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Diagnosis of Tuberculosis in Macaques, Using Whole-Blood In Vitro Interferon-Gamma (PRIMAGAM) Testing

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During the fall of 2001, a tuberculosis outbreak caused by *Mycobacterium bovis* occurred in a conditioned colony of rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) macaques at Stanford University School of Medicine. During this outbreak, we evaluated the diagnostic performance of a new in vitro tuberculosis screening test (PRIMAGAM). The PRIMAGAM test measures the interferon-gamma (IFNγ) response to purified protein derivatives (PPDs) of *M. bovis* and *M. avium*. On the basis of the results of the last test administered before necropsy, the PRIMAGAM test had good sensitivity (68%) and excellent specificity (97%), compared with the disease status, as determined by the presence or absence of gross and/or histologic lesions indicative of tuberculosis. By contrast, sensitivity and specificity of the tuberculin skin test (TST) was 84 and 87%, respectively. Both tests suffered from intermittent positive and negative reactions on repeat testing. Overall, however, there was no significant difference ($P = 0.09$, McNemar's χ^2-test) and moderate agreement ($\kappa = 0.52$) between these two tests. Lastly, the IFNγ response to bovine PPD was significantly lower in infected cynomolgus macaques. Moreover, each test failed to detect tuberculosis in three cynomolgus macaques. Fortunately, they were different animals; therefore, we recommend the parallel use of the TST and PRIMAGAM test for maximal overall sensitivity in a tuberculosis screening program, especially for cynomolgus macaques.

Tuberculosis is a major health concern in research primate colonies. The disease is difficult to diagnose, and since shedding of bacteria precedes development of immunologic reactivity (5), an infection may go undetected for weeks or even months. Immunologic reactivity is commonly assessed, using the tuberculin skin test (TST), which detects delayed-type hypersensitivity (DTH) to tuberculin antigens. The concentration of antigen required to detect reactivity is higher in nonhuman primates than in humans (11, 20). However, the exact quantity or number of tuberculin units (TUs) is not defined in the commercially available antigen preparation for nonhuman primate testing. Moreover, as a crude preparation, the antigenic component is poorly defined, and this may result in decreased specificity due to cross reactivity to antigenic components that are shared between pathogenic and non-pathogenic mycobacteria (12, 13, 28). False-negative reactions also are a problem, and can result from incorrect placement or interpretation of skin tests, insufficient antigen concentration (11, 27, 31), anergy secondary to advanced infection (16-18, 33), immunosuppression (9), isoniazid therapy (10), or concurrent infection with immunosuppressive viruses (23).

In vitro lymphocyte stimulation assays have been documented to distinguish between tuberculosis-infected and non-infected rhesus macaques (4, 22), with greater sensitivity than that of the TST (3). A more recent refinement of this lymphocyte stimulation assay is seen in the commercially available PRIMAGAM test. This test kit detects immunologic reactivity to tuberculin antigens through the measurement of interferon-gamma (IFNγ) production in whole blood samples. Interferon-gamma is a critical cytokine in the cell-mediated immune (CMI) response to tuberculin antigens (6, 7). The test offers the advantage of being quantifiable and, therefore, avoids the subjectivity of observer interpretation. However the PRIMAGAM assay has only recently been adapted to work with macaque specimens. Moreover, recent results suggest that the IFNγ response to tuberculin antigens may not be as robust in cynomolgus macaques (15), which may impinge on the diagnostic performance of the test. Therefore, during a recent outbreak of tuberculosis in a conditioned colony of rhesus and cynomolgus macaques, we evaluated the diagnostic performance of the PRIMAGAM test and compared this test with the TST.

