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Infection at the Subcellular Level

II. Distribution and Fate of Intravenously Injected Brucellae Within Phagocytic Cells of Guinea Pigs

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Cells of *Brucella melitensis* strain 16 M were labeled with ^{32}P. When injected into normal guinea pigs, labeled, viable bacteria were taken up and inactivated in liver and spleen during the 60 min after infection. Both uptake and inactivation increased if brucellae were coated with antibrucella antibody. Neither viability nor radioactivity were lost when labeled brucellae were incubated for 60 min in vitro with normal guinea pig blood, liver homogenates, or in defined medium. Incubation for 12 h with antibrucella rabbit immunoglobulin G similarly was innocuous. Livers were removed from infected animals at various times up to 60 min after injection and were separated into subcellular fractions. The numbers of total (determined by radioactivity measurements) and viable brucellae as well as the acid phosphatase activity in the various fractions were determined. Total bacteria and acid phosphatase activity were progressively transferred from the mitochondrial plus light mitochondrial (M + L) fraction to the nuclear (N) fraction. Viability of brucellae declined more rapidly in the N fraction than in other fractions. Examination of M + L fractions by isopycnic centrifugation showed a decrease in viability of both free brucellae and those in particles. The results indicated the formation of bacteria-containing heterolysosomes which progressively increased in size and in which brucellae were inactivated. The antibrucella activity of phagocytes of guinea pig liver in vivo appeared to be greater than that of peritoneal macrophages from immune rabbits or of bovine leukocytes studied in vitro.

An important property of certain bacterial parasites, notably those responsible for tuberculosis, leprosy, and brucellosis, is their ability to survive and even to replicate intracellulary within phagocytic cells. For the most part, research on host-parasite interactions at the cellular level has been carried out by using leukocytes or suspensions of macrophages isolated from the peritoneal cavity or the bronchial tree (12, 14). Although these investigations have yielded valuable information, they tell little about how the fixed phagocytes of liver and spleen interact with the bacteria that they remove from the bloodstream (1, 3, 8, 13). As discussed by Furth et al. (6), the functional characteristics of cells in the mononuclear phagocyte system (MPS) may vary depending upon localization of the cells in the body. Since the outcome of an infection may depend upon clearance of bacteria by fixed macrophages, investigations of these cells in vivo will further the understanding of infectious diseases.

Certain characteristics of brucellae make them well suited for in vivo studies to determine how phagocytic cells deal with ingested bacteria. Brucellae grow slowly, plate easily, and have little endotoxic activity (9), and some strains are not damaged by serum factors. Braude (3) and Moulton and Meyer (11) described the development of brucellosis in guinea pigs and emphasized the role of the reticuloendothelial system or MPS (6), but they did not quantitate the fate of the injected bacteria, a matter of crucial importance for the outcome of the infection.

In a previous report (7), we demonstrated that most of the brucellae injected intravenously into guinea pigs were removed from the blood by liver and spleen, and that the bacteria in the liver were localized within the vacuolar apparatus of phagocytic cells. The present

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investigation describes the fate of brucellae within this subcellular system of liver phagocytes.

MATERIALS AND METHODS

Procedures described previously. Most of the procedures used in these investigations were described previously (7). Infection of male guinea pigs with *Brucella melitensis* 16 M, grown in a defined medium (ROS-Tris) containing ^{32}P, was effected by intravenous injection of the washed bacteria via the penile vein. In some experiments, brucellae coated with rabbit antibody against *B. abortus* were used. Blood, liver, and spleen from animals sacrificed at various times after inoculation were taken for study. Livers were homogenized and fractionated into nuclear (N), mitochondrial plus light mitochondrial (M + L), and microsomal plus soluble (P + S) fractions (5). The M + L fractions were subjected to isopycnic centrifugation in sucrose density gradients. Samples of organs and fractions were assayed for acid phosphatase, viable brucellae, and total (radioactive) brucellae. In all investigations, a single animal was studied at each time point.

