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"AMERICAN CUTANEOUS LEISHMANIASIS: A MODEL FOR VACCINE DEVELOPMENT AGAINST HUMAN DISEASE"

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EXECUTIVE SUMMARY

The purpose of the project was to evaluate the development of cutaneous leishmaniasis and immune response to *Leishmania (Viannia) panamensis* in the squirrel monkey (*Saimiri boliviensis*), as a non-human primate model for vaccine development against human American Cutaneous Leishmaniasis (ACL); and to compare this animal model with the cell mediated immunity (CMI) and clinical manifestations of cutaneous leishmaniasis, occurring naturally in patients from Guatemala. During the project several activities were accomplished, being mainly the following:

- Cultures of *Leishmania (Viannia) panamensis* were produced for antigens for vaccination and to evaluate CMI and humoral immune (HI) responses in non-human primates, and for challenging inocula to induce infections in primates and golden hamsters.
- The 29 non-human primates (*Saimiri boliviensis*) fully adapted to the animal facilities at the Universidad del Valle de Guatemala (UVG) and increased in body weights (average > 47%). Four animals died, however, during the period of experimentation due to intestinal bacterial infections.
- Determination of challenge dose of *L. (V.) panamensis* ($1x10^6$ and $1x10^7$ promastigotes) was done in two young adult squirrel monkeys, inoculating each both at the tip of the nose (TN) and at the dorsal base of the tail (DBT), for a total of 112 wk. Lesions persisted for 43 wk at the TN and 48 wk at the DBT.
- Vaccination trials with BCG+KLV, KLV and BCG alone and challenge inoculation of vaccinated and control monkeys were done with $2x10^7$ promastigotes each at the TN and at the DBT, and follow-up of lesion development was concluded after 112 wk post challenge inoculation (PCI). A bimodal lesion development was observed: whereas BCG+KLV provided up to 95% suppression [over control] and KLV 75% and BCG 78% at the TN, BCG+KLV and KLV 100% but BCG none at the DBT, during 33 to 52 wk PCI. Thereafter, lesions reappeared at the TN and persisted up to 112 wk PCI in some monkeys from all groups inoculated but having 43%, 56% and 52%, suppression of lesion development, respectively.

- Delayed-type hypersensitivity response to tuberculin and leishmanin skin test antigens were done, being highly manifested to tuberculin but having minimal response to leishmanin at 6, 9, and 18 wk PCI.
- Concentration-response curves for PHA and soluble *Leishmania* antigen (SLA) were done on Histopaque separated peripheral blood mononuclear cells (PBMNC) and preinfection baseline data determined. Several post infection and post vaccination immune determinations were done, where response to PHA was high and consistently observed but none to SLA or other antigens at various concentrations.
- Initial tests for measuring squirrel monkey cytokines (Interferon-τ, IL-2, IL-4, IL-5 and GM-CSF) were done on supernatant from PBMNC stimulated with PHA, PMA-Ionomycin, PPD, and SLA, but the results were consistently negative.
- Western-blot analysis of squirrel monkey IgG antibodies to SLA were done after SDS-PAGE electrophoresis, for preinfection and several post vaccination and post challenge infection determinations. Consistently, there were none or very few week bands recognized in all uninfected monkeys, whereas a strong response to a major 87, 79, 75, 41-48, 34-38, 26, and 20-21 kDa antigens of *Leishmania* were observed after vaccination or infection with promastigotes.

These results showed that typical cutaneous leishmanial lesions were induced experimentally with $2x10^7$ *L. (V.) panamensis* promastigotes at the tip of the nose (TN) and at the dorsal base of the tail (DBT) in all vaccinated (BCG+KLV, KLV), BCG alone, and unvaccinated squirrel monkeys, although cutaneous lesions developed preferably at the tip of the nose and lasted for more than two years in some monkeys. The progression, regression and type of cutaneous lesions were similar to the monkeys previously infected to determine the dose inocula. Protective immunity induced by BCG+KLV or BCG alone had a bi-phasic effect not only on the size but also on the development of experimentally induced cutaneous leishmanial lesions, the proportion of infected animals and the mean duration of cutaneous leishmanial lesions, as compared to the unvaccinated challenge-control animals. In spite that cutaneous leishmanial lesions developed and progressed at the two sites of inoculation in all vaccinated animals, BCG+KLV had greater capacity in inducing a protective response (only one third of animals were infected at 44 WPCI), albeit not complete and sterilizing, for at least up to 52 WPCI

at the TN, than in monkeys vaccinated with KLV or BCG alone, where two thirds and one-half, respectively, of animals were infected at 44 WPCI. Thereafter, however, cutaneous lesions persisted in some animals of all inoculated groups at 112 WPCI (when the experiment was terminated), where an equal proportion of infected animals were observed in the vaccinated groups [BCG+KLV, KLV and BCG alone], but were still lesser (one third to less than one half) than the unvaccinated challenge-control animals. The bimodal-type cutaneous lesion development observed in the BCG+KLV and KLV vaccinated animals suggests that either the initial vaccination regime should be modified (eg., vaccine with higher protein concentration) or that another booster inoculation should be given later, at least, prior to 52 WPCI to maintain the protective status in the animals. Interestingly enough, cutaneous leishmanial lesions developed only at one half of the observed rate in the animals vaccinated with BCG alone, thus reflecting the immune potentiating capacity of BCG. Further trials should be done to improve on the vaccination regimes tested, based on these preliminary but promising results.

The skills and awareness that will remain in Guatemala as a result of the Project are the capabilities of setting up and running an animal facility for non-human primates, a well equipped laboratory setup for conducting other kind of work on leishmaniasis (from culture of parasites to antigen preparation) and determination of immune response (by CMI and humoral immunity). The Project provided enough infrastructure and human resource capabilities to engage in future work, especially that oriented at molecular biology techniques in leishmaniasis and other tropical diseases.

RESEARCH OBJECTIVES

The Project was conducted to evaluate the development of cutaneous leishmaniasis and immune response to *Leishmania (Viannia) panamensis* in the squirrel monkey (*Saimiri boliviensis*), as a non-human primate model for vaccine development against human American Cutaneous Leishmaniasis (ACL).

The problem addressed was to determine the effectiveness of a crude killed vaccine (KLV) of *L. (V.) panamensis* with or without BCG to induce protective immunity against ACL in this non-human primate model, as well as to monitor vaccine-induced protection and basic cell- mediated (CMI) and humoral immune (HI) responses, after immunizations with crude antigens. This Project was important for development since it wanted to compare the results from this animal model with the cell mediated immunity (CMI) and clinical manifestations of cutaneous leishmaniasis in patients from Guatemala. Cutaneous leishmaniasis continues to be a serious health problem in many tropical countries in the world, where no effective vector control measures are available due to the nature of transmission of this disease. Therefore, only few alternatives are left to control this disease in which either treatment has to be provided to infected individuals, although it is very costly (more than $100.00 per treatment), or preventative measures are implemented such as immuno prophylaxis or vaccination. In many countries of the world, such as Iran, Brasil, and Venezuela, great efforts are being done to determine the best prophylactic measures for cutaneous leishmaniasis in human beings.

The innovative aspects of this Project were to evaluate a crude killed vaccine which already had been proven to be partially effective in human beings (e.g., in Brasil) but needed comparison of the route of immunization of the antigen, given with or without BCG (a potent immune adjuvant) and the immunoblot assays for determining which molecular weight antigens were recognized by anti-leishmanial antibodies during vaccination and after challenge-exposure.

Due to some changes in the original proposal objectives, the ongoing work received financial support from both a grant from the CDR-Israel Program of the Agency for International Development, USAID, USA, and the Tropical Diseases Research Program of the World Health Organization (TDR/WHO) to conduct vaccination trials for *L. braziliensis* in squirrel monkeys. Support from the USAID was for a total of U.S. $200,000.00 ($148,310.00 for the Universidad del Valle de Guatemala and $51,690.00 for the Hebrew University of Jerusalem), for the period July 1989 to December 1993. The USAID and TDR/WHO projects did not duplicate financially with each other in any aspect. On the contrary, more information was gained for vaccination trials where both projects complemented the findings of each other. In addition, some common elements in both projects, such as the acquisition of squirrel monkeys and the furnishing of the animal house facility, trained personnel, and development of basic procedures, benefitted each other. Therefore, having both projects complementing but not duplicating each other, including expenses, resulted in greater benefit to the small scale research operation at the Universidad del Valle de Guatemala, although the support from USAID as well as that from TDR, did not cover fully the total cost of the projects.

(N.B. Aspects of this report which are in common with the TDR/WHO sponsored project and those by USAID which are relevant for comparisons in vaccination strategies are presented in this final report, and when submitted to TDR/WHO, the appropriate recognition of USAID will be made).

METHODS AND RESULTS

Twenty seven young adult squirrel monkeys (*Saimiri boliviensis*) were divided into five groups: One group of 6 were vaccinated with crude killed *Leishmania* promastigotes (KLV) + BCG and were challenge-exposed (CE) with infective promastigotes [*]; a second group of 6 received KLV only and were CE; a third group of 6 received BCG only and were CE; a fourth group of 4 did not receive KLV or BCG but were CE; and a fifth group of 5 nothing. The first two groups served as unvaccinated/CE and uninoculated controls, respectively, for CMI and HI responses. Brazilian-type vaccine of *L. (V.) panamensis* was prepared and each animal received 1200 ug N protein of antigen, given via intradermal inoculation in three separate monthly doses, either mixed with 200 ug BCG (first dose) and 10 ug (second and third doses), or BCG alone; KLV alone was given via intramuscular inoculation at three weekly intervals. At 4 weeks after the last booster injection, monkeys were CE each with $2x10^7$ live promastigotes of *L. (V.) panamensis*, at the tip of the nose and at the dorsal base of the tail. Evaluation of lesion development and *in vitro* CMI and HI responses were done at 4-5 wk intervals post infection (PI), during 112 wk, while Montenegro skin testing was done at 4, 6, 9, and 18 wk PI.

Prior to vaccination trials, two young adult squirrel monkeys were each inoculated with $1x10^6$ or $1x10^7$ infective promastigotes, at the tip of the nose and at the dorsal base of the tail, to validate the model and to determine the natural progression and regression of cutaneous leishmanial lesions (USAID funded Project).

[*] TDR/WHO funded Project.-

i. Cultivation of *Leishmania* spp. for preparation of leishmanial antigens and inocula for inducing infections in non-human primates and golden hamsters:

Leishmania (Viannia) panamensis (MHOM/PA/83/(WR539)) and *L. mexicana* (MHOM/GT/87/G074/ (WR917)) promastigotes were continuously grown in Schneider's Drosophila medium (Gibco Laboratories, Grand Island, NY) supplemented with 20% fetal bovine serum, as described before. A typical growth curve for *L. (V.) panamensis* is illustrated in Figure I.1.

ii. Adaptation of the non-human primates (*Saimiri boliviensis*) to the animal facilities at the Universidad del Valle de Guatemala:

The squirrel monkeys (*Saimiri boliviensis*, roman arch) recuperated the sizeable ($\geq$ 58%) loss in body weight since when initially arrived at the Universidad del Valle de Guatemala. Average body weights over time are illustrated in Figure I.2, where a continuous increase occurred from 863 ($\pm$ 173) grams in December 1992 to 1022 ($\pm$ 181) grams in February 1995 [average > 47%], as compared to 542 ($\pm$ 148) grams in January, 1991 (Miami) and 697 ($\pm$ 124) grams in December, 1990 (Peru). Four animals died, however, during the period of experimentation due to intestinal bacterial infections.

iii. Determination of challenge dose of *L. (V.) panamensis* ($1x10^6$ and $1x10^7$ promastigotes) in two squirrel monkeys, inoculated each both at the tip of the nose and at the dorsal base of the tail (USAID supported project):

Prior to vaccination, two young adult squirrel monkeys were each inoculated intradermally at the tip of the nose (TN) and at the shaven dorsal base of the tail (DBT) with $1x10^6$ (monkey # 095) or $1x10^7$ (monkey # 186) live promastigotes of *L. (V.) panamensis*, respectively, after the 5th. subculture and harvested during late log growth phase (5 - 6 days of culture). Promastigotes were washed in 0.1 M phosphate-buffered saline (PBS), pH 7.2, suspended and counted to adjust to the required inocula concentration. Cutaneous leishmanial lesions were

measured at perpendicular diameters and the resulting area ($\pi r_1 r_2$) was determined, for a total follow-up of 112 weeks post inoculation (wk PI).