Materials and Methods

Non-human primates. Fifty-eight adult male feral cynomolgus macaques (*Macaca fascicularis*) of Mauritius origin, and 21 adult male, and one adult female rhesus macaques (*Macaca mulatta*) were individually housed in three physically separated animal holding facilities at Stanford University, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All macaques were antibody negative for *Cercopithecine Herpesvirus* 1 (B-virus), and simian retroviruses (simian immunodeficiency virus [SIV], simian T-lymphotropic virus [STLV], and simian retrovirus type D [SRV]). These tests were performed during quarantine, and in the case

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of the B-virus, serologic testing was done on an annual basis. Animal use protocols were reviewed and approved by the Institutional Animal Care and Use Committee in accordance with the *Guide for the Care and Use of Laboratory Animals*.

Tuberculin skin test. Intradermal testing, using mammalian tuberculin (Lot 415A, Synbiotics Corporation, San Diego, Calif.), was performed every other week in the eyelid or abdomen as per standard protocols (25). Reactions were measured at 24, 48, and 72 h after tuberculin administration. Intrapalpebral reactions were scored according to the California Primate Research Center/Oregon Primate Research Center grading system (25). Abdominal skin reactions were scored on a 0 to 5 grading system, as described by Staley and co-workers (29). Reactions of grade 0 to 2 were considered negative results, those of grade 3 were suspect, and those graded 4 or 5 were considered positive results.

The PRIMAGAM test. Within 24 h of its collection, heparinized whole blood was processed according to the manufacturer's recommendation. Briefly, 0.5- to 1.0-ml aliquots of whole blood, in 24-well culture plates, were stimulated with two drops of either nil antigen control, purified protein derivatives of *Mycobacterium bovis* (bPPD) and *M. avium* (aPPD), or mitogen control (concanavalin A [ConA]). After overnight incubation at 37°C and 5% CO_2 in a humidified atmosphere, the concentration of IFNγ in the supernatant plasma of each aliquot was determined by enzyme immunoassay. Three methods were used to analyze the IFNγ response: OD_{450} difference between the bPPD- and aPPD-stimulated samples, OD_{450} difference between the bPPD-stimulated sample and nil antigen control, and the OD_{450} ratio between the bPPD-stimulated sample and the ConA control. Receiver operating characteristic (ROC) curves were generated to compare the three methods and to establish threshold cutoffs between infected and non-infected macaques.

Necropsy, and histologic and microbiological examinations. Sixty-nine of the 80 exposed macaques were humanely euthanized by administration of a barbiturate overdose, and were necropsied under appropriate biosafety conditions to prevent contamination of personnel or the environment. Histologic examination was performed on most (17/25) of the macaques with gross lesions, and many (24/44) of the macaques without lesions. Additionally, acid-fast staining was performed on many (18/41) of the specimens obtained for histologic examination. Lastly, lesion-containing tissues from various macaques were frozen or directly processed for bacterial isolation and characterization by use of standard methods.

Analysis of test results. The efficacy of the tuberculosis screening tests was evaluated by calculating sensitivity (Se), specificity (Sp), positive-predictive value (PPV), and negative-predictive value (NPV) (26). A McNemar's χ^2-test was used to probe for difference in the observed proportions between the TST and PRIMAGAM results, and a Kappa test (κ) was used to determine the degree of agreement between the methods. For continuous variables, statistical differences were determined by use of analysis of variance (ANOVA) or paired sample *t*-test. Friedman and Mann-Whitney non-parametric analyses were performed to determine differences between the various observation times and different test sites for the TST, respectively.

Results

Brief description of an outbreak of tuberculosis in a macaque colony. Eighty macaques (22 rhesus and 58 cynomolgus) were involved in an outbreak of tuberculosis at Stanford University. All monkeys had completed an unremarkable quarantine at the non-human primate importer's facility. Additionally, all had successfully completed a 31-day quarantine period at Stanford University, which included a minimum of three negative TST results, and had been in the conditioned colony for at least two months. Infection was initially identified in a pair of coughing rhesus macaques on September 28, 2001 that were strong (grade 4) TST reactors. Animals that had been in contact with the infected rhesus macaques were quarantined pending successful completion of five consecutive TSTs administered at two-week intervals. Positive reactors (rhesus and cynomolgus macaques) were euthanized, and necropsies were performed. Several TST-negative cynomolgus macaques also were euthanized and necropsied.

