Pathologic changes associated with brucellosis experimentally induced by aerosol exposure in rhesus macaques (*Macaca mulatta*)

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Objective—To develop an aerosol exposure method for induction of brucellosis in rhesus macaques (*Macaca mulatta*).

Animals—10 adult rhesus macaques.

Procedure—8 rhesus macaques were challenge exposed with 10^2 to 10^5 colony-forming units of *Brucella melitensis* 16M by use of an aerosol-exposure technique, and 2 served as control animals. All macaques were euthanatized 63 days after challenge exposure. Gross and microscopic lesions, bacterial burden in target organs, and histologic changes in tissues were evaluated.

Results—Grossly, spleen weights were increased in exposed macaques, compared with spleen weights in control macaques. Histologically, there was inflammation in the liver, kidneys, spleen, testes, and epididymides in exposed macaques. The spleen and lymph nodes had increased numbers of lymphohistiocytic cells. Morphometrically, the spleen also had an increased ratio of white pulp to red pulp. Areas of hepatitis and amount of splenic white pulp increased with increasing exposure dose.

Conclusions and Clinical Relevance—Pathologic findings in rhesus macaques after aerosol exposure to *B melitensis* are similar to those observed in humans with brucellosis.

Impact for Human Medicine—These results may aid in the development of a vaccine against brucellosis that can be used in humans. (*Am J Vet Res* 2004;65: 644–652)

Brucellosis is caused by a gram-negative coccobacillus of the genus *Brucella*. Brucellae cause infertility and abortion in sheep, goats, pigs, cattle, and dogs. Of the 6 recognized species, only *Brucella melitensis*, *B suis*, *B abortus*, and *B canis* cause disease in humans, most of whom become infected following exposure to infected animals or animal products. Brucellae can cause disease after inhalation or ingestion and can enter the body through breaks in the skin. Although brucellosis in humans results in a systemic febrile illness, there are few reports[1-4] describing pathologic changes of the respiratory tract.

Data from other studies[5,6] have revealed that at least 2 vaccines that are candidates for use in humans are immunogenic and protective against respiratory challenge in mice. A number of studies[7-11] have indicated that nonhuman primates are susceptible to infection with virulent or attenuated strains of *Brucella* organisms administered by oral, SC, or respiratory routes. Infected primates become bacteremic, and infection of the liver; spleen; kidneys; and mesenteric, inguinal, and axillary lymph nodes is evident up to 8 weeks after inoculation.[10] However, histopathologic changes in affected animals were not described in any of those studies.

The objective of the study reported here was to further define an aerosol-exposure method for nonhuman primates to determine whether gross and histologic changes associated with brucellosis in nonhuman primates would be similar to those described for infected humans. Furthermore, we attempted to determine whether aerosol challenge exposure of rhesus macaques would be a suitable model for evaluation of candidate vaccines.

Materials and Methods

Animals—Ten adult male rhesus macaques (*Macaca mulatta)* were used in the study. All macaques were considered to be healthy on the basis of unremarkable results of a physical examination, CBC counts, and routine serum biochemical analyses (activities of γ-glutamyltransferase, alanine transaminase, aspartate transaminase, lactate dehydrogenase, alkaline phosphatase, creatinine kinase, and amylase and concentrations of calcium, phosphorus, chloride, potassium, cholesterol, triglycerides, glucose, BUN, albumin, and total bilirubin). Additionally, all macaques had negative results when tested for tuberculosis (purified-protein-derivative skin test), simian immunodeficiency virus (immunofluorescent antibody test), and simian retrovirus (immunofluorescent antibody and polymerase chain reaction tests). Titers for anti-*B melitensis* 16M lipopolysaccharide IgG antibodies were < 1:160, as determined by use of an ELISA.

Approximately 3 weeks before challenge exposure, a telemetry device[a] was surgically implanted in the left flank of each macaque. All animals were observed at least twice daily by qualified personnel to assess general health, humane treatment, and husbandry considerations. Temperature, body weight, and general appearance were recorded daily begin-

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ning 10 days before challenge exposure and continuing until the macaques were euthanatized.

All macaques were housed in a temperature-controlled room in separate cages with an additional containment designed to prevent potential aerosolization of organisms within the animal room. Cages were placed in a clear vinyl negative-pressure room that provided a mean of 100 air changes/h; exhaust air was filtered by use of a **high-efficiency particulate air (HEPA)** filter. Macaques were fed a diet consisting of a standard commercial biscuit formulated for monkeys; biscuits were fed twice daily, and fresh fruit was provided several times each week. Water was available ad libitum. Macaques were housed, cared for, and maintained in accordance with established guidelines.[12]

Bacteria and challenge exposure—*Brucella melitensis* 16M was obtained from another investigator[b] and passaged once in mice. An isolated bacterial colony was obtained from the spleens of inoculated mice and cultured in broth[c] at 37°C. The culture was divided into aliquots, which were added to a solution of 10% sterile glycerol-90% broth[c]; frozen; and stored at −70°C. A vial of this primary stock culture was thawed 2 days before challenge exposure, and contents were inoculated by use of a sterile culture swab onto plates containing agar.[d] Plates were incubated at 37°C for 48 hours; plates were then scraped and washed twice with saline (0.9% NaCl) solution by use of centrifugation. Bacteria were adjusted spectrophotometrically to the approximate desired number of **colony-forming units (CFUs)** per milliliter in saline solution.

Each macaque was anesthetized for the aerosol challenge exposure by administration of tiletamine hydrochloride-zolazepam[e] (6 mg/kg, IM) by use of a 1-mL tuberculin syringe and 25-gauge sterile needle. Respiratory minute volumes were measured by whole-body plethysmography[f] immediately before challenge exposure. Because plethysmography was performed on anesthetized macaques immediately before the aerosol exposure, all macaques were considered to be at the same time point. The minute volume also remained sufficiently constant after anesthetic administration to enable us to reliably estimate the dose of inoculant by use of plethysmography.

