Comparative Medicine
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Vol 53, No 2
April 2003
Pages 165-172

Antigen Recognition by Serum Antibodies in Non-human Primates Experimentally Infected with *Mycobacterium tuberculosis*

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Tuberculosis is a significant threat to non-human primates and their caretakers. The diagnosis of tuberculosis in living non-human primates is currently based on the tuberculin skin test, which is cumbersome and sometimes inaccurate. Development of an accurate serodiagnostic test requires identification of the key antigens of *Mycobacterium tuberculosis* involved in antibody production. When sequential serum samples obtained from 17 cynomolgus, rhesus, and African green monkeys up to seven months since experimental infection with *M. tuberculosis* Erdman were screened for antibody against purified proteins of *M. tuberculosis*, three highly seroreactive antigens were identified. One protein, ESAT-6, reacted with sera from all infected animals. Two additional proteins, α-crystallin and MTSA-10, were recognized by sera from approximately 90% of infected animals. Time course analysis of antibody production indicated that the earliest response was usually to ESAT-6 alone or to ESAT-6 and other antigen(s). These results provide experimental evidence of the potential value of ESAT-6 as an antigen for use in serodiagnosis of tuberculosis in non-human primates.

Infectious diseases are a substantial threat to non-human primates (NHP) and to humans who handle or care for them during breeding and use their tissues in experimental studies. Guidelines instituted by the Centers for Diseases Control require that all imported NHP used for research purposes undergo a quarantine period of at least 31 days in the United States. During quarantine, animals are tested to detect diseases that could be transmitted to humans (1-3).

Tuberculosis is one of the most economically devastating diseases of NHP (4). It usually presents itself as a progressive, often fatal disease. Since animals at early stages of infection may not manifest clinical signs of disease, tuberculosis may spread quickly through a colony before it is detected. Diagnosis of tuberculosis in living NHP is based on skin testing by intradermal injection of mammalian old tuberculin (MOT) into the eyelid (5). For release from quarantine, animals must have negative results of least three consecutive tuberculin skin tests performed at two-week intervals (6). Extensive skin testing is also conducted throughout experimental and breeding procedures. Any NHP that tests positive to tuberculin during quarantine is considered to be potentially infected and infectious. A positive tuberculin skin test is followed by euthanasia of the infected animal and by histologic and microbiological investigations at necropsy to confirm tuberculosis. All exposed NHP undergo repeated skin testing until three consecutive negative test results are obtained (3).

Although tuberculosis control measures are based on tuberculin skin test results, it is widely recognized that a negative tuberculin skin test in NHP does not reliably indicate lack of infection. Infected animals may exhibit false-negative test results at early stages of infection as well as at advanced stages of disease (7), or, may be intermittently positive when repeatedly tested (8). Concomitant infections, such as measles (9), or treatment with isoniazide (10) may also cause anergy to the test. To further complicate the interpretation of skin test results, it has been reported that animals sensitized with non-tuberculous mycobacteria (11, 12) or administered Freund's complete adjuvant (13) may test positive to tuberculin. Thus, alternative diagnostic approaches are needed.

Serologic testing constitutes an attractive diagnostic method because it is rapid, easy to perform, and non-invasive. However, despite earlier, encouraging results in experimentally infected rhesus macaques (14, 15), lack of a standard antigen and reports of a limited ability of ELISA-based tests to discriminate between infected and non-infected animals (7) have discouraged use of serologic methods to diagnose tuberculosis in NHP. Use of molecular biology techniques and the availability of the *M. tuberculosis* genome sequence (16) may now promote rational antigen selection and lead to effective serodiagnostic tests for tuberculosis in NHP.

The search for antigens for serodiagnosis of tuberculosis in NHP should benefit from the progress made in human tuberculosis. The antibody response to tuberculosis in humans targets multiple antigens of *M. tuberculosis*, presumably because of genetically determined differences in antigen presentation and of differences in antigen recognition by antibodies at various stages of infection and disease (17-19). As a result, many seroreactive antigens of *M. tuberculosis* have been described (18, 20-22). We used 14 *M. tuberculosis* proteins described as seroreactive in human tuberculosis to characterize the antibody response of NHP during the course of experimental infection

Received: 8/05/02. Revision requested: 10/01/02. Accepted: 12/10/02.
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Supported in part by NIH AI-36989 (MLG).