Determination of total and viable brucellae within various compartments. A means of tracing the injected brucellae and determining their viability was required to evaluate their fate in various tissues and subcellular compartments. In previous studies (8, 10, 13), viable counts alone were used to describe both distribution and killing of bacteria. However, an unambiguous analysis of simultaneous redistribution and antibacterial action in different tissue or subcellular compartments cannot be made unless the number of viable and total bacteria in each of the compartments is obtained. For a two-compartment system, different biological states can give rise to the same distribution of viable bacteria, expressed in percentages. Expression of our data as the percent of total bacteria recovered from each compartment, and the percent of bacteria that are viable in each compartment, can distinguish whether the bacteria merely were redistributed into different compartments, were selectively inactivated or killed in a specific site, or were both redistributed and inactivated. This approach is particularly desirable in these experiments because, depending on the size of the animal and variations in dosage of bacteria and in organ phagocytic activity, considerable differences were found in the quantities of brucella that accumulated in the various organs. We wished to compare events occurring in different animals at different intervals after injection of the bacteria. The use of percentage values made such comparisons possible.

The number of viable bacteria in a compartment was determined by plate counting. The total number of bacteria was obtained from radioactivity measurements once the radioactivity associated with a single bacterium in each individual experiment was determined. The validity of the latter procedure rested upon the assumptions that (i) all bacteria in the inoculum were uniformly labeled; (ii) the radioactivity associated with each bacterium remained attached until the brucellae reached a subcellular compartment; and (iii) once within a given compartment, radioactivity did not leak out in spite of death of the bacteria. The validity of the first assumption depended on the observation that the difference between mean microscopic and viable counts of brucellae growing in the presence of 1.0 μCi of ^{32}P per ml was only 9% (H. Guerra, Ph.D. thesis, Baylor College of Medicine, Houston, Tex., 1970), and on the assumption that isotope was incorporated equally by all viable brucellae. Evidence was provided that the second assumption was valid by data indicating that incubation for 60 min in a variety of media did not result in loss of either viability or radioactive label (Table 1). The validity of the third assumption was not evaluated.

Coating of brucellae with antibody. Immunoglobulin (Ig) G was obtained by electroconvection (E-C Apparatus Corp., Philadelphia, Pa.) from the serum of a rabbit bled 4 days after the second injection of heat-killed *B. abortus* 456 (1.0 ml of a 3.2% vol/vol suspension) that was given 22 days after a first injection of the same bacteria (0.5 ml of a 32% vol/vol suspension). The purity of the IgG was determined by immunoelectrophoresis by using polyvalent antisera to whole rabbit serum.

Different concentrations of brucellae were incubated with various dilutions of the purified IgG, in the presence of fresh guinea pig serum, for up to 12 h at 37 C. No evidence of antibody and complement-

TABLE 1. *Viability and specific radioactivity of brucellae after in vitro incubation in various media*

Suspension medium	0 min[a]		60 min[a]			
	Viable count/ml	Counts per min per brucella	Viable count/ml	Percent of 0-min value	Counts per min per brucella	Percent of 0-min value
ROS-Tris	1.83×10^8	2.19×10^{-6}	1.65×10^8	90.5	2.24×10^{-6}	100.2
Blood[b]	1.63×10^8	2.30×10^{-6}	1.90×10^8	116.6	2.04×10^{-6}	88.8
Liver, pH 5.0[c]	1.89×10^8	2.46×10^{-6}	1.82×10^8	96.5	2.06×10^{-6}	83.7
Liver, pH 7.0[d]	2.77×10^{8e}	1.85×10^{-6} [e]	2.52×10^8	91.0	1.93×10^{-6}	104.0

[a] Differences between values obtained at 0 and 60 min were evaluated by *t* test and were found to be statistically not significant in all cases.
[b] Fresh heparinized blood from normal guinea pig.
[c] Homogenate contained 1 g of guinea pig liver per 2 ml of saline, with acetate buffer.
[d] Homogenate contained 1 g of guinea pig liver per 2 ml of saline, with phosphate buffer.
[e] Viable counts at 0 min were contaminated; values given were those obtained at 20 min of incubation.

mediated bacteriolysis could be demonstrated by colony counts performed at intervals during incubation of brucellae with antibody. For in vivo studies with antibody-coated bacteria, brucellae were incubated with nonagglutinating concentrations of IgG at 37 C for 15 min prior to injection.