Two macaques were assigned to each group. Groups of macaques were scheduled to receive 0, 10^2, 10^3, 10^4, or 10^5 CFUs of bacteria, respectively. The aerosol challenge dose was suspended in 10 mL of broth.[c] The aerosol (mass median aerosol diameter, 1.2 mm) was generated by a 3-jet collision nebulizer.[13,14] The aerosol challenge method used in this study was designed to deliver droplets to all lobes of the lungs. Each macaque was separately exposed to the aerosol (head only) in a dynamic aerosol chamber. Each exposure was 10 minutes, and the samples of the aerosol were continuously obtained by use of an all-glass impinger.[g] For each macaque, the aerosol concentration of bacteria was calculated by plating dilutions of a sample from the all-glass impinger onto plates containing agar,[d] and inhaled doses were then calculated. After challenge exposure, the coat of each macaque was wiped with 70% (vol:vol) isopropyl alcohol and all macaques were then housed in separate cages in biosafety level 3 conditions.

Collection of blood samples for bacterial culture—Blood samples were collected weekly for bacterial culture. Macaques were anesthetized by administration of tiletamine-zolazepam[e] (6 mg/kg, IM) prior to each blood collection. Blood samples were collected from a saphenous or femoral vein. The collection site was cleaned with iodine solution[h] and allowed to air-dry. After the site was dry, iodine solution[h] was applied to the venipuncture site, beginning at the center and moving outward in concentric circles to the periphery of the site; the iodine solution was allowed to air-dry before collection of blood samples. All blood samples were obtained by use of 21- to 23-gauge, 1- to 1.5-inch sterile needles and 3.3-mL whole-blood tubes containing sodium polyanethol sulfonate.[i]

Quantification of bacterial culture of blood samples—One milliliter of blood was equally divided onto 2 plates containing agar.[d] The remaining blood (up to 2.2 mL) was added to a commercially available bacterial culture system.[j] Colony counts on agar plates and the commercially available bacterial culture system were quantified and totalled. Positive colonies identified in the commercially available bacterial culture media were assigned a value of < 1 because the sensitivity of that assay was greater than the sensitivity of the agar plates, which were estimated as having a threshold of detection of 2 CFUs/mL.

Euthanasia—All macaques were euthanized 63 days after challenge exposure. First, a deep plane of anesthesia was induced by administration of tiletamine-zolazepam[e] (9 mg/kg, IM). A combination product[k] (390 mg of pentobarbital/mL and 50 mg of phenytoin/mL) was then administered (60 mg of pentobarbital sodium/kg; intracardiac) by use of an 18-gauge needle. Macaques were auscultated to determine cessation of heartbeat and breathing.

Necropsy and tissue collection—Organs and tissues from control and inoculated macaques were examined grossly during necropsy, and specimens were collected for histologic examination. Tissues were collected from the axillary, inguinal, cervical, submandibular, mesenteric, and mediastinal lymph nodes; testes; epididymides; liver; spleen; lungs; bone marrow of the proximal portion of the femur; hair-covered skin; tonsils; tongue; larynx; thyroid and parathyroid glands; trachea; heart; mediastinum; right brachial plexus; kidneys; adrenal glands; bladder; prostate gland; stomach; duodenum; pancreas; jejunum; ileum; ileocecal junction; cecum; proximal and distal portions of the colon; right sciatic nerve (including skeletal muscle); nares; oral mucocutaneous junction; eyes; brain; cervical spinal cord; and pituitary gland. Samples of urine and bile were also collected during necropsy. Tissue samples were preserved in neutral-buffered 10% formalin. For histologic and morphologic analysis, tissue sections were processed in an automatic tissue processor.[l] Sections (5 µm thick) of paraffin-embedded tissues were then stained with H&E in accordance with standard protocols. Histologic sections were assigned a score on the basis of relative severity of lesions (no lesions, minimal, mild, moderate, or severe), in which minimal lesions reflected only a slight change and severe lesions reflected extensive architectural involvement.

Bacterial culture of tissue samples—Tissue samples (approx 1 g) of spleen, liver, lungs, blood, and epididymides from all macaques were aseptically collected during necropsy and cultured to detect *Brucella* spp. Solid tissue samples were weighed, ground in manual tissue grinders with 1 mL of PBS solution, serially diluted in PBS solution, and plated on agar.[d] Plates were incubated for 72 hours at 37°C. Colonies of *Brucella* spp were identified on the basis of characteristic morphologic features. Suspicious colonies were further characterized by use of a slide-agglutination test that used anti-*B melitensis* antiserum.[m]

Immunohistochemical analysis—Immunohistochemical analysis was performed on all tissue samples collected during necropsy. Briefly, 5-µm-thick paraffin-embedded tissue sections were mounted on glass slides,[n] deparaffinized, and rehydrated.[15,16] Endogenous peroxidase activity was blocked by addition of 0.3% hydrogen peroxide in distilled water and incubated at 21°C for 30 minutes. After slides were washed with PBS solution, nonspecific binding of secondary reagent

was blocked by addition of normal horse serum[o] to all specimens and incubated at 21°C for 20 minutes. Rabbit polyclonal antisera[p] used as a primary antibody were directed against *B abortus* lipopolysaccharide. Slides were incubated with the primary antibody at 21°C for 1 hour. Tissue sections were washed with PBS solution, and secondary antibody (horse anti-rabbit IgG[q]) was applied; slides were incubated for 1 hour. Slides were washed with PBS solution and treated with peroxidase-conjugated streptavidin[r] (1:250 dilution), incubated for 30 minutes, washed with PBS solution, incubated with a chromagen,[s] and counter stained with hematoxylin.[t]

Morphometric analysis—Prior to collection of tissue samples for embedding in paraffin, at least 3 random sections of spleen and liver were collected from each macaque. Sections of spleen were transected longitudinally along the median plane to provide at least 1 longitudinal section from each macaque for morphometric analysis of splenic compartments. Sections of liver were collected at random sites from at least 3 hepatic lobes. The H&E-stained sections of tissue were examined by use of a microscope[u] and color video camera.[v] Images were entered into a computer[w] by use of image analysis software.[x] Area (number of pixels on an image) of the white pulp and marginal zone in sections of spleen and inflammatory foci in sections of liver on each slide were examined for each macaque at each time point. The marginal zone was defined as the zone of macrophages and some lymphocytes that surround the periarteriolar lymphoid sheath. Area of the red pulp was calculated by measuring the total spleen area and subtracting the white pulp and marginal zone areas. Total area of each tissue section was measured so that ratios and percentages could be calculated.

