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Outbreak of *Mycobacterium bovis* in a Conditioned Colony of Rhesus (*Macaca mulatta)* and Cynomolgus (*Macaca fascicularis*) Macaques

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We describe a tuberculosis outbreak caused by *Mycobacterium bovis* in a conditioned colony of rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) macaques. Animals in five rooms were exposed, but most (16/27) infections were confined to the room that housed a mixed population of cynomolgus and rhesus macaques. In this room, rhesus (8/8) and cynomolgus (10/11) macaques naturally exposed to *M. bovis* were infected at nearly identical rates (Fisher exact test, 2-tailed $P = 1$). The clinical signs of disease and pathologic lesions in infected macaques, however, were moderately different between the two species. Rhesus macaques were more likely (5/8) to exhibit clinical signs of persistent coughing and inappetance, and had more severe pulmonary lesions. By contrast, clinical signs of disease were seen in only 1 of 19 cynomolgus macaques, and overall, the pulmonary lesions were often focal and less severe, although some still had severe involvement of the lungs similar to that seen in rhesus macaques. These differences should be taken into consideration when developing or evaluating a tuberculosis-screening program. On the basis of observations made during this outbreak, we recommend that alternative screening methods such as the PRIMAGAM test and the ESAT-6 ELISA, be incorporated into the screening program to aid in the identification of infected animals.

Simian tuberculosis is a devastating bacterial disease caused by two species of aerobic facultative intracellular bacilli, *Mycobacterium tuberculosis* and *M. bovis* (1). Outbreaks of tuberculosis have been documented in nonhuman primate (NHP) research colonies almost as long as primates have been used as experimental models (10, 16). Most tuberculosis infections are caused by *M. tuberculosis* (13, 14); however, there appears to be no difference in the disease manifestation caused by either organism (15, 18). In early experimental colonies, the high incidence of tuberculosis prompted development of a cutaneous test for the detection of tuberculosis (16). Prior to 1960, the incidence of tuberculosis in captive primates remained as high as 33% (10, 13). In the 1970s, federal regulations imposed restrictions on importation of nonhuman primates (NHPs) and mandated quarantine of NHPs by primate importers (6). These restrictions were augmented in the 1980s and early 1990s with guidelines for screening NHPs for tuberculosis (3, 9). As a result of these efforts, the incidence of tuberculosis has been decreased to approximately 0.5% in contemporary colonies (3, 5).

Clinical signs of disease are not a prominent feature of simian tuberculosis. When present, they are often non-specific (e.g., persistent coughing and weight loss [1, 2]). In both species, tuberculosis results principally in thoracic lesions (15, 18, 19, 22), which are characterized by enlarged caseous tracheobronchial lymph nodes, discrete pulmonary granulomas, and granulomatous pneumonia.

The course of the disease may vary depending on the species of macaque. Rhesus macaques develop an acute, progressive, and often fatal form of tuberculosis (17). By contrast, cynomolgus macaques appear to be more resistant or at least able to contain the infection in subclinical form, thus mimicking latent infection in humans (2, 17, 21). Having latently infected animals in a colony may increase the difficulty in detecting tuberculosis. This is more of a problem now than in the past because cynomolgus macaques are now the most commonly imported nonhuman primates (3, 5). Here we describe a naturally acquired outbreak of tuberculosis to highlight species-specific differences in disease manifestation. Companion articles discuss the efficacy of standard and alternative tuberculosis screening methods (8, 12).

Chronologic Case Reports

All of the macaques involved in the outbreak were individually housed in five animal holding rooms (Fig. 1). These animal rooms were located in three separate buildings at Stanford University School of Medicine, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC). All animal use protocols had been reviewed and approved by the Institutional Animal Care and Use Committee in accordance with the *Guide for the Care and Use of Laboratory Animals*.

On September 6, 2001, an adult male rhesus macaque (No.

