Serum-mediated Immune Cellular Responses to *Brucella melitensis*  

IV. Infection of Macrophages Under Anaerobic Conditions  

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Received for publication 29 August 1968

Immune mechanisms active against *Brucella* were studied under conditions of oxygen deficiency. *B. melitensis* grew in rabbit serum-Tyrode medium flooded with N₂ and CO₂ gas mixtures. Immune sera from rabbits injected with *B. melitensis* strain Rev I possessed growth-inhibitory activity that operated in anaerobic environments against Rev I and virulent strain 6015. When mixed with macrophages, immune sera mediated even greater inhibition of bacterial growth and slowed the spread of infection throughout the tissue culture. Although under anaerobic conditions the rate of phagocytosis was reduced, the macrophages in immune serum killed significant percentages of *Brucella*, suggesting that an antibacterial mechanism had been activated. Sonic extracts of macrophages prepared and tested under anaerobic conditions depressed the growth rate of strain Rev I. The extracts, however, exhibited no immediate killing capacity when tested in Tyrode solution. A factor from serum was required for depression of the growth rate.

Prior studies on the development of the state of immunity to *Brucella* infection revealed that injection of the vaccine strain of *B. melitensis* Rev I produces macrophages of increased resistance to the toxic degenerative effects of virulent *Brucella* strains. The demonstration of this change required cultivation of oil-induced peritoneal macrophages in the presence of a nonspecific serum factor which remains in anti-*Brucella* serum after absorption with killed *Brucella* (5). In addition to this change, immunization was shown to be followed by the appearance of serum factors that accelerate the ability of macrophages to ingest *Brucella* into drug-resistant sites and facilitate inhibition and killing of *Brucella* (17). Some of these serum factors are related to the presence of mercaptoethanol-sensitive globulins (presumably 19S), formed in maximal titer between the second and third week postimmunization, but others are related to heat-stable materials that appear later.

The immunity induced by the living vaccine must eventually be understood in relation to a wide range of physiological conditions within which various cell-serum factors exert a net effect expressed as control of the growth of virulent *Brucella* cells. These defenses may involve (i) outright destruction and digestion of bacilli, (ii) reduction of the growth rate by sequestering bacilli to areas of limited nutrient or gaseous supply, (iii) induction of self-destruction mechanisms, such as phage lysis or faulty wall synthesis, or (iv) metabolic alterations which render bacilli less toxic and less capable of intracellular growth.

Unfortunately, the means by which intracellular killing of *Brucella* cells is achieved by phagocytes is still as poorly defined as are the changes that enable a virulent organism to persist in chronic infections with granuloma formation. The present study is concerned with the effects of decreased oxygen tension on *Brucella*-macrophage interactions.

The O₂ tension in air is around 155 mm of Hg, whereas that of venous blood has been reported to average 70 mm of Hg (6). Further gradients between blood and tissues occur, dependent upon the physiological demands. These gradients are achieved primarily through O₂ uptake from the lung surface and only in a negligible way from the other body surfaces. Under various conditions, the oxygen supply to tissue sites can be severely limited. In chronic granulomatous infection states, the circulation may be impeded by the formation of dense infiltrations of mononuclear cells, exudation of fibrin, and hemorrhagic reactions. In the case of tuberculosis, the O₂ concen-
tration in the center of tubercles has been reported as being close to zero (20). Similar relationships are probably applicable to the foci of Brucella infections. Relative anaerobiosis might, therefore, have considerable bearing on the functional level of immunity in this infectious disease.

We wish to present information on the growth of Rev I cells and the more virulent cells of B. melitensis 6015 under restricted O2 supply in serum media. The effects of immune serum and of peritoneal macrophages of rabbits on the fate of bacilli under anaerobic conditions will be described.

MATERIALS AND METHODS

Bacterial strains. The strains used were B. melitensis Rev I and the virulent B. melitensis 6015. Preparation of strain Rev I for immunization (Trypticase Soy Agar, BBL) and for growth studies (Brucella Agar, Albimi Laboratories, Inc., Flushing, N.Y.) has been described (17; Ralston and Elberg, J. Bacteriol.).