Statistical analysis—Quantitative data were expressed as the mean ± SD for each group. Differences between groups were analyzed by use of the Student *t* test. Differences were considered significant at values of $P < 0.05$.

Results

Bacterial culture of blood samples—Analysis of the number of bacteria delivered by aerosol exposure revealed a linear increase in inocula as predicted by the experimental design. Actual dose inhaled by each macaque was 0 CFUs (saline control group), 1.25×10^2 CFUs and 2.55×10^2 CFUs (group 10^2), 3.60×10^3 CFUs and 3.04×10^3 CFUs (group 10^3), 9.60×10^4 CFUs and 1.02×10^5 CFUs (group 10^4), and 1.45×10^5 CFUs and 3.34×10^5 CFUs (group 10^5). Bacteremia began on day 21 after inoculation in the high-dose group (10^5 CFUs; **Table 1**). On day 28 after inoculation, 6 of 8 inoculated macaques were bacteremic. The highest number of infected macaques was on days 28 and 35 (6/8 inoculated macaques had viable *B melitensis* in blood samples). The intensity of bacteremia was low(< 1 CFUs/mL) in several macaques, with a maximum of > 1,000 CFUs/mL of blood in 1 macaque that received 10^3 CFUs. One control macaque was bacteremic on day 35. The 2 macaques that received 10^3 CFUs were bacteremic from days 28 to 63 after inoculation.

Necropsy—The most striking macroscopic finding in the macaques was splenomegaly. The cut surface of the enlarged spleens appeared normal in color but had numerous 1- to 2-mm pale white foci suggestive of hyperplasia of the white pulp. The ratio of spleen weight to body weight was significantly greater in inoculated than in control macaques (**Fig 1**). The highest ratio of spleen weight to body weight was evident in macaques that received 10^4 CFUs. One macaque that had been inoculated with 3.34×10^5 organisms had a markedly enlarged testis and epididymis with grossly palpable, firm nodules ranging from 2 to 10 mm in

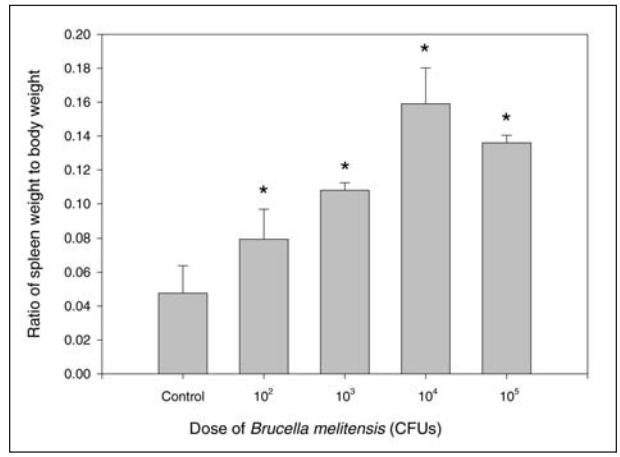

Figure 1—Mean ± SD ratio of spleen weight to body weight in rhesus macaques (*Macaca mulatta*) 63 days after aerosol inoculation with saline (0.9% NaCl) solution (control group) or various doses of *Brucella melitensis*. There were 2 macaques/inoculation group. Notice the increasing ratio of spleen weight to body weight with increasing inoculation dose. *Value differs significantly ($P < 0.05$) from value for the control group. CFUs = Colony-forming units.

Table 1—Bacteremia in rhesus macaques (*Macaca mulatta*) aerosol challenge exposed with saline (0.9% NaCl) solution or various doses of *Brucella melitensis*

Macaque	Inoculation dose (No. of CFUs)	Days after inoculation										
		0	3	7	14	21	28	35	42	49	56	63
1	0	0	0	0	0	0	0	12	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	10^2	0	0	0	0	0	0	0	0	0	0	0
4	10^2	0	0	0	0	0	0	0	0	0	0	0
5	10^3	0	0	0	0	0	< 1	< 1	> 1,000	285	6	1
6	10^3	0	0	0	0	0	< 1	< 1	1	< 1	30	3
7	10^4	0	0	0	0	0	< 1	12	< 1	0	< 1	0
8	10^4	0	0	0	0	0	3	< 11	0	0	0	0
9	10^5	0	0	0	0	1	< 1	< 1	< 1	0	0	0
10	10^5	0	0	0	0	2	3	1	0	0	0	0

Results reported are number of colony-forming units (CFUs) per milliliter of blood. Culture plates were incubated at 37°C for 72 hours, and *Brucella* colonies were identified morphologically. Confirmation of atypical bacterial colonies was accomplished by use of a slide-agglutination test that used anti-*B melitensis* antiserum. Day 0 = Day of aerosol inoculation.

diameter (**Fig 2**). A few of the superficial (axillary, inguinal, submandibular, or superficial cervical), bronchial, and mesenteric lymph nodes appeared slightly enlarged, but this finding was highly variable and did not appear to correlate with the exposure dose. The macroscopic appearance of the remaining organs was unremarkable.