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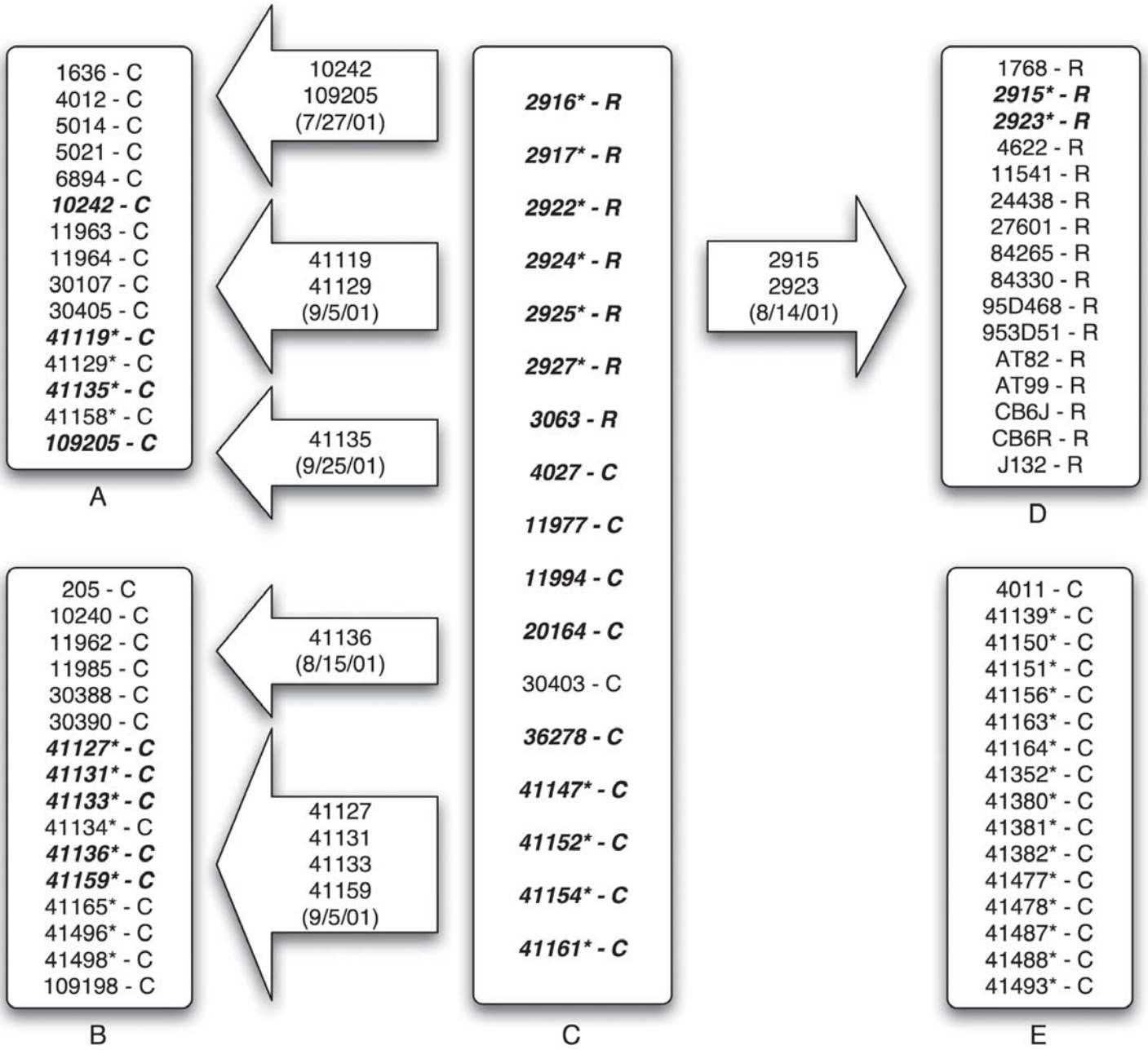

Figure 1. Graphical representation of where infected macaques were housed on October 2, 2001. Animal identification numbers are used to represent the 52 cynomolgus (C) and 28 rhesus (R) macaques that were potentially exposed to *Mycobacterium bovis*. Arrows are used to represent the transfers of macaques between holding rooms (labeled A-E) in the 3 months preceding the outbreak. These arrows contain the animal identification number(s) and date of transfer. Animals from the most recent shipment of cynomolgus and rhesus macaques are identified by a superscript asterisk symbol.

2916) presented with persistent cough and depressed appetite. This animal was part of a shipment of 10 rhesus macaques purchased from a commercial vendor (Primate Products, Miami, Fla.) that had acquired them from a domestic pharmaceutical company. These macaques successfully completed a 31-day quarantine at Stanford University. During quarantine, the macaques were given a tuberculin skin test (TST) in the eyelid with mammalian tuberculin (Lot 415A, Synbiotics Corporation, San Diego, Calif.) every other week as per standard protocols (20). Also during quarantine, blood was collected and the serum was processed to detect antibodies against *Cercopithecine Herpesvirus* 1 (B-virus), simian immunodeficiency virus (SIV), simian T-lymphotropic virus (STLV), and simian retrovirus type D (SRV/D). These macaques were TST negative and virus antibody free and were released from quarantine on April 27, 2001.

A complete physical examination, including blood collection and thoracic radiography, was performed on rhesus 2916 on September 6, 2001. This examination revealed a cough that could be elicited by minimal tracheal palpation, and leukocytosis (16,500 cells/μl) characterized by mature neutrophilia and monocytosis. Tracheobronchitis was tentatively diagnosed, and the animal was treated with enrofloxacin (5 mg/kg of body weight, i.m., q 12 h) for 7 days. The problems (coughing, decreased appetite) resolved following this course of antibiotics and nutritional supplementation.

