Brucella melitensis Rev I has been shown to be an effective immunogenic and protective agent in goats, sheep, monkeys, mice, guinea pigs, and rabbits (1). The mechanism by which it leads to immunity is under investigation in our laboratories.

Organisms such as *B. melitensis*, which are capable of prolonged intracellular survival, cause the appearance of phagocytic cells with altered capacities (7, 21, 32). Some of these altered capacities are (i) increased though often transient ability to restrict bacterial growth (8, 16, 21, 26), (ii) prolonged capacity to withstand the toxic effects of pathogenic organisms (8, 12, 31), and (iii) alteration of the physiology of the bacterium so as to limit its destructive and invasive activities (11, 30).

Previous work in our laboratories has emphasized that at least one activity of the macrophage from *Brucella*-immune hosts is mediated by a heat-stable factor in immune sera (8). This material, mixed with macrophages from immune animals, confers a heightened resistance to the toxic degenerative effects of the organism. The protection, once invoked, is nonspecific in that macrophages resist the toxic action of unrelated genera (for example, *Salmonella*, *Mycobacterium* sp.). In principle, these results are in accord with numerous other observations of heightened cellular protection against intracellular organisms (22). However, there appear to be differences with respect to the absolute requirement for immune serum in mediating the various responses. Mackaness, Blanden, and Collins (23), Fauve (9), and others (22) found that the immune macrophage functions independently of immune serum, whereas Jenkins et al. (19) and Turner et al. (34) found that immune globulins, particularly cytophilic antibodies, impart protective activity to macrophages.

In the present study, we are concerned with an aspect of initial ingestion. Dihydrostreptomycin sulfate (DHSM) was used as a test for successful storage of bacilli by macrophages. This in vitro activity of the macrophage is greatly stimulated by immune serum, but until the various serum factors are identified, we designate them simply as macrophage-stimulating factors (MSF), to indicate that, they enable the macrophage to store bacilli within streptomycin-protected loci. Our results indicate that, when bacilli are removed to intracellular areas, they grow at rapid rates in both normal and immune macrophages in the presence of large extracellular amounts of the drug.

MATERIALS AND METHODS

Bacterial culture. The vaccine of *B. melitensis* strain Rev I was used throughout. This strain was derived from a streptomycin-dependent mutant which reverted to independence. It is inhibited by 5 μg of DHSM per ml and is characterized by marked attenuation of pathogenicity, slow growth on agar media, small colony formation, and excellent immunizing properties. A smooth culture which had been passaged twice through guinea pigs for 6 weeks, then cultured on Trypticase Soy (TS) Agar (BBL) and preserved by lyophilization, was used for the primary seed source. For the stock culture, material from the dried state was reconstituted in distilled water, placed on the desired medium, and incubated for 5 days at 37 C. For each experiment, a transfer was made from a stock culture which had undergone from 3 to 4 passages on Brucella Agar (Albimi Laboratories, Inc., Flushing, N.Y.). The rough-smooth characteristics of brucellae were determined by the usual methods.

Immunization of rabbits. White, male rabbits (approximately 8 to 12 weeks of age and weighing 4 to 4.5 lb) were immunized by subcutaneous injection of 10^9 smooth bacilli, suspended in ZøBell solution (36) from 5-day growth on TS Agar, prepared directly from reconstituted lyophilized vaccine.

Preparation of peritoneal exudates. Rabbits received intraperitoneal injections of 50 ml of sterile mineral oil (Klearol, Sonneborn Chemical Co., Inc., distributed by L. H. Butcher, Hayward, Calif.). (Klearol preparations have a significant quantity of material absorbing in the ultraviolet region, maximum 270 mμ. The nature of this material is unknown, and we do not know in what way, if any, the macrophages have been affected. We note that the absorption spectrum in the infrared region is similar to that of Nujol, the mineral oil recommended for use in spectroscopic analysis.) After 5 days, the animals were sacrificed by injection of air in the ear vein. The macrophages were suspended in chilled modified Tyrode solution (27), the oil was separated, and the preparations were centrifuged at 250 $\times$ *g* for 15 min at 4 C. The cells were resuspended in a small volume (10 ml) of modified Tyrode solution (*p*H 7.4). Total counts were made in a Petroff-Hauser chamber in the presence of 0.2% eosin, according to the dye exclusion technique of Hanks and Wallace (14) for cell viability. The macrophages were washed at least once prior to use, but often received three to four washes in 30 to 50 ml of chilled Tyrode solution.

Preparation of rabbit serum. Serum was obtained by cardiac bleeding, except where noted, from rabbits that had not received oil. The blood was allowed to clot for 1 to 2 hr at 37 C, followed by 24 hr at 4 C; then the serum was drawn off, centrifuged, and sterilized by filtration through a 0.45-μ membrane (Millipore Corp., Bedford, Mass.). The serum was stored frozen, at -10 C, for periods of from 1 week to many months. In some experiments, comparisons were made between serum which had been stored at 4 C, and serum which was freshly prepared on the day macrophage cultures were infected.

Tissue culture medium. The medium was composed of 60% Tyrode solution and 40% serum, as in previous studies (27). All water was obtained from a deionized and glass-distilled source. Tyrode solution was sterilized at 10-fold the desired concentration by filtration through 0.45-μ membranes and was diluted with sterile distilled water just prior to use. The *p*H was adjusted by the addition of phosphoric acid, in concentrations not exceeding 0.001 M.

Phagocytosis of bacteria. For phagocytosis, macrophages were adjusted to 1.1×10^7/ml in culture media. In a typical experiment, 2.7 ml of macrophages was placed in a 12-ml polypropylene round-bottomed centrifuge tube to which 0.3 ml of bacterial suspension containing 10^9 cells/ml was added. The tube was stoppered tightly and was incubated at 37 C for 1 hr. Between 30 and 40 min, the samples were swirled gently to mix unattached bacilli and macrophages. After this phagocytic interval, the samples were chilled for 5 min and were centrifuged for 15 min at 250 $\times$ *g*. The supernatant layer was removed with a fine capillary pipette. In early experiments, it was passed through a 5-μ membrane to separate any resuspended macrophages; but this procedure was found to retain clumps of bacilli. It was later omitted, and unfiltered material from the upper layer was assayed for nonadherent bacilli. The macrophages were resuspended to volume in the Tyrode-serum mixture and then were diluted to 5×10^5/ml. Samples (1 ml) were then placed in 10 sterile, flat-bottomed, glass vials (17 $\times$ 60 mm), 5 of which contained flying cover slips (5 $\times$ 5 mm).

The number of bacteria adherent to macrophages was estimated from: input *B* per ml minus free *B* per ml, where *B* = bacilli by plate count. This calculation assumes that no killing occurs in the extracellular fluids during exposure to macrophages, and provides an estimate of the maximal number of adherent (and/or ingested) bacilli.