Histologic examination—Aerosol challenge exposure with *Brucella* organisms was associated with signs of disease in several organs (**Table 2**). Lesions were graded on the basis of relative severity from no lesions to severe changes. Histologically, the marginal zone in spleens from inoculated macaques contained increased numbers of lymphohistiocytic cells characterized by moderate to abundant eosinophilic cytoplasm (**Fig 3**). Additionally, the spleen from a macaque inoculated with 10^4 CFUs had a focally extensive area of inflammation that expanded and disrupted the surrounding architecture of the splenic red pulp. This focus of inflammation contained high numbers of histiocytic cells characterized by abundant fibrillar to granular eosinophilic cytoplasm admixed with lesser numbers of lymphocytes. Frequently intermixed with these cells, and slightly more abundant peripherally, were pyknotic nuclei or karyorrhectic debris indicative of cellular necrosis. There were only a few polymorphonuclear cells associated with areas of inflammation. Interestingly, *Brucella* antigen was not detectable in the area of inflammation as determined by immunohistochemical analysis.

Histologically, the liver of inoculated macaques was characterized by hepatitis containing multifocal to coalescing aggregates that consisted predominantly of lymphocytes and histiocytes with a lesser number of neutrophils (**Fig 4**). Histiocytes often contained a moderate to abundant amount of flocculent to granular eosinophilic cytoplasm. These areas of inflammation appeared to be random but were sometimes located periportally and adjacent to central veins such that the inflammatory process partially obstructed the hepatic vasculature. Inflammatory foci varied in size from a few cells to aggregates measuring 150 to 200 µm in diameter. Smaller foci appeared to be located within the hepatic sinusoids, whereas larger foci frequently disrupted the normal hepatic architecture. In some areas, inflammatory cells expanded the sinusoids, entrapping hepatocytes that had a condensed hypereosinophilic cytoplasm with pyknotic nuclei or karyorrhectic debris. Similar-appearing hepatocytes were also located adjacent to the areas of inflammation, sug-

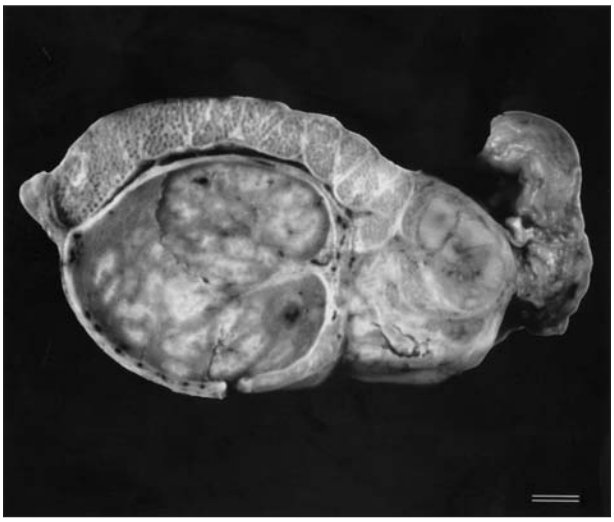

Figure 2—Photograph of the cut surface of a testis and epididymis obtained from a rhesus macaque 63 days after aerosol inoculation with 3.34×10^5 CFUs of *B melitensis*. Notice the multiple nodules of granulomatous inflammation. Bar = 0.7 cm.

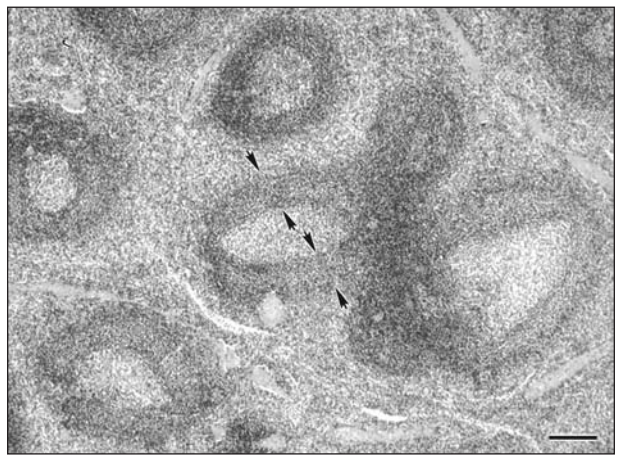

Figure 3—Photomicrograph of a section of spleen obtained from a rhesus macaque 63 days after aerosol inoculation with 1.45×10^5 CFUs of *B melitensis*. Notice there is a diffuse increase in the amount of white pulp marginal zone (region between arrows). H&E stain; bar = 270 µm.

Table 2—Histologic lesions in various tissues obtained from rhesus macaques 9 weeks after aerosol inoculation with saline solution or various doses of *B melitensis*

	Inoculation dose (No. of CFUs)									
Lesions	**0**	**0**	**10^2**	**10^2**	**10^3**	**10^3**	**10^4**	**10^4**	**10^5**	**10^5**
Splenic hyperplasia	0	1	2	2	2	2	2	2	3	2
Hepatitis	0	1	1	2	2	3	3	3	2	2
Nephritis	0	2	0	0	1	1	1	2	1	2
Lymph node hyperplasia	0	0	0	0	2	0	0	0	2	2
Epididymitis	0	0	0	0	2	0	0	0	0	4
Pneumonia	0	0	0	0	3	0	0	0	0	2
Orchitis	0	0	0	0	0	0	0	0	0	4
Splenitis	0	0	0	0	0	0	1	0	0	0

Histologic lesions were scored as follows: 0, no lesions; 1, minimal lesions; 2, mild lesions; 3, moderate lesions; 4, severe lesions reflecting extensive architectural involvement.

