Mycobacterium tuberculosis ESAT-6 antigen immunogenicity in Owl Monkeys

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Abstract

Several ESAT-6-based vaccines have been widely studied in different animal models, presenting potent ability to induce both cellular and humoral responses. As ESAT-6 is a well-characterized mycobacterial antigen, its capacity to induce immune responses in a nonhuman primate model has been evaluated. The immunization of recombinant ESAT-6 (rESAT-6) in *Aotus nancymaae* monkeys has elicited a strong cellular response, not just to rESAT-6, but also to the native protein present in *Mycobacterium tuberculosis* culture filtrate proteins measured as [^{3}H]thymidine incorporation in lymphocyte proliferation assays. High humoral response was also observed, having antibody titers of 1:12,800 directed towards rESAT-6. The protein's multi-epitope nature was further demonstrated since several peptide sequences were specifically recognized at both cellular and humoral level. The high immunogenicity observed, as well as the relatively high characterization of the *Aotus'* immune system at molecular level, are two advantage to propose *Aotus nancymaae* as animal model for studying *M. tuberculosis* infection; however, our results reveal an animal-to-animal variation in response to vaccination, this could be a disadvantage.

Keywords: Owl monkey, animal model, *Mycobacterium tuberculosis* ESAT-6, immune response.

Resumen

La única vacuna disponible contra la tuberculosis es la cepa *Mycobacterium bovis* BCG, que ofrece una eficacia protectiva variable (0%-80%), siendo urgente un nuevo agente profiláctico. Se han evaluado diversos candidatos a vacuna contra este patógeno, en los modelos animales de experimentación convencionales (murino, cobayo, conejo), obteniéndose información básica sobre el efecto de la vacuna en la carga bacterial frente a un reto infeccioso, así como también la reducción o prevención de la patología en los pulmones u otros órganos blanco; además de los aspectos relacionados con la respuesta inmune hacia el *Mycobacterium tuberculosis*. Los primates no humanos tienen ventajas sobre los modelos convencionales en la evaluación de vacunas, de hecho se ha verificado el comportamiento de agentes terapéuticos en humanos después de haber sido medida la capacidad protectiva de éstos en monos con tuberculosis inducida. Los primates mas estudiados en la infección por micobacterias son el cynomulgus, y el rhesus, observándose que estos animales mantienen la infección en un estado subclínico, muy similar a la tuberculosis humana donde el 90% de la población infectada mantiene la infección en un estado latente. Dado que el modelo animal debe semejar el

comportamiento de las proteínas estudiadas en el ser humano, el mono *Aotus* puede representar ventajas en la investigación de tuberculosis por ser un primate con aproximadamente un 90% de similitud al humano en cuanto a las moléculas del sistema inmune estudiadas hasta hoy. La proteína ESAT-6 de (early secretory antigenic target 6 kD) de *Mycobacterium tuberculosis* es un componente minoritario del filtrado de cultivo de corto tiempo (CFP), ha sido genética y químicamente caracterizada e induce una potente respuesta inmunogénica del tipo TH1. Este antígeno es secretado durante la fase inicial de crecimiento siendo fuertemente reconocido por animales y humanos infectados por *Mycobacterium tuberculosis*, este hecho hace que su inclusión en una futura vacuna anti-tuberculosis por subunidades y en pruebas de inmunodiagnóstico. En este trabajo se estudió la respuesta inmune del mono *Aotus* frente al antígeno micobacteriano ESAT-6 en forma recombinante (rESAT-6). El antígeno fue inmunizado junto con el adyuvante Montanide 720 produciendo una fuerte respuesta humoral y celular, no solo hacia rESAT-6 sino también hacia la proteína nativa presente en el filtrado de medio de cultivo de *Mycobacterium tuberculosis.* La respuesta celular fue medida por la incorporación de [^{3}H]timidina en ensayos de linfoproliferación. La respuesta humoral se analizó por ensayos de ELISA e inmunoblot, obteniéndose altos títulos de anticuerpos (hasta de 1:12,800) dirigidos rESAT-6. Se demostró la naturaleza multiepitope de este antígeno ya que en general péptidos que mapean la proteína fueron específicamente reconocidos tanto a nivel celular como humoral. Se observó además variación en la respuesta a la vacunación entre animal y animal, siendo una desventaja para un modelo de experimentación en cuanto a la predicción de la respuesta. Este estudio se constituye en un primer paso de evaluación del mono *Aotus* como modelo animal en tuberculosis.

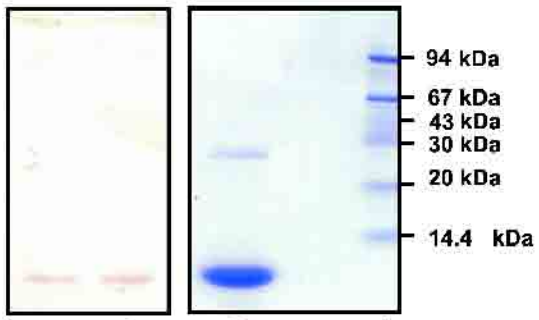
Palabras claves: Mono *Aotus*, modelo animal, *Mycobacterium tuberculosis* ESAT-6, respuesta inmune.