Table 1. Antigen recognition by serum antibodies of non-human primates experimentally infected with *Mycobacterium tuberculosis*

Antigen	Responders	
	n	%
38-kDa	3	17.6
α-crystallin	15	88.2
ESAT-6*	17	100
19-kDa	3	17.6
MTC28	1	5.9
MPT63	1	5.9
GS	4	23.5
MPT64	3	17.6
MTSA-10	15	88.2
MS	0	0
MPT53	0	0
SOD	13	76.5
Rv 1974	8	47.1
Rv 3871	1	5.9

Results of multi-antigen print immunoassay (MAPIA) experiments are shown. Number (n) and proportion (%) of antibody responders among 17 experimentally infected animals are indicated. SOD = superoxide dismutase; GS = glutamine synthetase; MS = malate synthetase. Rv numbering is according to the *M. tuberculosis* genome nomenclature (16).
*One African Green monkey (95m017) gave a very weak response to the ESAT-6 antigen.

with *M. tuberculosis*. We report that three proteins in the panel—ESAT-6, MTSA-10, and α-crystallin—elicited strong antibody responses in three species of monkeys.

Materials and Methods

Antigens. Fourteen recombinant antigens of *M. tuberculosis* (Table 1) were purified to near-homogeneity from *Escherichia coli* by use of published protocols (23).

Experimental infection. 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*. Animal work was conducted using Biosafety Level-3 operating procedures and policies in a BSL-3 facility with approval of and oversight by the Institutional Environmental Health and Safety Office. Rhesus and African green monkeys were obtained from closed breeding colonies; cynomolgus monkeys were feral of Mauritius origin. Animals were in comprehensive conditioning programs and were immunized against tetanus, measles, hepatitis A, and rabies. The macaques routinely tested negative for B virus, simian immunodeficiency virus (SIV) and simian T-cell leukemia virus 1 (STLV-1) by ELISA and simian retrovirus (SRV) by immunofluorescence. The African green monkeys similarly tested negative for simian agent (SA)-8, SIV, and STLV-1 by ELISA and for SA-11 by immunofluorescence.

The animals were quarantined for eight months after arrival at the BSL-3 facility, and were evaluated extensively for the absence of tuberculosis. Seventeen animals (six cynomolgus, six rhesus, and five African green monkeys) were infected intratracheally with 100 colony-forming units (CFU) of *M. tuberculosis* Erdman. The experiment was terminated at or around day 240. Animals that developed signs of disease (severe coughing, anorexia, or depression) were euthanized earlier. Evaluations included intrapalpebral and intradermal abdominal testing with MOT every other week; thoracic and extrathoracic radiography; bacteriologic culture and polymerase chain reaction (PCR) testing of tracheal wash specimens; evaluation of hematologic and serum biochemical panels and erythrocyte sedimentation rates; investigational ELISA and lymphocyte proliferation assays for tuberculosis; and complete physical examination. Tuberculosis was confirmed at necropsy by histologic examination of major organs, acid-fast or fluorescent staining of infected tissue, culture methods, and bacterial nucleic acid amplification.

Amplification and detection of *M. tuberculosis* ribosomal RNA from infected tissues were carried out by use of a commercial kit (Amplified MTD, Gen-Probe, Inc., San Diego, Calif.). Identification of tuberculous and major non-tuberculous mycobacteria from cultures was performed by use of AccuProbe tests (Gen-Probe, Inc.). Fifty one uninfected cynomolgus monkeys housed at a separate institution were used as a negative-control group. The control animals all had negative results of three consecutive tuberculin skin tests performed at days 1, 13, and 30 during quarantine and at six-month intervals over the next two years.

Sera. Serial serum samples were obtained from experimentally infected animals at times indicated in the Results section. Control sera were taken from 51 uninfected cynomolgus macaques after they were held for quarantine plus two years. All serum samples were stored frozen at –80°C.