Necropsy results. Macaques that had gross lesions, such as enlarged tracheobronchial lymph nodes with caseous centers, discrete granulomas in the lungs, and/or miliary foci in abdominal viscera, were considered infected with mycobacterial species. Macaques with microscopic lesions consisting of granulomatous inflammation, caseous necrosis, and abundant multinucleate giant cells, with or without acid-fast bacilli, also were considered infected. This was the standard by which the disease state was determined, and against which the tuberculosis screening tests were compared. Gross and/or histologic lesions indicative of tuberculosis were seen in 27 (eight rhesus and 19 cynomolgus macaques) of the 69 macaques that were necropsied. The infective agent, which was isolated from characteristic lesions of rhesus (n = 3) and cynomolgus (n = 2) macaques, was identified as *M. bovis* at multiple diagnostic laboratories.

Tuberculin skin testing. We compared the TST scores from 10 skin test-positive macaques (three rhesus and seven cynomolgus macaques) at various observation times (24, 48, and 72 h). All tests were administered on the same date, the reactions were scored by the same observer, and all 10 macaques had gross and/or histologic lesions indicative of tuberculosis at necropsy, which was performed shortly (median, 13.5 days; 97.9% confidence interval [CI], seven to 21 days) after the TSTs were administered. The reactivity at the various observation times ranged from grades 2 to 5. There was no significant ($P = 0.325$, Friedman test) difference in the rank assigned to a skin test reaction between the various observation times. Moreover, at any given observation time, there was no significant difference (two-tailed $P > 0.10$, Mann-Whitney test) between rhesus and cynomolgus macaques.

We also compared TST reactivity in 33 macaques (six rhesus and 27 cynomolgus macaques) that received palpebral and abdominal intradermal tuberculin injections at the same time. The same observer scored all tests, and the prevalence of tuberculosis in this population was 49%. There was no significant difference (two-tailed $P = 0.5$, McNemar's χ^2-test) between the two tests, and very good agreement ($\kappa = 0.88$).

Most (20/27) of the infected macaques were identified at the onset of the outbreak; consequently, there was a large decrease in prevalence between the first (39%) and second (13%) test as infected animals were culled from the population. However, there were seven infected cynomolgus macaques that tested negative on at least their first test. Three subsequently had positive results by at least their last test. By contrast, cynomolgus macaques 10242 and 41127 had six negative bi-weekly TST re-

Table 1. Diagnostic performance of the tuberculin skin test (TST)

Cutoff	TST	Necropsy		PV (%)
		Positive	Negative	
Grade 3	Positive	23	6	79
	Negative	4	36	90

The sensitivity and specificity of the TST were 85 and 86%, respectively. The predictive value (PV) of a positive result was 79%; the PV of a negative result was 90%. The disease prevalence was 39%. This analysis was performed on the last TST results before necropsy for a total of 69 macaques. The median time interval between the last TST test and necropsy was 11 days (97.1% confidence interval [CI], nine to 21 days). The cutoff for a positive test result was a grade-3 or higher reaction at 24, 48, or 72 h.

sults before necropsy; cynomologus macaque 41135 had four negative bi-weekly TST results before necropsy; and cynomolgus macaque 41159 had a grade-3 reaction on the second TST followed by two more negative test results before necropsy. These macaques received their last test four (10242, 41135, and 41159) or eight (41127) days before necropsy. Lesions in these falsely negative animals were not obviously different from those in the other infected cynomolgus macaques. Fortunately, these four macaques tested positive on the PRIMAGAM test.

Since this was a spontaneous outbreak of tuberculosis, we could not establish when these macaques were infected, and therefore, some of the initial negative reactions may have been attributable to the macaques being tested too soon after they became infected, or even prior to becoming infected. To minimize this potential bias, we analyzed the diagnostic performance of the last test results before necropsy (Table 1). This analysis indicated that the TST had very good sensitivity (85%) and specificity (86%). To allow direct comparison between the TST and the PRIMAGAM test (see PRIMAGAM results), we eliminated the results of five macaques that were only tested with the TST. The TST sensitivity (84%) and specificity (87%) in this sample population (64 macaques) were essentially unchanged.