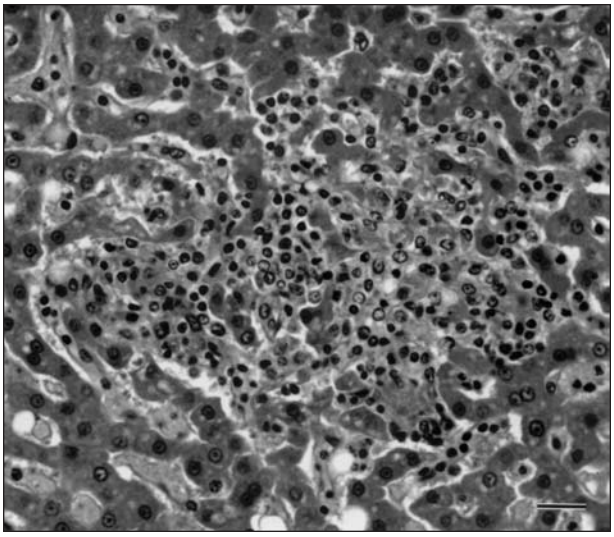

Figure 4—Photomicrograph of a section of liver obtained from a rhesus macaque 63 days after aerosol inoculation with 3.60 X 10^3 CFUs of *B melitensis*. Notice the lymphohistiocytic inflammatory infiltrate expanding the sinusoids mixed with loss of hepatocytes. H&E stain; bar = 60 μm.

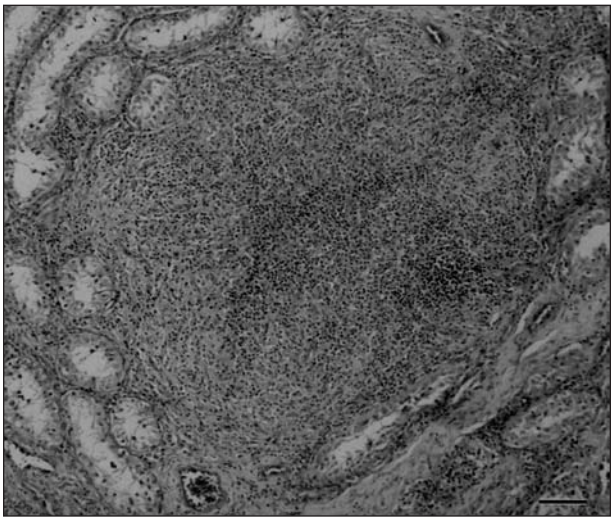

Figure 5—Photomicrograph of a section of the testis in Figure 2. Notice the seminiferous tubules are replaced by a granulomatous inflammatory cell infiltrate. H&E stain; bar = 270 μm.

gestive of active expansion of inflammation and additional entrapment of hepatocytes. Sections of liver also contained low numbers of periportal lymphocytes and plasma cells that often surrounded the bile ducts and blood vessels. The remaining sections of liver were considered to be normal in appearance.

Histologically, the enlarged testis from 1 macaque (10^5 CFUs) had severe granulomatous orchitis (**Fig 5**). Inflammation extended from the fibrous connective tissue tunic throughout the testis, replacing 70% to 80% of the normal architecture. Multifocal to coalescing areas of inflammation surrounded and separated the seminiferous tubules. Central areas of inflammatory foci contained abundant granular to flocculent eosinophilic cellular debris admixed with numerous pyknotic nuclear remnants and karyorrhectic debris. These areas were surrounded by a zone containing predominantly large polygonal cells (epithelioid macrophages) admixed with lymphocytes and neutrophils. Neovascularization was often evident as small, plump, endothelium-lined blood vessels arranged perpendicular to the inflammatory foci and containing few RBCs. The remaining seminiferous tubules within and adjacent to areas of inflammation lacked evidence of spermatocyte production or contained degenerating cells representative of preexisting spermatocyte lineage. The ipsilateral epididymis from that macaque also had histologic evidence of a similar chronic granulomatous inflammatory response within the epididymal intertubular fibrous connective tissue, which separated and surrounded the tubules. Remaining tubules in noninflamed areas lacked evidence of intraluminal maturing spermatocytes and were often filled with abundant amounts of granular to flocculent eosinophilic cellular debris admixed with pyknotic and karyorrhectic cells.

Sections of the kidneys from several macaques had histologic evidence of mild, multifocal nephritis. This inflammation was characterized by moderate numbers of lymphocytes and histiocytes admixed with eosinophilic cellular debris that expanded the interstitium to surround and separate mildly compressed renal tubules. The inflammation was predominantly paravascular, although in some sections of renal tissues, the inflammatory cells were arranged in a paraglomerular location.

Sections of pulmonary tissue from 1 macaque (10^3 CFUs) had mild, multifocal to coalescing interstitial pneumonia characterized by moderate numbers of lymphocytes, plasma cells, and neutrophils admixed with numerous, random multinucleated cells, edema fluid, pyknotic nuclei, or karyorrhectic and cellular debris. Multinucleated giant cells were often distended by intracytoplasmic acicular (lipid) clefts. This inflammation extended into and expanded the border interstitium in a perivascular pattern, mildly disrupting adjacent pulmonary architecture. The macaque with the enlarged testis also had minimal, focally extensive pneumonia characterized by numerous intra-alveolar eosinophils, macrophages, a few neutrophils, a small amount of eosinophilic proteinaceous edema fluid, and cellular debris. Alveolar septae in the area of inflammation were slightly thickened by congestion, minimal edema, and low numbers of intravascular inflammatory cells. The cause of inflammation in both of these macaques was not evident. The lungs of the remaining macaques contained histologic changes: minimally hyperplastic parabronchial lymphoid tissue, mild edema indicated by dilated parabronchial and parabronchiolar lymphatic vessels, and parabronchial accumulation of anthracosilicotic material that was considered to be experimentally unimportant.

Several macaques had evidence of lymphoplasmacytic gastritis associated with silver-stained organisms in gastric glands; these organisms were morphologically consistent with *Helicobacter* sp. Sections of tissues from the remaining organs collected during necropsy were considered to be within normal limits for adult rhesus macaques.