Peritoneal macrophages. Procedures for harvesting and culturing of oil-induced macrophages into chilled modified Tyrode solution (no calcium) have been reported (17). Briefly, two methods were used: (i) infection for 1 hr at 37 C of 107 rabbit macrophages per ml suspended in serum-Tyrode medium, the Brucella macrophage ratio generally being set at 10:1; and (ii) infection of the portion of macrophages that fixed to glass within 3 hr after placing 5 X 106/ml in small glass vials.

Immunization schedules. Injections of Rev I in rabbits for production of immune sera and macrophages were performed according to three schedules: (i) single dose, 106 organisms; (ii) single dose, 108 organisms; and (iii) multiple doses, 106, 108, and 1010 organisms, given at weeks 1, 2, and 4. Immune serum was collected from 2 to 12 weeks postimmunization. It was filtered through 0.45-μm membranes (Millipore Corp., Bedford, Mass.) and used at once or stored at low temperatures. Macrophages were collected from 3 to 18 weeks postimmunization.

Determination of the extent of phagocytosis and of numbers of intracellular and extracellular bacilli. Methods for recovery of intracellular bacilli, counts of viable macrophages, and intracellular staining have already been described (17; Ralston and Elberg, in press). The number of phagocytized bacteria was estimated as the difference between counts of total organisms and those recovered in extracellular fluids after 1 hr of exposure.

Control of gaseous environment. A 1-ml amount of test mixture was introduced into a glass vial (16 X 60 mm) capped with a rubber serum stopper. The sample was flooded with the appropriate gas mixture through sterile hypodermic needles serving as entrance and exit ports. All gases were of a quality equivalent to Liquid Carbonic custom gas mixtures (General Dynamics Corp., New York, N.Y.). All mixtures were passed through sterile cotton filters prior to flooding of test samples. Each sample was flooded with a minimum of 500 ml of gas over a period of 1 to 2 min, as measured by a gas-flow meter. Gaseous mixtures consisted of 95% air + 5% CO2, 1% O2 + 5% CO2, and 94% N2, 95% N2 + 5% CO2, and 100% N2. These mixtures are referred to in the results as air + CO2, 1% O2 + N2 + CO2, N2 + CO2, and N2, respectively. The acidity of the medium was readjusted to pH 7.4 by the insertion of microdoplets of 7.5% NaHCO3 in some experiments. The initial pH was adjusted to 7.8 to 8.0 prior to gassing, to compensate for the increased acidity generated by CO2.

Preparation of macrophage extracts. Oil-induced peritoneal macrophages of rabbits were collected by the usual technique in modified Tyrode solution. They were washed four times in 30 to 50 ml of Tyrode solution and then adjusted to contain 2 X 108 macrophages/ml in Tyrode solution buffered to pH 7.0 with 0.01 m K2H/KH2PO4. Glass vials (16 X 60 mm) containing known amounts of macrophages were flooded with N2 + CO2 mixture and subjected to 60 sec of ultrasonic vibration at 90 w in a Branson model W1850 sonic disrupter equipped with a water-cooled jacket supplied with coolant at 4 C. More than 99.9% of the macrophages were disrupted by this procedure.

The disrupted macrophages were frozen at -20 C for periods ranging from 2 hr to 5 days, after which they were thawed and sedimented at 140 X g to remove debris. The supernatant layer was designated macrophage extract. Macrophages for this procedure were harvested from normal animals and from rabbits immunized for 5 and 8 weeks.

Tests of macrophage extracts. Macrophage extracts were diluted in serum-Tyrode medium to an equivalent of 5 X 106 cells/ml. Amounts of 1 ml of extract dilution (pH 7.4) were mixed with 0.2-ml amounts of Rev I cells (at a concentration of 108/ml) in glass vials. The samples were flooded with N2 + CO2 and were incubated at 37 C. At intervals, samples were removed from replicate vials for plate counts of bacterial survivors on Trypticase Soy Agar. The supernatant solutions of the washed macrophages used in preparing the extracts produced no agglutination of test bacilli at a 1:2 dilution. Extracts of both normal macrophages and immune preparations produced no visible clumping recorded at 1:1 agglutination at a 1:80 dilution. To minimize error due to possible clumping in test mixtures, bacterial suspensions were subjected to 60 sec of ultrasonic vibration in some of the tests. This period of time was found to produce no loss of bacilli in control suspension.