Multi-antigen print immunoassay (MAPIA). Serum IgG antibodies against antigens of *M. tuberculosis* were detected, using the MAPIA, a method that involves use of nitrocellulose membranes as a solid phase (24). Purified proteins were immobilized on nitrocellulose membranes (Protran BA83, Schleicher & Schuell, Dassel, Germany) as narrow bands by use of a semi-automatic airbrush-printing device (Linomat IV, CAMAG Scientific, Inc., Wilmington, N.C.). Coating antigen concentrations ranged from 0.05 to 0.5 mg/ml in phosphate-buffered saline (PBS), pH 7.4. The antigen-coated nitrocellulose sheet was cut into 4-mm-wide strips, which were blocked for one hour in PBS plus 0.05% Tween (PBS-T) and 1% non-fat skim milk. Strips were then incubated for one hour with serum samples diluted 1:50 in the same blocking buffer. After washing with PBS-T, strips were incubated for one hour with alkaline phosphatase-labeled anti-human IgG antibody (Sigma Chemical Co., St. Louis, Mo.) diluted 1:2,000 in PBS-T, and washed again with PBS-T. Enzyme activity was visualized by incubating strips for 10 min with 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium phosphatase substrate (Kierkegaard & Perry Laboratories, Gaithersburg, Md.). Strips were rinsed in distilled water to stop the reaction. The entire procedure was performed at room temperature. The presence of a visible band was scored as a positive result. Positive- and negative-control sera were used in all experiments.

Enzyme-linked immunosorbent assay. Four proteins (ESAT-6, α-crystallin, MTSA-10, and 38-kDa protein) were used as coating antigens. Polystyrene microtitration plates were coated with antigen in 0.1*M* carbonate-bicarbonate buffer, pH 9.6, at 4°C overnight. Coating antigen concentrations ranged from 2 to 4 μg/ml. Plates were washed extensively with PBS-T, blocked with PBS-T containing 1% skim milk for two hours at room temperature, then washed again with PBS-T. Serum samples were used at a 1:50 dilution in PBS-T containing 1% skim milk. Serum was incubated at room temperature for one hour. Plates were washed extensively with PBS-T, then were incubated for 30 min at 37°C with rabbit anti-human IgG antibody conjugated to horseradish peroxidase (Dako, Glostrup, Denmark) diluted 1:20,000 in PBS-T containing 1% skim milk. Plates were washed with PBS-T, and horseradish peroxidase activity was assayed by using a TMB (3,3',5,5'-tetramethylbenzidine)

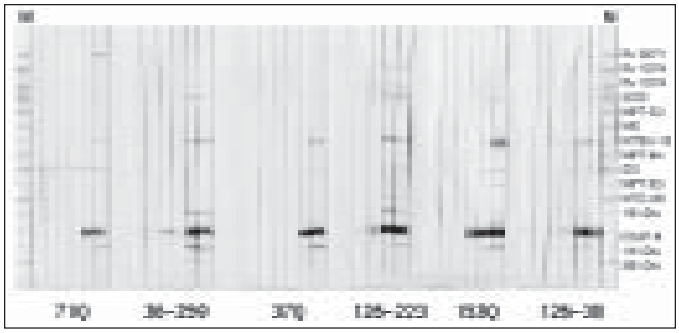

Figure 1. Antigen screening by use of multi-antigen print immunoassay (MAPIA) with sera from NHP experimentally infected with *M. tuberculosis*. Four to six sera from each of 17 infected animals were tested against 14 recombinant *M. tuberculosis* antigens. Results are shown for six animals. Each set of strips corresponds to one animal; animal identifiers are shown at the bottom of each set. For each animal, the leftmost strip was incubated with pre-immune serum (day 0). Names of antigens printed on the membrane are indicated on the right of the figure. SOD = superoxide dismutase; GS = glutamine synthetase; MS = malate synthetase. Strips marked "M" were incubated with a mouse monoclonal antibody directed against the polyhistidine tag of the recombinant proteins.

peroxidase kit (Bio-Rad Laboratories, Hercules, Calif.). Optical density was measured at 450 nm (OD_{450}) in a microtitration plate reader (Spectra Shell, Tecan Systems, Inc., San Jose, Calif.).

Results

Antigen screening. Four to six sera from each infected animal were tested against 14 *M. tuberculosis* purified antigens by use of MAPIA to identify those eliciting an antibody response in infected monkeys (Table 1 and Fig. 1). The highest proportion of responses was to antigens ESAT-6, α-crystallin**,** and MTSA-10 (100, 88, and 88% of infected animals, respectively). Superoxide dismutase (SOD) and Rv1974 also gave a high number of responders; however, the intensity of the response to these two antigens was weak. All remaining antigens were recognized by few, if any sera. The 38-kDa protein, which is the most seroreactive protein antigen in human tuberculosis (25, 26), elicited a weak antibody response in only three of the 17 animals.

The five most seroreactive antigens (ESAT-6, α-crystallin, MTSA-10, Rv1974, SOD) were tested by MAPIA against sera from 51 non-infected control animals. SOD and Rv1974 gave a high background (data not shown), and were not investigated further. In contrast, no reaction was detected with ESAT-6, α-crystallin, and MTSA-10 (data not shown). These results indicate that, as previously observed (27), the extensive skin testing to which all animals were subjected prior to experimental infection (at least 10 tuberculin skin tests per animal) had no detectable effect on the antibody response to the antigens tested.