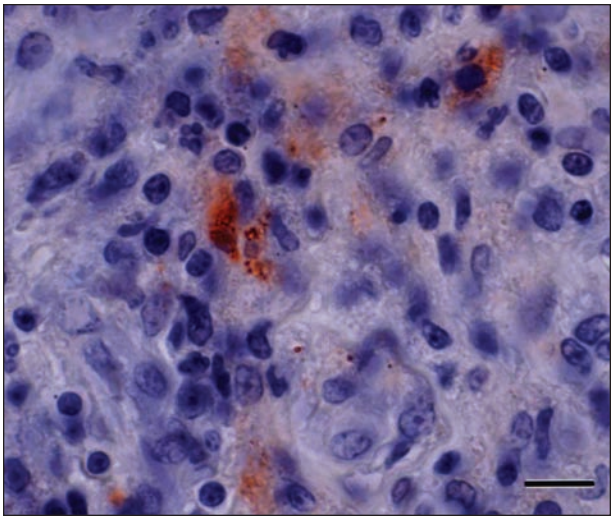

Figure 6—Photomicrograph of a section of the testis in Figure 2. Abundant *Brucella* antigen (red coloration) indicates histiocytic cells in areas of inflammation. AEC chromogen labeling with hematoxylin counterstain; bar = 20 µm.

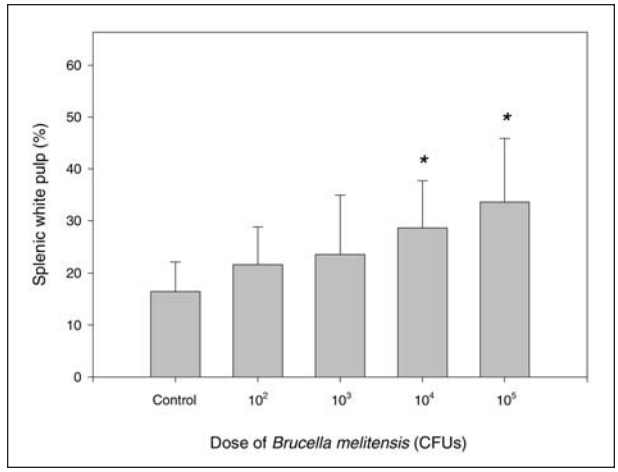

Figure 7—Mean ± SD percentage of white pulp in spleens obtained from rhesus macaques 63 days after aerosol inoculation with saline solution or various doses of *Brucella melitensis*. There were 2 macaques/inoculation group. Notice the increased amount of splenic white pulp with increasing inoculation dose. *See* Figure 1 for key.

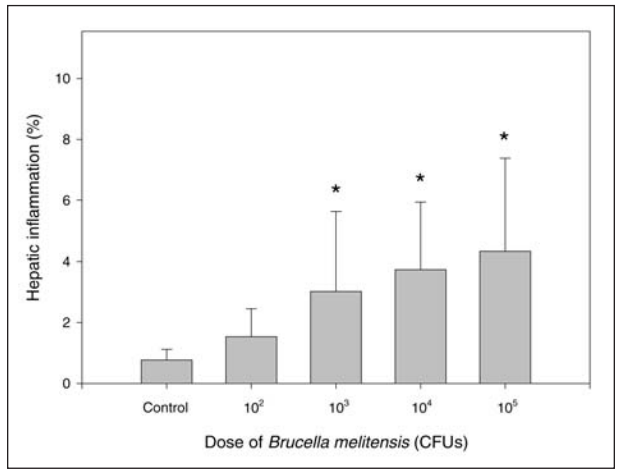

Figure 8—Mean ± SD percentage of hepatic inflammation in livers obtained from rhesus macaques 63 days after aerosol inoculation with saline solution or various doses of *B melitensis*. There were 2 macaques/inoculation group. Notice the increased amount of hepatic inflammation with increasing inoculation dose. *See* Figure 1 for key.

Immunohistochemical analysis—Of the tissues examined, only cells morphologically consistent with phagocytic cells in areas of testicular inflammation contained evidence of *Brucella* antigen (**Fig 6**). These cells contained intracytoplasmic red coloration that varied from small (1 or 2 µm in diameter) foci to a few irregular, slightly larger foci that ranged up to 3 or 4 µm in diameter. *Brucella* antigen was not detected in other tissues by use of immunohistochemical analysis.

Bacterial culture of tissue samples—Culture of tissue samples collected during necropsy revealed viable organisms from at least 1 site in all macaques inoculated with at least 10^3 CFUs (**Table 3**). Viable organisms were also cultured from tissues obtained from a control macaque that was housed in the same room with challenge-exposed macaques (Table 3). The spleen most consistently yielded *Brucella* organisms (5/8 *Brucella*-inoculated macaques), followed by the liver, lungs, and blood samples. *Brucella* organisms were recovered only from the epididymis of the macaque with the enlarged testis and epididymis.

Morphometric analysis indicated that the percentage of white pulp in spleen sections increased with increasing dose of inoculum (**Fig 7**). The percentage increase was significantly greater in macaques inoculated with ≥ 10^4 CFUs. Similarly, the total area of hepatitis increased with increasing dose of inoculum (**Fig 8**). The increase was significantly greater in macaques inoculated with ≥ 10^3 CFUs, compared with the area in the control macaques.