Test for ingestion of bacilli: sensitivity to DHSM. At the first hour after admixture of bacilli and macrophages, assays of the intracellular and extracellular bacilli were performed. The latter estimate provided a measure of the firmness of attachment of bacilli to the macrophage surface. Then 50 μg of DHSM per ml was added. Assays of the total viable counts were made after incubation at 37 C. In addition, flying cover slips were stained to determine the percentage and extent of infection in the macrophages. Loss of viable bacteria from those macrophages present immediately after the initial attachment period provided a measure of the quantity of bacteria remaining susceptible to the drug, a value which is superimposed upon kiiling due to the lethal activities of the macrophage, as discussed in Results.

Estimation of intracellular bacteria. Vials containing infected macrophages were chilled in ice. This caused the cells to round up. They were swabbed off the floor of the vials with soft rubber policemen, prepared by cutting numerous fimbriae into thin-walled gum tubing, 5 mm outside diameter. The intracellular bacteria were released by 1:100 dilution into distilled water with vigorous pipetting. The released bacilli were then diluted further in ZøBell solution and were plated in triplicate on TS Agar. Samples of free

bacteria were obtained by filtration of a portion of the original infected macrophage suspension through 5-μ membranes.

When the macrophages were resuspended, viable counts of their bacilli content were made. From any given infected suspension, therefore, we could determine the number of intracellular bacilli per macrophage and also observe the distribution of infection within individual macrophages by the stain technique.

Intracellular stain technique. A modified Macchiavello stain was used as previously reported (27). In these tests, the material was dried in air but was not fixed in methanol, because we found that this solvent reduced the number of visible bacteria in samples removed during the early hours after infection. For each sample, a minimum of 100 macrophages in representative areas were observed under oil immersion with a Tiyoda microscope. The macrophages containing no bacilli, those containing 1 to 10, 10 to 100, and hundreds of bacilli, and those filled to bursting were recorded.

Absorption of serum. Absorption was carried out with heat-killed *Brucella* (100 C for 20 min) and with washed viable cells. For this purpose, bacteria were grown for 5 days on TS agar, washed four times in distilled water, and resuspended to 10^{10}/ml, according to standard turbidimetric curves for a Klett photoelectric colorimeter equipped with a 660-mμ filter. One portion was then heated and a second sample was stored at 4 C (viable cells). For absorption, 10 ml of the suspension was sedimented, the supernatant layer was removed, and 10 ml of immune or normal serum was added. Three successive absorptions were carried out at 4 C for 2 hr, after which the sera were filtered through 0.45-μ pads. No agglutinating antibody remained when the sera were tested with a standard suspension of killed *B. abortus*.

Heat-inactivation of serum. Samples were heated at 65 C for 15 min.

RESULTS

The first observations of differences between immune and normal sera stemmed from attempts to use DHSM to control extracellular growth of *Brucella*. With the Rev I bacilli, we found evidence that ingestion does not always immediately follow attachment of bacilli to cells. After the first hour of exposure at 37 C, many bacilli remained loosely bound to the macrophages; these bacilli could be centrifuged with the macrophages while still adherent to the cells, but were often released from the surface when the infected macrophages were resuspended in fresh medium.

Ingestion by macrophages from normal rabbits: effect of early addition of streptomycin. When normal macrophages were infected with Rev I in the absence of DHSM, extensive growth of bacilli occurred (Fig. 1). Immune serum reduced the rate. When the same macrophage suspension was treated with 50 μg of DHSM per ml 1 hr after infection and resuspension, extensive bacterial death occurred in cultures maintained in normal serum, in contrast to significant survival and growth in immune serum. At the time of resuspension of the macrophages in fresh medium, just prior to addition of DHSM, assays were made of the total and free bacilli. The free bacilli constituted 65% of the total in the normal serum system, but only 16% in the immune serum system. Inspection of stained preparations showed that only 2% contained bacilli at the first hour in normal serum, whereas 67% contained bacilli when infected in immune serum (Table 1). Many of these bacilli could be seen within well-defined vacuoles.

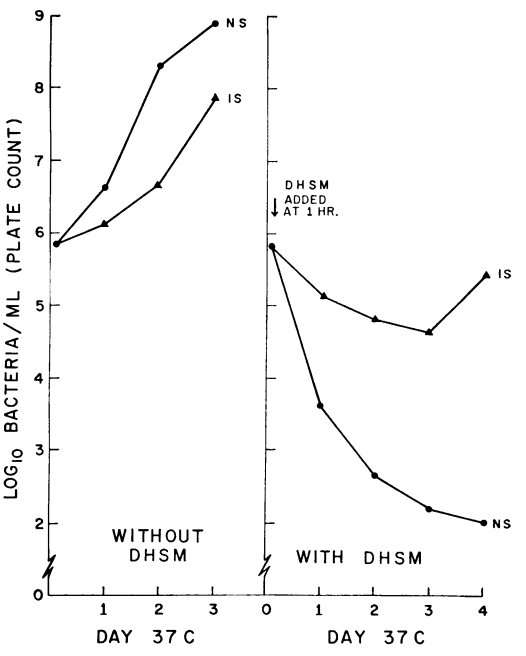

FIG. 1. *Growth of Rev I in macrophages from normal and immune rabbits in the presence and absence of DHSM. Macrophages were exposed to Rev I for 1 hr at 37 C in normal serum or immune serum removed from rabbits 6 weeks after immunization. Immune macrophages were from animals 16 weeks postimmunization. After centrifugation to remove free bacilli, the macrophages were placed in fresh media. One portion (left side) received no DHSM. A second series (right side) received 50 μg of DHSM per ml. Plate counts were made of bacterial survivors at the indicated intervals. Counts at zero-hour (1 hr postinfection) were made prior to addition of DHSM.*

The curves of bacterial survival are a composite of intracellular growth, death of bacilli in loci accessible to DHSM, and death due to macrophage-derived agents. In the presence of 50 μg of DHSM per ml, no extracellular growth was

TABLE 1. *Distribution of stained Brucella melitensis Rev I within normal macrophages infected in normal and immune sera*

Day of infection	Drug	Normal serum					Immune serum[a]				
		Percentage of macrophages stained					Percentage of macrophages stained				
		0[b]	+	++	+++	++++	0	+	++	+++	++++
0	DHSM[c]	98	2	0	0	0	33	56	11	0	0
1		87	13	0	0	0	22	53	25	0	0
2		72	24	4	0	0	33	65	2	0	0
0	None	98	2	0	0	0	33	56	11	0	0
1		20	53	27	0	0	37	47	16	0	0
2		2	38	52	2	0	14	50	35	1	0

[a] Immune serum was pooled from rabbits 6 weeks after immunization.
[b] Key: 0 indicates percentage of macrophages with no bacilli; +, 1–10 bacilli; ++, 10–100; +++, 100 to many hundred; ++++, filled to bursting.
[c] DHSM (50 μg/ml) was added 1 hr after infection; resuspension in fresh media.

possible. Extensive bacterial death was produced when DHSM was added during the early hours after infection. The data become more meaningful when used in conjunction with stained preparations and direct counts of macrophage survival. The curves, unless otherwise noted, have been transposed to common origins, the initial points representing the bacterial survivors after the first hour of infection. The data do not show differences in adherence or in killing prior to adding drug, both of which are also factors in the macrophage-bacterium interaction.