A follow-up examination on September 14 revealed persistent mild monocytosis (734 cells/µl), with otherwise normal hematologic results.

On September 19, another adult male rhesus macaque (No. 2922) from the same shipment in the same animal holding room (C) was coughing. Abnormalities were not detected on examination, except for moderate monocytosis (1,440 cells/µl). Enrofloxacin therapy was initiated, and the clinical signs of disease resolved. An adult male cynomolgus macaque (No. 11994) was also heard coughing at the time of the examination, but this animal was not treated; its condition was monitored, and a change was not observed.

On September 26, two adult male rhesus macaques (No. 2915 and 2923) in a different room (D) were reported to be coughing. These macaques were from the previously mentioned shipment of rhesus macaques, and had been housed in room C since their release from quarantine (April 27, 2001) and until their transfer (August 14, 2001). This transfer occurred after the quarterly TSTs were administered (August 10, 2001) to the macaques in room D; the TST results for these animals were negative. Room D contained a stable, long-standing colony of 14 rhesus macaques. According to laboratory personnel, the two new macaques began coughing approximately 3 weeks after their relocation.

A complete physical examination of rhesus 2915 and 2923 identified persistent cough but no other relevant abnormalities. We suspected an outbreak of tuberculosis and administered a TST to both coughing monkeys. The 48-h (September 28, 2001) observation revealed marked palpebral swelling and erythema in both animals. These animals were immediately removed from room D and were isolated in a separate room. They were euthanized and given a complete postmortem examination on October 3 and 10.

Between September 28 and October 2, TSTs were administered to 20 rhesus and 58 cynomolgus macaques in five animal holding rooms (A-E) that had been exposed to infected animals. All 78 macaques were quarantined pending successful completion of five consecutive bi-weekly TSTs. Any skin test reactors were immediately removed and euthanized, and the testing proceeded until five consecutive negative bi-weekly TSTs were completed.

The cynomolgus macaques involved in this outbreak were all Mauritius-origin, feral, adult males obtained from the same supplier (Charles River Laboratories, Houston, Tex.). They had an unremarkable quarantine at the primate importer's facility and successfully completed a 31-day quarantine at Stanford University (i.e., they were all TST negative and free of antibodies against simian retroviruses [SIV, STLV, and SRV/D]). These animals were introduced into the conditioned colony in March 1999, February 2000, August 2000, or on August 1, 2001. Many of these macaques were used in experimental investigations of organ transplant immunology and pharmacokinetic evaluations of immunosuppressive drugs. Macaques were periodically transferred between as many as four animal holding rooms (A, B, C, and E) in two buildings (Fig. 1) to maintain an appropriate pool of animals for pharmacokinetic and organ transplantation studies in rooms A and B. Pharmacokinetic studies were short to moderate in duration and rarely exceeded 10 days. Organ transplantation studies rarely exceeded 60 days during this period and were always terminal.

Table 1. Distribution of tuberculosis-associated lesions in infected rhesus and cynomolgus macaques

	Lesion distribution (%)				
Species	Lung	TBNL	Spleen	Liver	Kidney
Rhesus	5/8 (63)	8/8 (100)	5/8 (63)	5/8 (63)	1/8 (13)
Cynos	15/19 (79)	15/19 (79)	11/19 (58)	11/19 (58)	2/19 (11)

TBLN = tracheobronchial lymph node; Cynos = cynomolgus macaques.

In 1994 during a review of our primate health surveillance program by the veterinary staff, an unwritten exclusion from quarterly TST was granted for immune-suppressed animals to avoid potential experimental interference and because of the dubious value in screening these animals for tuberculosis via tests reliant on active immune function. As described at that time, the exclusion would involve few immune-suppressed animals on short-term studies. Over time, however, this exclusion policy was incorrectly interpreted and expanded, and by September 2001, none of the macaques used by this laboratory had been tested for tuberculosis since their release from quarantine. This failure to screen all macaque populations for tuberculosis on a routine basis may have contributed to delays in detecting infected animals. Therefore, we have since retrained the veterinary staff and the research group to ensure that all macaques (even those that may be immune-suppressed) are tested quarterly.

Results

Disease transmission. All rhesus macaques (6/6) and all but one cynomolgus macaque (10/11) in room C were determined to be infected on the basis of the postmortem identification of tuberculosis lesions, thus documenting a nearly identical (Fisher exact test, 2-tailed $P = 1$) attack rate (i.e., number of diseased animals/number of animals at risk at the beginning of the outbreak) between these species. Eleven macaques in rooms A, B, and D also were infected. These animals represented 11 of the 12 macaques transferred from room C between July 27 and September 24, 2001 (Fig. 1). Four cynomolgus macaques also were transferred from room C in the month of June, but none had evidence of tuberculosis on necropsy. Therefore, all infected macaques had been housed in the same room between July and September, and it is here that they were likely infected.