Time course of the antibody response against selected antigens. We next characterized, by use of ELISA, the time course of the antibody response during experimental infection. Four antigens were chosen on the basis of the antigen screening test results. Three—ESAT-6, α-crystallin**,** and MTSA-10—were selected because they were the most seroreactive antigens. A fourth protein, the 38-kDa antigen, was chosen as a representative of antigens showing low seroreactivity. Data obtained for each animal are presented in Fig. 2. The time course was shortest (less than two months) for African green monkeys (Fig. 2, panel A) because these animals developed rapid, severe disease and were euthanized within two months after infection. Duration of the experiment and, therefore, antibody time course measurements, ranged between 70 and 226 (median, 147) days for rhesus monkeys (Fig. 2, panel B) and between 168 and 237 (median, 227) days for cynomolgus monkeys (Fig. 2, panel C). Control sera from 51 non-infected animals gave low OD_{450} readings (MTSA-10: 0 to 0.06 [range], 0.001 [median]; α-crystallin: 0 to 0.32 [range], 0.006 [median]; and ESAT-6: 0 to 0.54 [range], 0.0015 [median]).

The ELISA data analysis indicated that one animal was antibody negative (African green 95m017; Fig. 2, panel A). The low ELISA readings obtained for this animal with ESAT-6 were consistent with the weak signals observed with the same antigen in the MAPIA (Table 1). The remaining 16 animals strongly reacted to ESAT-6, α-crystallin**,** and/or MTSA-10. As expected, the serologic activity of the 38-kDa antigen was overall poor.

Analysis of the pre-immune sera indicated that three animals had slightly increased antibody levels ($OD_{450} \geq 0.5$) to the antigens tested. African green monkey 95m009 and cynomolgus monkey 66-646 had OD_{450} readings between 0.5 and 1 for anti-α-crystallin antibody, and African green monkey 95m018 had antibody levels to α-crystallin and ESAT-6 corresponding to OD_{450} between 1.0 and 1.5. Review of signs of disease and radiographic and microbiological findings for the three animals did not provide an explanation for these results.

In most animals, the antibody time course was characterized by a sharp rise in antibody levels during the first 30 to 60 days of infection. Animals examined for at least 90 to 120 days tended to stabilize their antibody levels. Further follow-up (120 days or more) was often characterized by a slow decrease of antibody levels. Examples are cynomolgus monkeys 126-30, 126-223, and 126-345. An exception to the aforementioned general pattern was observed in cynomolgus monkey 66-646. Antibodies in this animal peaked twice during the first half of the experiment, then stabilized at different levels for each antigen (high for α-crystallin, intermediate for ESAT-6, and low for MTSA-10).

Time to antibody detection. Early diagnosis is needed to prevent unrecognized, tuberculous NHP from being released from quarantine. Thus, time to antibody detection is an important parameter of assay evaluation. To determine time to antibody detection, we chose for each animal the earliest time point at which serum gave an ELISA reading ≥ 0.5 with at least one antigen. Four animals were excluded from this analysis, one (African green monkey 95m017) because it never mounted an antibody response and three (African green monkeys 95m009 and 95m018, and cynomolgus monkey 66-646) because they were already seropositive at the time of infection. Of the 13 animals analyzed, both African green monkeys (95m008 and 95m019) and one cynomolgus monkey (36-259) were seropositive by day 30 of infection. The four other cynomologus and all six rhesus monkeys tested positive between day 30 and day 60 of infection (Table 2). The earliest serum reactivity was directed to ESAT-6 alone in eight animals and to ESAT-6 plus one or both additional proteins in the remaining five animals.