Introduction

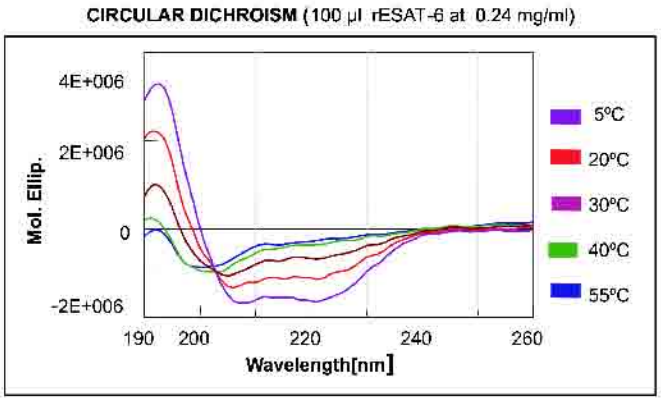
The only available anti-tuberculosis vaccine is based on the *Mycobacterium bovis* BCG strain giving variable protective efficacy (0%-80%), making developing a new prophylactic agent an urgent priority (1-3). Great advances have been made in knowledge regarding *Mycobacterium tuberculosis*, including deciphering the genome's sequence (4), bioinformatics analysis *in silico*, proteomic studies directed towards characterizing and determining mycobacterial protein function and immunogenicity (5-8), the mycobacterium's cell wall structure and morphology (9,10) as well as the role of the main cellular populations, cytokines and chemokines involved in *Mycobacterium tuberculosis* infection (11,12).

Possible vaccine candidates against this pathogen have been developed having diverse characteristics such as recombinant vaccines, auxotroph vaccines and DNA vaccines (13-19). Orme I. *et. al* have reported that about 50% of the 170 candidates or combinations of candidates which have been tested fall into the "subunit vaccine category", reflecting increasing knowledge regarding protein make-up of the bacillus and increased access to techniques helping identify immunogenic proteins (20). These candidate vaccines have been especially evaluated in mouse, guinea-pig and rabbit models which have provided basic information about the vaccine's effect on bacterial load regarding infectious challenge and reducing or preventing pathology in the lungs and other target organs as well as some aspects related to the immune response to *Mycobacterium tuberculosis* (21-25).

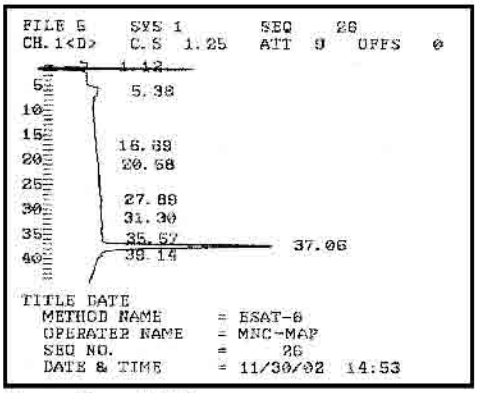
Nonhuman primates appear to have significant advantages over conventional laboratory animals in terms of modelling pulmonary tuberculosis for purposes of vaccine evaluation (26). The usefulness of tuberculosis induced in monkeys for developing different therapeutic agents has been verified when predicting these drugs' behaviour in humans (27,28). Non-human primate studies have demonstrated that these animals



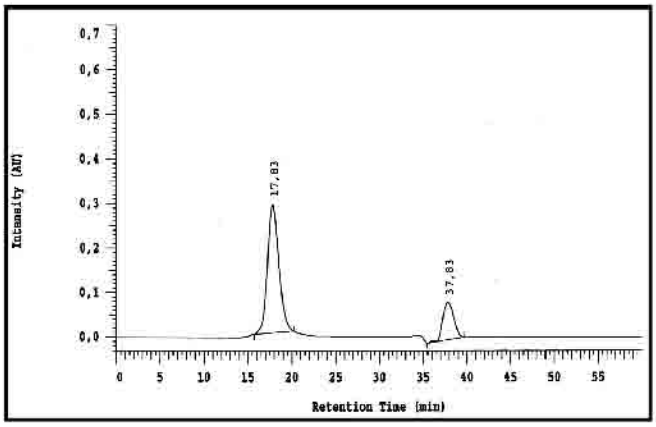
1. Strip immunoblot of induced r-ESAT-6-DH5α-pQE30
2. Strip immunoblot of purified fraction (rESAT-6)
3. Purified rESAT-6
4. MWM



A.

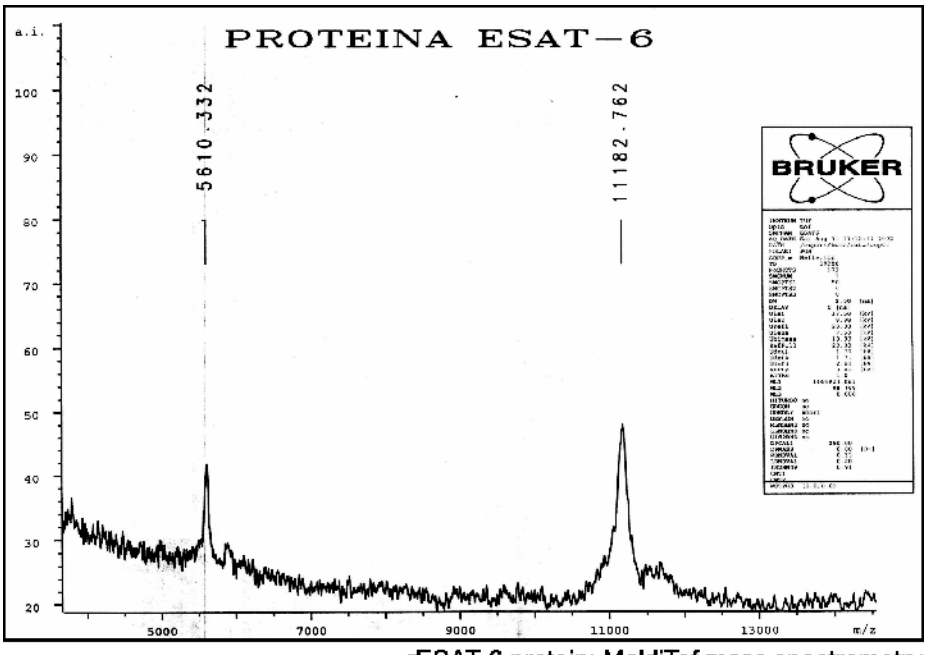


Reverse Phase–HPLC
Column: C-18 (Merck)
Injected: 50 µlml of rESAT-6 (0.24 mg/ml)
Program: 0-70% ACN in 45 min.
Flow: 1 ml/min Detection: 210 nm UV