Effect of addition of streptomycin at intervals after phagocytosis. The data in Table 1 suggested that, in the presence of immune serum, more bacilli were contained within macrophages by the first hour after mixing bacteria and phagocytes. To obtain additional information on the ingestion process, we added DHSM at intervals of several hours. An analysis of this kind was possible because the slow growth rate of Rev I reduced the error due to extracellular multiplication. For infection, 10^7 macrophages from normal rabbits were mixed with 10^8 bacilli for 1 hr at 37 C; then the mixtures were chilled, centrifuged, and diluted in fresh medium to 5×10^5 normal rabbit macrophage (NRM)/ml. Samples were treated immediately (1 hr postexposure) or within 7 and 24 hr, and plate counts were made of the bacterial survivors. When the DHSM was added in normal serum, increasing numbers of bacteria grew intracellularly (Fig. 2). At 24 hr, DHSM had little effect on the count, indicating that by this time the bacteria were largely within protected loci. The data thus supported our conclusion that ingestion, as defined by establishment of loci capable of excluding DHSM, required considerable time.

In the presence of immune serum, a higher proportion of bacteria were within the macrophages at 1 hr and, consequently, addition of DHSM had less effect. Stained preparations also showed that a larger percentage of macrophages had been infected in immune serum (Fig. 3). On the fourth day of culture in this serum, the percentages of infection in macrophages treated at 1, 7, and 24 hr were very similar, whereas in normal serum increasing percentages of infected macrophages appeared, according to the time of DHSM addition.

Effect of increasing concentrations of DHSM. Macrophages from normal rabbits were parasitized for a period of 1 hr at 37 C; then they were resuspended in fresh medium and incubated for 6 hr at 37 C, at a level of 5×10^5 NRM/ml, before adding the drug. During this interval, the macrophages were afforded further opportunity to ingest bacteria that had been absorbed to the surface during the first hour. Additions of increasing concentrations of DHSM decreased the count. This occurred for a period of 48 hr and was followed by a period of rapid bacterial growth, the rates of which were comparable to those occurring in untreated samples. The net yield of bacteria, as measured by plate counts of survivors, decreased with each increasing level of drug (Fig. 4). Stained preparations revealed that the percentage of infected macrophages also progressively decreased as the concentration of the drug was raised (Fig. 5). The observed decrease in bacterial count was not correlated with increasing percentages of dead macrophages, because direct counts of macrophages surviving at 4 days indicated that those exposed to 500 μg of drug per ml had survived to extents similar to or greater than those treated

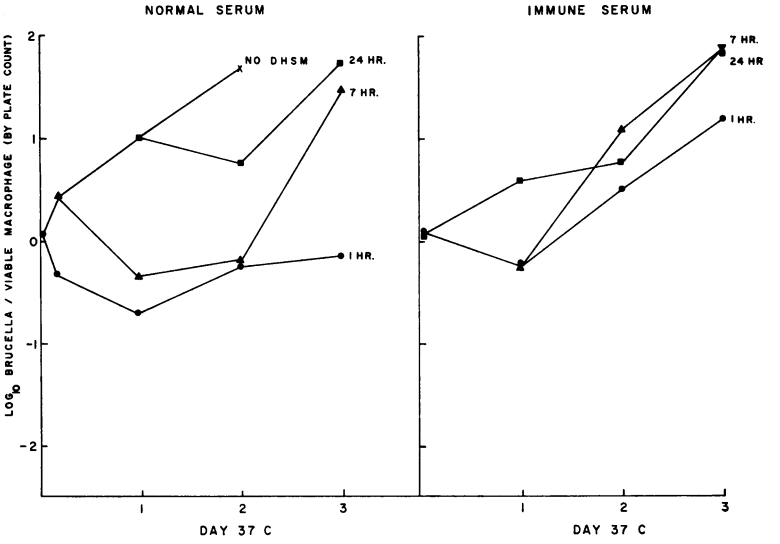

FIG. 2. *Effect of DHSM added at intervals on normal macrophages infected with Rev I in normal and immune sera. Normal macrophages were infected for 1 hr at 37 C in normal rabbit serum and in a pooled immune serum removed from rabbits 8, 12, and 16 weeks postimmunization. After centrifugation to remove free bacteria, the macrophages were resuspended in fresh medium, diluted, and treated with 50 μg of DHSM per ml at the indicated intervals. Counts were made of viable bacterial and macrophage survivors, on which calculations of the bacteria per viable macrophage were based.*

with 5 μg of drug per ml (Table 2). The results of this test indicated that the macrophage populations might be heterogeneous, some cells or some areas of cells being capable of faster ingestion and greater exclusion of DHSM. This might be thought of as the establishment of a threshold level of killing action, a point repeatedly noted by those warning of the dangers implicit in using this drug to control "extracellular" growth (13, 22).

When infection was carried out in immune serum, the differences between drug concentrations were much reduced, indicating that the MSF had minimized differences resulting from the possible heterogeneous condition of the macrophage population (Fig. 6).

From the preceding tests, we concluded that ingestion of Rev I requires a prolonged interval. The following observations indicate that, during the time after attachment to phagocytes, a high proportion of bacilli remain in exposed loci. (i) Large numbers of bacilli remained loosely attached and were easily released from macrophages when resuspended in fresh media. (ii) Of those bacilli originally adherent to macrophages, many could not be seen within well-defined vacuoles in stained preparations. (iii) As time for the completion of ingestion progressed, more bacteria became protected from the bactericidal levels of DHSM. (iv) Eventually, intracellular bacilli became protected from high levels of drug. The addition of immune serum containing active MSF hastened this process.

Differences in ingestion rates of immune and normal macrophages. Because of the shortened interval brought about by MSF in previous tests of uptake and ingestion of Rev I, we selected a period of 1 hr at 37 C as a standard test condition for comparisons of macrophages from immune and normal sources. In these tests, we compared macrophages from rabbits immunized for different periods. These included samples from animals in which the agglutinin titer had fallen to low levels, and samples from animals whose spleens had just cleared the immunizing infection, in which the agglutinin titer had reached a peak (18 to 21 days postimmunization). In addition, since a number of other published studies have indicated the transient nature of macrophages with enhanced bactericidal and bacteriostatic properties (9, 21), we reinjected rabbits (which had received primary injections several months before) with booster amounts of Rev I intravenously 8 days prior to harvesting donor macrophages. In Fig. 7 and in Tables 3 and 4, data are presented for macrophages removed from rabbits 18 days and 16 weeks after immunization, for macrophages removed from rabbits that had been immunized 16 weeks pre-

viously and had also received a recent intravenous booster, and for macrophages removed from normal controls. The macrophages were infected in the presence of immune and normal sera according to the usual procedure. The immune serum had been collected from animals 6 weeks after immunization and had been stored at −10 C; the normal serum had been stored for a slightly longer interval at −10 C. At 1 hr, 50 μg of DHSM per ml was added to the resuspended infected macrophages. It is noteworthy that immune serum enhanced bacterial survival in all macrophage suspensions. This was accompanied by an increased percentage of macrophages containing stainable bacilli.