Intradermal testing. As stated in Materials and Methods, intradermal testing with MOT was performed every other week of infection in the eyelid and on the abdomen. Intrapalpebral reactions were scored according to the 0 to 5 grading system (28). Reactions of grade 0 to 2 were considered negative, those of grade

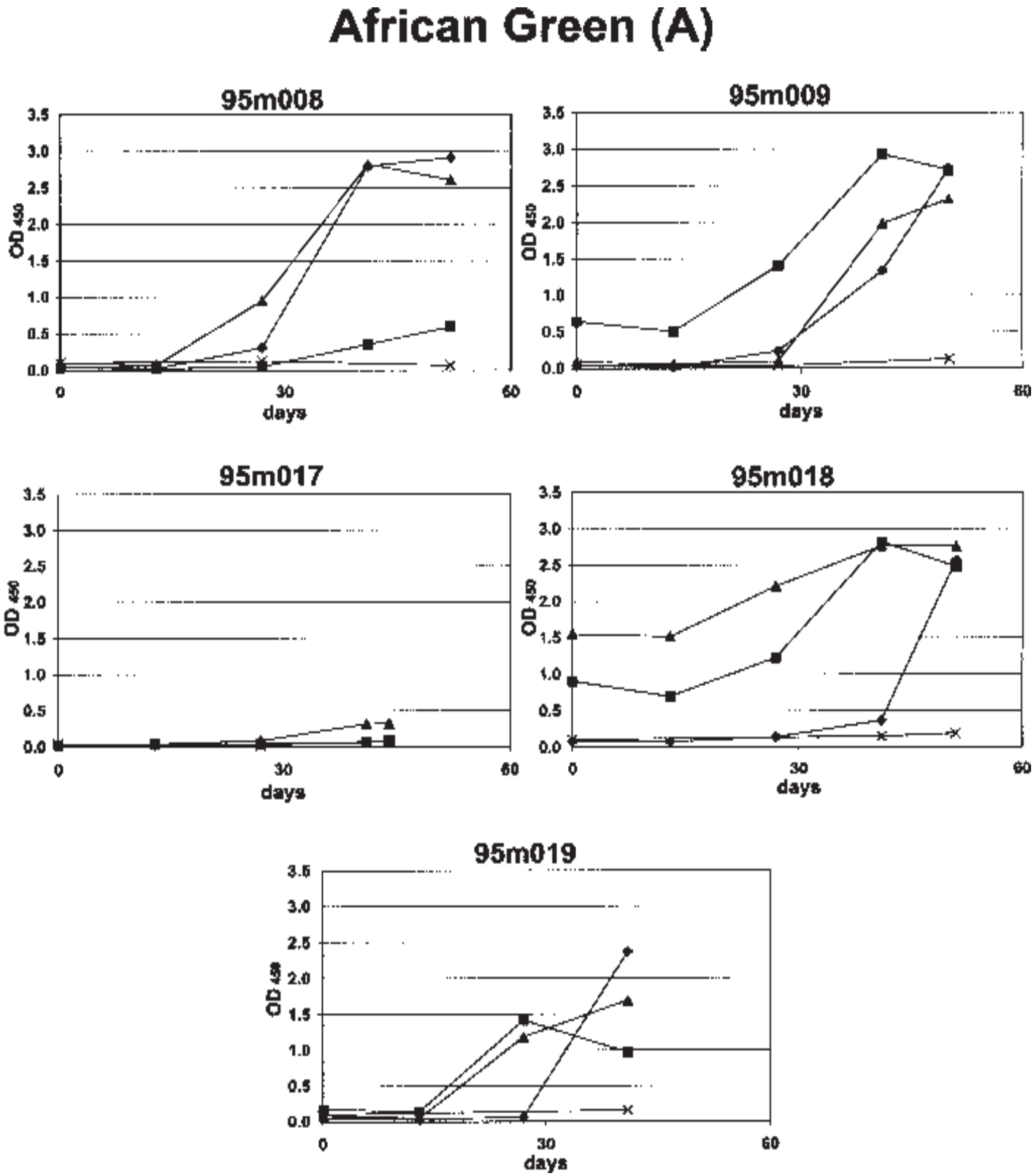

Figure 2. Time course of the antibody response to experimental infection with *M. tuberculosis*. Serial serum samples from 17 experimentally infected NHP were analyzed by ELISA for antibodies against four antigens of *M. tuberculosis* (ESAT-6, a-crystallin, MTSA-10, and the 38-kDa antigen). Each panel represents one species of NHP, as indicated. Each graph represents one animal. Antigens are indicated as follows: ESAT-6 (▲); α-crystallin (■); MTSA-10 (◆); and 38-kDa antigen (✕).