Size Exclusion Chromatography
Column: Superdex 10/30 Peptide.
Injected: 100 µl of rESAT-6 (0.24 mg/ml)
Flow: 0.5 ml/min in ACN 20% Detection: 210 nm UV

B.



rESAT-6 protein: MaldiTof mass spectrometry

Figure 1. rESAT-6 characterizing. (A) Clonation, expression and purification of rESAT-6: gel electrophoresis, immunoblot, HPLC chromatography and circular dichroism. (B) *Maldi-Tof mass spectrometry.*

Table I. ESAT-6 synthetic peptide sequences

Peptide*	Amino-acid start position	Sequence	Amino-acid final position
12033	1	MTEQQWNFAGIEAAASAIQG	20
12034	21	NVTSIHSLLDEGKQSLTKLA	40
12035	41	AAWGGSGSEAYQGVQQKWDA	60
12036	61	TATELNNALQNLARTISEAG	80
12037	76	ISEAGQAMASTEGNVTGMFA	95

*FIDIC peptide number

are susceptible to tuberculosis when challenged (29,30).

According to reports, a significant percentage of *Macaca fasicularis* (cynomolgus monkeys) challenged with low *M. tuberculosis* doses were able to maintain the infection in a sub-clinical state, very similar to human tuberculosis where 90% of the infected population have latent infection. Other studies have shown that non-human primates can be partially protected by vaccination with BCG and display a delayed-type hypersensitivity (DTH) to prior contacts with mycobacterial antigens (27,31-32). *M. tuberculosis* culture filtrate protein (CFP) immunogenicity and safety in rhesus and baboon monkeys have been investigated; results showed a short-lived immunity at both humoral and cellular responses, with low IFNγ production levels (33,34).

ESAT-6 (early secretory antigenic target 6 kD) is a short-term culture filtrate component; it is a genetic and chemically characterized protein inducing potent TH1 responses (35-41). This antigen is secreted during mycobacterial initial growth phase, being strongly recognized in different species infected with *M. tuberculosis* (humans, bovines and mice); this means that its inclusion in a future anti-tuberculosis subunit vaccine and in current immuno-diagnostic tests is well-founded (39,42-44).

The *Aotus* owl monkey's immune response to rESAT-6 is studied in this work. Immunization with the recombinant antigen together with Montanide 720 adjuvant produces very good humoral and cell-specific responses, even for a long period of time. It was observed at humoral immune response that ESAT-6 protein present in *Mycobacterium tuberculosis* CFP was recognized by *Aotus* sera immunized with rESAT-6. Efficient *in vitro* stimulation of lymphocytes with both recombinant protein and CFP was determined in proliferation assays, indicating that a cell-mediated immune response was being generated.

Materials and Methods

Immunogen and antigens.

rESAT-6 was expressed in DH5α-pQE$_{30}$ prokaryote system (QIAGEN), was purified by affinity chromatography using a Metal Chelate Affinity-Ni^{2+} resin and imidazol gradient. rESAT-6 was characterized by immunoblot, HPLC (reverse phase and size exclusion chromatography), Maldi-Tof mass spectrometry and circular dichroism, Figure 1. The *Mycobacterium bovis* BCG live vaccine (Lyophilized, Inter Vax, Canada) was also used as reactivity control for *in vivo* experiments.

Crude *Mycobacterium tuberculosis* H37Rv culture filtrate protein (CFP) produced by the National Institutes of Health, National Institute of Allergy and Infectious Diseases (Contract N01-AI-75320, kindly donated by Dr. J. Belisle at Colorado State University) was used in the *in vitro* immunological assays. ESAT-

Table II. Immunized *Aotus* monkey groups

Group	*Aotus*	Immunogen	Days
I	1, 2, 3, 4, 5	(rESAT-6)	0, 30, 60
II	5, 6, 7, 8, 9, 10	*M. bovis* BCG	0
III	11, 12, 13, 14, 15	Placebo (PBS)	0, 30, 60

Immunogens were dissolved in PBS (I, II, and III) and emulsified 30:70 with Montanide ISA720 adjuvant (I and III).
M. bovis BCG was not emulsified in Montanide ISA720 adjuvant

6 protein 20-amino-acid long peptides were synthesized by solid phase methodology (53), purified by HPLC and characterized by mass spectrometry. Peptide numbers and sequences are shown in Table I.

Animals.

A total of 15 healthy *Aotus nancymaae* owl monkeys (having been previously orally deparasitized with Ivermectin) were selected based on immunoblots of the sera analyzed having minimum or null mycobacterial recognition (*Mycobacterium tuberculosis* H37Rv lysate). Monkeys were kept at our institute's experimental primate station in Leticia (Amazon) in accordance with CCAC (Committee on Care and Use of Laboratory Animals, US) guidelines and Colombian National Institute of Health guidelines for the use of laboratory animals, supervised by the Colombian Wildlife Corporation (CorpoAmazonia).