There was no evidence of disease transmission outside of room C. All cynomolgus macaques in rooms A, B, and E that were exposed to tuberculosis-infected animals were euthanized, and only those animals that were relocated from room C between July and September were determined to be infected on postmortem examination. Similarly, in room D, only the two rhesus macaques relocated from room C in August were infected. Of the other 14 exposed rhesus macaques in room D, nine are still alive and have shown no reactivity on either the TST or PRIMAGAM test (10); two have been transferred to another academic institution. The remaining three animals were euthanized at the end of their experimental protocol (June 11, 2002; July 1, 2002; and December 13, 2002); on postmortem examination, lesions associated with tuberculosis could not be identified.

Pathologic examination. Postmortem examination of 11 rhesus macaques was performed by a veterinary pathologist. Eight of these animals were determined to be infected; necropsies were performed between October 3, 2001 and November 19, 2001 (Table 1). Enlarged lymph nodes (tracheobronchial and, in-

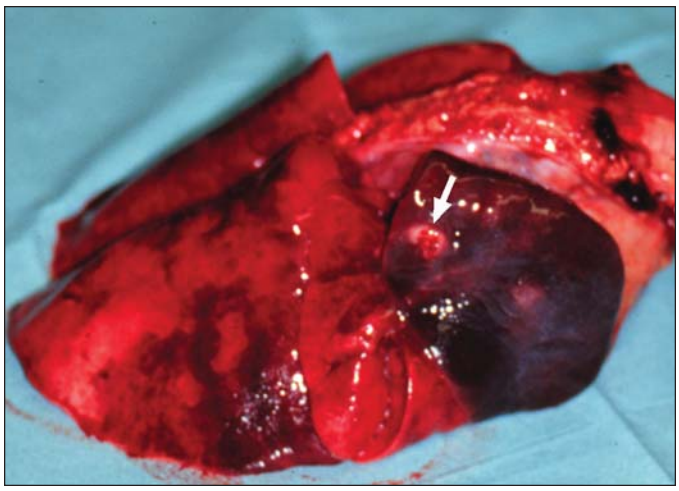

Figure 2. Severe consolidation and hemorrhage typical of the pulmonary lesions seen in rhesus macaques at necropsy. Prominent raised yellow/grey nodules (granulomas) were abundant, particularly in the right cranial lobe (arrow).

frequently, pancreatic lymph nodes), with gray-green, soft, cheesy, somewhat crumbly centers (caseous necrosis) were the most prominent lesions observed. Palpably firm nodules, areas of parenchymal consolidation, and/or gross evidence of bronchopneumonia with airway exudates were typical of the lung lesions (Fig. 2). Splenic, hepatic, or renal miliary nodules (granulomas) were less frequently identified (Table 1). The rhesus macaques with the extrapulmonary lesions were the last of the infected rhesus macaques to be euthanized (starting in late October) and, hence, may have been infected for a longer period.

Microscopically, pulmonary lesions ranged from scattered, discrete, small, organized granulomas, some with central clusters of neutrophils (pyogranulomas), to coalescing areas of caseous necrosis surrounded by granulomatous and lymphoplasmacytic inflammation (Fig. 3A and 3B). In two rhesus macaques, the airways were severely distended (bronchiectasis) and filled with abundant mucus, neutrophils (mucosuppurative exudate), and occasionally, necrotic debris, indicating rupture of the tubercles into the airways (Fig. 3C). In the most severely affected animals, there was extensive pulmonary edema and hemorrhage.

The histoarchitecture of affected lymph nodes was often effaced by large areas of amorphous, eosinophilic, granular caseous necrosis surrounded by dense infiltrates of epithelioid macrophages, multinucleated giant cells (Langhans' giant cells), and fewer fibroblasts, lymphocytes, and plasma cells (Fig. 4A). "Normal" lymph node architecture was barely recognizable in the most severely affected nodes. An acid-fast stain was used to aid in detection of organisms, and although extremely sparse, acid-fast bacilli were found within giant cells in all affected lymph nodes and in most lung lesions (Fig. 4B).

Postmortem examinations were also performed by the same pathologist on 58 cynomolgus macaques. Nineteen of these macaques were diagnosed with tuberculosis; necropsies were performed between October 3, 2001 and December 14, 2001 (Table 1). Caseous granulomas were observed in many infected cynomolgus macaques, and were especially prominent in the tracheobronchial lymph nodes. Nodes in three animals were less severely affected and contained discrete granulomas of various sizes. Fewer epithelioid macrophages and multinucleated giant