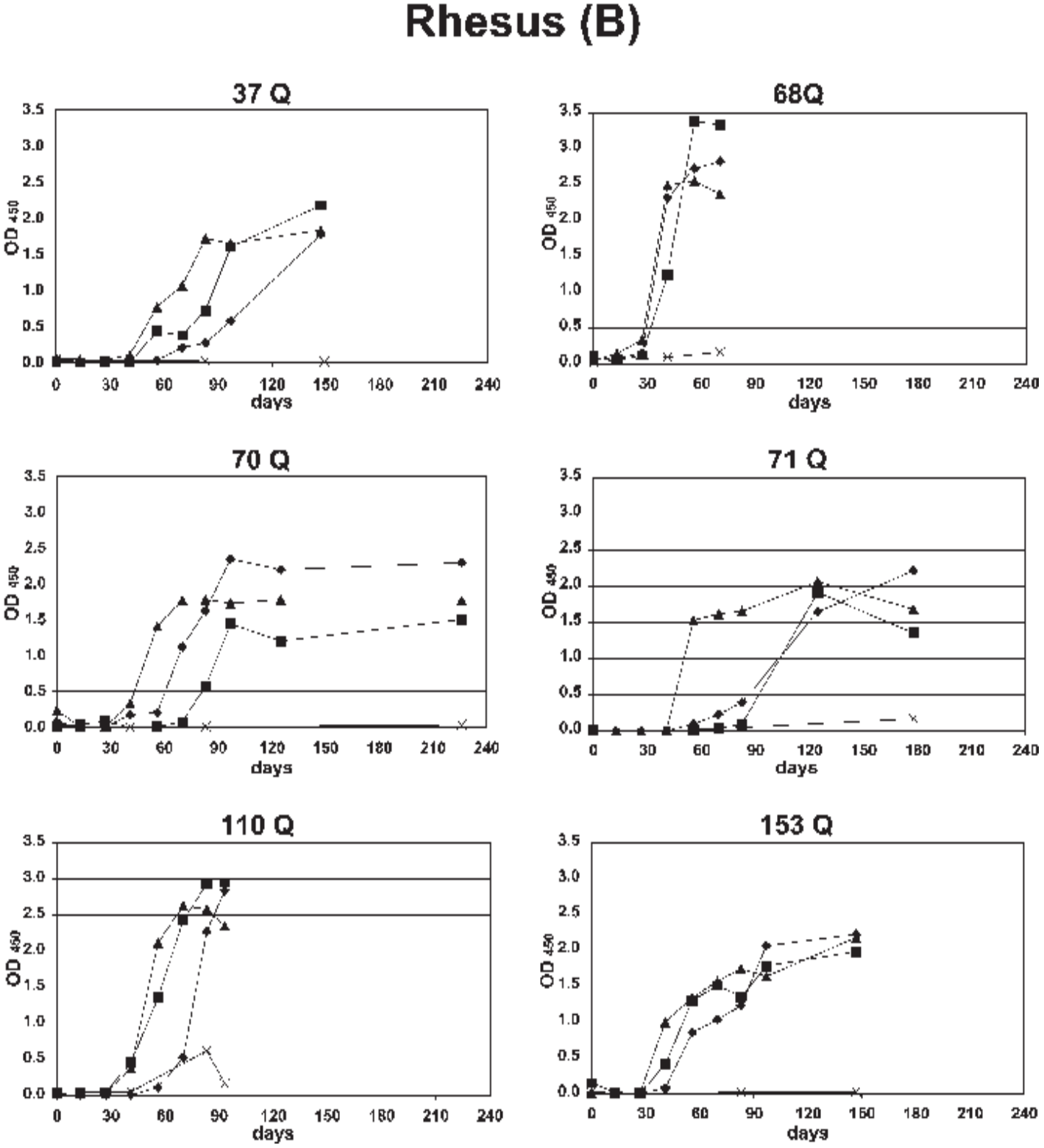

Figure 2. (cont.).

3 were suspect, and those graded 4 or 5 were considered positive. In African green monkeys, results of intrapalpebral testing at week 4 post-infection were positive in three of five animals, suspect in one animal, and negative in one animal. All animals tested negative during week 6 post-infection. In rhesus monkeys, intrapalpebral reactions were positive or suspect between week 4 (6/6 positive) and week 8 (3/6 positive and 2/6 suspect) post-infection. Afterward, intrapalpebral reactions gradually waned, with all animals testing stably negative from week 14 onward.