$1x10^6$ inoculum (monkey # 095).

A. Tip of the nose: Leishmanial lesions were detected as a primary nodule until 5 WPI, reaching maximum size (35.74 mm^2) at 10 WPI. The primary lesion persisted for 43 WPI, but being smaller in size (from 3.74 to 13.95 mm^2). Ulceration of the primary nodule was observed from 7 - 28 WPI. Two nodular satellite lesions (3.14 and 4.91 mm^2) were observed at only 32 WPI and rapidly regressed (Figure I.3A).

B. Dorsal base of the tail: Leishmanial lesions were detected as a primary nodule at 3 WPI, reaching maximum size (50.27 mm^2) at 5 WPI. The lesion persisted for 36 WPI, varying in size from a nodule to a large plaque (47.12 to 75.40 mm^2) and then decreasing (from 14.92 to 4.91 mm^2). Ulceration of the primary nodule was observed at 7 WPI, progressing to an erythematous plaque with subsequent healing and scarring. One nodular satellite lesion (35.19 mm^2) was observed from 7 to 17 and 36 to 40 WPI which rapidly ulcerated and regressed (Figure I.3B).

$1x10^7$ inoculum (monkey # 186).

A. Tip of the nose: Leishmanial lesions were detected as a primary nodule at 3 WPI, reaching maximum size (2[illegible].73 mm^2) at 7 WPI. The primary lesion persisted for 43 WPI, but being smaller in size (from 0.79 to 12.82 mm^2). Ulceration of the primary nodule was observed from 5 to 10 WPI. Two nodular satellite lesions (3.14 and 7.85 mm^2) were observed at 23 WPI, one which persisted for an additional 20 wk (reaching maximum size of 34.62 mm^2 at 37 WPI) and the other for only 17 wk (reaching maximum size of 9.62 mm^2 at 40 WPI), and then both regressed (Figure I.4A).

B. Dorsal base of the tail: Leishmanial lesions were detected as a primary nodule at 3 WPI, reaching maximum size (27.88 mm^2) also at 3 WPI. The lesion persisted for 36 WPI, varying in size from a nodule to a large plaque (from 17.67 to 52.23 mm^2) and then rapidly decreasing in size. Ulceration of the primary nodule was observed at 5 - 7 WPI, progressing to an hypochromic plaque with subsequent healing and scarring. Three nodular satellite lesions (17.32, 19.64 and 26.39 mm^2) were observed, one from 5 to 48 WPI and the other two from 5 to 12 WPI, changing from a nodule to a hypochromic or erythematous plaque (Figure I.4B).

These results demonstrated that the two inoculating doses of *L. (V.) panamensis* promastigotes were able to induce typical leishmanial lesions at the tip of the nose and at the dorsal base of the tail of squirrel monkeys. No apparent differences were observed in the development, progression and regression of the primary nodules at either site of inoculation, although the total lesion area (based on primary and satellite lesions) at the tip of the nose (Figure I.5A) and at dorsal base of the tail (Figure I.5B) was greater with the $1x10^7$ inoculum, and more satellite lesions of longer persistence were observed. Interestingly, these results were similar to previous reports of experimental infections in squirrel monkeys with *L. (V.) panamensis* (Luján et al., 1990. J. Parasitol., 76: 594), but in the present situation only naive ("virgin") animals were infected, in contrast to animals which had recovered from a previous experimental infection with *L. donovani*. Moreover, leishmanial lesions were demonstrated to develop also at the tip of the nose, in comparison with the dorsal base of the tail, the only site where it had been done before.

$1x10^6$ and $1x10^7$ inocula in golden hamsters.

Twenty two male golden hamsters (*Mesocricetus aureatus*), 11 for each dose, were simultaneously inoculated each at the dorsal base of the tail, in parallel with the corresponding inoculating doses of the

squirrel monkeys. All animals (100%) developed typical leishmanial nodular lesions, as early as 1 WPI, reaching maximum size (average from 40.40 and 159.44 mm^2) between 4 - 7 WPI. Only single nodular lesions were observed, however, with no significant differences in lesion size (average 40.84 and 45.16 mm^2) between the two inocula doses ($1x10^6$ and $1x10^7$) after 7 - 10 WPI, when the experiment was terminated (Figure I.6).

iv. Acquisition of BCG from the Guatemalan Ministry of Health; commercial tuberculin:

A freeze-dried glutamate of BCG containing $1.5x10^6$ viable BCG per 0.1 mL was obtained (Japan BCG Laboratory, 2-8-8 Kohjimachi, Chiyoda-ku, Tokyo, Japan), but after an unforeseeable and unexpected delay. In spite of having made previous arrangements with the Guatemalan Ministry of Health to acquire BCG in February, 1992, their stock was rapidly depleted and new vaccine was available after July 1992. This caused a serious delay (more than 5 months) in the proposed schedule for vaccination of the squirrel monkeys, when it was finally initiated in August 1992.

Tuberculin, mammalian, human isolates of *Mycobacterium tuberculosis* was obtained commercially from Coopers Animal Health, Inc., Kansas City, KS, USA.

v. Vaccination trials of squirrel monkeys with BCG+KLV, KLV and BCG. Included in this report are also the results of monkeys vaccinated with KLV only (USAID supported project):

a. Determination of vaccination and control groups of squirrel monkeys.

Based on the limited number of available squirrel monkeys (29), the following groups were established to accommodate vaccination and control groups, in accordance to the established objectives of both the TDR/WHO and USAID projects (Modabber & Luján, December 1991):

Group	No.	BCG	KLV	Challenge	Comments
1	6	+	+	+	id[a)] inoculation BCG+KLV
2	6	-	+	+	im[b)] inoculation KLV
3	6	+	-	+	id inoculation BCG control
4	4	-	-	+	normal control for challenge inoculation
5	5	-	-	-	normal control for *in vitro* CMI and HI tests of immune response
6	2	-	-	+	to determine the challenge dose inoculum (= Group 4)

a) id = intradermal
b) im = intramuscular

Before initiating experimental procedures, the squirrel monkeys were assigned to a particular group after three-times, blinded randomization. Each group consisted of males and one female, and no significant differences (ANOVA, $p>0.05$) were observed between the mean proliferative response of PBMNC to PHA in the groups (see later sections on *in vitro* tests of immune response).

b. Vaccination dose and protocol.

BCG+KLV (TDR/WHO).

Killed *Leishmania* vaccine (KLV) (="NLCV"= "non living crude vaccine") consisted of *L. (V.) panamensis* promastigotes, cultured as described before. Promastigotes were washed three times in sterile PBS, subjected to six cycles of freezing and thawing, disrupted briefly with sonication (10 seconds at 4C), and merthiolate was added to a final concentration of 1:10,000. Protein was determined (8.875 mg/mL) (Coomasie blue, Bio-Rad Laboratories) and diluted accordingly to adjust the inoculating dose (0.4 mg) in 0.05 mL.

BCG was diluted in sterile physiological saline to 0.2 mg in 0.1 mL for the primary inoculating dose. For each of the two subsequent booster doses, it was diluted to 0.01 mg in 0.1 mL.

KLV and BCG were thoroughly mixed prior to inoculating the squirrel monkeys (Group 1). Each animal received 0.15 mL of BCG+KLV intradermally using a tuberculin needle (27 G) and syringe on the shaven upper scapular crest. The first dose was given on the right side, and each of the subsequent booster doses (at 4 week intervals), on the left side.

KLV (USAID).

KLV was thoroughly mixed prior to inoculating the squirrel monkeys (Group 2). Each animal received 0.2 mL of KLV by deep intramuscular inoculation using a tuberculin needle (25 G) and syringe on the thigh of the leg. The first dose was given on the right leg, and each of the subsequent booster doses (at 1 week intervals), on the left side.

BCG only (TDR/WHO).

BCG was diluted in sterile physiological saline to 0.2 mg in 0.15 mL for the primary inoculating dose. For each of the two subsequent booster doses, it was diluted to 0.01 mg in 0.15 mL. Animals (Group 3) were inoculated as described before.

c. Reactions to vaccination with BCG+KLV and BCG alone.

The reactions to the primary inoculation of BCG+KLV and BCG alone included the formation of an ulcerated nodular lesion and right axillary adenopathy. The mean lesion area (mm^2) was greater at 4 weeks post vaccination (WPV) (BCG+KLV = 50.18; BCG = 29.43), and decreasing subsequently at 8 WPV (BCG+KLV = 30.35; BCG = 5.71), 12 WPV (BCG+KLV = 12.52; BCG = 0.00), with healing and scar formation at 16 WPV (Figure I.7). Ulcerated nodules and right axillary adenopathy were observed in all six animals of Group 1 (BCG+KLV), but in only four of Group 3 (BCG only).

The reactions to the first booster inoculation of BCG+KLV and BCG alone included the formation of an ulcerated nodular lesion and left axillary adenopathy, but to a much lesser degree than the primary inoculation. The mean lesion area (mm^2) was greater at 4 WPV (BCG+KLV = 35.11; BCG = 8.12), and decreasing subsequently at 8 WPV (BCG+KLV = 33.99; BCG = 1.57), with healing and scar formation at 12 WPV. Ulcerated nodules were observed in five animals and left axillary adenopathy in only one of Group 1 (BCG+KLV); ulcerated nodules were observed in two animals and left axillary adenopathy in only one of Group 3 (BCG only).

The reactions to the second booster inoculation of BCG+KLV and BCG alone were minimal, where only five animals of Group 1 (BCG+KLV) developed a small scar (mean lesion area, mm^2) at 4 WPV was 6.62. No reactions were observed in all animals of Group 3 (BCG only).

vi. Challenge inoculation of vaccinated and control monkeys with $2x10^7$ promastigotes each at the tip of the nose and at the dorsal base of the tail:

Squirrel monkeys (Groups 1 - 4) were each inoculated intradermally at the tip of the nose (TN) and at the shaven dorsal base of the tail (DBT) with $2x10^7$ live promastigotes of *L. (V.) panamensis*, as described before, 4 wk after the last booster inoculation of vaccinated animals. Evaluation of lesion development was done at 4 - 5 wk intervals during 112 wk, when the experiment was terminated.

a. Development of cutaneous lesions.

A. Tip of the nose: Leishmanial lesions developed in all monkeys from the four groups but differences in the rates of appearance, disappearance and type of lesion were observed.

Group 1. BCG+KLV: A primary nodule developed at 2 weeks post challenge inoculation (WPCI) in all monkeys, reaching maximum size (group mean = 31.10 mm^2) at 4 WPCI and started to regress (group mean = 20.71 mm^2) until 23 WPCI. The primary lesion persisted for 112 WPCI in two animals, 69 WPCI in two, 18 WPCI in one, and 14 WPCI in another one; ulceration of the primary nodule was observed in 5 monkeys from 4 - 18 WPCI and scar formation in 4 monkeys from 13 up to 41 WPCI. One monkey developed a very large cutaneous lesions (= "tapir nose"?) and satellite lesions in four. The mean total lesion area is illustrated in Figure I.8A.1. Photographs of monkeys # 175 and # 218 who still had leishmanial lesions at 112 WPCI are shown in Appendix I.1.A1.

Group 2. KLV: A primary nodule developed at 2 WPCI in only one monkey, and in all monkeys reaching maximum size (group mean = 43.23 mm^2) at 7 WPCI and started to regress (group mean = 26.83 mm^2) until 75 WPCI. The primary lesion persisted for 112 WPCI in two animals, 69 WPCI in two, 18 WPCI in one, and 14 WPCI in another one. Ulceration of the primary nodule was observed in 3 monkeys from 7 - 13 WPCI and scar formation in 3 monkeys from 14 up to 41 WPCI. Three monkeys developed very large cutaneous lesions (= "tapir nose"?) and satellite lesions in four. The mean total lesion area is illustrated in Figure I.8A.2. Photographs of monkeys # 116, # 141 and # 231 who still had leishmanial lesions at 112 WPCI are shown in Appendix I.1.A2.