Table 3—Bacterial culture of tissue samples obtained from rhesus macaques 9 weeks after aerosol inoculation with saline solution or various doses of *B melitensis*

	Inoculation dose (No. of CFUs)									
Tissue	**0**	**0**	**10^2**	**10^2**	**10^3**	**10^3**	**10^4**	**10^4**	**10^5**	**10^5**
Spleen	39	0	0	0	9	78	220	350	15	0
Liver	0	0	0	0	16	25	0	6	2	0
Lung	0	0	0	0	10	250	0	16	0	0
Blood	0	0	0	0	1	3	0	0	0	0
Epididymis	0	0	0	0	0	0	0	0	0	> 1,000

Results reported are number of CFUs per gram of tissue or number of CFUs per milliliter of blood. Tissue specimens were collected by use of sterile technique, ground, resuspended in PBS solution, and plated on agar. Blood samples were directly plated on agar. Cultures were incubated for 72 hours, and *Brucella* colonies were identified morphologically.

Discussion

Analysis of the results reported here documents that aerosol exposure of rhesus macaques to 10^2 to 10^5 CFUs of *B melitensis* 16M leads to dose-related hepatic and splenic inflammation with persistence of bacteria in tissues of the mononuclear phagocyte system for at least 9 weeks. Interestingly, although the maximal tissue response in liver and spleen was evident with the highest bacterial inoculum, no brucellae were detected in these tissues at the time of necropsy from 1 of the macaques in this group, and only a few organisms were detected in the other macaque in this group. In contrast, the spleen and liver from macaques that received lower doses of inoculum more often yielded brucellae when cultured after necropsy. In our experience, there is a clear dose relationship between *Brucella* inoculum and anti-*Brucella* antibody titer. In the study reported here, macaques challenge exposed with higher doses of inoculum had antibodies earlier and higher final titers than macaques challenge exposed with fewer organisms. Analysis of these data suggests that higher doses of inoculum may lead to an earlier and more robust immune response that more rapidly eliminates bacteria from the liver and spleen.

The finding that 1 of 2 control macaques not inoculated with *Brucella* organisms but housed in the same room with inoculated macaques and became infected is consistent with our observation that both macaques challenge exposed with the lowest dose of inoculum contracted brucellosis. It is most plausible that infection of the control macaque was via aerosol transmission when inoculated macaques were brought into the common housing room immediately after aerosol exposure. Both control macaques probably were infected, with 1 macaque having positive results for bacterial culture of blood samples and spleen tissues and the other macaque developing antibody titers, indicating infection but to a differing degree. It would be difficult to estimate the dosage for either of the control macaques, although the number of bacteria isolated during bacterial culture was consistent with that seen in macaques inoculated with 1×10^4 CFUs. In our experience, bacterial culture and antibody data are the most reliable information, yet they must be examined in context. We believe it would be difficult to explain how the control macaque could have received 1×10^4 *Brucella* organisms when it required 30 minutes in an aerosolization chamber to deliver a similar amount to inoculated macaques. It is highly probable that the macaque was more susceptible to infection for some unknown reason.

Inflammatory changes were consistently observed in macaques exposed to brucellae, compared with the results for control macaques. These findings suggest that exposure of rhesus macaques to brucellae leads to a multiple-organ disease that principally targets the mononuclear phagocyte system,[17,18] which is similar to brucellosis in humans.

Gross lesions have been described in rhesus macaques orally infected with *B abortus*, *B suis*, and *B melitensis*.[11] In that study, macaques were often inoculated more than once and sometimes with both *B abortus* and *B melitensis*. Additionally, the macaques used were not reportedly certified to be free of background diseases that may have hindered interpretation of the macroscopic findings. Finally, the macaques were euthanatized only after they appeared to be ill. This may have resulted in a failure to identify subtle lesions in target organs at earlier time points. In the study reported here, we believe that the enlarged spleens evident during necropsy were related to the brucellae challenge exposure. Although there was histologic evidence of inflammation in several organs, most changes were not evident macroscopically.

Spleen weight (relative to body weight) increased in inoculated macaques, compared with values for control macaques. The ratio of splenic white pulp to splenic red pulp had a similar increase in inoculated macaques. Splenomegaly has been described as a sequela of *Brucella* infection in rodents.[19] Splenomegaly has been reported[11] in rhesus macaques after inoculation with *B abortus*. Splenomegaly has also been reported[20] in stumptail macaques (*Macaca arctoides*) with experimentally induced *B canis* infection. In another study[16] conducted by our laboratory group, we reported that splenic white pulp-to-red pulp ratios increased in mice challenge exposed intranasally with *B melitensis*. Similar findings in the study reported here require further characterization to ascertain the specific cell populations and subpopulations of the splenic white pulp responsible for the morphologic changes detected. Also, investigation of quantitative changes in red and white pulp during the course of infection from onset to resolution may provide insights on the ability of brucellae to stimulate the host response. Furthermore, the fact that 1 macaque in this study developed granulomatous splenitis in the red pulp is interesting in view of a similar finding in stumptail macaques infected with *B canis*.[20]

Histologic examination revealed that hepatitis increased in severity with increasing *Brucella* inoculum. Hepatic lesions ranging from aggregations of hyperplastic Kupffer cells and intrasinusoidal lymphocytes to granulomatous inflammation were also seen after experimentally induced *B canis* infection in stumptail macaques.[20] The results reported here support this finding in that there was an apparent chronologic variation in lesions evident in the liver. In affected macaques, some hepatic lesions were small and consisted of a few intrasinusoidal lymphocytes in contrast to other areas in which larger aggregates of lymphocytes and a mixture of histiocytes and neutrophils expanded and replaced hepatic architecture. This variation may reflect intermittent hepatic inoculation after periodic bacteremia. We were not surprised to see evidence of hepatitis because the liver is a documented target organ for humans and other animals with brucellosis. Hepatitis clinically manifested as an increase in activity of transaminases is evident in approximately half of the people with brucellosis, although substantial hepatic disease is evident in only a small percentage.[21] More extensive time-course studies for our model could further elucidate this finding and provide clinically useful prognosticators in cases of hepatic involvement during brucellosis.

Finally, we also observed low numbers of paraportal lymphocytes and plasma cells surrounding bile

ducts and blood vessels in sections of liver from control and exposed macaques. This inflammation was considered an experimentally unimportant background change.