RESULTS

Growth of Brucella under anaerobic conditions. Members of the genus Brucella are primarily aerobic organisms (9) and will grow maximally in broth cultures shaken in air. Working with strain Rev I, we found that rabbit serum (40%) provided the necessary hydrogen acceptors for growth under N2 + CO2 in serum-Tyrode medium. Growth of Rev I and of a virulent
B. melitensis (strain 6015) was compared in normal serum-Tyrode medium buffered to pH 7.4 with 0.01 M KH$_2$PO$_4$. [This concentration increased the K$^+$ concentration from between 0.0026 and 0.0126 M to a level intermediate between those reported for plasma and cells (13).] Both strains grew at decreasing rates as O$_2$ was progressively removed from the environment (Fig. 1). Rev I was also capable of growing in normal serum-Tyrode medium in atmospheres flooded with pure N$_2$, but this growth was at a much lower rate than in comparable samples to which 5% CO$_2$ had been added.

Macrophage infection under anaerobic conditions. The viability of macrophages was determined by direct counts in the presence of 0.2% eosin-Tyrode solution. By this criterion, macrophage survival in cultures flooded with N$_2$ + CO$_2$ was comparable to that in controls incubated in air. Use of a staining technique (modified Machiavello stain), however, provided visual evidence of morphological differences. Macrophages incubated anaerobically often showed peripheral condensation of cytoplasmic material, irregular, ill-defined membranes, and vacuolated nuclei. These changes resembled the effects of anoxia on cultured lymphocytes, described recently by Trowell (21).

Macrophages supported growth of Rev I under anaerobic conditions in the presence of amounts of dihydrostreptomycin sulfate (DHSM, E. R. Squibb & Sons, New York, N.Y.) sufficient to inhibit extracellular growth. To initiate infection, normal rabbit macrophages in normal serum were exposed to Rev I in air for 1 hr (bacterium to macrophage ratio = 27). Since our previous studies had shown that removal of Rev I to streptomycin-protected loci often requires many hours (17), the macrophages were incubated in their respective gaseous environments for 24 hr to allow large numbers of bacilli to reach intracellular loci before the DHSM (50 µg/ml) was added. By 24 hr, over 90% of the anaerobic population was infected, and intracellular bacterial growth progressed until, by 96 hr, the macrophages contained by calculation about 1,580 bacilli per viable cell, a 13-fold increase over the number of bacilli present at the start of the infection. In air, the infection spread more slowly, being limited to approximately 83% of the population, but the total viable count rose 36-fold, to a calculated 5,500 per viable macrophage (Fig. 2). During this period, the macrophages survived in greater numbers under anaerobiosis than under aerobic conditions.

Brucella inhibition by macrophage-serum combinations. The combination of immune serum and macrophages was inhibitory, even when the serum itself produced insignificant effects on the bacilli (Fig. 3). Immune rabbit macrophages (4th week postimmunization) were infected in immune serum (5th week postimmunization) for 1 hr in air (bacterium to macrophage ratio = 2), after which they were suspended in fresh medium and exposed to N$_2$ + CO$_2$. A count taken 72 hr later showed an 85-fold restriction in comparison with

![Fig. 1. Growth of B. melitensis Rev I and 6015 in air and under anaerobic conditions in normal serum-Tyrode medium.](http://jb.asm.org/Downloaded from http://jb.asm.org)
Fig. 2. Intracellular distribution of Brucella in macrophages infected in normal serum under aerobic and anaerobic conditions. Figures in parentheses indicate viable brucella per surviving macrophage. Di-hydrostreptomycin (50 µg/ml) was added 24 hr after infection.

that in serum alone. Stains of these macrophages revealed that infection had been limited to less than 11% of the population. Viable extracellular bacilli were kept to levels approaching 1% of the total bacteria, indicating a sustained phagocytic and killing process.