Group 3. BCG only: A primary nodule developed at 2 WPCI in all monkeys, reaching maximum size (group mean = 29.94 mm^2) at 7 WPCI and started to regress (group mean = 17.27 mm^2) at 18 WPCI. The primary lesion persisted for 112 WPCI in two animals, 75 WPCI in one, 44 WPCI in one, 13 WPCI in one, and 7 WPCI in another one. Ulceration of the primary nodule was observed in 3 monkeys from 4 - 18 WPCI and scar formation in 3 monkeys from 14 up to 33 WPCI. One monkey developed a very large cutaneous lesions (= "tapir nose"?) and satellite lesions in three. The mean total lesion area is illustrated in Figure I.8A.3. Photographs of monkeys # 169 and # 108 who still had leishmanial lesions at 112 WPCI are shown in Appendix I.1.A3.

Group 4. Unvaccinated challenge control: A primary nodule developed at 2 WPCI in 2 monkeys, and in all monkeys at 4 WPCI, reaching maximum size (group mean = 50.73 mm^2) at 7 WPCI and started to regress (group mean = 21.15 mm^2) at 75 WPCI. The primary lesion persisted for 112 WPCI in two animals and 69 WPCI in another one [one animal who had lesions died at 9 WPCI]. Ulceration of the primary nodule was observed in 2 monkeys from 4 - 7 WPCI. One monkey developed a very large lesion (= "tapir nose"?) and satellite lesions in two. The mean total lesion area is illustrated in

Figure I.8A.4. Photographs of monkeys # 224 and # 096 who still had leishmanial lesions at 112 WPCI are shown in Appendix I.1.A4.

Group 5. Unvaccinated unchallenged control: Photographs of monkeys # 151 and # 142 who did not have any leishmanial lesions are shown in Appendix I.1.A5.

B. Dorsal base of the tail: Leishmanial lesions developed in all monkeys from the four groups but differences in the rates of appearance, disappearance and type of lesion were observed.

Group 1. BCG+KLV: A primary nodule developed at 2 weeks post challenge inoculation (WPCI) in all monkeys, reaching maximum size (group mean = 39.19 mm^2) at 7 WPCI and started to regress (group mean = 16.24 mm^2) at 14 WPCI. The primary lesion persisted for 14 WPCI in one animal, 13 WPCI in four, and 7 WPCI in another one. Ulceration of the primary nodule was observed in all monkeys from 2 - 13 WPCI. Three monkeys developed satellite lesions. The mean total lesion area is illustrated in Figure I.8B.1.

Group 2. KLV: A primary nodule developed at 2 WPCI in two monkeys, in all monkeys at 4 - 5 WPCI, reaching maximum size (group mean = 37.61 mm^2) at 9 WPCI and started to regress (group mean = 21.65 mm^2) at 18 WPCI. The primary lesion persisted for 14 WPCI in two animals, 13 WPCI in two, 9 WPCI in one, and 7 WPCI in another one. Ulceration of the primary nodule was observed in all monkeys from 2 - 13 WPCI. All monkeys developed satellite lesions. The mean total lesion area is illustrated in Figure I.8B.2.

Group 3. BCG only: A primary nodule developed at 2 WPCI in 3 monkeys, in all at 4 WPCI, reaching maximum size (group mean = 49.04 mm^2) at 7 WPCI and started to regress (group mean = 20.59 mm^2) at 23 WPCI. The primary lesion persisted for 41 WPCI in one animal, 18 WPCI in one, 14 WPCI in one, 13 WPCI in two, and 7 WPCI in another one. Ulceration of the primary nodule was observed in all monkeys from 4 - 18 WPCI. Five monkeys developed satellite lesions. The mean total lesion area is illustrated in Figure I.8B.3.

Group 4. Unvaccinated challenge control: A primary nodule developed at 2 WPCI in 3 monkeys, and in all monkeys at 4 WPCI, reaching maximum size (group mean = 46.31 mm^2) at 9 WPCI and started to regress (group mean = 41.76 mm^2) at 23 WPCI. The primary lesion persisted for 23 WPCI in two animals and 9 WPCI in two [one animal who had lesions died at 9 WPCI]. Ulceration of the primary nodule was observed in 3 monkeys from 7 - 14 WPCI. Three monkeys developed satellite lesions. The mean total lesion area is illustrated in Figure I.8B.4.

Needle aspirates from cutaneous lesions.

Needle aspirates from cutaneous lesions to isolate *Leishmania* parasites was not routinely done in order to not to disrupt the natural development of the lesions. At 75 WPCI, however, aspirates and cultures were done in seven monkeys who had nodular lesions at the TN [Group 1: # 175 and # 218; Group 2: # 116 and # 231; Group 3: # 166 and # 169; and Group 4: # 224]. Needle aspirates from each monkey were placed in Schenider's Drosophila medium. Twenty days afterwards the cultures were positive and viable promastigotes were present from the aspirates of three monkeys [# 231, # 169, and # 224]. Unfortunately, the rest of the cultures became contaminated with yeasts and it was difficult to ascertain the presence of promastigotes.

These results demonstrated that $2x10^7$ *L. (V.) panamensis* promastigotes were able to induce typical cutaneous leishmanial lesions at the tip of the nose (TN) and at the dorsal base of the tail (DBT) in all vaccinated

(BCG+KLV, KLV), BCG alone, and unvaccinated squirrel monkeys. The progression, regression and type of cutaneous lesions were similar to the monkeys previously infected to determine the dose inocula. Cutaneous lesions persisted for longer time, however, when the squirrel monkeys were inoculated at the TN than at the DBT.

A bimodal-type of lesion development was observed at the TN in the monkeys vaccinated with BCG+KLV and KLV, where a rapid progression of cutaneous lesions was observed between 7 and 14 WPCI, regressing thereafter to a minimal lesion size by 42 - 52 WPCI, but progressing again in some monkeys until 112 WPCI (Figures I.8.A1 and I.8.A2); in contrast, lesion development in the monkeys vaccinated with BCG only and the unvaccinated challenge-infected monkeys progressed rapidly between 7 and 14 WPCI, moderately regressed thereafter until 52 - 75 WPCI but persisted in some monkeys until 112 WPCI (Figures I.8.A3 and I.8.A4). A similar behavior of lesion development was observed at the DBT, but typical nodular lesions had regressed between 42 - 75 WPCI in all animals from the four groups experimentally infected (Figures I.8.B1 to I.8.B4).

$2x10^7$ inoculum in golden hamsters.

Twenty one male golden hamsters were each simultaneously inoculated at the dorsal base of the tail, in parallel with the inoculating dose of the squirrel monkeys. In 81.25% of the animals a typical leishmanial nodular lesion developed as early as 1 WPCI, and in all animals (100%) at 4 WPCI, reaching maximum size (56.25 mm^2) at 9 WPCI, when the experiment was terminated (Figure I.9).

b. Assessment of vaccine induced protection.

To determine the capacity of the tested vaccines to induce protection and to make comparisons between the groups, with BCG alone and the unvaccinated challenge-control animals, the development of cutaneous nodules only was considered since the nodule is the typical leishmanial lesion. Moreover, since differences were also observed in the development, progression and regression of cutaneous lesions at the TN and at the DBT, comparisons were made by grouping the results from the two inoculated sites together and by analyzing each inoculated site separately, for each group of animals.

Combined development of cutaneous nodules at both the tip of the nose plus at the dorsal base of the tail of vaccinated and unvaccinated monkeys.

At first, an apparent difference was observed in the development, progression and regression of primary nodules in the BCG+KLV monkeys (Group 1), at both sites of inoculation, where the mean total nodule area at the TN plus at the DBT was larger (19 - 32 mm^2) initially (at 2 - 4 WPCI) but it then became smaller (17 - 22 mm^2) in size (at 9 - 13 WPCI) than that of the other groups of animals (Group 3: 24 - 26 mm^2; Group 2: 31 - 38 mm^2; Group 4: 30 - 44 mm^2). In addition, ulceration of the nodule occurred more frequently and appeared earlier in the BCG+KLV group, probably indicating a faster inducing healing process. More weeks of observation on the course of infection were needed, however, to determine if protective immunity was induced or not in the vaccinated groups, or if the $2x10^7$ inoculum was able to break immunity. The mean total nodule area of monkeys with BCG+KLV (Group 1) was smaller than that of the unvaccinated challenge-control (Group 4) ($p<0.05$, Mann Whitney U - Wilcoxon Rank Sum W Test) from 13 to 52 WPCI. In the monkeys vaccinated with BCG only (Group 3), from 13 to 23 WPCI only ($p<0.05$), and for those with KLV (Group 2) from 23 to 33 WPCI only ($p<0.05$). Significant differences in mean total nodule size were also observed between monkeys in Groups 1 and 3, from 9 - 18 WPCI and from 41 - 52 WPCI ($p<0.05$); no mayor differences ($p>0.05$) were observed, however, between each of the other groups. The smallest mean total nodule size was present at 52 WPCI (Group 1: 0.48 mm^2; Group 3: 5.78 mm^2; Group 4: 11.36 mm^2; Group 2: 14.67 mm^2). Thereafter, the mean total nodule size

was rather the same in all groups (2 - 14 mm^2) from 62 to 112 WPCI, where lesions developed similarly in the BCG+KLV, KLV, BCG only, and unvaccinated challenge-control monkeys (Figure I.10.1).

To determine the magnitude of vaccine induced protection, the percent nodule development was calculated for each group of vaccinated animals by relating the mean total nodule area of the vaccinated (V) group to that of the unvaccinated challenge-control (UC) group, by the following formulae:

$$\text{percent (\%) nodule development} = \frac{\text{mean total nodule area V - mean total nodule area UC}}{\text{mean total nodule area UC}} \times 100$$

As illustrated in Figure I.10.2, an unexpectedly higher development of cutaneous nodules occurred in monkeys vaccinated with BCG+KLV (Group 1), from 2 - 4 WPCI, being 69 to 173 % higher than the UC (Group 4); similarly, monkeys vaccinated with BCG alone (Group 3) had also an increased nodule development, 24 to 33 % higher, while monkeys vaccinated with KLV (Group 2) manifested a reduced nodule development, 16 to 61 % lower, for the same period, as compared to the UC. As time progressed, the nodules in monkeys from Group 1 became smaller, being 10 to 96 % less than the UC, from 5 to 52 WPCI; similarly, nodules in monkeys from Group 3 were 11 to 60 % and in monkeys from Group 2, 21 to 79 % less than the UC. Thereafter, the percent nodule development was rather similar between the vaccinated groups, from 62 to 112 WPCI, being between 22 and 70 % less than the UC. At the end of the experiment, however, a reversion was observed for monkeys from Group 1, where the mean total nodule area was 42 % higher than the UC, while that for monkeys from Groups 3 and 2 remained reduced to 52 and 56 % less, respectively.

Independent development of cutaneous nodules at the tip of the nose and at the dorsal base of the tail of vaccinated and unvaccinated monkeys.

A more clearly and distinct development, progression and regression of the mean total nodule area for each of the vaccinated and unvaccinated challenge-exposed groups, at the TN (Figure I.10.1.A) or at the DBT (Figure I.10.1.B); and the percent nodule development of vaccinated groups in relation to the unvaccinated challenge-control, at the TN (Figure I.10.2.A) or at the DBT (Figure I.10.2.B), is illustrated. A summary of the mean (± SD) total nodule area, % nodule development, and % of monkeys with cutaneous nodules, for each group at selected WPCI, is presented in Table I.I.A for the TN and in Table I.I.B for the DBT. The mean duration of cutaneous leishmanial lesions at the TN was 47 (± 45) wk for the BCG+KLV monkeys, 54 (± 46) wk for the BCG alone, 65 (± 39) wk for the KLV alone, as compared to 97 (± 20) wk of the UC; at the DBT was 11 (± 2) wk for the BCG+KLV monkeys, 15 (± 11) wk for the BCG alone, 11 (± 3) wk for the KLV alone, as compared to 17 (± 7) wk of the UC.