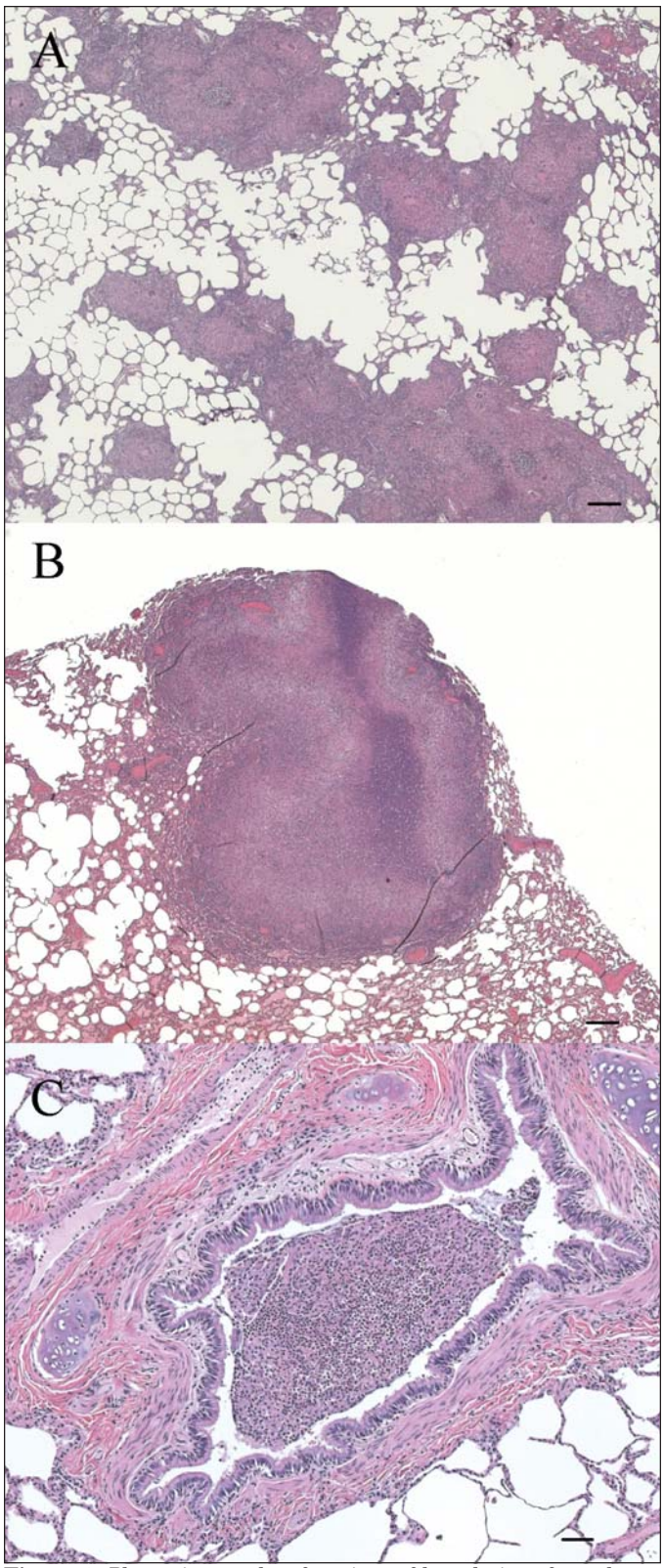

Figure 3. Photomicrographs of sections of lung lesions from rhesus macaque. (A) Numerous discrete granulomas and pyogranulomas seen in some of the affected rhesus (bar = 0.2 mm). (B) Large discrete granuloma consisting of abundant caseous debris surrounded by granulomatous and lymphoplasmacytic inflammation (bar = 0.2 mm). (C) Large airway contains suppurative and granulomatous inflammation admixed with mucus (bar = 0.05 mm). H&E stain.