Involvement of the reproductive tract in 1 macaque was evident as granulomatous orchitis and epididymitis. It is generally recognized that brucellosis in human males results in localization to the reproductive tract in approximately 2% to 10% of reported cases. *Brucella canis* infection in 4 stumptail macaques resulted in lymphocytic aggregation in the seminal vesicles in 2 animals.[20] Acute endometritis was evident in another stumptail macaque in the same study.[20] Our study did not include female macaques. The reason for localization of brucellae to the male reproductive tract in many animal species, including humans, is not yet known.

To our knowledge, immunohistochemical detection methods have not been applied to rhesus macaques with aerosol-induced *B melitensis* infection. We detected *Brucella* antigen in the macaque with the enlarged testis that contained chronic inflammation, but we did not find it in the other tissues in inoculated macaques. On the basis of our bacterial culture results, it is likely that the number of organisms in other target organs was below the limit of detection for the immunohistochemical technique used. The sections of tissue cultured also differed from those embedded in paraffin and examined histologically; subsequently, variation in detection may have reflected spatial differences of bacterial distribution within organs.

The paucity of pulmonary changes in challenge-exposed macaques in the study reported here was of particular mention because the route of exposure was via aerosol. In another study,[11] investigators reported pneumonia, but the cause was apparently verminous in origin. Although we observed some inflammation in the lungs of several macaques, we believe that the lesions may have represented nonspecific incidental background changes. Inflammatory changes were evident in only 2 macaques and varied qualitatively between the 2 animals. Moreover, we did not observe *Brucella* antigen in areas of inflammation and cultured *Brucella* sp from the lungs of only 1 macaque (inoculated with 10^3 CFUs) with pulmonary inflammation. A study of longer duration after bacterial inoculation may be required to fully elucidate whether pulmonary lesions develop after aerosol challenge exposure to *Brucella* organisms in rhesus macaques. Substantial pulmonic involvement is an uncommon feature of brucellosis in humans.[22] Pulmonary involvement in humans with brucellosis may vary from focal granulomas and pneumonitis to pleural effusion and empyema. In contrast to its rarity as a cause of pulmonary disease in humans, *B abortus* is commonly isolated from bronchopneumonic lungs of aborted bovine fetuses.[23] Pulmonary infection has also been described in bison, elk, and a harbor seal.[23-28] Enumeration of *B abortus* in the lungs of infant mice after experimentally induced infection has also been reported,[29] although detailed pathologic findings in target organs were not described in that study.

Culture of viable brucellae in tissues collected during necropsy revealed a pattern for the organisms to localize to organs rich in reticuloendothelia, most notably the spleen and liver (Table 3). The spleen appeared to have the highest number of organisms. We were surprised to find culturable organisms in the lungs of 3 macaques, 2 of which had no evidence of pulmonic inflammation. It is possible that the organisms isolated from the lungs resided within phagocytic cells without inciting an inflammatory response. However, we cannot exclude the possibility that the positive results for culture of lung tissues reflected bacteremia because blood samples obtained at the time of necropsy from 2 of these 3 macaques also yielded brucellae. The high number of organisms cultured from the enlarged testis of the macaque was consistent with inflammation seen grossly and histologically.

The method of inoculation of rhesus macaques reported here resulted in a number of pathologic features seen in humans with brucellosis, suggesting that it may be useful as a tool for evaluation of candidate vaccines. In addition, this method of inoculation may provide insights into the immunopathologic processes of the host response to aerosol exposure to *Brucella* organisms.

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[a]Model No. CTA-D70, Data Sciences Int, St Paul, Minn.
[b]*Brucella melitensis* 16M, provided by Dr. Gerhardt Schurig, Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Va.
[c]Brucella broth, Difco Laboratories, Detroit, Mich.
[d]Brucella agar, Difco Laboratories, Detroit, Mich.
[e]Telazol, Fort Dodge Laboratories, Fort Dodge, Iowa.
[f]Buxco Biosystem XA, Buxco Electronics, Sharon, Conn.
[g]AGI-30, Ace Glass Inc, Vineland, NJ.
[h]Sepp applicator, Medi-Flex Hospital Products Inc, Overland Park, Kan.
[i]Full draw BD vacutainer SPS tube, Becton-Dickinson, Franklin Lakes, NJ.
[j]Septi-Check blood culture system, Becton-Dickinson, Franklin Lakes, NJ.
[k]Euthasol, Delmarva Laboratories, Midlothian, Va.
[l]Tissue-tek VIP, Miles Scientific, Mishawake, Ind.
[m]Catalogue No. 010944, S. A. Scientific, San Antonio, Tex.
[n]Esco Superfrost Plus slides, Erie Scientific, Portsmouth, NH.
[o]Normal horse serum, Vector Laboratories, Burlingame, Calif.
[p]Antisera used as a source for primary antibody was directed against *Brucella abortus* lipopolysaccharide, provided by Dr. Apurba K. Bhattacharjee, Department of Bacterial Diseases, Walter Reed Army Institute of Research, Silver Springs, Md.
[q]Universal anti-mouse and anti-rabbit IgG (H&L) made in horses, Vector Laboratories, Burlingame, Calif.
[r]Horseradish peroxidase streptavidin, Vector Laboratories, Burlingame, Calif.
[s]AEC substrate, Vector Laboratories, Burlingame, Calif.
[t]Vector hematoxylin, Vector Laboratories, Burlingame, Calif.
[u]Zeiss Axiophot microscope, Carl Zeiss, Oberkochen, Germany.
[v]Model DXC 960 MD, Sony Corp, Tokyo, Japan.
[w]Dimension XPS M200s Pentium, Dell Computer Corp, Round Rock, Tex.
[x]Zeiss image version 3.0, Media Cybernetics, Silver Springs, Md.

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