Vaccination of squirrel monkeys with BCG+KLV, KLV and BCG alone proved to be effective in reducing not only the size but also the development of experimentally induced cutaneous leishmanial lesions, the proportion of infected animals and the mean duration of cutaneous leishmanial lesions, as compared to the unvaccinated challenge-control animals. In spite that cutaneous leishmanial lesions developed and progressed at the two sites of inoculation in all vaccinated animals, BCG+KLV had greater capacity in inducing a protective response (only one third of infected animals at 44 WPCI), albeit not complete and sterilizing, for at least up to 52 WPCI at the TN, than in monkeys vaccinated with KLV or BCG alone, where two thirds and one-half, respectively, of animals were infected at 44 WPCI (Table I.I.A). Thereafter, however, cutaneous lesions persisted in some animals of all inoculated groups at 112 WPCI (when the experiment was terminated), where an equal proportion of infected animals were observed in the vaccinated groups [BCG+KLV, KLV and BCG alone], but were still lesser (one third to less than one half) than the unvaccinated challenge-control animals.

The bimodal-type cutaneous lesion development observed in the BCG+KLV and KLV vaccinated animals suggests that either the initial vaccination regime should be modified (eg., vaccine with higher protein concentration) or that another booster inoculation should be given later, at least, prior to 52 WPCI to maintain the protective status in the animals. Interestingly enough, cutaneous leishmanial lesions developed only at one half of the observed rate in the animals vaccinated with BCG alone, thus reflecting the immune potentiating capacity of BCG. Further trials should be done to improve on the vaccination regimes tested, based on this preliminary but promising results.

vii. Tests of immune response in vaccinated, challenge-infected monkeys:

a) Delayed-type hypersensitivity (DTH) response to tuberculin and leishmanin skin test antigens.

Tuberculin skin test antigen. Prior to infection all animals were negative on repeated tuberculin skin testing with 0.1 Ml of *Mycobacterium tuberculosis* old mammalian tuberculin (Coopers Animal Health Inc.). When animals from all Groups were skin tested on the right eyelid at 4 WPCI, only those in the BCG+KLV (Group 1) and BCG alone (Group 3) responded with a positive DTH reaction (Figure 10). This corresponded also to skin testing after 4 months of primary BCG+KLV or BCG alone inoculations. DTH reactions were classified as follows: 0 = no reaction; 1+ = slight erythema or edema; 2+ = erythema and edema; 3+ = marked erythema and edema, rough surface and exudate; and 4+ = eyelid closed shut with ulcer or necrosis. DTH reactions were manifested as early as 24 hours, although maximum reaction occurred at 48 to 72 hours (mean score reaction Group 1 = 2.2 and Group 3 = 1.9 at 72 hours). In 3 animals of each Group, a 3+ reaction score was recorded, while the rest manifested either a 1+ or 2+ individual score reaction.

Leishmanin skin test antigen. When animals from all groups were skin tested on the eyelid with 0.1 mL of leishmanin (*L. major*) skin test antigen (Pasteur Institute of Iran, lot 109) on the left eyelid at 4 WPCI, none responded. Skin testing was repeated with 0.1 mL of *L. (V.) panamensis* skin test antigen ($1x10^6$ promastigotes in 0.5% phenol-saline) at 6, 9, and 18 WPCI, where a summary of the results was as follows:

Group of monkeys	6 WPCI		9 WPCI		18 WPCI	
	% animals + ¶	mean score reaction *	% animals +	mean score reaction	% animals +	mean score reaction
BCG+KLV	66	0.5, 0.7	33	0.2, 0.1	83	0.8, 0.4
KLV	83	0.8, 0.5	33	0.3, 0.2	100	1.1, 0.5
BCG	83	0.7, 0.3	50	0.3, 0.1	83	1.1, 0.7
UCC	75	0.8, 0.5	66	0.3, 0	100	1.3, 0.7
normal	0	0, 0	0	0, 0	0	0, 0

¶ percent positive animals at 48 - 72 hs after skin testing;
* at 48 and 72 hs after skin testing.

b) Concentration-response curves for PHA and soluble *Leishmania* antigen (SLA) on Histopaque separated peripheral blood mononuclear cells (PBMNC); establishment of several preinfection baseline data; post vaccination and post challenge inoculation data.

Prior to vaccination and inducing experimental infections in the squirrel monkeys, concentration- response curves for PHA and SLA were elaborated on Histopaque separated peripheral blood mononuclear cells (PBMNC). After standardizing procedures for isolation and culture of PBMNC from 3 mL of monkey blood, cell recoveries were $\geq$ 93%, initial viabilities $\geq$ 94%, and after 6 days in culture $\geq$ 73%. Optimal final culture concentrations for PHA were 5 - 10 ug/mL (Figure I.12) with background counts of methyl-tritiated thymidine between 1374 to 18265 cpm for a total of four days of culture. No significant response was observed to SLA (2.5, 5, and 10 ug/mL) (Figure I.13) where background counts were between 455 to 6097 cpm for a total of six days of culture.

A whole-blood assay was tested using 100 uL of whole blood per well mixed with 100 uL of RPMI 1640 media supplemented with 15 % heat-inactivated human AB positive serum, and the appropriate concentrations of mitogen and soluble antigens. This test, however, did not produce significant response to PHA and SLA.

Response to phytohaemagglutining (PHA) mitogen during pre vaccination and post challenge inoculation trials.

Several baseline data were collected at 8 - 12 week intervals. Information was available for six pre vaccination times (April and November 1991, and January, April, June, and August 1992) since two additional points (June and August 1991) were lost due to power shortages in Guatemala and incubator failure.

Proliferative responses of individual squirrel monkeys's PBMNC to PHA (10 ug/mL) (expressed as net counts per minute) were varied over time, and major differences were observed between all animals, some of them being high and others low responders. Prior to vaccination, the group mean ($\pm$ SE) responses, however, were relatively similar ($\log_{10}$ net cpm = $\log_{10}$ mean stimulated - $\log_{10}$ mean unstimulated, ANOVA, $p > 0.05$) between groups (e.g., net cpm for June 1992: Group 1 = 115349 $\pm$ 29751; Group 2 = 150748 $\pm$ 21609; Group 3 = 131900 $\pm$ 13907; Group 4 = 170949 $\pm$ 47258; and Group 5 = 147860 $\pm$ 17317).

In addition, the mean responses to PHA (10 ug/mL) for each group over time were not significantly different during pre vaccination times (ANOVA, $P > 0.05$). After vaccination, however, BCG+ KLV (Group 1) and BCG alone (Group 3), had decreased (ANOVA, $p < 0.05$) responses one and two months after primary and first booster inoculations with BCG (Sep and Oct 1992), but were rapidly regained at higher levels (Nov 1992). Whether if BCG inoculation induced a transient immunosupression to PHA with a subsequent enhancement, cannot be completely assured, but in none of the other groups (KLV, unvaccinated challenge control, and uninoculated monkeys) this phenomenon was observed. After challenge infection, the response of PBMNC to PHA from each group of monkeys was evaluated on 13 different times, from 4 to 94 WPCI. The individual and group mean response was high but extremely varied where no significant differences could be determined, as illustrated for each group of monkeys in Figure I.14 A-E.

Response to soluble *Leishmania* antigen (SLA) during pre vaccination and post challenge inoculation trials.

Similarly, several baseline data were collected for response to SLA. Proliferative responses (expressed as stimulation indices, S.I.) of individual squirrel monkeys' PBMNC were varied over time, and major differences were observed between all animals, some of them being high and others low responders. Prior to vaccination, the group mean ($\pm$ SE) responses, however, were relatively similar ($\log_{10}$ S.I. = $\log_{10}$ mean stimulated / $\log_{10}$ mean unstimulated, ANOVA, $p > 0.05$) between groups (e.g., S.I. for June 1992: Group

1 = 0.83 ± 0.16; Group 2 = 0.85 ± 0.25; Group 3 = 1.18 ± 0.27; Group 4 = 1.22 ± 0.49; and Group 5 = 1.30 ± 0.41).

In addition, the mean responses to SLA (10 ug/mL) for each group over time were not significantly different during pre vaccination times, neither after post vaccination and challenge inoculation. After challenge infection, the response of PBMNC to SLA from each group of monkeys was evaluated on 13 different times, from 4 to 94 WPCI. The individual and group mean response was low but extremely varied where no significant differences could be determined, as illustrated for each group of monkeys in Figure I.15 A-E.

Response to other antigens after post challenge inoculation trials.

Other antigens, such as PPD (10 ug/mL) and SLO (streptolysin O) (1.25 ug/mL), and soluble L. donovani antigen (SLMA and MONGI) (5 ug/mL), were obtained from Dr. Thomas Nutman and Dr. Frank Neva, Laboratory of Parasitic Diseases, National Institutes of Health, Bethesda, MD. The results, however, to all of these antigens at various times tested were consistently low (S.I. < 2.5) although at some few occasions a positive and sizeable response was observed.

The major problem encountered during the evaluation of the *in vitro* cell-mediated immune response was the increasingly high response of PBMNC when incubated with media alone (control wells). Therefore, even if a high response was obtained when PBMNC were stimulated with any of the antigens tested, the resulting net counts per minute or the stimulation index were negative or low. Several approaches were done trying to correct for the high background of unstimulated cells, such as testing different batches of the human AB positive serum supplement or using the serum supplement at different concentrations (eg., 5-15%), but the background remained consistently high. Another possible explanation for the low *in vitro* response, in spite of the high background of unstimulated cells, could have been that there were not enough number of sensitized lymphocytes to *Leishmania* antigen in the isolated PBMNC when placed in the culture wells, during vaccination and challenge infection trials. Finally, other possibilities could have been that the assays were evaluating the peripheral immune response and not the *in situ* immune response, where the cutaneous leishmanial lesion developed, progressed and finally regressed in some monkeys, or that during the progression of cutaneous infection in the squirrel monkeys a state of immuno suppression could have been induced, thus reflected in the low or negative *in vitro* tests of cell mediated immune response. In a way, these results were comparable to the low or negative DTH response to leishmanin skin test antigen.

c) Tests for measuring squirrel monkey cytokines (Interferon-τ, IL-2, IL-4, IL-5 and GM-CSF) on supernatant from PBMNC stimulated with PHA, PMA-Ionomycin, PPD, and SLA (USAID supported project):

Culture supernatant of PBMNC alone, PHA and SLA stimulated were tested for the presence of released soluble cytokines, by collaboration from Dr. Thomas Nutman, LPD, NIH. IL-2 was measured in an IL-2 CTLL bioassay after thymidine incorporation; IFN-τ, IL-4, IL-5 and GM-CSF on ELISA based tests using monoclonal-specific capture and signal antibodies to human IFN-τ, IL-4, IL-5 and GM-CSF. In spite of testing supernatant from 20 different squirrel monkeys after vaccination and challenge infection, none or low levels of the cytokines were detected. An experiment was done where culture supernatant (as described above) and from PMA/IONOMYCIN, PPD and SLO stimulated PBMNC were included, to determine whether the low levels of cytokines measured were due to lack of cross reactivity between the monoclonal antibodies to human cytokines and squirrel monkey T-cell growth factors, or because the cytokines were not induced by the specific culture conditions. Unfortunately, these other tests were also negative, but soluble cytokines were detected on supernatant cultures of control PBMNC from uninfected human volunteers.

d) Western-blot analysis of squirrel monkey IgG antibodies to SLA under reducing conditions of SDS-PAGE electrophoresis, for several preinfection baseline and post vaccination and post challenge infection determinations (USAID supported project):

Western-blot analysis of squirrel monkey IgG antibodies [sera diluted 1:50] to SLA were done under reducing conditions of SDS-PAGE electrophoresis, for preinfection baseline and several post vaccination and post challenge infection determinations. Consistently, there were none or very few week bands recognized in all uninfected monkeys, whereas a strong response to a major 87, 79, 75, 41-48, 34-38, 26, and 20-21 kDa antigens of *Leishmania* were observed after vaccination or challenge infection with promastigotes; other bands in the 50 to 80 kDa range were also recognized. An illustration of the molecular weight bands recognized by sera from monkeys in all groups, at selected times, is shown in Figure I.16 A-F. At preinfection (-24 WPCI) none or very few weeks bands were detected, demonstrating only some degree of cross reaction to constitutive natural antigens (Figure I.16.A); after vaccination of the corresponding groups of monkeys but prior to challenge infection (time 0 WPCI), 5/6 monkeys in the BCG+KLV group, 5/6 monkeys in the KLV group, 4/6 monkeys in the BCG group, and none in the unvaccinated challenged and unchallenged control monkeys recognized any distinct major bands (Figure I.16 B); thereafter, at 9, 18, 44 and 75 WPCI, the majority of the vaccinated as well as the unvaccinated challenge control monkeys recognized the molecular weight bands described before; none of the unvaccinated unchallenged control (normal) monkeys did recognize any significant bands at all times (Figure I.16 C-F). Interestingly, some bands appeared or disappeared or increased and decreased in intensity, as time progressed. A preliminary assessment of bands which disappeared as time progressed was done and apparently correlates with those monkeys in which the primary nodular lesion healed (or cured) faster, for example, monkey # 121 in the BCG+KLV group, monkey # 147 in the KLV group, monkey # 226 in the BCG group, and monkeys # 096 and # 103 in the unvaccinated challenge control group. Finally, some molecular weight proteins appeared to be specific to Leishmania antigens during infection and others to BCG after vaccination [these results are now being analyzed].

viii. Tests of immune response in non vaccinated, primary course of infection squirrel monkeys (Group 6) (USAID supported project).