Combinations of immune serum and immune macrophages showed the highest bacterial survival, the rate being particularly high with macrophages collected at 18 days. Macrophages from rabbits which had been immunized for 16 weeks exhibited a slightly enhanced bacterial survival, suggesting a better ability to ingest Rev I. These differences between immune and normal macrophages, however, were not detected in stained infected preparations.

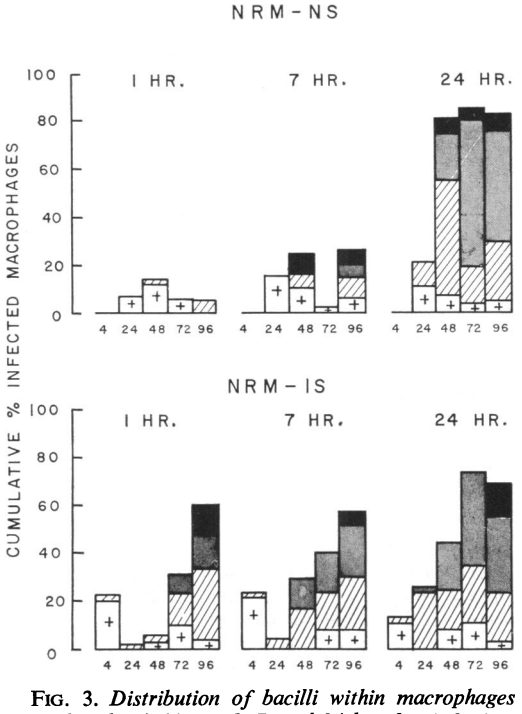

FIG. 3. *Distribution of bacilli within macrophages treated with DHSM at 1, 7, and 24 hr after infection. Procedure by which macrophages were infected is reported in the legend to Fig. 2. Macrophages were stained by a modified Macchiavello technique. A total of 100 macrophages were observed. Distribution of infection is as summarized in Fig. 5. NRM-NS, normal rabbit macrophage-normal serum; NRM-IS, normal rabbit macrophage-immune serum.*

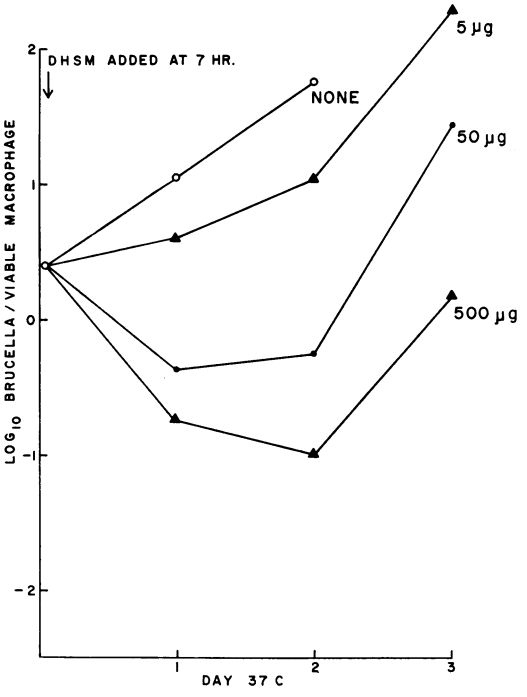

FIG. 4. *Effect of increasing concentrations of DHSM on Brucella yield in infected normal macrophage-normal serum cultures. Curves show bacterial survival per macrophage from the first hour after infection and resuspension in fresh medium. DHSM was added 7 hr after infection.*

We tested preparations of macrophages removed at 4, 8, and 16 weeks after immunization. When compared with normal macrophages in normal serum, the immune cells showed increased bacterial survival in the presence of 50 μg of streptomycin per ml (Fig. 8). However, when the same cell preparations were tested with immune serum, the differences were no longer apparent. It is possible that the immune macrophage preparations had been contaminated with traces of immune serum, either in the fluids or absorbed to the surface of the mammalian cells.

Failure of inhibition of intracellular bacilli by immune and normal macrophages. One surprising finding of these studies was that macrophages that have ingested bacilli within drug-protected loci do not cause extensive killing (we observed net growth). To obtain a comparison of the relative rates of intracellular growth in immune and normal systems, we performed the following experiment. Ingestion by normal and immune macrophages (6 weeks postimmunization)

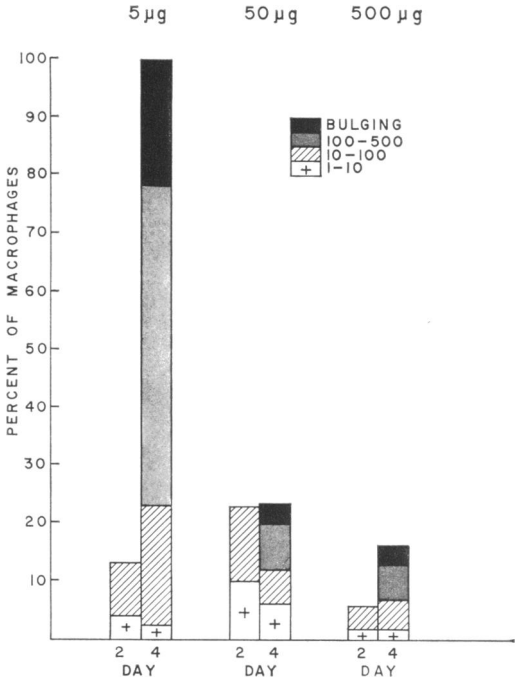

FIG. 5. *Distribution of Rev I in normal macrophages treated with various concentrations of DHSM. Cumulative distribution of infection within 100 macrophages in stained samples removed at day 2 and 4 after infection with Rev I is shown. Source of macrophages and procedure for infection were as reported for Fig. 4.*

TABLE 2. *Survival of infected normal rabbit macrophages in normal serum treated with DHSM*

Concn of DHSM	Percentage of macrophages surviving after				
	4 hr	24 hr	48 hr	72 hr	96 hr
μg/ml					
5.0	82	64	52	51	53
50.0	82	73	62	48	39
500.0	82	70	77	34	32

was allowed to proceed for 6 hr past the preliminary period for adherence to macrophages, to ensure that bacilli which were only loosely attached had an opportunity to be transported to intracellular loci. Then DHSM was added at three levels, 10, 50, and 250 μg/ml. Extensive bacterial multiplication occurred intracellularly in both normal and immune macrophages, irrespective of the drug concentration, indicating that a large proportion of bacilli had been removed to protected loci in the hours prior to exposure. When the data were plotted so as to compare the relative growth rates at each drug level (Fig. 9), it became apparent that the bacteria grew similarly in the two systems. Therefore, macrophages removed from rabbits 6 weeks postimmunization were not endowed with significantly enhanced mechanisms for restricting growth, even in the presence of immune serum. The primary contribution of the immune serum was to increase the rate at which macrophages could establish drug-protected sites, as is evident in curves of survival following addition of DHSM at even earlier intervals (Fig. 10).