A total of six false-positive reactors also were identified; however, because there was usually only one false-positive reactor found on any given test date, the TST specificity was never < 97% on any given test date. All false-positive reactors were cynomolgus macaques. Moreover, these were weak positive reactions (grade 3) that occurred only once on repeat testing, except in macaque 30404. This animal had a strong TST reaction (grade 4) and a positive PRIMAGAM test result on its last test before necropsy; however, gross or histologic lesions were not identified.

The PRIMAGAM test. As expected, the bPPD-stimulated IFNγ response was significantly ($P < 0.0001$) greater than baseline response in the infected macaque population (Fig. 1). The aPPD-stimulated IFNγ response was also significantly ($P < 0.0001$) greater than the baseline response (Fig. 1). However, comparing the two antigens, the bPPD-stimulated IFNγ response was significantly ($P < 0.0001$) greater. Therefore, although cross-reactivity existed between these antigens, infected macaques responded most strongly to the PPD of the infecting mycobacterial species.

According to the manufacturer's recommendation, > 0.05 OD difference in the IFNγ response between the bPPD and aPPD stimulation (bPPD-aPPD) groups and the bPPD and nil antigen control (bPPD-Nil) groups should be considered indicative of tuberculosis infection. Within the infected population, the bPPD-aPPD and bPPD-Nil results were significantly ($P < 0.05$) greater in the rhesus macaques (Fig. 2). Moreover, the IFNγ response was specific to the tuberculin antigen (bPPD), since there was no

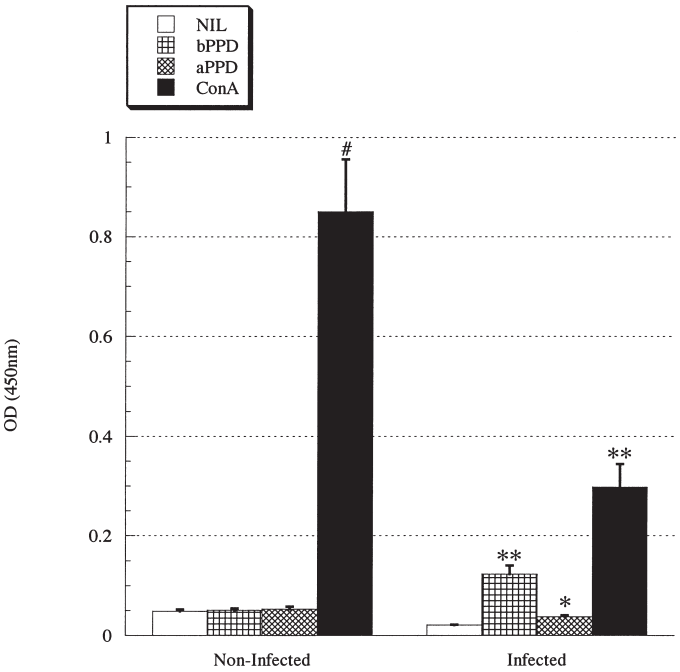

Figure 1. Differences in the interferon-gamma (IFNγ) response between tuberculosis-infected and non-infected macaques. Whole-blood aliquots from 25 tuberculosis-infected and 39 non-infected macaques were stimulated with nil antigen control (NIL), bovine PPD (bPPD), avian PPD (aPPD), and mitogen control (concanavalin A [ConA]). After overnight incubation, the plasma supernatant was separated from each aliquot, and the IFNγ responses were determined by enzyme immunoassay. Mean and SEM of the OD_{450} values from each treatment group are plotted. The IFNγ responses of stimulation groups within the infected macaque population that have a single superscript asterisk symbol are significantly ($P < 0.0001$) higher than baseline (NIL) values. Those with two superscript asterisk symbols also are higher than the aPPD-stimulated IFNγ response. Within the non-infected population, only the ConA stimulation group had significantly ($P < 0.0001$) greater IFNγ response as denoted by the superscript (#) symbol.

significant difference in the IFNγ response between the ConA and aPPD stimulation groups ($P = 0.2447$), or the ConA and nil antigen control ($P = 0.3421$) groups.