Two squirrel monkeys were each inoculated with *L. (V.) panamensis* to determine the infective dose inoculum (see section iii).

Tuberculin and leishmanin skin test antigens. The two animals were negative to repeated skin testing with PPD and with leishmanin (*L. major*) skin test antigen (Pasteur Institute of Iran, lot 109), at 40 WPI. When *L. (V.) panamensis* skin test antigen was used at 41 WPI, a weak DTH response (score = I+) was observed in both animals ($1x10^6$ and $1x10^7$ inoculum) between 48 and 72 h.

PHA mitogen. The group mean average (± SE) response to PHA (10 ug/mL) over time remained relatively unchanged (ANOVA, $p>0.05$), except when initially tested the time in which the animals arrived at the Universidad del Valle de Guatemala (Apr /91), where the values were lower (78447 ± 12803 cpm), to those obtained afterwards (e.g., 274212 ± 27412 cpm) on repeated testing. The results of the S.I. for the two monkeys as a group average over time, before and after primary inoculation, are illustrated in Figure I.17 A.

Soluble leishmanial antigen (SLA). No significant response was observed in either animal to varying concentrations of SLA before inoculation with *L. (V.) panamensis*. Significant response (S.I. $\geq$ 2.5), however, were detected until 28 WPI in the monkey inoculated with $1x10^6$ promastigotes (e.g., S.I. = 5.7 for 10 ug/mL), persisting at 32 WPI (S.I. = 3.0) and subsequently increasing at 36 WPI (S.I. 8.7). For the monkey inoculated with $1x10^7$ promastigotes, a response was observed only at 28 WPI (S.I. = 3.9) but

later decreasing to S.I. = 1.6 and 1.9 at 32 and 36 WPI, respectively. A small response to 5 ug/mL (S.I. = 2.5), however, was observed at 32 WPI, but later decreased to a S.I. = 1.3. The results of the S.I. for the two monkeys as a group average over time, before and after primary inoculation, are illustrated in Figure I.17 B.

Response to other antigens. Similar results were obtained as described before for the other groups of monkeys.

Tests for measuring squirrel monkey cytokines (Interferon-τ, IL-2, IL-4, IL-5 and GM-CSF) on supernatant from PBMNC stimulated with PHA, PMA-Ionomycin, PPD, and SLA (USAID supported project). Similar results were obtained as described before for the other groups of monkeys.

Western-blot analysis of squirrel monkey IgG antibodies to SLA under reducing conditions of SDS-PAGE electrophoresis, for several preinfection baseline and post infection times (USAID supported project): Similar results were obtained as described before for the unvaccinated unchallenged control group of monkeys.

ix. Determination of normal hematological and biochemical parameters of squirrel monkeys, body weight and corporal dimensions, blood and intestinal parasites, and intestinal pathogenic bacteria.

During the course of the project determinations of normal parameters and periodic surveillance of the health status of the animals (as indicated before), was done on a routine basis, following established procedures.

x. Treatment of infected monkeys with antimonium salt of N-methyl glucamine.

At the end when the experiment was terminated at 112 WPCI, ten squirrel monkeys still had some indication of cutaneous leishmanial lesions at the tip of the nose, ranging from a slightly elevated nodule to a raised ulcerated nodule. For illustration, photographs of selected monkeys are provided in Appendix I. Figures I.A1 - A5. All ten monkeys were each treated with 30 mg/Kg/day [mg of total salt base, as recommended by Dr. Byron Arana, Medical Entomology Research and Training Unit/Guatemala, MERTU/G, CDC] for a total of five days via intramuscular inoculation of the antimonium salt of N-methyl glucamine (Glucantime®, Rhone-Poulenc Rorer). Twenty days after treatment was completed, the lesions had regressed significantly and scar formation was evident in those initially ulcerated.

IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

The findings from this Project will be useful in Guatemala and other developing countries to introduce prophylactic or vaccination procedures for American cutaneous leishmaniasis in human beings, especially when a crude killed vaccine is combined with BCG. Hopefully, the results will be used by other investigators in the region who are studying various combinations of vaccine induced protection in human beings, especially in clinical trials in Guatemala, Venezuela and Brazil. Larger scale trials in non-human primates and initial trials in human beings are urgently needed to validate the results derived from this Project.

The Project had a great impact on local human resource capability and development, infrastructure and laboratory build-up, and strengthening project management at the Center for Health Studies at the Universidad del Valle de Guatemala. Now that the Project is complete, there are many new capacities built up and left behind which were not present at the start of this Project. There is enough local expertise for implementing and measuring tests of immune response (e.g., SD-PAGE, immunoblotting, ELISA, cytokine measurement, delayed-type hypersensitivity, etc.). The next step will be to implement the molecular biology oriented assays, such as the polymerase chain reaction (PCR), in studies on tropical parasitic and infectious diseases. Moreover, these techniques have already been implemented, and an example of such effort is the recently concluded Seminar/Workshop on PCR techniques applied to diagnosis of infectious and tropical diseases in Central America, held in Guatemala from July 3-7, 1995.

The scientific capabilities of collaborating country scientists have also improved as result of this Project, where continuation of this and other projects on leishmaniasis has been possible with the support provided.

PROJECT ACTIVITIES/OUTPUTS

MEETINGS ATTENDED AND HELD FOR ENTIRE PROJECT

Feb 1989 *"Immunologic aspects of cutaneous leishmaniasis."* "VI Course on Actualization of Parasitic Diseases," Guatemalan Society of Parasitology and Tropical Medicine, Guatemala.

Jul 1989 *"Vector borne diseases: Leishmaniasis,"* in the Round Table "Vector Borne Diseases: Leishmaniasis, Malaria, and Onchocerciasis." II National Seminar on Laboratory Techniques and Medical Technology, Guatemala.

Aug 1989 *"Research activities in tropical diseases,"* in the Biology Seminar, Universidad del Valle de Guatemala, Guatemala.

May 1990 *"Comparison between extracts of Leishmania braziliensis braziliensis and L. mexicana mexicana by polyacrylamide gel electrophoresis (SDS-PAGE)."* III Latinamerican Congress of Tropical Medicine and IX National Congress of Parasitology, México City, México.

Jun 1990 *"Use of DNA probes for the identification of parasites."* I National Cytology Symposium, Guatemala.

Sep 1990 *"Cellular immune response to American cutaneous leishmaniasis in ma.' Guatemalan adults, before and after treatment."* In the Meeting on "Vaccines Against Leishmaniases," Universidade Federal Minas Gerais, Belo Horizonte, Brazil.

Temporary Adviser (IMM-FIELDLEISH/WHO) in the Meeting "Vaccines Against Leishmaniases," University of Minas Gerais, Belo Horizonte, Brazil.

Oct 1990 Observer in the Meeting "I Ordinary Meeting of the Regional Committee for Primatology in the Americas", (PAHO/WHO), Battelle Seattle Conference Center, Seattle, WA, U.S.A.

"Immunopathology of cutaneous leishmaniasis," in the Immunopathology Seminar, Facultad de Ciencias Químicas y Farmacia, Universidad de San Carlos de Guatemala, Guatemala.

Apr 1991 *"Comparison between proteinic extracts of Leishmania braziliensis braziliensis, L. b. panamensis and L. mexicana mexicana by polyacrylamide gel electrophoresis (SDS-PAGE)."* I Central American Congress of Parasitology and Tropical Medicine and II National Congress of Parasitology and Tropical Medicine, Guatemala, Guatemala.

Sep 1991 Country Representative (ONUDI/PNUD) in the Project "Massive Production of Monoclonal Antibodies," Centro de Biotecnología, Instituto Butantán, Sao Paulo, Brazil.

Nov 1991 *"Immunologic diagnosis of onchocerciasis and cutaneous leishmaniasis,"* in the Workshop "Update on diseases transmitted by vectors," XLII National Congress of Medicine, Guatemala.

Dec 1991 Participant in the Meeting "Program Planning: Field Stations," Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control, Atlanta, GA, U.S.A.

Jan 1992 Country Representative (Guatemala) in the European Economic Community (EEC) / Central American Seminar - Workshop "Parasitic Diseases of Major Prevalence and Transmitted by Vectors in Central America", Tegucigalpa, Honduras.

Participant in the Meeting "The Foundations for Scientific and Technologic Development in Central América," Instituto de Relaciones Europeo - Latino Americanos, Secretaría Permanente del Tratado General de Integración Económica Centroamericana (SIECA), Guatemala, Guatemala.

May 1993 *"Experimentally induced infections with Leishmania (Viannia) panamensis in the squirrel monkey (Saimiri boliviensis) and evaluation of the cell-mediated immune response."* IV Latinamerican Congress of Tropical Medicine (CLAMT), Guayaquil, Ecuador.

"Antibodies to Leishmania (Viannia) panamensis in Guatemalan patients, as measured by the indirect ELISA test." IV Latinamerican Congress of Tropical Medicine (CLAMT), Guayaquil, Ecuador.

Jun 1993 *"Current Situation of Leishmaniasis in Guatemala."* XI Latinamerican Congress of Clinical Biochemistry, Acapulco, México.

Aug 1994 *"Central American Network for Cooperation in Tropical Diseases (REDCEN),"* X Years Celebration of the Ibero American Program for Development in Science and Technology (CYTED), Guatemala, Guatemala.

HONORS/APPOINTMENTS:

Jun 1989 Recipient of the decoration *"Medalla de Mérito Intelectual"* by the President of the Republic of Guatemala and by the Guatemalan Ministry of Defense, for personal contributions as an author and co-author in the book entitled "Cutaneous Leishmaniasis in Guatemala," resulting from the studies and control of cutaneous leishmaniasis in military personnel, as a member of the Commission for the Study of Leishmaniasis in Guatemala, Guatemala.

Jul 1991 Co-Founder of the "Fundación Guatemalteca de Primatología", Zoológico Nacional "La Aurora", Guatemala.

Sep 1991-Jun 1993 Adjunct Associate Professor, Department of Tropical Medicine of the School of Public Health and Tropical Medicine, Tulane University Medical Center, Tulane University, New Orleans, Louisiana, USA.

TRAINING:

Jan 1989 Immuno-blot Techniques, Department of Parasitology, Walter Reed Army Institute of Research, Washington, DC, USA.

Dec 1992 Interleukin Assays, Laboratory of Parasitic Diseases, National Institutes of Health, Bethesda, MD, USA.

PUBLICATIONS - THESIS

Alfaro Altuve, Luis Gustavo. 1994. "Determinación de Anticuerpos a Leishmania (Viannia) panamensis por Medio del Método de ELISA Indirecto". Tésis de Licenciatura, Departamento de Bioquímica, Facultad de Ciencias y Humanidades, Universidad del Valle de Guatemala, Guatemala, C.A.

PUBLICATIONS - BOOK CHAPTERS

Luján, R. 1991. *Inmunología de la leishmaniásis cutánea*, pp. 29-33. In: "La Leishmaniásis Cutánea en Guatemala," T.R. Navin (editor), Rhone-Poulenc Rorer.

PUBLICATIONS - ABSTRACTS

Mury, M.A., Luján, R., Guzmán, G.E., and Arana, F. 1990. Comparison between extracts of *Leishmania braziliensis braziliensis* and *L. mexicana mexicana* by polyacrylamide gel electrophoresis (SDS-PAGE). III Latinamerican Congress of Tropical Medicine and IX National Congress of Parasitology, México City, México.