The presence of mycobacteria in monkeys housed at the colony was evaluated by randomly taken samples. One hundred samples from different sources, gastric lavage, faecal material, soil, food and drinking-water were thus analyzed. Advantage was taken of the anaesthesia (35mg/kg weight ketamin, 0.1mg/Kg atropine and 1 mg/Kg diazepam, applied intra-muscularly) required for some procedures carried out on the 30 *Aotus* for also performing gastric lavage using a size 6 Levin probe (54). The samples were decontaminated by Petroff's technique (55). Ziehl–Neelsen (ZN) staining, and culturing in Bactec MIGT960 system and solid medium (Middlebrook 7H10) (for 6 weeks at 37°C) were then carried out. The tuberculin test was done in the eyelids of 15 *Aotus* previously positive for acid-fast bacilli (AFB) and/or rejected from the study during the selection process due to having some type of specific mycobacterial antigen recognition. A 100µl tuberculin dose (PPD RT23 SSI, Swtatens Serum Institute, Copenhagen) was intra-dermally administered.

Immunization.

Five monkeys were selected for each immunogen (Table II). 100µg rESAT-6 in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2P0_4) per monkey-dose were applied to the first group. The second group received only *Mycobacterium bovis* BCG; this was reconstituted in PBS, applied at 50µg per dose. The third group corresponded to the placebo (PBS plus adjuvant). Immunogens were emulsified with Montanide ISA 720 adjuvant (SEPPIC-France) in a 70:30 ratio by sonication when being applied. *Aotus* were immunized subcutaneously with 200µl emulsion in the abdomen three times at 30 day intervals (days 0, 30 and 60). Blood was collected immediately prior to the first injection, on day 30 and every month on three occasions. Monkeys were physically monitored for 180 days for developing local and systemic reactions.

Antibody response.

For enzyme-linked Immunosorbent assays (ELISAs), the antigens were diluted in PBS at 10µg/well (CFP) and 1 µg/well (rESAT-6 and peptides) and used for coating microtiter wells; the wells were blocked with 3% BSA. Serum samples (previously adsorbed with *E. coli* lysate proteins) were added at diferent dilutions; horseradish peroxidase (HRP)-conjugated goat anti-*Aotus* IgG (produced by FIDIC) was added to each well as second antibody. The reaction was developed using TMB Microwell Peroxidase Substrate System, A_{620nm} was read on a Labsystems Multiskan MJ ELISA reader (56).

For Western Blot analysis, CFP were separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), under reducing conditions on a 10-15% gradient gel. rESAT-6 was separated by 14% SDS-PAGE in Tris-Tricine system. The gels were electrophoretically transferred to nitrocellulose membrane (Trans-Blot Bio Rad). Blocking buffer (1% nonfat milk-TBS (10mM TrisHCl pH7.5, 150mM NaCl)-0.05% Tween 20) was used for saturating non-specific sites. Single nitrocellulose strips with nearly 50µg CFP or 1 µg rESAT-6 were incubated with monkey serum diluted 1:100 in blocking buffer. Strips were incubated with Alkaline phosphatase (AP)-labelled goat anti-*Aotus* IgG, BCIP/NBT (Promega) was used for detection (57).

Cellular proliferative response.

Monkeys were splenectomized on day 150 after the study was begun. Lymphocytes were obtained by preparing single-cell suspensions from spleens and then purified by Ficoll-Hypaque (ICN Lympho separation medium) gradient centrifugation. Cells were washed and suspended in RPMI 1640 medium containing HEPES, L-glutamine, antibiotic-antimycotic and 20% autologous serum. 100µl cell suspension containing 10^6 cells were plated onto 96-well flat-bottom plates, together with antigen (13). CFP (10µg/ml), rESAT-6 or peptides (1µg/ml) were used as antigens and 5µg/ml phytohemagglutinin (PHA, DIFCO) or RPMI 1640 as positive control and non-stimulated control respectively. Each stimulation was performed in triplicate wells. Plates were incubated at 37°C in 5% CO_2 for 72 h. [^{3}H]thymidine (1µCi/well; Amersham) was added for the final 16-18 hours incubation. Cells were harvested onto filters (Glass Fibre Strips 240-1, Cambridge Tech.) by using a cell harvester; filters were dried and cells were counted in a scintillation counter. Results are reported as stimulation index (SI): a two-fold increase in counts per minute over non-stimulated control were considered positive .

Results

Mycobacterial presence.

No signs of tuberculous infection were observed; few alcohol-resistant bacilli were found in those randomly-chosen samples taken; only 8 positive specimens were isolated in the population analyzed in BACTEC culture and verified by ZN: 4 came from gastric lavage, 1 from soil and 3 from faecal material. DNA isolated from cultured samples was further analyzed by PCR (54), being negative for *M. tuberculosis* complex mycobacteria (data not shown). The tuberculin test was done in the eyelids of 15 *Aotus* previously positive for AFB and/or rejected from the study during the selection process due to having some type of specific mycobacterial antigen recognition; no induration or positive reaction was detected 24 to 72 hours following application.

Aotus selection and immunization.