Retrospectively, we compared the two methods (bPPD-aPPD and bPPD-Nil) of analyzing the PRIMAGAM results with an alternate method of calculating the IFNγ response on the basis of the ratio between the bPPD and ConA treatment groups (bPPD:ConA). All three methods of analyzing the PRIMAGAM test results were able to discriminate between infected and non-infected macaques. Moreover, from ROC curves, we determined that the bPPD-aPPD and bPPD-Nil methods produced the fewest false reactions at threshold cutoff values of 0.01 and 0.009 OD, respectively.

A total of 223 PRIMAGAM tests were performed on the quarantined macaque population between October 8, 2001 and February 26, 2002. Initially the PRIMAGAM test was selectively used to confirm or refute the diagnostic predictions of the TST. Beginning on November 12, the remaining macaques were screened by use of both tests simultaneously. After December 10, six more rounds of PRIMAGAM testing were performed, but truly positive animals were not identified.

Most infected macaques tested positive on their first PRIMAGAM test. However, we identified 11 cynomolgus macaques that tested negative on at least their first test, but had

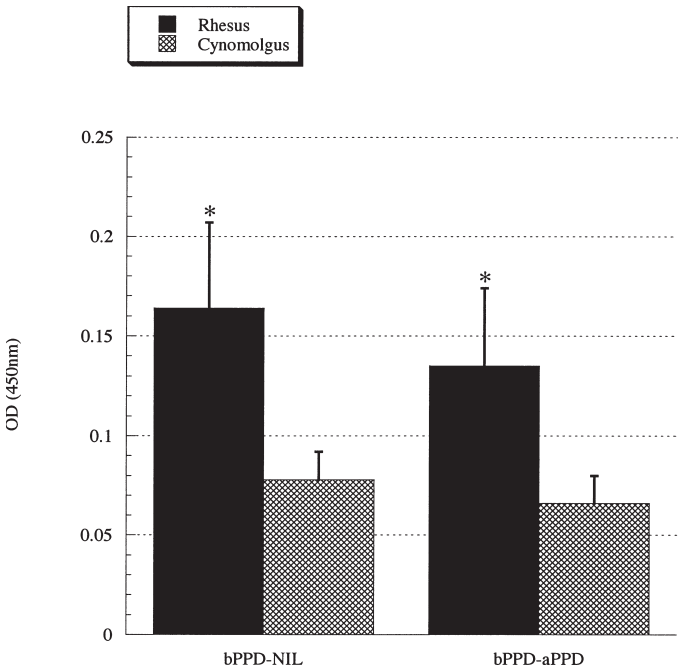

Figure 2. Differences in the IFNγ response between tuberculosis-infected cynomolgus and rhesus macaques. Whole-blood aliquots from 18 tuberculosis-infected cynomolgus macaques and seven tuberculosis-infected rhesus macaques were stimulated with nil antigen control (NIL), bovine PPD (bPPD), avian PPD (aPPD), and mitogen control (ConA). After overnight incubation, the plasma supernatant was separated from each aliquot, and the IFNγ responses were determined by use of enzyme immunoassay. Mean and SEM of the OD (450 nm) difference between the bPPD and aPPD treatment groups (bPPD-aPPD) and the bPPD and NIL treatment groups (bPPD-NIL) are plotted. The asterisk symbols indicate significantly ($P < 0.05$) greater IFNγ response by rhesus macaques.

characteristic tuberculosis lesions at necropsy. Eight of these false-negative reactors were positive on follow-up PRIMAGAM testing. However, three macaques never converted into positive reactors. Macaque 109205 only received one PRIMAGAM test before it was euthanized. This animal was TST positive and PRIMAGAM negative. The other two macaques were negative on two (41152) or three (41161) follow-up PRIMAGAM tests. These animals were TST positive on the date of the first PRIMAGAM test.