RESULTS

Uptake of injected brucellae by liver and spleen of guinea pigs. Individual animals injected with radioactively labeled brucellae, with or without a coating of antibody, were sacrificed at 20, 40, 50, and 60 min after injection. Table 2 shows the data for blood, liver, and spleen, the tissues that contained about 95% of the bacteria found in a variety of organs after intravenous injection (7). As can be seen, the percent of the inoculum recovered from these organs was variable, probably due to extravasation into the connective tissue of the penis of different amounts of the inoculum during injection. Despite these difficulties, uptake of bacteria was evident in all animals. However, even at 60 min, 40% of uncoated bacteria recovered from the blood, liver, and spleen remained in the circulation. Other studies demonstrated that at 315 min, the blood contained 4.1% of the viable bacteria recovered, the liver contained 46.6%, and the spleen contained 49.2% (H. Guerra, Ph.D. thesis). Coating the brucellae with antibody increased uptake, especially by liver.

Fractionation of livers of guinea pigs injected with brucellae. Figure 1a and b show, respectively, the distribution of acid phosphatase activities and total (radioactive) bacteria in the fractions obtained from livers of guinea pigs injected with brucellae. Some of these data were presented previously (7). Both acid phosphatase and bacteria appeared to concentrate in the N fraction, at the expense of the M + L fraction, as the time between injection of the brucellae and sacrifice of the animal was increased. Little change in acid phosphatase content of the P + S fraction was observed, and very few bacteria were found in this fraction except at 50 min, when faulty technique may have placed bacteria belonging to the M + L fraction in the P + S fraction. Figure 1c gives the proportion of viable brucellae in the various fractions. The decrease in viable bacteria in the N and M + L fractions might be due to killing or to modification of the brucellae so that they did not grow on media that was not osmotically protective. We did not use media of the latter type and, therefore, refer to the bacteria that failed to produce colonies on our media as inactivated rather than killed. These inactivated bacteria appeared to accumulate in the L fraction with increasing time after injection. In two experiments with antibody-coated brucellae, the proportion of inactivated bacteria in the fractions was larger than for brucellae not coated with antibody (Fig. 1c).

Density gradient centrifugation of M + L

TABLE 2. *Distribution and viability in blood, liver, and spleen of brucellae after intravenous injection into guinea pigs*

Time after injection (min)	Inoculum ($\times 10^8$)	Inoculum recovered from blood, liver and spleen (%)[a]	Blood			Liver			Spleen		
			No./g ($\times 10^8$)	Proportion in tissue[b] (%)	Percent viable[c]	No./g ($\times 10^8$)	Proportion in tissue[b] (%)	Percent viable[c]	No./g ($\times 10^8$)	Proportion in tissue[b] (%)	Percent viable[c]
Brucellae not coated with antibody											
20	123.3	4.5	0.18	72.0	36.9	0.11	20.2	53.2	0.61	7.8	ND[d]
40	123.3	20.0	0.13	13.0	80.7	0.82	47.3	42.2	8.87	39.7	ND
50	50.7	96.8	0.77	41.7	109.7	1.43	38.4	35.1	16.30	19.9	64.6
60	65.7	72.3	0.83	40.5	86.7	2.50	57.9	25.0	2.05	1.6	ND
Brucellae coated with antibody[e]											
20	57.5	8.1	0.004	3.2	16.1	0.26	82.3	31.3	1.20	14.5	38.0
40		7.8	0.013	11.0	22.1	0.14	55.6	28.5	1.58	33.4	37.8

[a] Determined from the radioactivity in each organ and the radioactivity per brucella in the inoculum.
[b] Radioactivity in tissue divided by sum of radioactivity in blood, liver, and spleen.
[c] Number of viable bacteria, times 100, divided by total number of bacteria in the same tissue, as determined from radioactivity measurements.
[d] ND, Not determined.
[e] Brucellae were incubated with rabbit IgG antibody for 15 min at 37 C prior to injection of bacteria.