Two parameters were taken into account in the process of selecting animals for this study: optimum state of health (indicated by clinical evaluation) and minimum or null sera recognition of *M. tuberculosis* H37Rv proteins in Western blot assay. Sera adsorption with *E. coli* proteins allowed specific recognition of mycobacterial proteins which is why this procedure was done prior to the immunoblot and ELISA

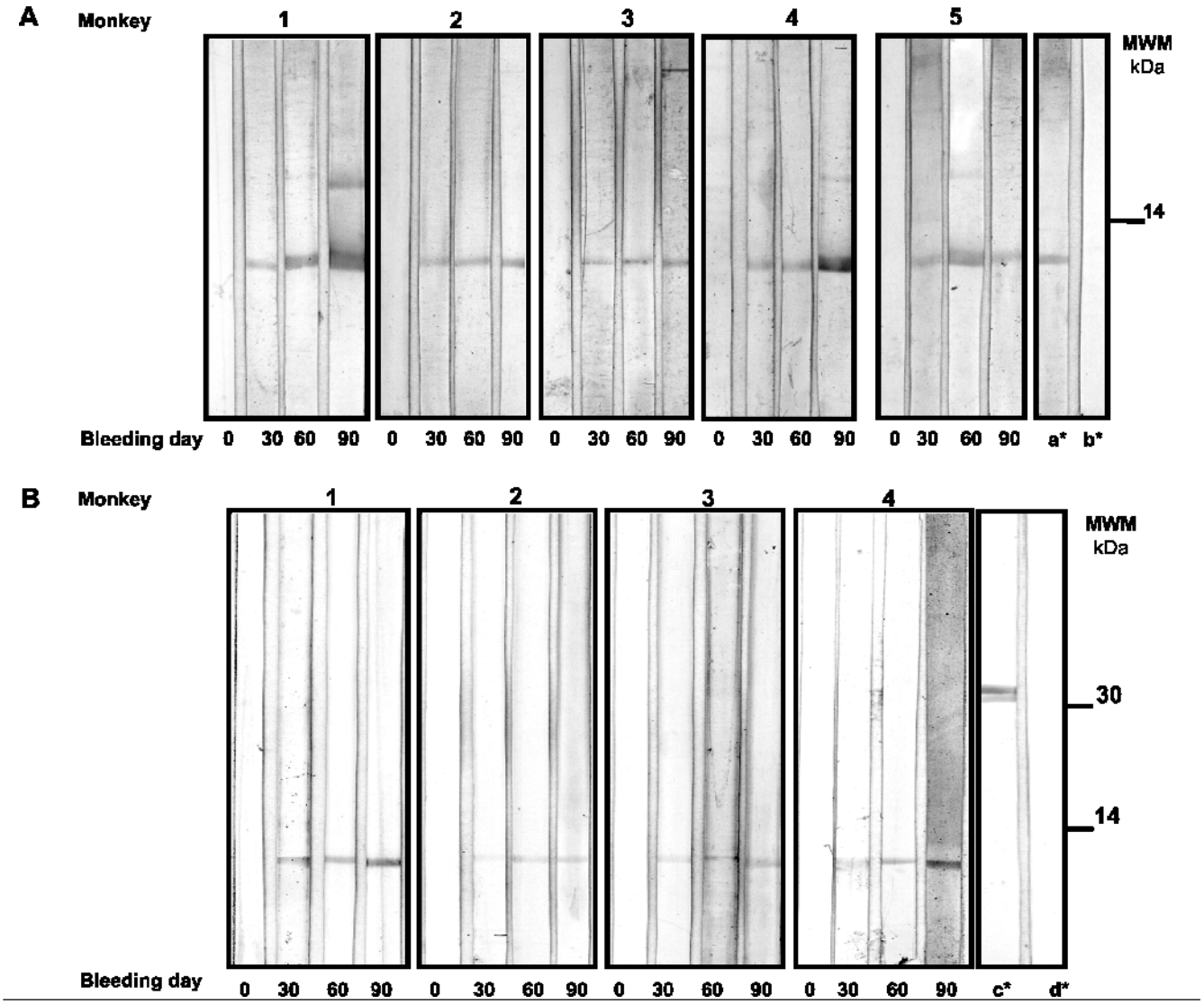


Figure 2. Western blot analysis showing the binding of antibodies from immunized *Aotus* monkeys with rESAT-6. (A) Results of immunodetection in rESAT-6. (B) *Mycobacterium tuberculosis* crude CFP (B). Primary antibodies 1:100 dilution sera. Assay controls: a* strip incubated with anti-polyHistidine (Sigma), then anti-mouse IgG-PA (ICN.); c* strip incubated with anti-Ag85B, then anti-rabbit IgG-PA (ICN); b* and c* reagent control for A and B, respectively.

analyses. Monkeys were immunized on three times, presenting satisfactory clinical analysis. No evidence was seen of nodules or erythema at the application site in animals immunized with rESAT-6; similar results were obtained in the placebo monkeys. *Aotus* group II received a single dose of BCG; nodulations were observed which were immobile to the touch at the application site compromising skin and subcutaneous tissue, Table II.

Humoral immune response.

Immunoblot and ELISA were used for determining rESAT-6 and CPF antibody recognition by monkey sera. Antisera obtained from group I animals at various times after the first and subsequent immunizations were able to strongly bind rESAT-6 dose-dependently for most animals, Figure 2A. Except for Monkey 5, which displayed very low recognition (data not shown), native ESAT-6 present in CFP was also recognized, even from the first bleeding. There was less recognition intensity, however, out of the whole protein pattern found in CFP (more than 100 proteins), specificity was confirmed as the only band being recognized had the same molecular mass as ESAT-6, Figure 2B.