Despite our failure to demonstrate significant growth restriction by macrophages under conditions where DHSM had been added to inhibit extracellular bacilli, we nevertheless observed

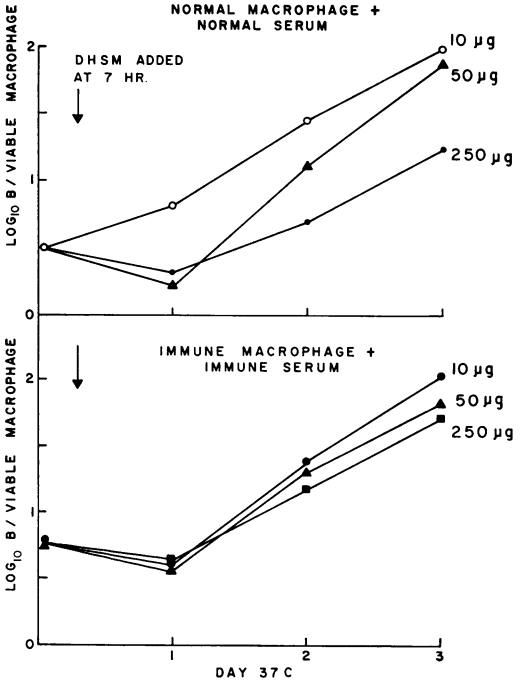

FIG. 6. *Effect of increasing concentrations of DHSM on intracellular bacilli in immune and normal macrophages. Drug was added at 7 hr postinfection. Macrophages from immune animals were collected 6 weeks after immunization. Infection of immune macrophages was performed in pooled immune serum collected 3 weeks postimmunization. Infection was carried out for 1 hr at 37 C, followed by centrifugation, dilution in fresh medium, and further incubation for 6 hr, when DHSM was added. Calculations of bacteria per viable macrophage were based on plate counts of bacterial survivors and direct counts of macrophages made at the indicated intervals. Plots of total bacteria per sample were essentially the same shape; differences in macrophage death for each test condition were relatively minor.*

that in the absence of DHSM combinations of immune serum and macrophages consistently restricted the total amount of bacterial increases as compared with the yields obtained in normal serum-macrophage combinations.

Ingestion-promoting factors in normal sera. Normal rabbit sera that had been stored frozen for periods of 1 week to several months showed a low capacity to promote ingestion of attached Rev I bacilli. The residual count of the bacilli recovered at the first hour in resuspended mixtures generally decreased by log-fold amounts for 48 to 72 hr, occasionally rising on the fourth day. Heating at 65 C for 15 min did not change

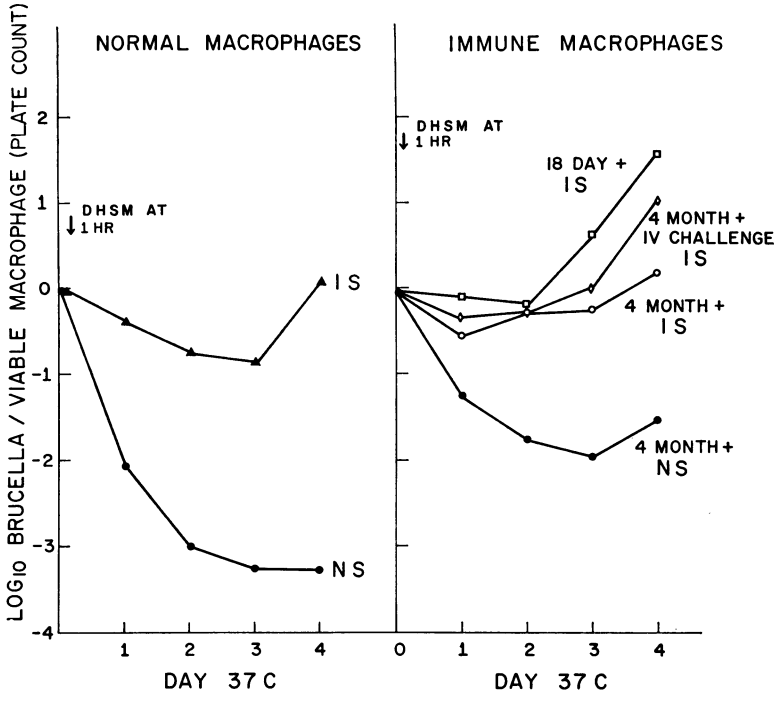

FIG. 7. *Survival of Rev I bacilli in macrophages tested at intervals following primary immunization or following a secondary booster injection. DHSM was added 1 hr after infection at 37 C. Immune serum was collected 6 weeks after primary immunization. Macrophages were infected for 1 hr at 37 C. After centrifugation and dilution in fresh media, each macrophage was treated with 50 μg of DHSM per ml. Surviving macrophages were derived from plate counts and from direct counts of bacteria and macrophages at the indicated intervals. IS, immune serum; NS, normal serum.*

TABLE 3. *Distribution of bacilli in macrophages from animals immunized for various periods*[a]

Macrophage source	Percentage of macrophages in normal serum					Percentage of macrophages in immune serum[b]									
						6-week					Autologous				
	0[c]	+	++	+++	++++	0	+	++	+++	++++	0	+	++	+++	++++
Normal	98	2	0	0	0	33	56	11	0	0	—	—	—	—	—
Immune (16 week)	100	0	0	0	0	39	59	2	0	0	—	—	—	—	—
Immune (18 day)	100	0	0	0	0	81	19	0	0	0	57	43	0	0	0
Immune (16 weeks) + booster[d]	100	0	0	0	0	72	26	2	0	0	77	73	0	0	0

[a] Counts made 1 hr after infection at 37 C.
[b] From rabbits 6 weeks after immunization.
[c] See footnote *b*, Table 1.
[d] Intravenous injection of 10^9 organisms 3 days prior to giving oil for induction of macrophages.

the course of this downward trend (Fig. 11). Normal rabbit sera collected in a similar manner but tested immediately without freezing contained various levels of a heat-labile material that promoted ingestion. Such sera did not always promote better attachment or increased initial killing of bacteria prior to adding the drug. They simply caused rapid enclosure of the bacilli. This activity was drastically reduced by heating the sera at 65 C for 15 min. In comparative studies of the role of immune serum, it is important that the possible presence of various amounts of this factor be considered.

Properties of MSF in immune sera. Immune serum collected 5 to 7 days after immunization contained some MSF activity. This material was stable to storage at 4 C for 12 weeks. Three weeks after immunization, immune serum contained both a heat-stable and a heat-labile factor (Fig. 11). After absorption of this serum with heat-killed Rev I cells or with viable bacilli, the MSF activity was reduced approximately to the level of stored normal serum, suggesting that both factors had been removed (Fig. 12). At 14 to 16 weeks, a heat-stable component of MSF activity was present. Heating of such serum, tested immediately after collection or after storage at −10 C, produced a slight increase in survival.