Similar to our analysis of the TST, we chose the last test results before necropsy to determine the diagnostic performance of the PRIMAGAM test (Table 2) to minimize any potential bias. The PRIMAGAM test had good sensitivity (68%) and excellent specificity (97%). If we consider intermittent positive results that occurred prior to the last test as sufficient indication of tuberculosis, the apparent sensitivity would be 88%. As expected, decreasing the threshold cutoff (i.e., > 0.01 OD) also increased the sensitivity (88%) (Table 2). Furthermore, there was no significant difference between the PRIMAGAM test and the TST (two-tailed $P = 0.09$, McNemar's χ^2-test) and moderate ($\kappa = 0.52$) or good ($\kappa = 0.64$) agreement, depending on the threshold cutoff (Table 3).

Variable results were seen on repeat testing. Specifically, four infected cynomolgus macaques and two infected rhesus macaques tested positive on one date, then negative on at least one test afterward. We suspected that these animals were infected and that the subsequent negative reactions may have been due to deterioration of the blood sample or immunosuppression. Therefore, we retrospectively determined the difference between the ConA and nil treatment groups as a way of confirming our suspicions. We hypothesized that, if the difference between the ConA and nil treatment groups did not exceed the cut-off threshold of a positive result in the tuberculin antigen treatment group (bPPD-Nil > 0.05 OD), the sample may have been invalid. We identified 21 (9%) of the 223 PRIMAGAM tests in which the difference in the IFNγ response between the ConA and nil treatment groups was equal to or less than 0.05 OD. Only one of the six aforementioned infected macaques was included in this group of potentially invalid results. However, a cynomolgus macaque (109205) that was receiving immunosuppressive treatments as part of a renal transplant protocol was captured under these criteria.

Table 2. Diagnostic performance of the PRIMAGAM test

Cutoff	PRIMAGAM	Necropsy		PV (%)
		Positive	Negative	
0.05 OD	Positive	17	1	94
	Negative	8	38	83
0.01 OD	Positive	22	3	88
	Negative	3	36	92

At the 0.05 OD threshold cut off, the sensitivity (Se) and specificity (Sp) of the PRIMAGAM test were 68 and 97%, respectively. The PV of a positive result was 94%; the PV of a negative result was 83%. Lowering the threshold cutoff for a positive result to 0.01 OD improved the sensitivity (88%) and negative PV (92%). The disease prevalence was 39%. This analysis was performed on the last PRIMAGAM test result before necropsy for a total of 64 macaques. The median time interval between the last PRIMAGAM test and necropsy was four days (96.7% CI, three to nine days).

Table 3. Agreement between TST and PRIMAGAM methods

TST	PRIMAGAM (0.05 OD)		PRIMAGAM (0.01 OD)	
	Negative	Positive	Negative	Positive
Negative	35	3	33	5
Positive	11	15	6	20

Moderate ($\kappa = 0.52$) or good ($\kappa = 0.64$) agreement was recognized between the TST and PRIMAGAM test when using a high (0.05 OD) or low (0.01) threshold cut-off, respectively. There was no significant difference in the observed proportions between the test results. This analysis was performed on the last TST and PRIMAGAM test results before necropsy for a total of 64 macaques.

Discussion

Mycobacterium tuberculosis and *M. bovis* are the principal etiologic agents responsible for tuberculosis in nonhuman primates (14). They are aerobic facultative intracellular bacilli that infect monocytes and macrophages. The outcome of that infection is dependent on the pathogenicity of the bacteria (i.e., its repertoire of virulence genes and products) and on the nature of the host immune response to these and other mycobacterial gene products (24). An effective immune response requires a coordinated interaction between T lymphocytes and macrophages. Specifically, the presentation of mycobacterial antigens by infected macrophages to Th1 lymphocytes results in the production of IFNγ and other cytokines, which activate macrophages and promote their microbicidal activity (6, 7). This CMI response to tuberculin antigens is also the basis of the TST and the PRIMAGAM test.