Mury, M.A., Luján, R., Guzmán, G.E., and Arana, F. 1991. Comparison between proteinic extracts of *Leishmania braziliensis braziliensis*, *L. b. panamensis* and *L. mexicana mexicana* by polyacrylamide gel electrophoresis (SDS-PAGE). I Central American Congress of Parasitology and Tropical Medicine and II National Congress of Parasitology and Tropical Medicine, Guatemala, Guatemala.

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Luján, R. 1993. Current situation of leishmaniasis in Guatemala. XI Latinamerican Congress of Clinical Biochemistry, Acapulco, México.

PROJECT PRODUCTIVITY

The Project did accomplish most of the proposed goals. Due to a series of unforeseeable problems, however, it took a much longer period of time for fulfilling the Project objectives and activities. For example, a delay in acquisition of the non-human primates from Peru, a longer than expected quarantine and recovery period, delays on acquiring BCG from the Guatemalan Ministry of Health, regular power shortages in the country which damaged the motor of the CO_2 incubator and caused loss of the lymphocyte cultures, frequent failure of the scintillation counter at the Dirección General de Energía y Minas, the lack of cross reactivity between the monoclonal antibodies to detect human cytokines and squirrel monkey cytokines, and many others which were mentioned during the management and yearly reports. In spite of these problems and the longer than needed time to finish, it can be said that the overall results of this Project were a success, especially the establishment of cutaneous leishmanial lesions at the tip of the nose of the squirrel monkeys, the bimodal-type of cutaneous lesion development in the vaccinated groups, and the partial protection obtained by BCG alone. In addition, the Western blot results demonstrated the recognition of some specific molecular weight proteins, detected only after vaccination and challenge infection, making them useful for specific diagnosis. The role of these antibodies on immune protection needs to be evaluated.

The Project did accomplish, in part, the expected relation and collaboration with the Israeli counterpart. The work and report submitted by Dr. Shoshana Frankenburg of the Haddasah University Hospital, The Hebrew University of Jerusalem, reflected the excellent work which they did in this Project. The cooperation could have been greater, however, if more effort in communicating would have been done between the two parties involved and if less unneeded delays on the mail system.

FUTURE WORK

The planned activities (without requesting additional funding) will be the determination of humoral (IgM and IgG) immune response by ELISA test and immunoblotting to *L. (V.) panamensis antigen* in plasma collected from the squirrel monkeys.

In addition, molecular biology techniques should be implemented to assist in the diagnosis of cutaneous leishmaniasis and for follow-up of cure, either after vaccination or drug therapy. Along these lines, a workshop on polymerase chain reaction (PCR) methodology has been planned for 3-7 July, 1995, for implementing PCR techniques in the diagnosis of infectious and parasitic diseases relevant in the Central American region.

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ATTACHMENTS

Tables I.I.A and I.I.B

Figures I.1 to I.17

Appendix I. Figures 1.A1 to 1.A5.

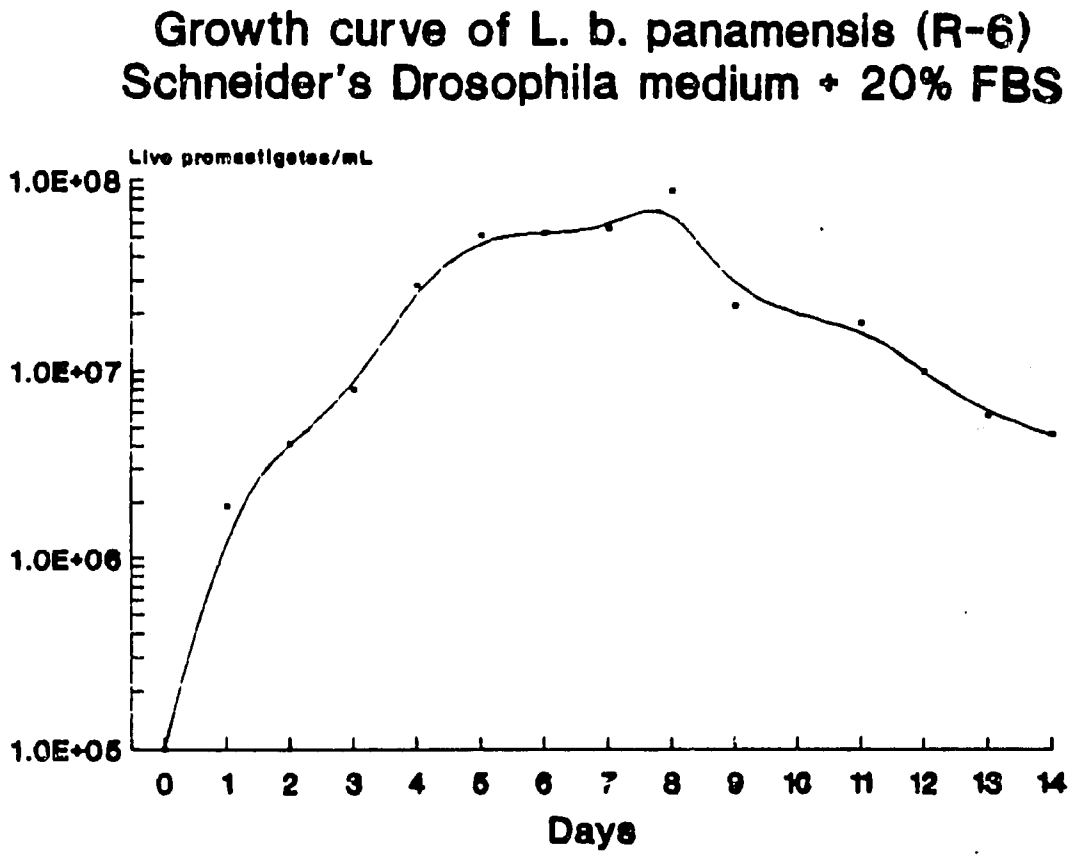


FIGURE I.1. Growth curve of live *Leishmania (Viannia) panamensis* (MHOM/PA/83/(WR539)) promastigotes per milliliter in Schneider's Drosophila medium supplemented with 20 % fetal bovine serum during 14 days of culture. Each point represents the mean of nine separate experiments.

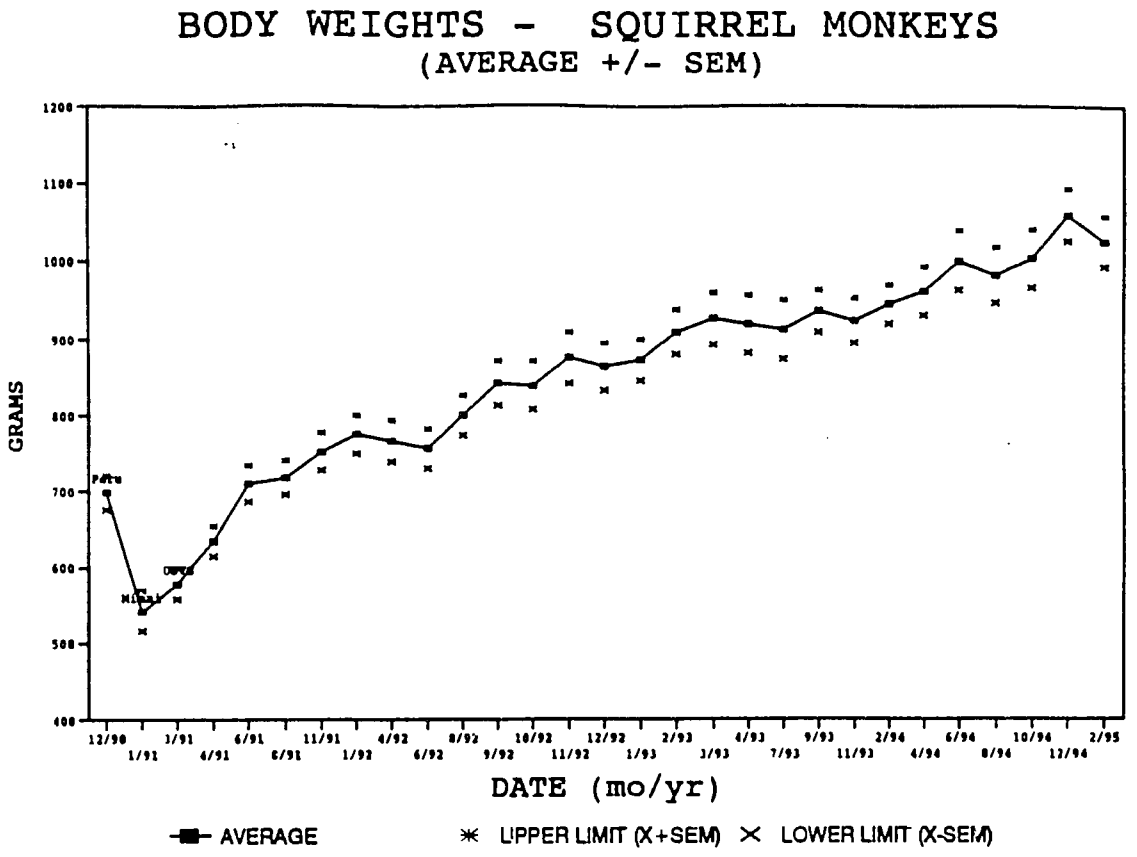
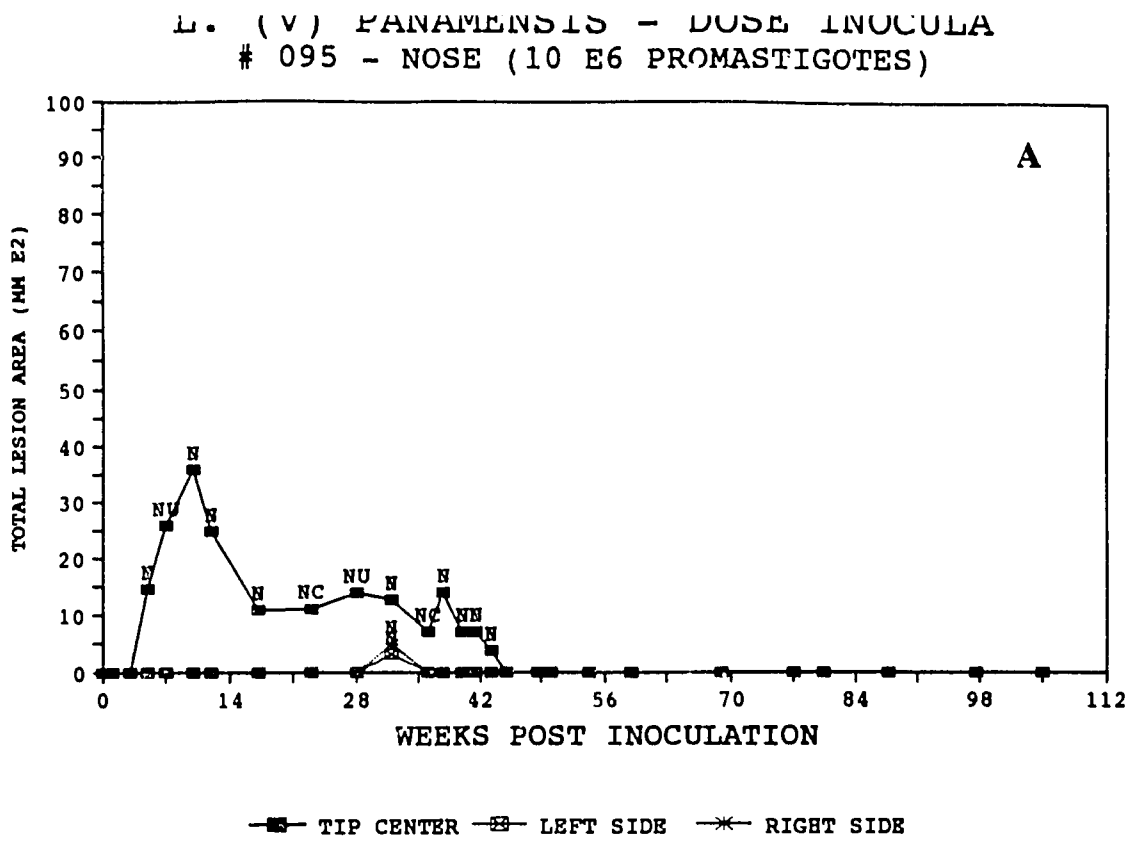


FIGURE I.2. Average body weights in grams of *Saimiri boliviensis* squirrel monkeys at the Universidad del Valle de Guatemala (UDVG). Peru = reported weights in Iquitos, Peru (12/'90); Miami = reported weights in Perrine Primate Center, USA (1/'91). Each point represents the mean (± SEM) of 25 - 29 squirrel monkeys during four years.