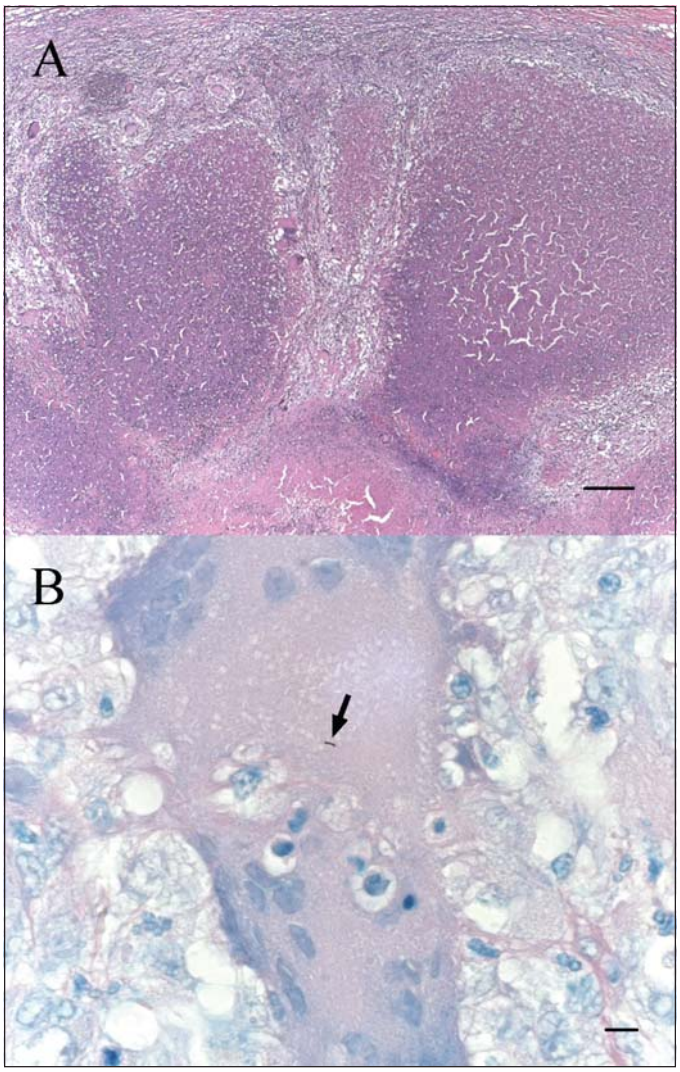

Figure 4. Photomicrographs of sections of a tracheobronchial lymph node from a rhesus macaque. (A) The histoarchitecture has been completely lost due to severe caseation (bar = 0.2 mm). H&E stain. (B) Giant cells of the Langerhans type have multiple nuclei and contain rare acid-fast bacilli (arrow; bar = 5 μm). Acid-fast stain.

cells were generally seen in the lymph nodes of cynomolgus macaques, and acid-fast bacilli were slightly more abundant within central areas of caseation in lymph nodes and lung lesions (Fig. 5).

Palpable pulmonary granulomas also were frequently seen in the cynomolgus macaques. These lesions generally consisted of coalescing caseous nodules surrounded principally by lymphocytes and plasma cells (Fig. 6). Epithelioid macrophages and giant cells were less frequently identified in this species. As with the rhesus macaques, tubercles were seen adjacent to and eroding through large airways in at least four cynomolgus macaques, and pulmonary edema and hemorrhage were present in the six most severely affected animals.

Extrapulmonary lesions in the infected cynomolgus macaques consisted of splenic and hepatic granulomas (11 animals), and renal granulomas (two animals; Fig. 7A). Microscopically, granulomas in the spleen of cynomolgus macaques were similar to lesions in the lungs (Fig. 7B), and acid-fast bacilli were detected in

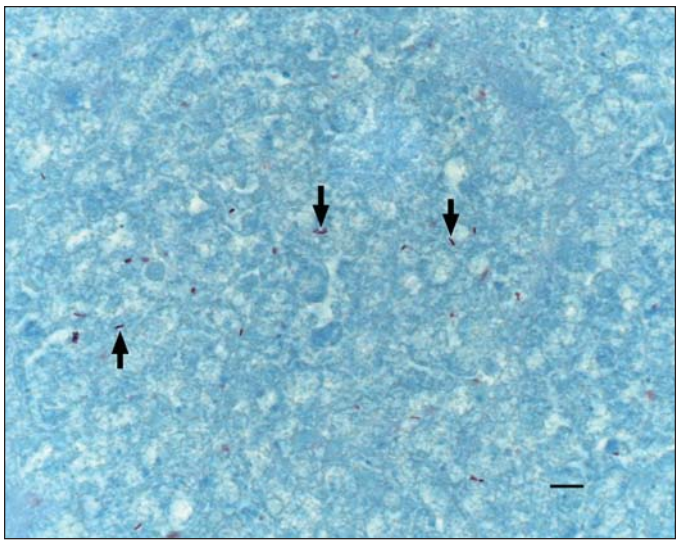

Figure 5. Photomicrograph of sections of lung and lymph nodes of cynomolgus macaques. Notice abundant acid-fast organisms (arrows) within caseous debris (bar = 5 μm). Acid-fast stain.

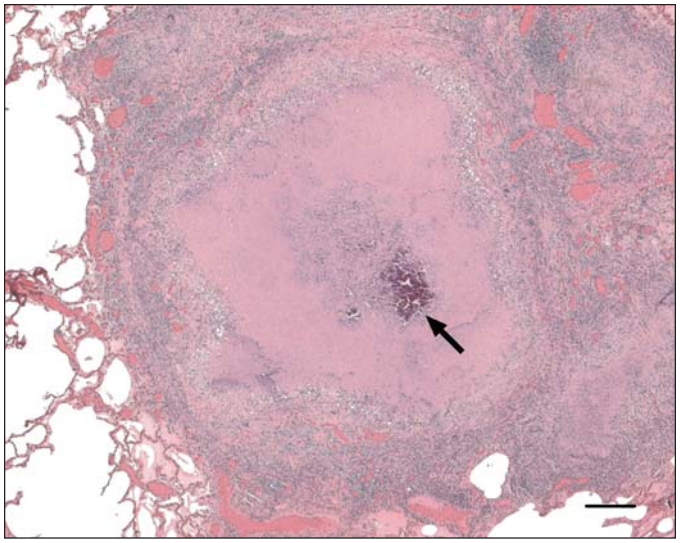

Figure 6. Photomicrograph of a section of a lung lesion from a cynomolgus macaque. Notice coalescing granulomas with a partially mineralized center (arrow; bar = 0.2 mm). H&E stain.

the spleen of three of these animals (not shown). By contrast, acid-fast bacilli were not observed in the tubercles found in the liver or kidneys.