Since both tests detect a CMI response to tuberculin antigens, it was not unexpected that our results indicated good agreement between the tests (Table 3). Obviously the threshold cutoff value

influenced this agreement; lowering the PRIMAGAM threshold cut-off improved the degree of agreement from moderate (κ = 0.52) to good (κ = 0.64). Similarly, had we set the cutoff for a positive TST at grade-4 reactivity, we would have seen good agreement with the low (κ = 0.66) and high (κ = 0.66) PRIMAGAM cutoffs. This flexibility of interpretation is a major benefit, especially for a quantifiable test such as the PRIMAGAM test. It is important because, in conditions of high disease prevalence, a high threshold cutoff is desirable to minimize false-positive reactions, but in conditions of low prevalence or routine monitoring, a low threshold cutoff is more desirable to minimize false-negative reactions. However, many factors other than modifying the interpretive criteria affect the sensitivity and specificity of these tests; these will be the focus of our discussion.

The TST detects CMI response to tuberculin antigens through the development of a visible DTH reaction in the skin of inoculated animals. Manifestations of this DTH reaction (i.e., erythema and swelling) may vary at different skin sites in the same animal, but typically reach maximal intensity by 24 to 48 h after inoculation (27). By contrast, results of a previous study of experimentally infected cynomolgus macaques that looked at only the 72-h time did not indicate correlation between abdominal skin test reactivity and disease progression (15). In our study we found no significant difference (two-tailed $P = 0.05$, McNemar's χ^2-test) in the DTH reaction between abdominal and palpebral skin test sites in infected and non-infected macaques, and no significant difference ($P = 0.325$, Friedman test) in the rank assigned to the palpebral skin test reaction at the various observation times in infected macaques. Therefore, we used the highest score at 24, 48, or 72 h when interpreting the results of the palpebral TST for optimal sensitivity. Using this criterion we identified three infected cynomolgus macaques and three non-infected cynomolgus macaques (false positive) that were skin test positive at the 24- and/or 48-h time, but not at the 72-h time. However, under circumstance of low disease prevalence this criterion, which is scoring 24-h reactions as positive, would likely increase the number of false-positive reactions.

The PRIMAGAM test is an in vitro assay of the CMI response. It involves use of PPDs of *M. bovis* and *M. avium* to distinguish between infection with *M. tuberculosis* or *M. bovis* and *M. avium* or other environmental mycobacteria on the basis of the principle that animals will mount a stronger immune response to antigens of the infecting mycobacterial species than to those of cross-reactive mycobacterial species. We found this to be true, in that macaques infected with *M. bovis* had significantly greater IFNγ response to bPPD than to aPPD (Fig. 1). It was also of interest to note that, within the infected population, rhesus macaques had a significantly greater response to bPPD and aPPD than cynomolgus macaques (Fig. 2), while maintaining the relative difference in the response between the tuberculin antigens (bPPD > aPPD). It is expected that infection with *M. tuberculosis*, which shares many of the same antigens with *M. bovis*, will likewise result in greater IFNγ response to bPPD over aPPD. In humans, where such comparison has been made, the IFNγ responses to *M. tuberculosis* or *M. bovis* PPDs were not significantly different in individuals with active or latent tuberculosis (30).

The PRIMAGAM test kit contains two stimulation antigens (bPPD and aPPD) and a nil antigen control to discriminate between the IFNγ response of infected and non-infected macaques than cynomolgus macaques . To this kit we added a mitogen control (ConA). In the human IFNγ assay (QuantiFERON) a mitogen control is used to detect immunosuppression or deterioration of the blood sample (8, 30). Unfortunately, since we did not determine the linear detection range for IFNγ in the PRIMAGAM test, we could not objectively set a minimal response to ConA. For example, the Centers for Disease Control and Prevention, in its interim recommendations for using and interpreting QuantiFERON, recommends that results be considered indeterminate if the difference between the mitogen and nil antigen treatments is < 1.5 IFNγ IU (19). Instead, we hypothesized that a failure to respond to the ConA control at a value that exceeded the cutoff threshold of a positive result in the tuberculin antigen treatment group (i.e., bPPD-Nil > 0.05 OD) indicated immunosuppression or deterioration of the blood sample. Using this criterion, we identified 21 (9%) test results in which the difference in the IFNγ response between the ConA and nil control treatment groups was equal to 0.05 OD. Eliminating these results would have only marginally (by 4%) improved the NPV. Therefore, our hypothesis that, if a sample does not respond to ConA, it will not respond to the tuberculin antigen, may not be valid. An alternative method of identifying deteriorated blood samples, or samples from immune suppressed animals remains to be identified.