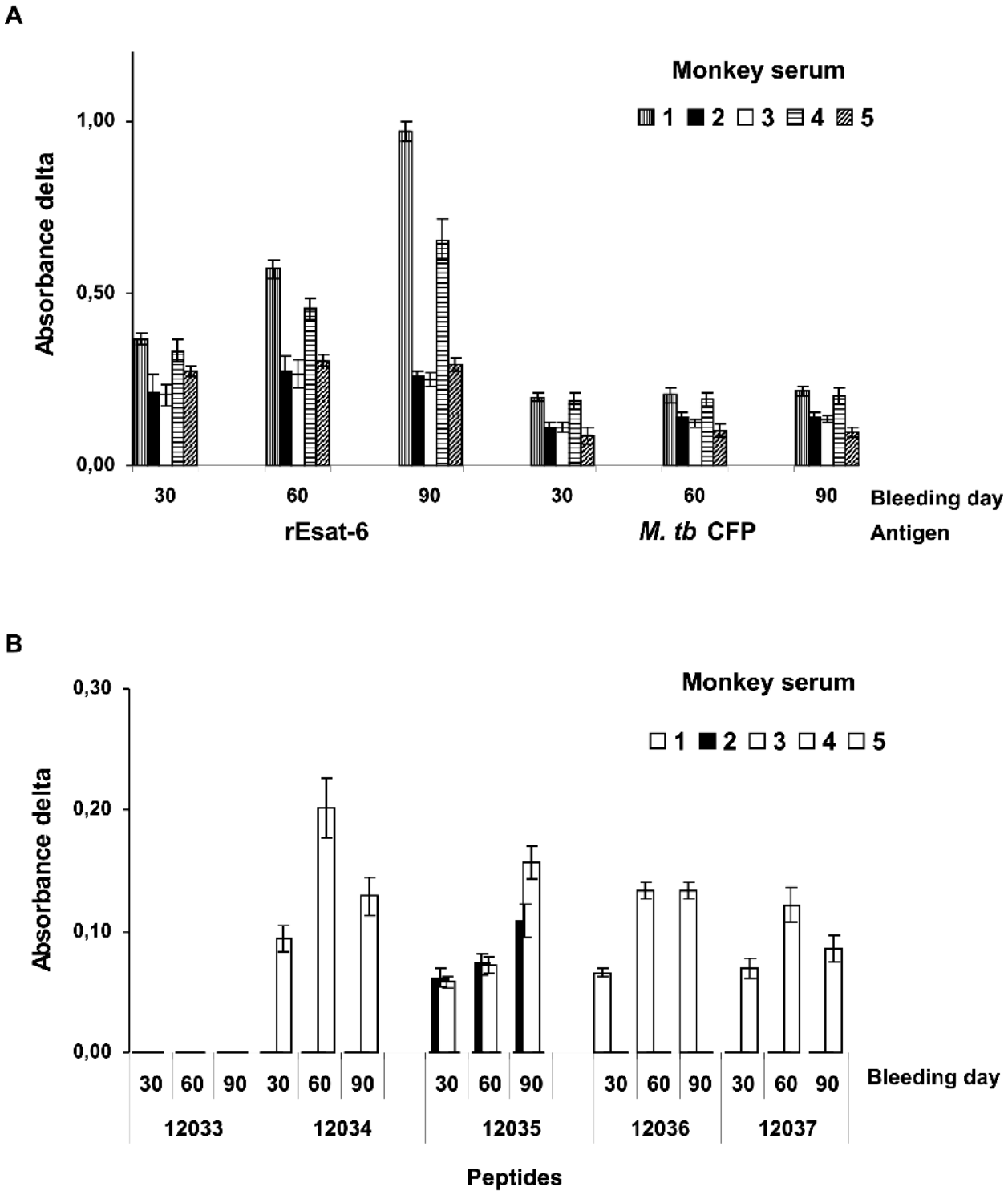


Figure 3. ELISA assay for IgG antibodies generated by immunization with rESAT. (A) CFP and rESAT. (B) 5 peptides mapping ESAT-6. 620nm absorbance delta, corresponding to the value obtained in each bleeding less pre-immune sera absorbance of each monkey regarding each antigen. Primary antibodies 1:100 dilution. Each bar represents the mean of triplicate values.

ELISA assays corroborated the foregoing; it was determined that the rESAT-6 antigen binding titer became progressively increased until obtaining titers between 1:800 and 1:12,800 for bleeding III (after 90 days). Antibody titers measured in CFP were generally constant in the different immunizations, 1:100 being the lowest value (Figure 3A). Studying the different peptide sequences mapping the protein showed varied