Low levels of the heat-stable component were present at 2 to 3 weeks. During this period, a peak of agglutinins sensitive to 2-mercaptoethanol was reached. Later, a 2-mercaptoethanol-resistant fraction appeared. Different antibodies

TABLE 4. *Percentage of bacteria released from attached sites on resuspended macrophages 1 hr after infection*

Macrophage source	Normal serum	Immune serum
	%	%
Normal rabbits	66[a]	17
Immunized (16 weeks)	100	16
Immunized (18 days)	nt	16[b]
Immunized (16 weeks) + booster[d]	nt[c]	1

[a] Per cent bacteria released from macrophages after resuspension in fresh medium at 1 hr post-infection; determined by plate counts of filtered material.

[b] Tested with autologous fresh serum, therefore oil-induced serum.

[c] Not tested.

[d] Intravenous injection of 10^9 organisms 3 days prior to giving oil for induction of macrophages.

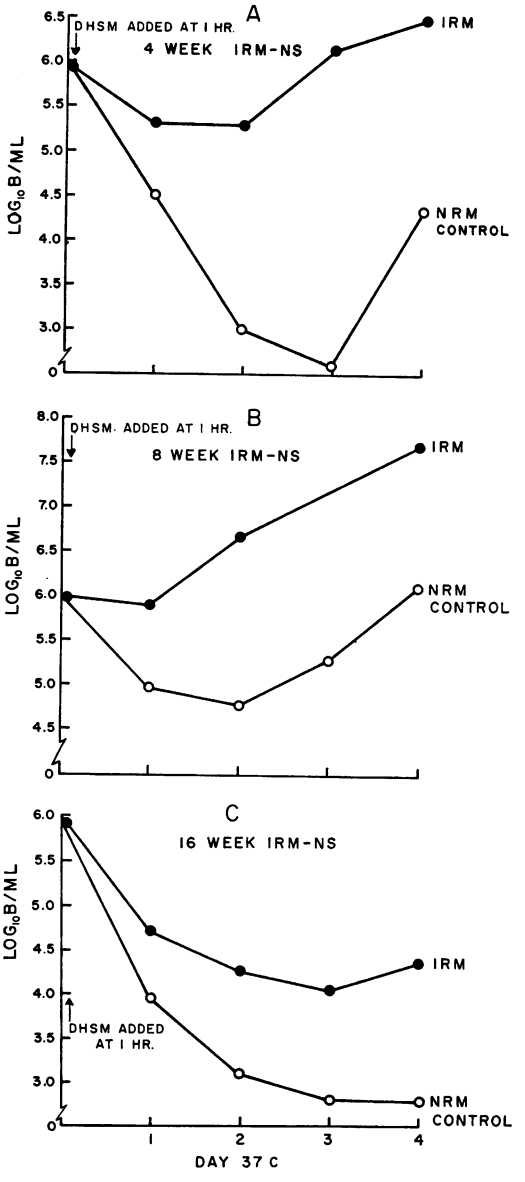

FIG. 8. *Survival of Rev I bacilli in immune and normal macrophages suspended in normal serum. DHSM was added at 1 hr at 37 C. Immune macrophages represent samples harvested at 4, 8, and 16 weeks postimmunization. Tests were made at different times. Serum was pooled from normal rabbits. Serum for curves A and C had been stored frozen, whereas that for curve B was collected shortly before use and was not frozen. Procedure for infection was essentially the same as in Fig. 7. IRM-NS, immune rabbit macrophage-normal serum; IRM, immune rabbit macrophage; NRM, normal rabbit macrophage.*

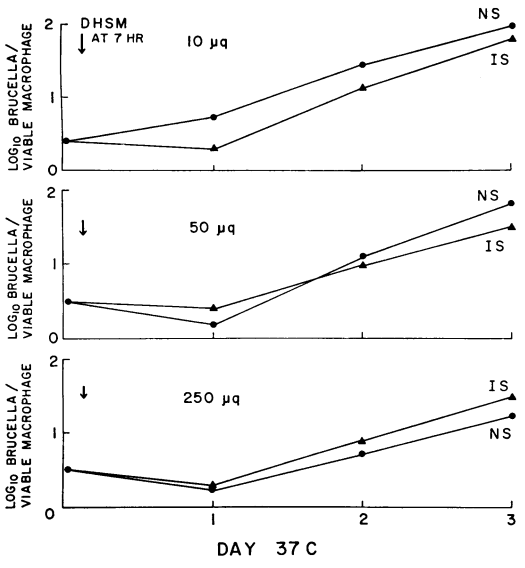

FIG. 9. *Intracellular survival of Rev I in immune and normal macrophage-serum systems treated with increasing concentrations of DHSM 7 hr postinfection. Data in this figure were obtained as for Fig. 6 and were replotted so as to compare infection in the immune and normal systems at each DHSM level. NS, normal serum; IS, immune serum.*

may play a role in MSF function. Further knowledge awaits physical separation of serum fractions.

Relationship of MSF to macrophage stability. Previous studies (8) have established that in the presence of a nonspecific serum factor infected macrophages are rendered highly resistant to the necrotizing effects of *Brucella*. If the death curves of bacilli produced by streptomycin are due to macrophage degeneration and lysis, accompanied by continued release of bacteria to the extracellular environment, one might predict that bacterial counts of infected normal macrophages in normal serum would fall rapidly in comparison with uninfected controls. This was not observed. Infected macrophages survived as well as, or better than, uninfected controls.

We have calculated the recovery of viable macrophages infected in normal serum that had been heated to destroy its "drug sparing" capacity. Despite the fact that extensive numbers of bacilli were released from the macrophages upon resuspension in fresh medium, the release was not accompanied by loss of macrophages (Table 5).

Another observation suggesting that differences in survival of macrophages were not due to toxic degeneration has come from studies of macrophage behavior following an infection in which bacterial growth had been allowed to proceed for 24 hr prior to the addition of DHSM. Under these conditions, in NRM-normal serum systems, the macrophages became more capable of excluding the drug than at the onset of infection, a fact not in accord with a concept of progressive toxic effects in the early stages of growth. It remains possible, however, that the toxic action of *Brucella* on macrophages is of a transient nature, being expressed as a delayed synthesis of membrane materials, rather than cellular lysis, under these conditions. MSF might hasten recovery of the damaged mechanism.

DISCUSSION

When brucellae are located intracellularly, "immune" macrophages treated with DHSM do not kill or restrict growth very differently from those of normal rabbits. This suggests that death, when it occurs, is simultaneous with the act of enclosure. Those bacilli which survive initial ingestion might then remain protected within the macrophage. Thus far, we have only observed growth inhibition with macrophages infected in the presence of immune serum but in the absence of any DHSM. Under these conditions, bacilli growing extracellularly are continually phagocytized and killed.

The question arises of whether the MSF of immune sera act in conjunction with the serum factor, previously reported to protect against macrophage destruction, to provide excellent sequestering of bacilli. Unfortunately, the present in vitro procedures for measuring ingestion have not allowed detection of increased survival of infected immune macrophage-serum systems.