Even more intermittent were the results of intrapalpebral

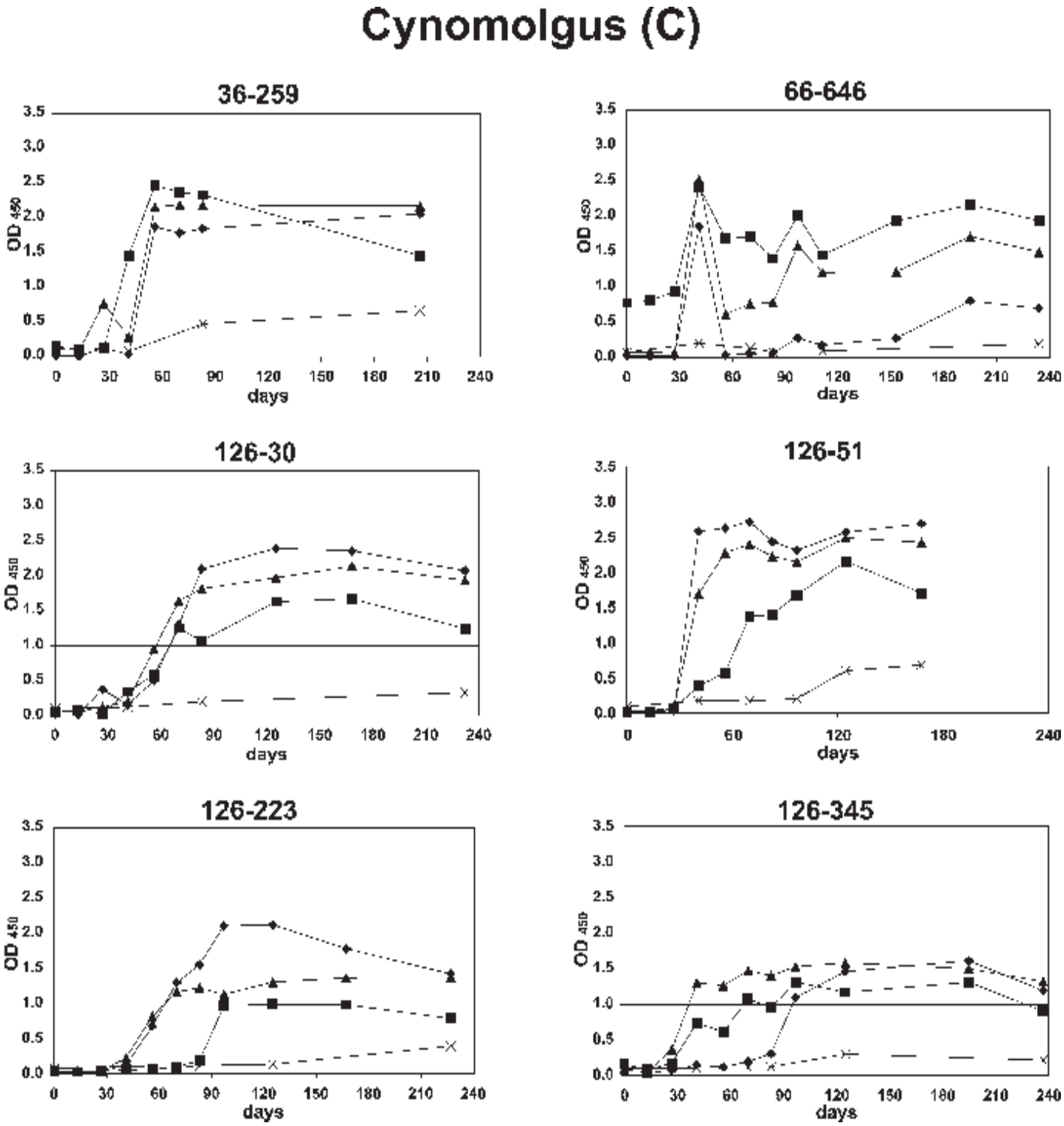

Figure 2. (cont.).

testing in cynomolgus monkeys. These animals tested positive at week 4 (6/6), positive (4/6) or suspect (2/6) at week 8, and positive (3/6), suspect (2/6), or negative (1/6) at week 22 post-infection. Intradermal reactions then waned to stably negative values. In all animal species, abdominal testing was found to be less sensitive than was intrapalpebral testing. Thus, NHP tended to become positive to intrapalpebral testing earlier than they did to the antibody test. However, levels of specific antibodies remained elevated throughout infection, whereas intradermal responses were intermittently positive on repeated testing, as reported (8).

Discussion

To our knowledge, the data reported here constitute the first characterization of the antibody response to a large number of purified *M. tuberculosis* proteins in experimentally infected NHP.