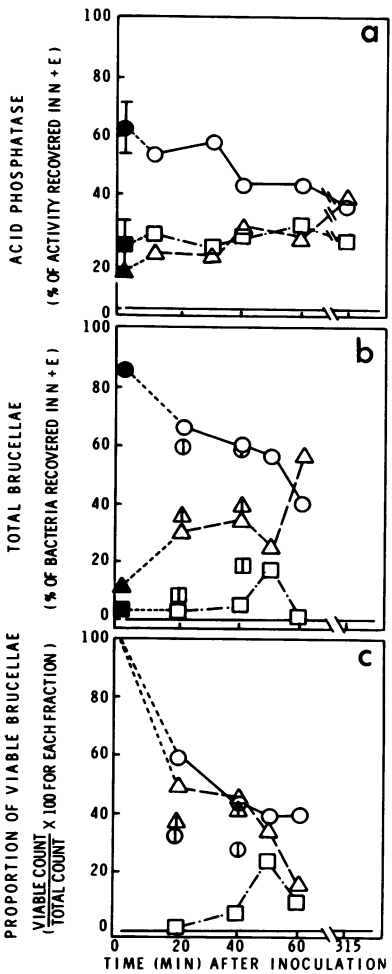

FIG. 1. *Distribution of acid phosphatase and brucellae in fractions from livers of guinea pigs injected with bacteria and sacrificed at various times after infection. Symbols: N, △; M + L, ○; and P + S, □. Solid symbols indicate control values; open symbols indicate values from animals injected with brucellae not coated with antibody; and symbols with vertical bar indicate values from animals injected with antibody-coated brucellae. (a) Distribution of acid phosphatase. Values at zero time were estimated from measurements on three mixtures of normal liver and brucellae. (b) Distribution of total brucellae, as obtained from radioactivity measurements. Values at zero time estimated from measurements on a mixture of labelled brucellae and normal liver. (c) Proportion of live bacteria in the fractions. Values at zero time are assumed to be 100%, since no inactivation of bacteria could be demonstrated. Protein distribution for these experiments were: N, 23.0% (±0.8 SD); M + L, 24.9% (±6.4 SD); and P + S, 52.3% (±6.6 SD); recoveries averaged 97.5% (±10.1 SD) of N + E.*

fractions of livers from guinea pigs injected with brucellae. The distribution of acid phosphatase and viable bacteria was determined at 10, 30, 40, and 60 min after injection of brucellae not coated with antibody, and at 40 min after injection of antibody-coated brucellae. These distributions were similar at all time points, and examples were presented previously (7).

The distributions of viable counts and radioactivity were determined at 20, 40, and 60 min after injection of non-antibody-coated brucellae and 20 and 40 min after injection of antibody-coated brucellae (Fig. 2).

Figure 3a summarizes the distribution of total bacteria in density gradients at the different time points studied. Regions of the density gradient are defined as previously (7). Region I contains the large granules derived from host cells, mainly mitochondria, peroxisomes, and lysosomes; region II is an intermediate zone; free bacteria equilibrate in region III. The distribution of total bacteria appeared not to change with time (Fig. 3a). The proportion of viable bacteria in regions I and III decreased at different rates, as shown in Fig. 3b. Experiments with antibody-coated bacteria yielded similar results.

DISCUSSION

This report constitutes one of the first attempts to describe the initial subcellular events of an infectious disease in vivo. The choice of both bacterium and host animal have been made so that the results obtained bear some similarity to the situation in man. Moreover, the application of techniques derived from the domain of cell biology in a carefully controlled experimental situation extends the work done by others in vitro and demonstrates that the function of the reticuloendothelial organs can be studied quantitatively in vivo by using infectious agents.

Uptake of brucellae by tissue phagocytes. The reticuloendothelial organs, liver and spleen, are active in the uptake of brucellae from the blood of normal guinea pigs. The average concentration (brucellae per gram) of bacteria in the liver was 2.5 times, and in the spleen 14.6 times, the average concentration in blood, for the experiments shown in Table 2. However, the uptake of brucellae is slow since even at 5 h after injection bacteria remain in the circulation (H. Guerra, Ph.D. thesis). Specific antibody, in typical opsonic action, speeds uptake, the greatest effect in liver being visible in the animal sacrificed at 20 min after injection of antibody-coated bacteria (Table 2).

Subcellular events after phagocytosis of brucellae. Our previous results (7), though conceivably affected by the use of viable counts in the distribution studies, and the data in Fig.

1 and 3 are consistent with the concept that brucellae are taken into phagocytic vacuoles or heterophagosomes which later fuse with acid hydrolase-bearing particles to form digestive vacuoles (heterolysosomes). In the latter particles, inactivation and degradation of the bacteria would be expected. The reduced proportion of viable bacteria in the N fraction may be the result of inactivation occurring in the heterolysosomes which, because of their size, have sedimented with this fraction (7).