In contrast, macrophages incubated in air supported more bacterial growth than their anaerobic counterparts. When treated with DHSM at 24 hr, they allowed extensive intracellular growth, but Brucella populations in macrophages placed under anaerobiosis underwent large decreases, suggesting that intracellular killing might have been enhanced (Fig. 4). However, because of our difficulties in the use of DHSM in this system (17), despite the delayed addition of drug, we cannot yet state whether the death of the Brucella cells was due to a host mechanism(s) or to drug action resulting from altered membrane permeability.

Phagocytosis under anaerobic conditions. In the above experiments, phagocytosis was initiated under aerobic conditions before the infected macrophages were exposed to N₂ + CO₂. In the following tests, phagocytosis was carried out in N₂ + CO₂ and compared with that in air. After the phagocytic interval (1 hr, 37 C) in immune serum-Tyrode medium, both sets of macrophages were centrifuged, resuspended in fresh medium, and then placed under N₂ + CO₂. A third set of macrophages was infected in normal serum (1 hr in air, then placed under N₂ + CO₂). Under
anaerobic conditions in immune serum, the macrophages took up fewer bacilli (94.2%) than in air (99.4%) but killed a slightly higher percentage (Table 1). Infection totally under N₂ + CO₂ resulted in a slightly greater limitation of growth (Fig. 5a) at 72 hr, correlated with a slower spread of infection through the macrophage population (Fig. 5b). Immune serum mediated better inhibition of Rev I than normal serum (Fig. 5a) and also delayed the spread through the macrophages (Fig. 5b), irrespective of whether the phagocytosis had taken place under aerobic or anaerobic conditions.

**Infection of macrophages fixed to glass.** In the above studies, infection was accomplished without any prior attempt to sort out macrophages with respect to possible functional differences. There has been recent evidence that leukocytes...
TABLE 1. Effect of immune serum on phagocytosis and killing by immune rabbit macrophages (14th week postimmunization) during the 1st hr of infection in air and under N₂ + CO₂

<table>
<thead>
<tr>
<th>Infection mixturea</th>
<th>Atmosphere</th>
<th>Unattached brucellae 1 hr after infection with 1.56 X 10⁴/ml</th>
<th>Brucellae recovered in macrophage pellet after 1 hr at 37 C</th>
<th>Phagocytosisb</th>
<th>Killingc</th>
<th>Macrophage recoveryd</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRM + NS</td>
<td>Air</td>
<td>3.0 X 10⁴</td>
<td>6.23 X 10⁴</td>
<td>87.0</td>
<td>38.5</td>
<td>83.0</td>
</tr>
<tr>
<td>IRM + IS</td>
<td>Air</td>
<td>9.5 X 10⁴</td>
<td>9.25 X 10⁴</td>
<td>99.4</td>
<td>40.6</td>
<td>98.1</td>
</tr>
<tr>
<td>IRM + IS</td>
<td>N₂ + CO₂</td>
<td>9.0 X 10⁴</td>
<td>5.63 X 10⁴</td>
<td>94.2</td>
<td>36.7</td>
<td>93.2</td>
</tr>
</tbody>
</table>

a IRM = immune rabbit macrophages; NS = normal serum; IS = immune serum.
b Immune macrophages from animals immunized with multiple injections were exposed to Rev I bacilli at a bacterium to macrophage ratio of 1.56 in IS (pool of sera from 3rd, 8th, and 12th weeks postimmunization) and NS. After 1 hr at 37 C, the macrophages were sedimented; the supernatant fluid was filtered through 5-μm membranes and assayed for nonattached bacilli.
c Killing of bacilli (B) = (1 - Bfree/Binput) X 100.
d Macrophage recovery = no. of viable macrophages after 1 hr at 37 C/no. of macrophages exposed to bacilli X 100.