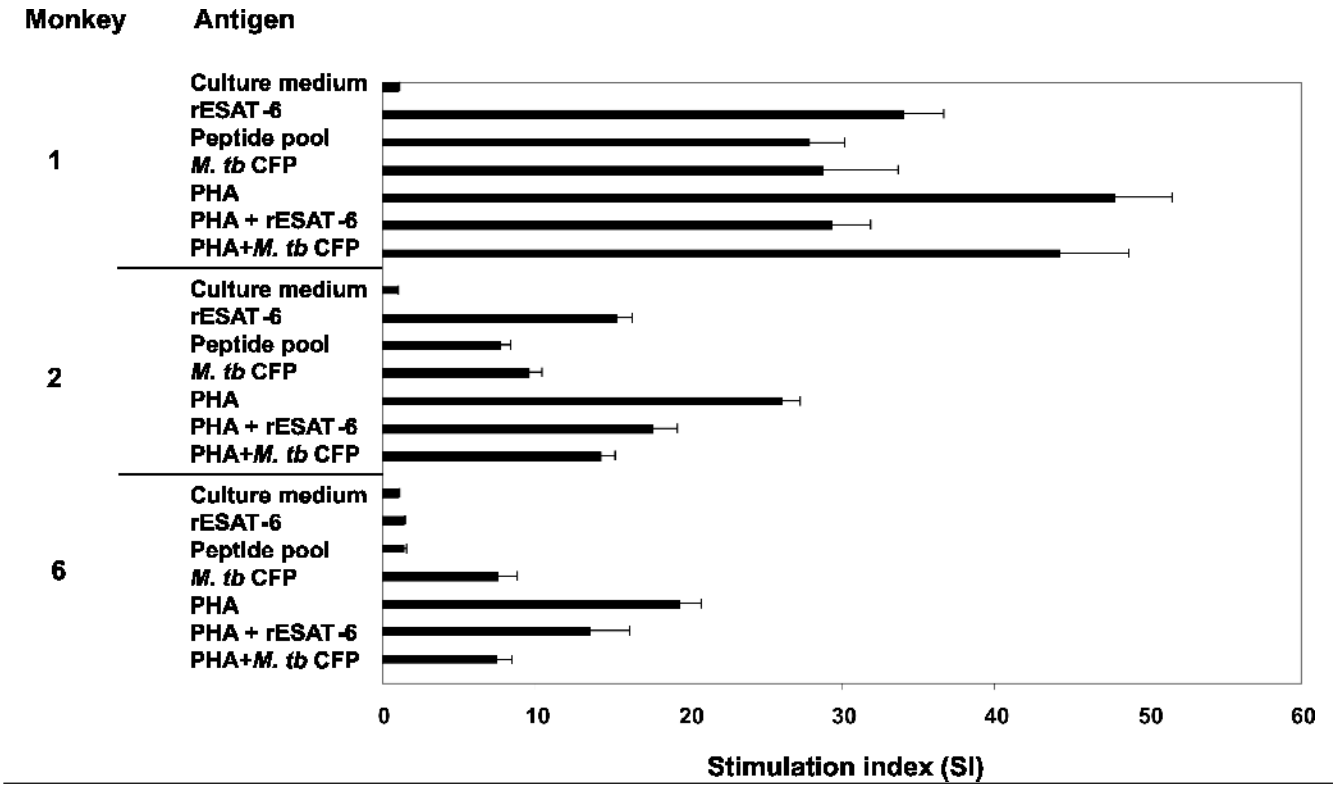


Figure 4. Proliferative responses of spleen-derived lymphocytes from *Aotus* immunized with rESAT-6 (Monkeys 1 and 2) and BCG (Monkey 6). [^{3}H]thymidine incorporation in response to *in vitro* stimulation with rESAT-6, the pool of ESAT-6 peptides, CFP and PHA (positive control) were measured 90 days after final immunization. Data are reported as SIs (fold stimulation over that of cells incubated in just medium). Results shown are representative of the immunized population. Each bar represents the mean of triplicate values ± sem

recognition for the different animals. Except for Monkey 5 (having no appreciable antibody titer values to any particular peptide), the other antisera recognized sequences throughout the whole protein, amino-terminal portion ($NH_{2\text{-ter}}$) not being recognized (Figure 3B). No significant rESAT-6 antibody titers were observed when sera from animals immunized with BCG or placebo were tested (data not shown).

Cellular immune response.

Cellular proliferative responses were assessed using animals' spleen lymphocytes. Figure 4 shows proliferation levels induced by rESAT-6, the pool of peptides mapping the protein, CFP and PHA in two representative experiments of rESAT-6 immunized animals (Monkeys 1 and 2). All immunized monkeys developed a proliferative cellular response to CFP, including monkeys immunized with BCG; Figure 4 shows Monkey 6 lymphoproliferation results which were typical of *Aotus* immunized with BCG. In all cases proliferation induced by PHA was greater than that found with the other antigens (recombinant and peptides), including that displayed by placebo monkey lymphocytes. Recombinant protein and peptide pool stimulation was specific in those animals immunized with just rESAT-6. The results show strong stimulation of antigens, even though being different for each monkey. SI values ranged from 15 to 34 for rESAT-6; 8 to 28 for the peptide pool; and 10 to 29 for CFP.

The proliferative response of cells from rESAT-6 immunized animals stimulated with peptides mapping the protein was lower than that observed when stimulation with the recombinant protein was carried out, Table IV. Contrary to what happens with humoral response, several monkeys recognized the protein's $NH_{2\text{-ter}}$ portion (peptide 12033) simulta-neously with the carboxy-terminal region ($COOH_{\text{-ter}}$), as observed in Monkeys 2 and 3. The other *Aotus* recognized the ($COOH_{\text{-ter}}$) region with peptides 12036 and 12037. None of the peptides tested was recognized by Monkey 5. Cells stimulated with peptides from the

Table III. rESAT-6 binding antibody titers obtained by ELISA assay

Bleeding day	Monkey serum dilution				
	1	2	3	4	5
30	1:1,600	1:400	1:400	1:800	1:400
60	1:6,400	1:1,600	1:800	1:1,600	1:800
90	1:12,800	1:1,600	1:800	1:12,800	1:800

Table IV. ESAT-6 peptide-stimulated splenocyte proliferation assays

Peptide	Stimulation Index (SI)			
	Monkey			
	1	2	3	5
12033	1.4±0.4	**5.2±0.8**	**2.9±0.3**	0.7±0.1
12034	0.8±0.2	0.8±0.5	1.7±0.3	1.1±0.4
12035	1.6±0.6	0.6±0.6	0.6±0.1	0.9±0.2
12036	**4.4±0.7**	**2.8±0.4**	**2.8±0.2**	0.6±0.1
12037	**9.6±1.7**	0.2±0.2	**3.3±0.1**	1.9±0.3
PHA + peptide pool	**18.5±1.6**	**8.1±0.9**	**6.5±0.6**	**17.5±2.1**

Monkey 4 died before the experiment ended.
Positive values were considered as having SI>2 (shown in bold and underlined).
Each result represents the mean of triplicate values ± sem.

protein's middle region (12034 and 12035) did not present any appreciable proliferation.