Microbiological examination. *Mycobacterium bovis* was isolated from aseptically obtained fresh or frozen pulmonary tissue or tracheobronchial lymph nodes of five representative animals (three rhesus and two cynomolgus macaques) by personnel at multiple diagnostic laboratories. Positive acid-fast cultures were tested by use of a routine anti-mycobacterial antibiotic sensitivity panel, and were sensitive to isoniazid and ethambutol and resistant to pyrazinamide. Spoligotyping confirmed the identity of the organism as *M. bovis* (11).

Discussion

We have described the clinical manifestations and postmortem findings of a large-scale outbreak of tuberculosis. However,

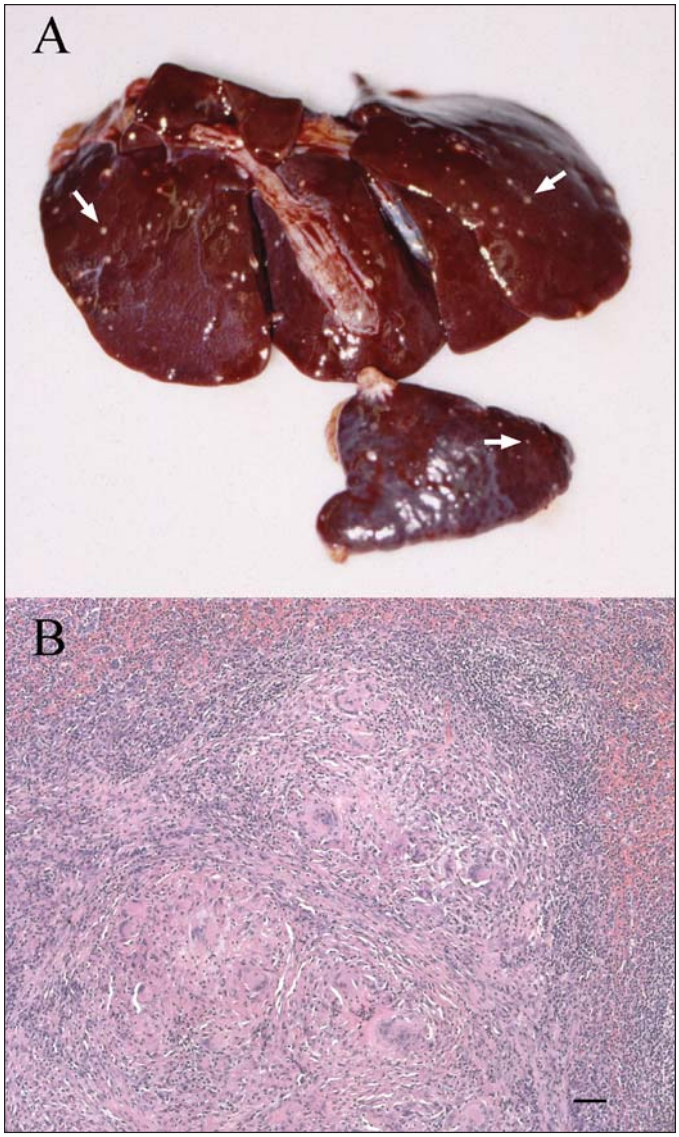

Figure 7. Extrapulmonary lesions in cynomolgus macaques: (A) Miliary disease characterized by 1- to 3-mm-diameter granulomas (arrows) in the liver and spleen of a cynomolgus macaque. (B) Granuloma in the spleen demonstrating the typical histologic features of the peripheral visceral granulomas (bar = 0.05 mm). H&E stain.

our ability to retrospectively identify the source and date of infection and to correlate the lesions observed at necropsy with species-specific differences was constrained by several factors: unknown infective dose, variability in the frequency of tuberculosis testing and clinical examinations, and difficulty in standardizing the reporting of clinical observations of a large quarantined population. A possible source of the outbreak was an animal from the new population of cynomolgus macaques introduced into the conditioned colony on August 1, 2001. Most cynomolgus macaques experimentally infected with low doses of *M. tuberculosis* develop TST reactivity by postinfection week 4 to 6 (2). All of the cynomolgus macaques recently introduced into the colony were TST negative and asymptomatic for tuberculosis during the quarantine period. The first post-quarantine TST was administered to these macaques concurrent with identification of the outbreak, approximately 12 weeks after the last negative quarantine test (July 9, 2001). The results of this post-quarantine TST indicated that 7 of the 32 cynomolgus macaques converted to positive reactors during this interim period. This finding suggests that these macaques became infected soon after their release from quarantine and, consequently, may not have been the source of the outbreak. A longer quarantine period with additional bi-weekly TSTs may have identified any infected individuals in this population. As a result of this outbreak, we have increased the duration of our quarantine period to 60 days, which allows sufficient time to administer five bi-weekly TSTs during quarantine.