Antigen concentration will affect the sensitivity of tuberculosis screening tests. For example, a positive DTH response in infected macaques requires between 250 (11) and 2,500 TUs (27). The current TST in macaques uses a dose range between 1,500 and 15,000 TUs (21). The concentration of TUs in the PRIMAGAM test is not defined. Since our results indicate that overall, the sensitivity of the PRIMAGAM test was lower than that of the TST, we naturally wonder whether increasing the concentration of the bPPD will result in any improvement in sensitivity. This question remains to be answered.

A prime benefit of the PRIMAGAM test is its high specificity. This may stem from the use of bovine and avian PPDs to distinguish between an immune response to tuberculosis-causing mycobacteria and an immune response to conserved epitopes of environmental mycobacteria, respectively. In the human and bovine counterparts of the PRIMAGAM (QuantiFERON and BOVIGAM, respectively) tests, the specificity has been increased by replacing the PPDs with recombinant proteins specific for *M. tuberculosis* and *M. bovis*, such as ESAT-6 (1, 2). However, this replacement has not improved the sensitivity of the test, and at least, in the BOVIGAM test, antigen replacement resulted in a small decrease in the sensitivity. Since the overall specificity of the PRIMAGAM test was excellent (only one false-positive result at the 0.05 OD cut-off), we believe that efforts to improve sensitivity would be more worthwhile. In the meantime, using the TST and PRIMAGAM test in parallel should improve the overall sensitivity. This was clearly documented in this outbreak. Similarly, the overall sensitivity in detecting *M. bovis* infection in cattle has been improved by using the BOVIGAM test and TST in parallel (34).

Lastly, there were clear species differences in the TST and PRIMAGAM test results. For example, rhesus macaques may be more prone to indeterminate results, since 29% of the PRIMAGAM results in this species had a ConA-Nil value less than or equal to 0.05 OD. A more important species difference

was that most false-negative responders to the TST and PRIMAGAM test were cynomolgus macaques. Similar variability in TST results have been seen in experimentally infected cynomolgus macaques, leading to the suggestion that this test is not a particularly reliable indicator of infection status (15, 32). These false-negative results may be a consequence of the significantly lower response to tuberculin antigens by cynomolgus macaques (Fig. 2), which may necessitate refinement of the interpretive criteria toward lower threshold cutoffs for this species. As such, the most sensitive PRIMAGAM threshold cutoff for cynomolgus macaques, as determined by ROC analysis, is the same as that previously mentioned (0.01 OD) for rhesus and cynomolgus macaques (data not shown). Therefore, we recommend that, in cynomolgus macaques, PRIMAGAM test results between the 0.01 and 0.05 OD cutoff should be considered suspect. In our experience most (4/6) of these suspect reactors converted to positive reactors (> 0.05 OD) two (2/6) or six (2/6) weeks later.

In conclusion, we found that the PRIMAGAM test had good sensitivity (68%) and excellent specificity (97%). Additionally, it is a quantifiable diagnostic test that allows clinicians to adjust the threshold cutoff depending on the disease prevalence. In that respect, we believe this report will provide the necessary information to permit clinicians to refine the interpretive criteria depending on disease prevalence and species (i.e., cynomolgus versus rhesus macaques). Lastly, we recommend parallel use of the TST and PRIMAGAM test for maximal overall sensitivity in a tuberculosis screening program.

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