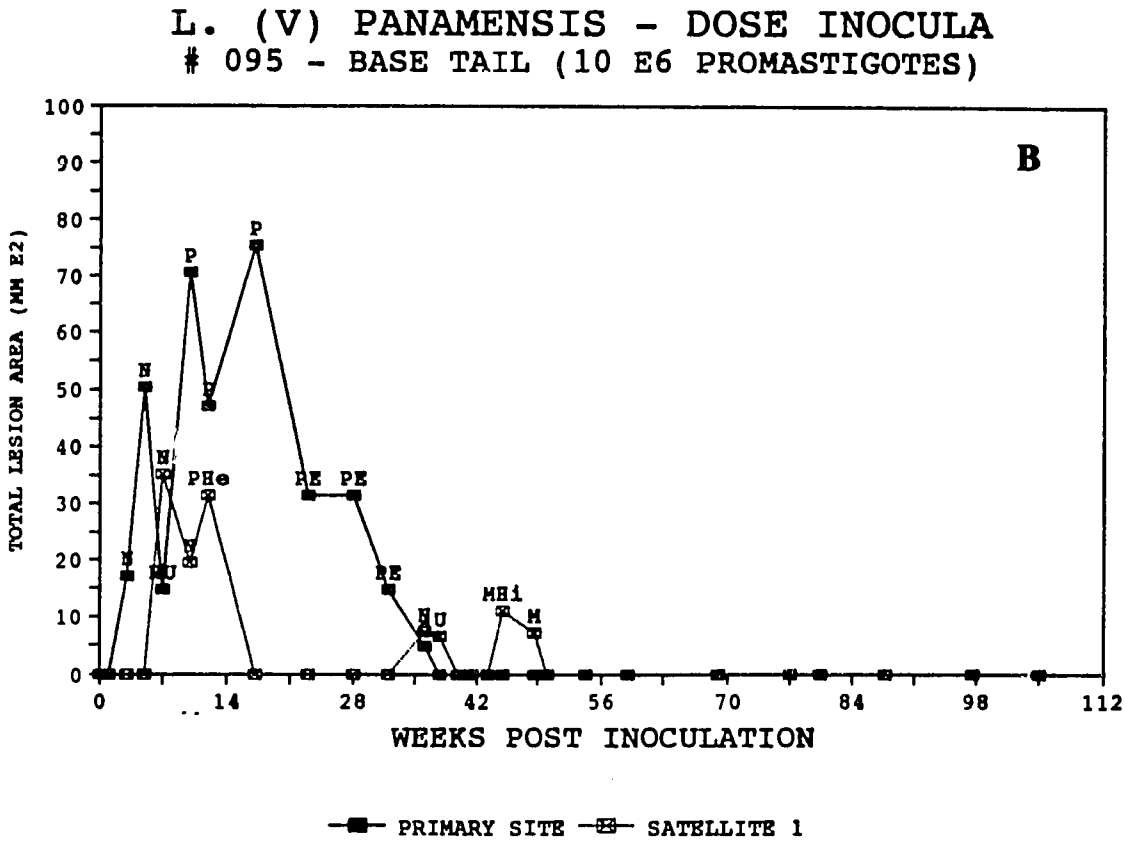
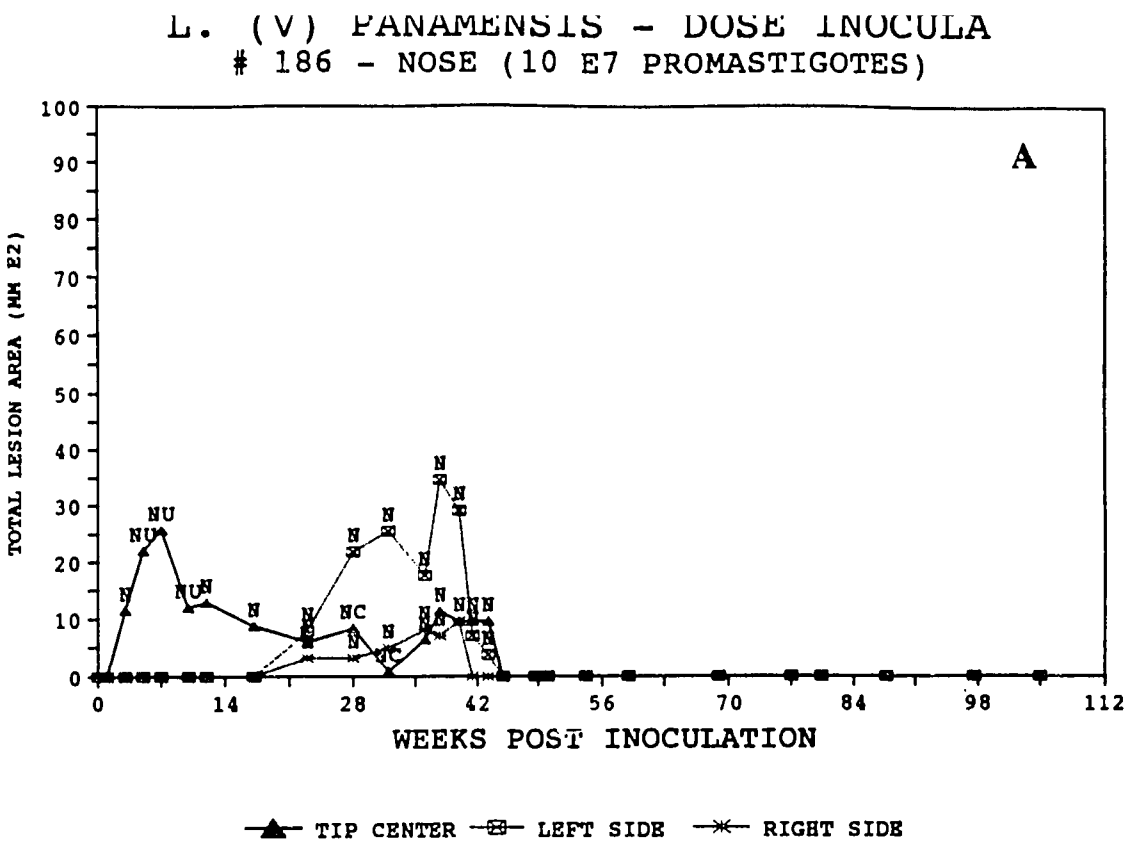


FIGURE I.3. Total area (mm^2) of experimental cutaneous leishmanial lesions in a squirrel monkey (44 wk) inoculated intradermally with $1x10^6$ promastigotes of *Leishmania (Viannia) panamensis*. (A) at the tip of the nose; (B) at the dorsal base of the tail. N = nodule; NU = ulcerated nodule; NC = scar; left side & right side = satellite lesions; P = plaque; He = hyperchromic; E = erythematous; U = ulcer; M = macula; Hi = hypochromic; satellite 1 = satellite lesions.



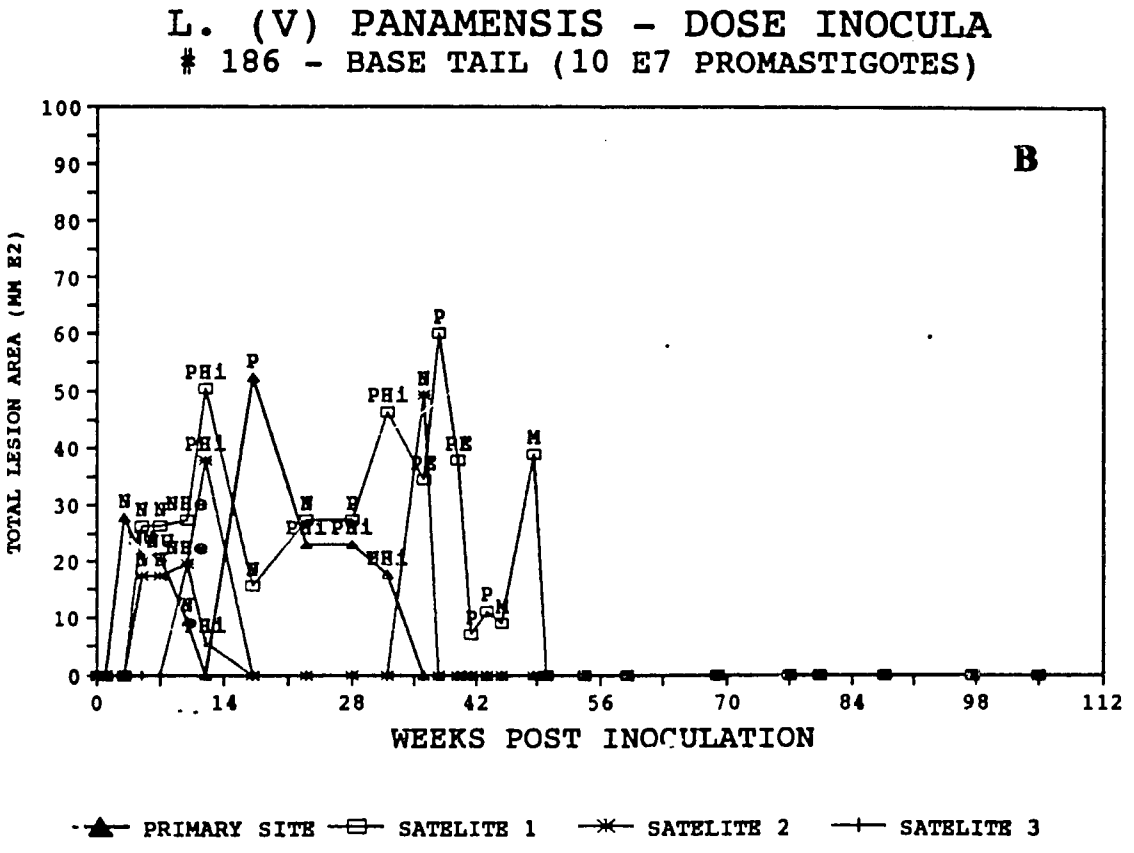
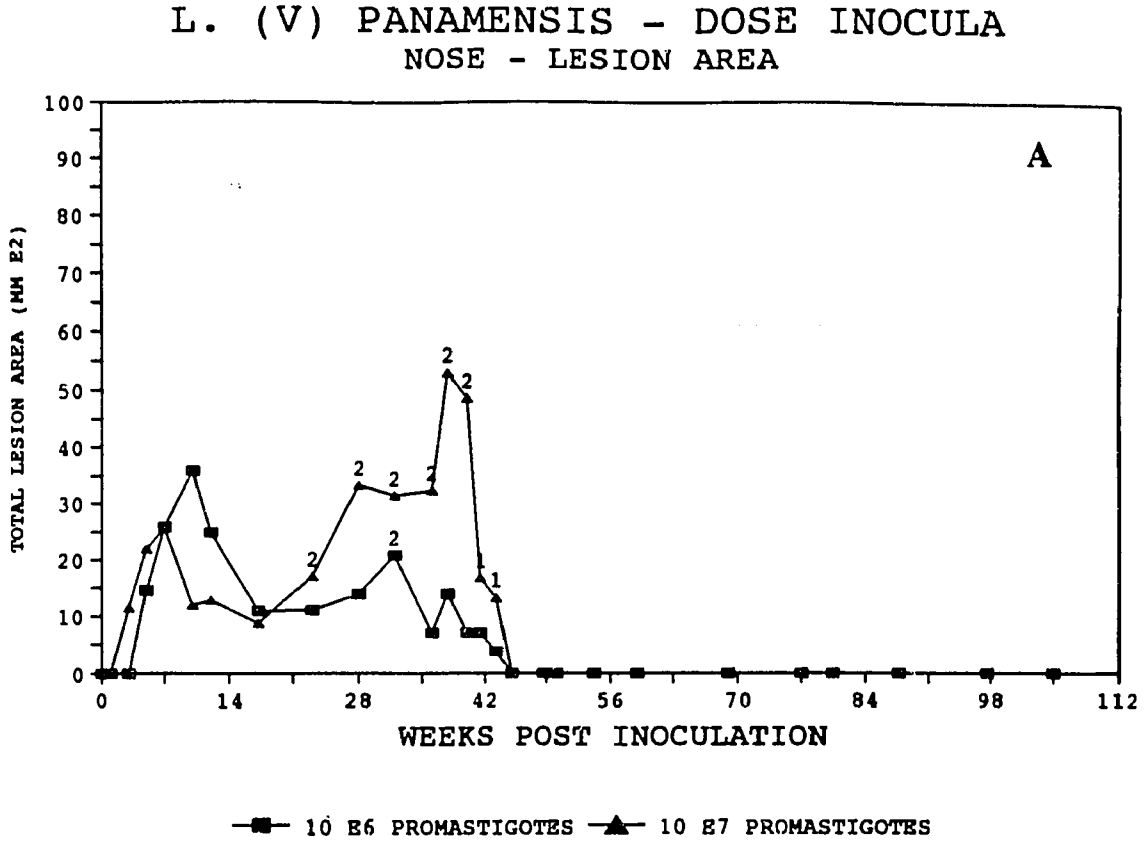