The time-dependent decrease in viability of

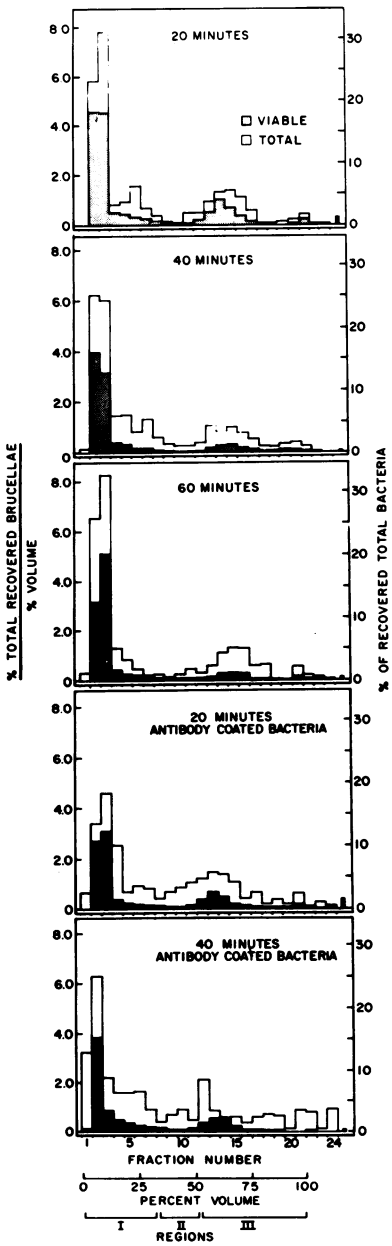

FIG. 2. *Distribution of total and viable brucellae after isopycnic density gradient centrifugation of M + L fractions of livers obtained from guinea pigs sacrificed at various times after intravenous injection of ^{32}P-labeled bacteria. Ordinate values on the left indicate the concentration of bacteria in various fractions. The abscissa gives the fraction numbers, volume distribution, and region designations. The area under the histogram associated with each fraction represents the percentage of brucella present in the fraction. These values can be obtained directly from the right-hand ordinate since the volume of each fraction is considered identical. The black bar to the right of each graph represents the magnitude, in percent, of the 2σ error of the background radioactive counts.*

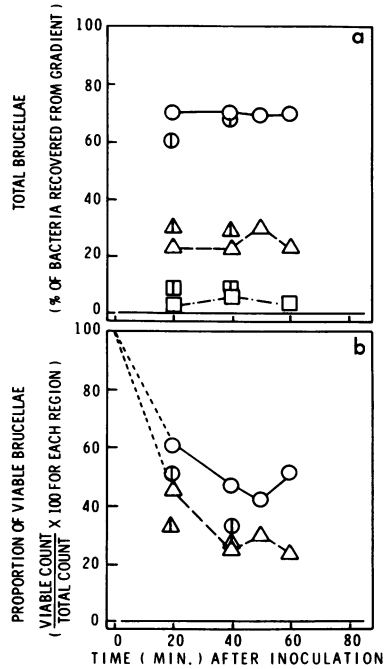

FIG. 3. *Distribution and viability of brucellae in different regions of the density gradient after centrifugation of M + L fractions obtained from the livers of guinea pigs injected with bacteria and sacrificed at various times after injection. Region I, with densities from 1.128 to 1.238, is indicated by circles (○); region II, with densities from 1.239 to 1.248, is indicated by squares (□); and region III, with densities from 1.249 to 1.279, is indicated by triangles (△). Open symbols represent values from animals injected with uncoated brucellae; symbols with a vertical bar represent values from animals injected with antibody-coated brucellae. (a) Distribution of total brucellae, as determined from radioactivity measurements. (b) Proportion of live bacteria in regions I and III. Region II is disregarded because of its low content of bacteria. Initial viability is considered to be 100%.*

the bacteria in region III of the density gradients, where free brucellae are found, may also be explained by assuming that inactivation of brucellae occurs in heterolysosomes of large size. As discussed by Deter and de Duve (4), particles of larger size are more susceptible to rupture during homogenization. An increase of the size of heterolysosomes with time, due to vacuolar fusion, would therefore favor rupture, with subsequent release of inactivated bacteria upon homogenization. These free bacteria would then sediment in the M + L fraction and equilibrate in region III of the density gradients (7), thereby increasing the proportion of nonviable bacteria found in this region.

Fate of phagocytized brucellae. The environment inside heterolysosomes of the liver appears to be considerably noxious to brucellae. One hour after injection, only 25% of the brucellae in liver remained viable. The spleen appears less effective in inactivating bacteria, since 65% remained viable at 50 min, and viability was high in both regions I and III in a corresponding density gradient experiment (H. Guerra, Ph.D. thesis).