A. GROWTH OF BRUCELLA IN MACROPHAGES

B. DISTRIBUTION OF INFECTION IN STAINED MACROPHAGES

Fig. 5. Infection of immune rabbit macrophages (14th week postimmunization) under anaerobic conditions in immune serum (IS) and normal serum (NS). Comparison of infection begun in air with infection begun in N₂ + CO₂. For explanation of section B, see legend for Fig. 4.

which adhere to glass exhibit excellent growth-inhibitory properties (8), and we have also reported that the Rev I strain was more effectively inhibited by adherent peritoneal macrophages than by the total population. This property was shown by both immune and normal rabbit macrophages, although the two types differed with respect to the proportion of each fixed to
growth was comparison macrophages immune macrophages growth of Brucella fixed macrophages, up took to phagocytosis, and concentration was carried to glass, serum to infected macrophages were washed to remove free bacilli and were returned to N₂ + CO₂ in fresh immune serum-Tyrode medium. A similar infection was carried out in air. The extent of growth limitation was measured by simultaneous comparison with macrophages infected in normal serum. Because we had observed earlier that heated serum contained a factor that caused inhibition of growth in the absence of macrophages, we used heated serum to stimulate normal and immune rabbit macrophages to phagocytize and restrain the growth of Brucella cells.

In these tests, the immune serum contributed to phagocytosis, killing, and growth restriction by fixed macrophages, and all three responses occurred very well under anaerobic conditions (Table 2, Fig. 6). During the first hour of infection, immune macrophages in immune serum took up and killed more bacilli than normal macrophages, but after this period, both kinds of macrophages became infected at similar rates. In comparison with normal serum, the Brucella growth was significantly depressed, indicating that immune serum enabled both immune and normal macrophages to sustain a prolonged growth inhibition. This inhibition was maintained under anaerobiosis. Unheated immune serum also promoted uptake and killing, and was effective under anaerobic conditions (Table 2), suggesting that both heat-labile and heat-stable serum factors are involved in the macrophage response. These in vitro tests show that the functions of the fixed portion of the macrophage population are not impaired by anaerobic conditions.

**Inhibition of Rev I by macrophage extracts.** Equal numbers of infected and uninfected macrophages were placed for 1 hr at 37°C, after which 0.45-μm filtrates of each were tested for their ability to limit growth under anaerobic conditions. Filtrates from both parasitized and uninfected immune cells reduced the growth in comparison with filtrates from normal macrophages. To determine whether immune macrophages contained agents which could act extracellularly, extracts prepared by ultrasonic disruption were tested against known numbers of Rev I bacilli.
The extracts were derived from uninfected macrophages adjusted to cell equivalents of $5 \times 10^9$/ml. When immune extracts were tested alone in Tyrode solution, they were not inhibitory. Immune extracts became inhibitory when they were combined with normal rabbit serum under anaerobic conditions (Fig. 7). They were not effective aerobically for reasons which remain obscure.

**DISCUSSION**

These studies of the anerobic infection of peritoneal cells indicate that macrophages survived and sustained infection by *Brucella* when the gaseous environment was replaced by N$_2$ and CO$_2$. Further, when O$_2$ was removed from the environment, immune serum mediated a sustained growth-restriction by infected macrophages, a response earlier described under aerobic conditions (5; Ralston and Elberg, J. Reticuloendothelial Soc., in press). This restriction was often far greater than that observed in the serum alone, suggesting that macrophages play an active role, perhaps by concentrating the serum into intracellular loci or by contributing additional inhibitory agents, or by a combination of these effects. Evidence that the macrophages contribute inhibitory agents was derived from our observation that growth could be inhibited by macrophage extracts mixed with serum-Tyrode medium.

In many of our tests with infected macrophages, the initial hour of phagocytosis was accompanied by killing of a high percentage of bacilli. This response was negligible when unseparated cell exudates from normal rabbits were infected in normal serum but became evident when that fraction of cells which fixed to glass was infected. When immune rabbit macrophages were infected in immune serum (both at the 3rd week postimmunization), the initial uptake of bacilli (which was usually far greater than in normal serum) was followed by a period of killing that lasted for 24 hr. The survivors of this treatment then multiplied, but at greatly reduced rates. Anaerobiosis did not obliterate these responses. The effects could be demonstrated in serum heated at 65°C for 15 min, indicating that heat-labile components of complement were not involved.