The present studies suggest that these two biological activities are expressed under very different cultural conditions. To demonstrate protection against macrophage degeneration, smaller numbers of macrophages per unit volume were employed in a small enclosed chamber, undoubtedly supplied with a different O_2-CO_2 ratio. These macrophages were infected by exposure to bacteria in fresh rabbit serum at 4 C, followed by washing and resuspension in aged serum. Demonstration of this cellular resistance also depended upon some degree of selection of a macrophage population, as a consequence of the trypsin-treatment and washing procedures prior to infection.

It is probable that more than one factor is concerned in these two phenomena. The material which confers resistance to macrophage degeneration is not absorbed by killed *Brucella*. It is heat-stable and acts nonspecifically (8).

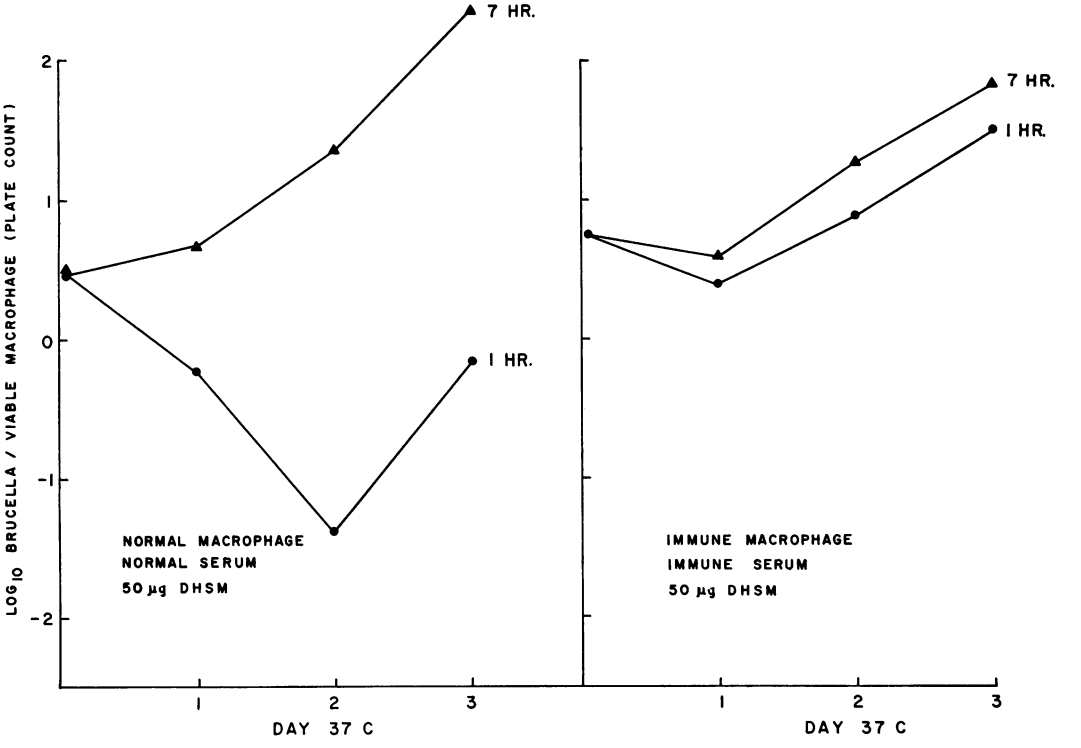

FIG. 10. *Survival of Rev I in immune and normal macrophage-serum systems. DHSM was added at 1 and 7 hr after infection at 37 C. Data were collected under the conditions described for Fig. 6. Curves show bacterial survivors collected at the first hour after injection.*

With respect to MSF, samples of early serum (3 weeks) contained quantities of a heat-labile substance, which is removed by absorption into killed *Brucella*, and a heat-stable factor, also removed by absorption. At later intervals, the serum contained primarily a heat-stable factor. Absorption studies have not been carried out, but we assume, at the moment, that this material represents increased quantities of the heat-stable absorbable fraction detected in the 3-week samples.

With respect to the cellular aspects of the immune response, our data indicate that MSF confers on normal macrophages an excellent capacity for enhanced storage, and in many tests we could not measure a significant difference between immune and normal macrophages in its presence. However, when immune macrophages were tested in normal serum, they were found to ingest bacilli at rates significantly higher than normal macrophages. In view of the possibility that the test suspensions contained traces of immune serum and of the even stronger possibility that the macrophages had been coated with cytophilic antibody, we cannot yet make a valid judgment as to the nature of this difference.

Streptomycin and other antibiotics have been used to control extracellular infection in many in vitro studies (18). Since the early observations of Eagle (6) that streptomycin might penetrate cells, there have been many warnings as to its usefulness in analyzing intracellular growth (22). It has been reported that conditions exist whereby it penetrates mammalian cells (28), inhibits intracellular growth (28), and, combined with other antibiotics, causes the production of L forms (15), reduces phagocytosis (24), and possibly alters the specificity of antibody (20). Yet, as shown in earlier studies, intracellular multiplication of walled bacilli can occur in the presence of high drug concentrations (27).

Experimental observations on the action of streptomycin on bacteria within mammalian cells range from those finding that no penetration occurred (2), that it was restricted to certain types of cells (3), or occurred under special conditions (33), to those stating that extensive penetration took place, resulting in intracellular death (13). Of the latter, final interpretation of the data requires that the mammalian cell be alive at the time bacterial death is measured and that the bacteria be located within the cell membrane.