An infected animal from the group of 10 rhesus macaques that were introduced into the conditioned colony on April 27, 2001 represented another potential source of this outbreak. When experimentally infected with *M. tuberculosis*, rhesus macaques develop clinical signs of disease (coughing) by postinfection week 6 to 8 (17). We observed clinical signs of disease in a few rhesus macaques as early as the first week of September, which suggests that they became infected after their release from quarantine (April 27, 2001). The infected animals were housed in two rooms (C and D) when the clinical observations were made, but had been housed in the same room a few weeks prior (i.e., before August 14, 2001). All but one of the macaques from room C were determined to be infected, but none of the macaques in room D that were exposed to the two rhesus macaques from room C became infected. We were fortunate that the infection did not spread to other rhesus macaques in room D. To prevent transmission of tuberculosis between macaque populations in the future, we have instituted a policy requiring a recent (approx. 2 weeks) negative TST result to approve an animal transfer.

Many cynomolgus macaques were also transferred between animal holding rooms (A, B, C, and E) during this period, including animals from the recently imported group of cynomolgus macaques (Fig. 1). Of particular interest was the transfer of cynomolgus macaque 3063, which was released from quarantine in August of 2000 and transferred several times between rooms A, B, and C. This macaque was housed in room C from April 27 to May 4 and again from September 21, 2001 until it was euthanized on November 9. It was TST positive on the first round of testing (September 28). More importantly, retrospective analysis of paired serum samples taken during quarantine (July 12, 2000) and necropsy (November 9, 2001) indicated that both samples from this animal had titer to a tuberculosis-specific antigen, 6-kDa early secretory antigenic target (12). Four other cynomolgus macaques also had pre- and post-outbreak titer, but those animals were classified as false-positive reactors because none of them had gross and/or histologic lesions indicative of tuberculosis. Therefore, it is possible that macaque 3063 may have been chronically infected but asymptomatic.

A substantial percentage of cynomolgus macaques experimentally infected intratracheally with low doses (approx. 25 CFU) of *M. tuberculosis* remained healthy and asymptomatic for many months after inoculation (2, 21). Similarly, we were able to identify clinical signs of disease in only one of our infected cynomolgus macaques during the outbreak quarantine period (2 to 3 months). Of particular interest to us was whether an asymptomatic cynomolgus macaque might have been the source of the outbreak. More specifically, did cynomolgus macaque 3063 reactivate a latent infection? This animal did not manifest clinical signs related to tuberculosis during the 15 months that it

was in our colony, and it had received an immune-suppressive treatment (December 27, 2000) and experimental immune-modulatory drug treatments (February 5 and 12, and May 8 and 20, 2001) as part of pharmacokinetic and pharmacodynamic studies. Unfortunately, due to limited data, we have been unable to determine whether this macaque reactivated a latent infection.

Another potential source for the outbreak may have been through contact with an infected human. This appears unlikely because of the occupational health program in effect that requires annual tuberculosis clearance for all personnel entering NHP holding or use areas. In addition public health authorities established a monitoring program at the time of the outbreak, and serial testing and evaluation of NHP-related personnel failed to identify any human disease at the time or in subsequent testing.

Ultimately, the source of this outbreak may never be unequivocally determined. Similarly, the reasons for the species-specific differences in disease manifestations are difficult to define. Results from experimentally infected cynomolgus and rhesus macaques, however, may provide some insight (2, 17). For example, the blood mononuclear cells of rhesus macaques produced nearly twice the amount of interferon-gamma (IFN-γ) as did those of cynomolgus macaques when tested for immune function following vaccination with bacillus Calmette-Guerin (BCG), yet when challenged with *M. tuberculosis*, vaccinated rhesus monkeys were minimally less susceptible to disease than were non-vaccinated animals, and vaccinated cynomolgus macaques were protected when challenged (17). In our naturally infected macaques, rhesus macaques consistently produced higher amounts of IFN-γ (8). Although IFN-γ has been reported to play a protective role in the cellular immune response against tuberculosis infection in mice and humans (4, 7), clearly, factors other than IFN-γ concentration also contributed to the differences in our rhesus and cynomolgus macaques and their overall disease pathogenesis.

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