The growth restriction was not due to exhaustion of nutrients from the macrophage cultures, because it was eventually followed by a rapid increase in viable count.

Although both cellular and serum elements are involved in the response of the rabbit to Rev I infection, the exact function of each in the in vivo disposal of bacilli remains to be clarified. A large number of observations on cellular responses to infection indicate that a range of nonspecific cross-protection occurs with respect to such organisms as *Listerella monocytogenes* and *Mycobacterium tuberculosis* (5, 14). There has been recent evidence that mice infected with the protozoan *Toxoplasma gondii* develop increased resistance to *Listerella* and *Salmonella* infection (18). The extent to which these responses can operate under conditions of decreased O$_2$ supply is largely unknown. The nature of the cross-protection would suggest that a general mechanism for control of growth can be evoked in certain cells.

In the case of tuberculosis, a number of observations have indicated that one consequence of the immune response is a forcing of bacilli into more anaerobic pathways of metabolism (12, 19). An agent, mycosupressin (22), isolated from the lungs of BCG-immunized mice, inhibits the respiration of tubercle bacilli. There have been suggestions (12) that the immunity induced by the BCG vaccine results in a slowing of growth and change to a form resistant to clearing mechanisms, rather than in an elimination of all bacilli. Thus, in chronic granulomatous diseases protection might be two-pronged: (i) development of a mechanism to kill and destroy rapidly growing...
bacilli and (ii) forcing of survivors into a slower rate of growth by agents that suppress oxidative metabolism or by tissue responses serving to exclude O₂ from the environment. In this respect, the more virulent strains of tubercle bacilli were shown to be more capable of anaerobic growth than the less virulent bacilli. Unfortunately, although there appear to be provocative indications that the in vivo residence of the tubercle bacillus is affected by relative anaerobiosis, corresponding information for Brucella is lacking. Most studies concerning O₂ deficiency have been concerned with rough-smooth variation and the survival of organisms in immune serum (1–3). The antisera of our present studies showed the multiplication of Brucella under both aerobic and anaerobic conditions. We do not yet know whether one or two or even multiple factors are concerned in this control, or whether the material(s) which acts to restrict growth in the absence of macrophages is the same factor that enables macrophages to add to the degree of inhibition of growth of Brucella. It is conceivable that antibodies may be found which exert their biological effects only when the organism is placed into oxygen-deficient conditions. (It is difficult to see how specific antibodies might play any role in cross-protection, unless they contribute nonspecific stimulation to the macrophages).

There have been numerous studies on the metabolic responses of leukocytes from various sources during phagocytosis and infection (4), but detailed information on the characteristic changes of cells from immune animals is still lacking, as are data concerning their function under conditions of changing oxygen supply. Recent studies by Iyer, Islam, and Quastel (11) and McRipley and Sbarra (15) on the mechanism of killing by polymorphonuclear leukocytes have led to the theory that under aerobic conditions peroxide formation contributes to the defense capacities of the cells. However, only a limited range of bacteria was affected, and the studies by Fitzgeorge, Keppie, and Smith (7) indicated that the virulence of Brucella was not correlated with its resistance to peroxides, making it evident that other mechanisms must be involved. That peritoneal macrophages possess a mechanism for killing under anaerobic conditions has been demonstrated by Nakae, Nakano, and Saito (16) in studies with Salmonella and Escherichia coli.

Our studies with strain Rev I appear to be among the first tests of the relative capacity of macrophages from animals immune to Brucella to kill and restrict growth under anaerobic conditions. The results indicate that immunity developed to the vaccine produces cellular and serum factors that operate over a wide range of gaseous O₂ supply. One or more mechanisms may be involved in these responses. It will be important to determine the extent to which this capacity applies, if at all, to the cross-protective immunity afforded to other intracellular infections.

ACKNOWLEDGMENTS

We are grateful to Joan Okimoto for excellent technical assistance.

This investigation was supported by Public Health Service grants AI-00022 and AI-1372 from the National Institute of Allergy and Infectious Diseases, and by a grant from the Veterinary Public Health Division of the World Health Organization.

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