Our results with liver may be compared with those obtained from experiments in vitro. We exclude those that were performed in the presence of antibiotics. Macrophages from the peritoneal cavities of immune rabbits inactivated 50% of phagocytized brucellae within 60 min (12); bovine blood leukocytes from normal animals yielded similar results (2). Our data indicate that, even in an animal as susceptible to brucellosis as the guinea pig (15), the phagocytic cells of the liver are capable of inactivating brucellae at least as well as the fully activated free macrophages of immune animals or polymorphonuclear leukocytes. Since brucellosis does become established in guinea pigs in spite of the activity of the reticuloendothelial system, one may speculate that, to do so, one of two alternatives must be operative: (i) there are privileged compartments in which sequestered brucellae remain unharmed, or (ii) the original population of inoculated bacteria is progressively inactivated until "modified" resistant bacteria appear. Perhaps certain cells of the spleen could represent such "privileged compartments." The destructive intravacuolar environment could represent a strong selecting force which would lead to the development of a population of "modified" brucellae.

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LITERATURE CITED

1. Benacerraf, B., M. M. Sebestyen, and S. Schlossman. 1959. A quantitative study of the kinetics of blood clearance of P^{32} labelled *Escherichia coli* and staphylococci by the reticuloendothelial system. J. Exp. Med. **110**:27-48.
2. Biozzi, G., B. Benacerraf, and B. H. Halpern. 1953. Quantitative study of the granulopectic activity of the reticuloendothelial system. II. A study of the kinetics of the granulopectic activity of the RES in relation to the dose of carbon injected. Relationship between the weight of the organs and their activity. Brit. J. Exp. Pathol. **34**:441-457.
3. Braude, A. I. 1959. Studies in the pathology and pathogenesis of experimental brucellosis. II. The formation of the hepatic granuloma and its evolution. J. Infect. Dis. **89**:87-94.
4. Deter, R. L., and C. de Duve. 1967. Influence of glucagon, an inducer of cellular autophagy, on some physical properties of rat liver lysosomes. J. Cell. Biol. **33**:437-449.
5. de Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans. 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. Biochem. J. **60**:604-617.
6. Furth, R. van, Z. A. Cohn, J. G. Hirsch, J. H. Humphrey, W. G. Spector, and H. L. Langevoort. 1972. The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. Bull. W.H.O. **46**:845-852.
7. Guerra, H., R. Deter, and R. P. Williams. 1972. Infection at the subcellular level. 1. Localization of intravenously injected brucellae in the vacuolar apparatus of cells of guinea pig liver. Infect. Immunity **5**:513-523.
8. Karthigasu, K., P. C. Reade, and C. R. Jenkins. 1965. The functional development of the reticuloendothelial system. III. The bactericidal capacity of fixed macrophages of foetal and neonatal chicks and rats. Immunology **9**:67-73.
9. Leong, B., R. Diaz, K. Milner, J. Rudbach, and J. B. Wilson. 1970. Some structural and biological properties of brucella endotoxin. Infect. Immunity **1**:174-182.
10. Mackaness, G. B., R. V. Blanden, and F. M. Collins. 1966. Host-parasite relations in mouse typhoid. J. Exp. Med. **124**:573-583.
11. Moulton, J. E., and M. E. Meyer. 1958. The pathogenesis of *Brucella suis* infection in guinea pigs. Lesions of the spleen, liver, testis, and articulations. Cornell Vet. **48**:165-195.
12. Ralston, D. J., and S. S. Elberg. 1969. Serum-mediated immune cellular responses to *Brucella melitensis* REV 1. II. Restriction of brucella by immune sera and macrophages. J. Reticuloendothel. Soc. **6**:109-139.
13. Rogers, D. E. 1960. Host mechanisms which act to remove bacteria from the blood stream. Bacteriol. Rev. **24**:50-66.
14. Smith, H., and R. B. Fitzgeorge. 1964. The chemical basis of the virulence of *Brucella abortus*. V. The basis of intracellular survival and growth in bovine phagocytes. Brit. J. Exp. Pathol. **45**:174-186.
15. Thorpe, B. D., R. W. Sidwell, and D. L. Lundgren. 1967. Experimental studies with four species of *Brucella* in selected wildlife, labroatory, and domestic animals. Amer. J. Trop. Med. **16**:665-674.