Table 2. Time to antibody detection

Monkey species	Seropositive* by	
	Day 30	Day 60
African green	2/2	NA
Rhesus	0/6	6/6
Cynomolgus	1/5	4/5

*An animal was defined as seropositive when antibody levels to at least antigen were ≥ 0.5 OD_{450} (refer to ELISA data in Fig. 2). NA = not applicable.

We document that: specific antibodies were present in serum between days 30 and 60 of experimental infection; the ESAT-6 protein reacted with sera from all infected NHP; two additional antigens, α-crystallin and MTSA-10, reacted with sera from approximately 90% of infected animals; and ESAT-6 (alone or with other antigens) was the earliest target of the antibody response in most animals. The ESAT-6 and MTSA-10 (also called CFP-10), the products of two adjacent genes of *M. tuberculosis* (29), are found in *M. tuberculosis* and virulent *M. bovis*, but not in the *M. bovis* Bacille Calmette-Guerin (BCG) (30). These genes are present only in few non-tuberculous mycobacteria, such as *M. kansasii*, *M. gastri* and *M. marinum*, as determined by Southern transfer and high-stringency hybridization (31, 32). They are notably absent from *M. avium*, the most common non-tuberculous mycobacterial species in the environment (33). This specificity for virulent tubercle bacilli makes ESAT-6 and MTSA-10 ideal candidates for development of immunodiagnostics for tuberculosis.

Our results are in agreement with current knowledge on the time course of antigen recognition during tuberculosis. A growing body of evidence indicates that ESAT-6 is a target of T-cell immune responses early in infection of cattle with *M. bovis* (34) and at subclinical stages of human tuberculosis (35, 36). The ESAT-6 protein is also one of the antigens most frequently recognized by serum antibodies at early stages of experimental tuberculosis in cattle (37). Since ESAT-6 and MTSA-10/CFP-10 are produced from the same transcript (29), it seems reasonable to assume that immune recognition of these two antigens should have a similar temporal pattern. The observed increase of levels of antibodies to α-crystallin in experimentally infected NHP is consistent with reports that antibodies to this protein [which is also called 16- or 14-kDa antigen] become detectable in humans during primary tuberculosis and among contacts of active tuberculosis cases (20). The poor serologic recognition of the 38-kDa protein observed at early times of infection in NHP is consistent with the widely accepted notion that the serologic response to the 38-kDa antigen in humans tends to correlate with advanced pulmonary tuberculosis (20).

Several questions remain open. One is whether it is possible to shorten the time to antibody detection. In the study reported here, antibodies were detected between day 30 and day 60 following intratracheal infection of NHP with 100 CFU of *M. tuberculosis*. Since time to antibody detection in natural infection may be longer due to a lower infecting dose, it is now necessary to monitor the course of the antibody response to *M. tuberculosis* antigens in animals exposed, by design or by accident, to tuberculous NHP. To decrease time to detection, it may become necessary to search for antigens eliciting antibody responses earlier than ESAT-6.

Another question is whether the antibody repertoire reflects the outcome of infection. As with humans, NHP develop forms of tuberculosis ranging from subclinical infection to fulminant disease (8). The antigenic make-up of tubercle bacilli varies with growth phase in the host (mouse) lung (L. Shi and M.L. Gennaro, in preparation). Consequently, it is expected that different antibody patterns be generated depending on whether *M. tuberculosis* growth is controlled by an effective host immune response (i.e., during subclinical, or latent, infection), or whether tubercle bacilli are allowed to actively multiply (active disease). The cynomolgus monkey model of tuberculosis recapitulates the spectrum of infection outcomes in NHP (8), making it possible to identify the antigens that are predominantly recognized by serum antibodies at each of the possible phases of *M. tuberculosis* infection. These antigens are expected to be appropriate reagents for diagnosis of NHP tuberculosis in its diverse presentations.

In conclusion, our analysis of humoral immunity in NHP experimentally infected with *M. tuberculosis* identified ESAT-6 as an *M. tuberculosis*-specific, "early" antigen that can be used for serodiagnosis of tuberculosis in NHP and possibly in other diagnostic immunoassays. The complex pathogenesis of tuberculosis warrants further research into antigen specificity of the antibody response to define the appropriate reagents for early diagnosis of NHP tuberculosis.

Acknowledgments

We wish to thank Ganga V. Kanaujia for assisting with some ELISA experiments; Cynthia West for technical assistance; Eddie Beck for assistance in figure preparation; and Karl Drlica and Sam Kayman for critical reading of the manuscript.

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