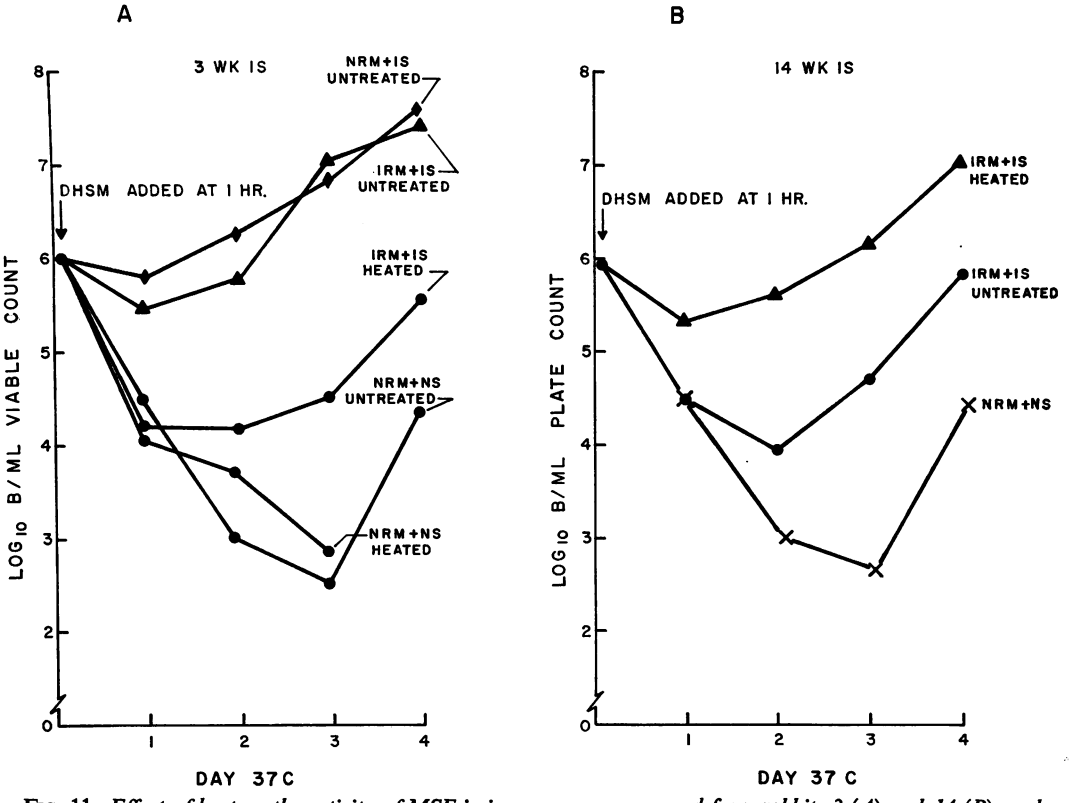

FIG. 11. *Effect of heat on the activity of MSF in immune serum removed from rabbits 3 (A) and 14 (B) weeks after immunization in comparison with stored normal serum. Immune macrophages were harvested 4 weeks post-immunization. Three-week immune serum had not been frozen; the 14-week serum had been stored frozen. Essentially similar results were obtained with 16-week fresh serum. Initial parasitism was carried out for 1 hr 37 C, and the macrophages were resuspended and diluted in fresh media prior to the addition of DHSM. Sera were heated for 15 min at 65 C where indicated. NRM + IS, normal rabbit macrophage plus immune serum; IRM + IS, immune rabbit macrophage plus immune serum; NRM + NS, normal rabbit macrophage plus normal serum.*

Brumfitt et al. (4) have pointed out that many investigators assume that attachment to the phagocyte is followed immediately by ingestion, and that in measuring drug effect such investigators have arbitrarily classified bacteria as being free and outside, or bound internally. In their studies with *Escherichia coli* Brumfitt et al. demonstrated a third and most important state, namely, attachment without immediate ingestion, a condition which is in complete accord with our results. These workers tested for drug uptake and determined that little, if any, drug was removed from a concentration of 200 μg/ml, despite the fact that extensive killing occurred.

Our data indicate that the use of streptomycin as a measure of the progress of ingestion provides a sensitive indicator of the state of the macrophage as it is taken from the rabbit.

When growth occurs in the presence of streptomycin, the simplest explanation is that the drug has not reached the bacteria within mammalian cells, but even in this case alternatives are possible. (i) The bacteria may have been rendered resistant to the drug by mutational events or may have been altered by phenotypic changes in their metabolism, allowing them to bypass or perhaps even inactivate the lethal effects (35). Examples of mutation to resistance have been well documented (17). Fong and co-workers have reported that *Mycobacterium tuberculosis* reisolated from immune macrophages possessed an increased resistance to streptomycin that disappeared on subculture on agar media (11). (ii) Conditions might prevail within the mammalian cell that mitigate against drug activity. For example, it has been known for a long time that streptomycin does not operate well under conditions of relative anaerobiosis (29) or in an acidic environment (5). In our experiments, no evidence of acidity developed when the system was exposed to air but, unless the

TABLE 5. *Macrophage survival after infection with Rev I for 1 hr at 37 C in immune and normal macrophage-serum systems*

System[a]	Recovery at 1 hr		Recovery at 24 hr		Relative growth of bacilli in the presence of 50 μg of DHSM per ml
	No.[b] of macrophages	Percentage	No.[b] of macrophages	Percentage	
N-NS	5.46×10^5	109	3.58×10^5	71.6	++++[c]
N-NS (heated)	6.22×10^5	125	4.86×10^5	97.5	—
I-IS[d]	6.58×10^5	132	3.80×10^5	76.0	++++
I-IS (heated)	5.68×10^5	111	4.06×10^5	81.0	+++

[a] N-NS, normal macrophage-normal serum; I-IS, immune macrophage-immune serum.
[b] Initial macrophage count was 5×10^5.
[c] See footnote *b*, Table 1.
[d] Fresh immune serum, collected 3 weeks postimmunization. Normal serum was freshly pooled. Macrophages from additional rabbits of this series were stimulated by this normal serum to an exceptionally high rate of ingestion, as evidenced by a high growth rate of the bacteria in the presence of DHSM.

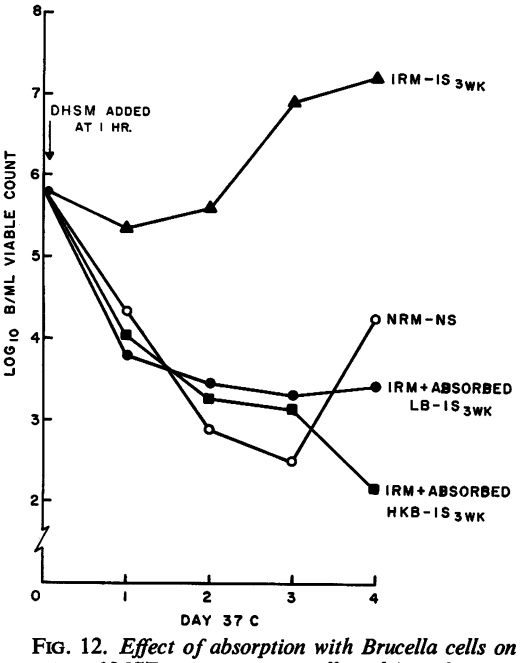

FIG. 12. *Effect of absorption with Brucella cells on activity of MSF present in sera collected 3 weeks postimmunization. Data for Fig. 12 were collected under the same conditions as those for Fig. 11. Macrophages from 4-week immune animals were infected and were compared with normal macrophages in stored normal serum. Serum absorption was performed as described in text. NRM-NS, normal rabbit macrophage-normal serum; IRM, immune rabbit macrophage; LB, live Brucella; HKB, heat-killed Brucella.*

medium was strongly buffered, it did become acidic when placed under CO_2.

Our observations that growth of Rev I occurred in the presence of 500 μg of drug per ml, although strongly indicative of exclusion, would be better supported by direct evidence that no drug had entered the macrophage. The organisms reisolated from such treated systems, however, retained their original level of streptomycin sensitivity (5 μg/ml) in the TS medium. Of the many studies (10, 16, 21, 25) utilizing streptomycin in similar systems, immune serum has never been found to produce the seeming paradox of increased bacterial survival.

In contrast to several other systems, the macrophage from Rev I-immune rabbits has not been shown to possess a heightened capacity to kill, at least once bacilli were firmly established within its protective membranes. The possibility must be considered that the peritoneal macrophages which we have tested merely (i) carry survivors to other sites in the intact animal where a separate cell population commits the final disposal, (ii) preserve bacilli within them until such time as a new population of macrophages appears, or (iii) require some as yet unknown factor for in vitro expression of this function. This problem is currently being investigated.

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