Discussion

Several years ago it was shown that the low mass secreted ESAT-6 protein was a molecule associated with memory effector TH1 lymphocytes in long-lived immunity to tuberculosis, having high interferon gamma (IFNγ) production levels (40,58). It is strongly recognized in *M. tuberculosis*-infected humans, leading to research into its use in diagnostic kits reflected in high percentages of sensitivity and specificity for infection caused by tuberculosis (44, 59-60). Studies aimed at vaccine development carried out in animals, including mice, guinea pigs, bovines and non-human primates have described strong humoral and cellular recognition. Several B- and T-epitopes have been recognized throughout the protein, depending on the species analyzed (43,61-63).

Immunogenicity and protection-inducing levels have been explored in diverse preparations such as DNA and recombinant ESAT-6 vaccines (13-14,17,64-65). These candidates have been able to induce cellular TH1 response and protection similar to that presented by BCG. Subunit vaccines (in which ESAT-6 potential has been evaluated) have also been reported; determining the modulating effect of the immune response of the adjuvant used in the immunization process, depending on the antigen. One study has shown that the ESAT-6-DDA combination (dimethyl dioctadecyl ammoniumbromide) does not induce any appreciable immune response in the murine model. When MPL (monophosphoryl lipid A) is incorporated into the preparation in different combinations, strong adjuvant activity can be observed for both cellular and humoral immune responses (42). The data presented in different reports indicate that protective immunity determined for different ESAT-6 vaccines is at the same level as BCG, a relevant result for a single *M.*

tuberculosis antigen; these results show high humoral and cellular response when recombinant protein was administered using Montanide 720 as adjuvant. Montanide 720 is a metabolizable mannide oleate in oil solution adjuvant which has been recently approved for use in humans. *Aotus* immunized with rESAT-6 or the placebo did not present nodules (as observed with animals immunized with BCG).

Different reports have indicated that ESAT-6 contains numerous epitopes recognized by a high percentage of individuals, depending on the sensitivity of the assay used (59,61,66). Different peptide sequences involved in humoral recognition were observed in *Aotus* monkeys, where almost the whole protein participates in antibody production. Interestingly none of the immunized animals' sera recognized the recombinant antigen $NH_{2\text{-}ter}$ portion. Likewise, low recognition of ESAT-6 $NH_{2\text{-}ter}$ region peptides by antibodies in tuberculous non-human primate sera (cynomulgus macaques, rhesus macaques, African Green monkeys) has been recently demonstrated in a study using overlapping peptides spanning the ESAT-6 protein sequence, while an important recognition towards the $COOH_{\text{-}ter}$ portion is observed even early in infection (63). At cellular response level, sequences mainly comprising the $COOH_{ter}$ fraction have induced proliferation in all immunized monkeys, while two of the immunized monkeys are able to respond towards the $NH_{2\text{-}ter}$ also. Similar results have been reported in previous studies using human cells (41,66-68). Cellular and humoral *in vitro* immune response stimulation were lower whenever peptides mapping ESAT-6 protein were used, suggesting that either conformational epitopes were present or that some epitopes were missing due to the use of non-overlapped peptides.

The *Aotus nancymaae* monkey has been recommended by the World Health Organization (WHO) as a model for malaria vaccine candidate evaluation (69-70). This has favored immune system molecule research leading to a high percentage of homology with humans being elucidated. Recent data has shown 78% to 95% homology for MHC-II molecules (histocompatibility Class-II complex) (45-52,71) which have been declared as having great importance in tuberculosis infection (23-24). Lymphocytes' proliferative *in vitro* response to mitogens in *Aotus nancymaae* has been analyzed, showing that it varies from monkey to monkey depending on the particular mitogen assessed (72); this was also seen in the present work. Varied cellular and humoral recognition was determined amongst different monkeys and even to different antigens; however, the results show that positive recognition values are antigen-specific, having no ambiguity regarding immune response.

It has been reported that all primates are susceptible to tuberculosis, although differences between species exist (e.g. Old World primate species such as rhesus monkeys are very sensitive to infection whilst New World species such as squirrel monkeys appear to be less susceptible) (27). There have been few reports recently regarding tuberculosis in New World Monkeys, even more so in the wild. A study isolating and identifying mycobacteria in New World primates maintained in captivity identified *M. chelonae* subsp. *abscessus* in one of the two *Aotus sp.* analyzed (54). Few monkeys were found to be positive for mycobacteria in the current study; all isolated mycobacteria were non-pathogenic, although Amazon province (Colombia) is tuberculosis pulmonary disease endemic area (73).

The tuberculin test was done in *Aotus* previously positive for AFB and/or having some type of mycobacterial antigen recognition; no induration or positive reaction was detected. This result (together with positive recognition of mycobacterial antigens) suggests that such animals were not infected by pathogenic mycobacteria, that low AFB detection in the analyzed population was due to *Aotus* having been in contact with environmental mycobacteria and that shared epitope cross-recognition was being generated.

Aotus develop a strong immune response when they were immunized with a mycobacterial antigen in a recombinant protein form (rESAT-6) emulsified with Montanide 720 as adjuvant. Immune response had multi-epitope characteristics and was seen to be different among the monkeys analyzed as similar human studies, but this behavior is a disadvantage for a reliable experimental model because de responses are not enough predictable. A significant number animals were analized and not any monkey were found to be positive for pathogenic mycobacteria or positive reaction to tuberculin test.

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