FIGURE I.4. Total area (mm^2) of experimental cutaneous leishmanial lesions in a squirrel monkey (44 wk) inoculated intradermally with $1x10^7$ promastigotes of *Leishmania (Viannia) panamensis*. (A) at the tip of the nose; (B) at the dorsal base of the tail. N = nodule; NU = ulcerated nodule; NC = scar; left side & right side = satellite lesions; P = plaque; He = hyperchromic; E = erythematous; U = ulcer; M = macula; Hi = hypochromic; satellite 1, 2, 3 = satellite lesions.



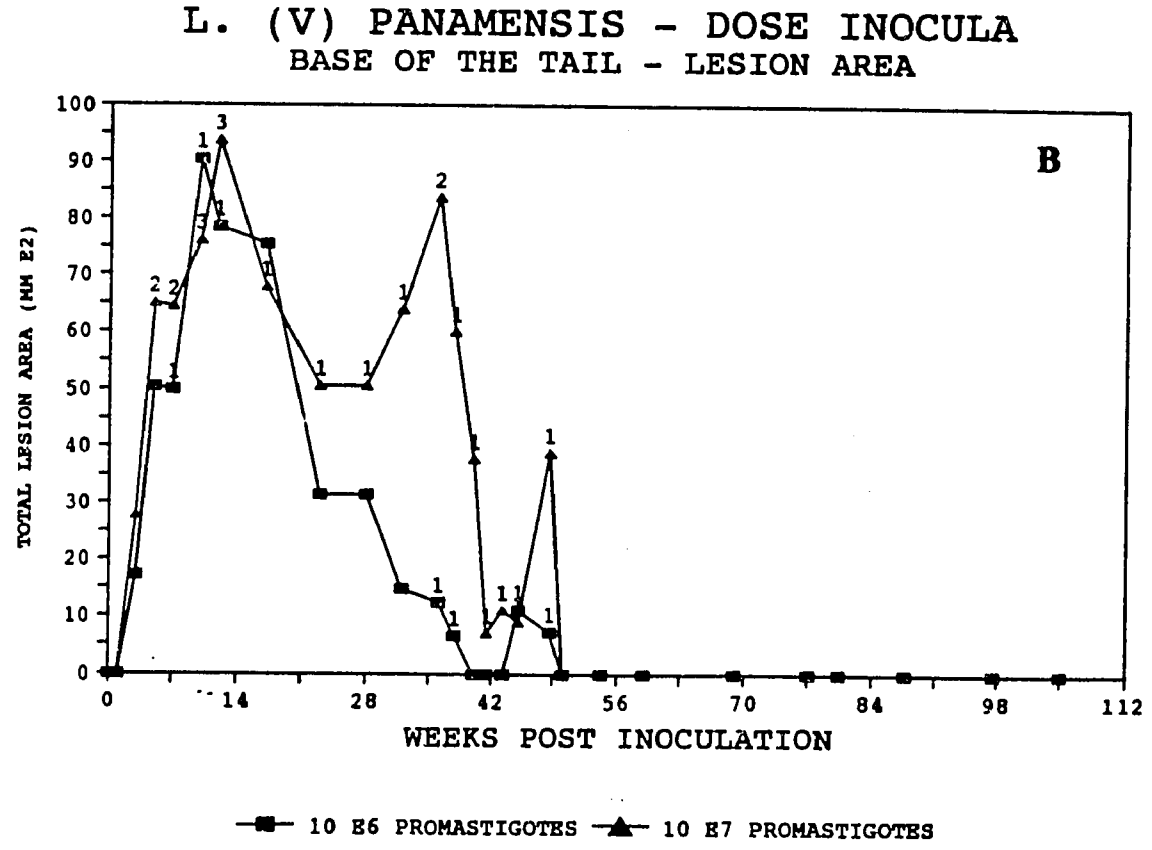


FIGURE I.5. Total area (mm^2) of progression and regression of primary and satellite lesions of experimental cutaneous leishmanial infections in squirrel monkeys (44 wk) inoculated intradermally with $1x10^6$ or $1x10^7$ promastigotes of *Leishmania (Viannia) panamensis*. *(A)* at the tip of the nose; (B) at the dorsal base of the tail. Superscript at each point indicates the number of satellite lesions.

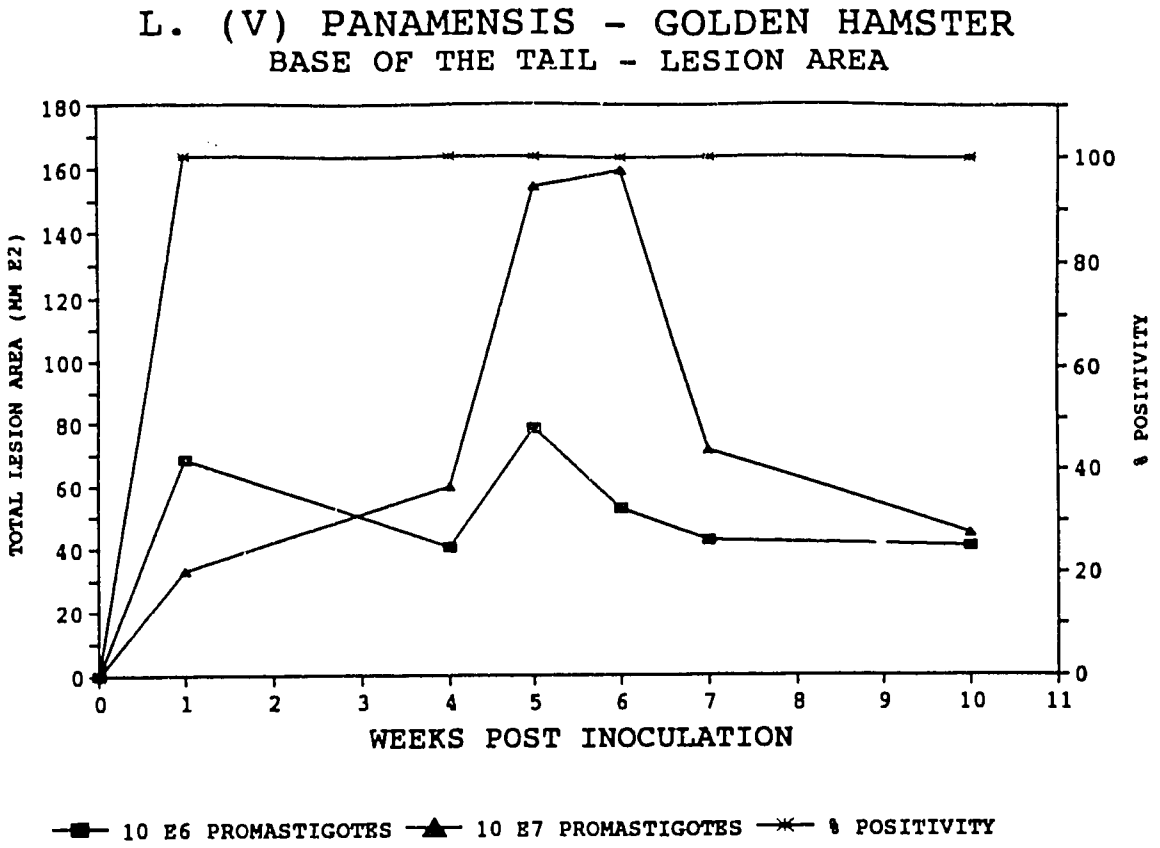


FIGURE I.6. Mean total area (mm^2) and percent positivity after experimental cutaneous leishmanial infections in hamsters (10 wk) inoculated intradermally with $1x10^6$ or $1x10^7$ promastigotes of *Leishmania (Viannia) panamensis* at the dorsal base of the tail, parallel to the primary infections induced in squirrel monkeys. Each point represents the mean of 11 hamsters for each group.

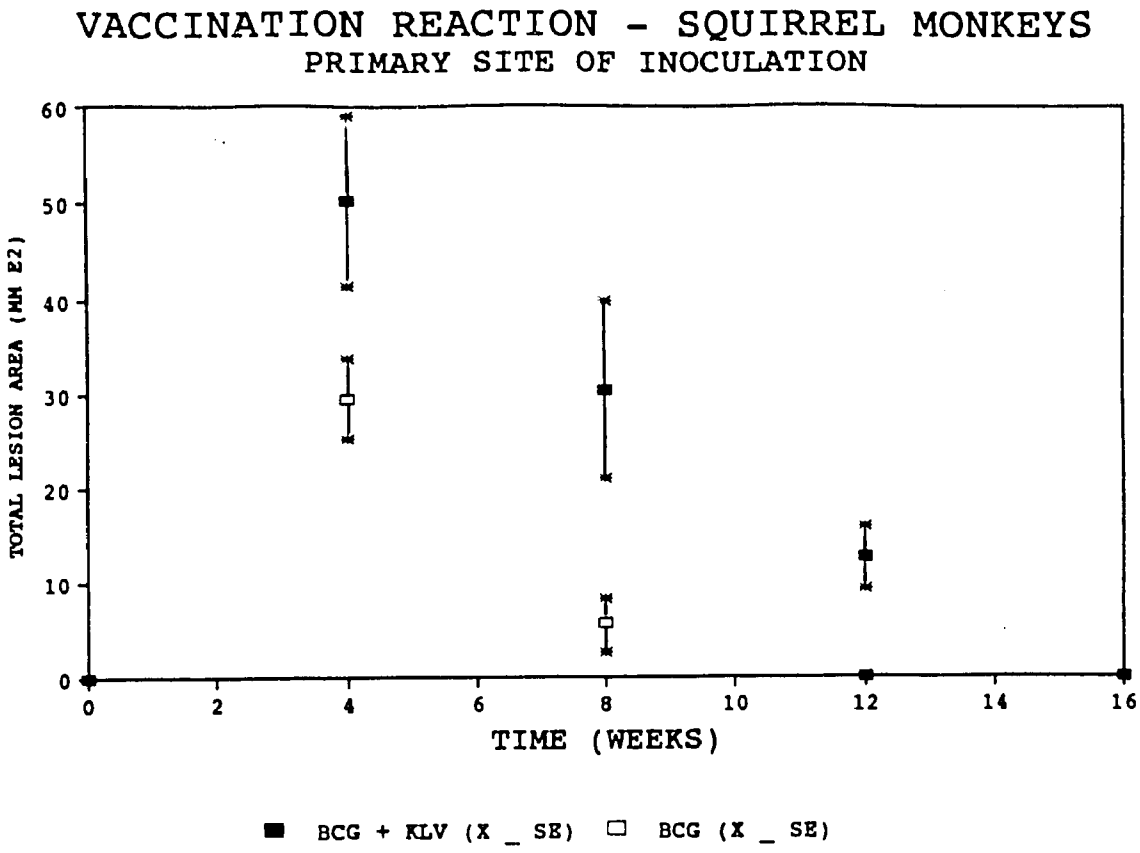
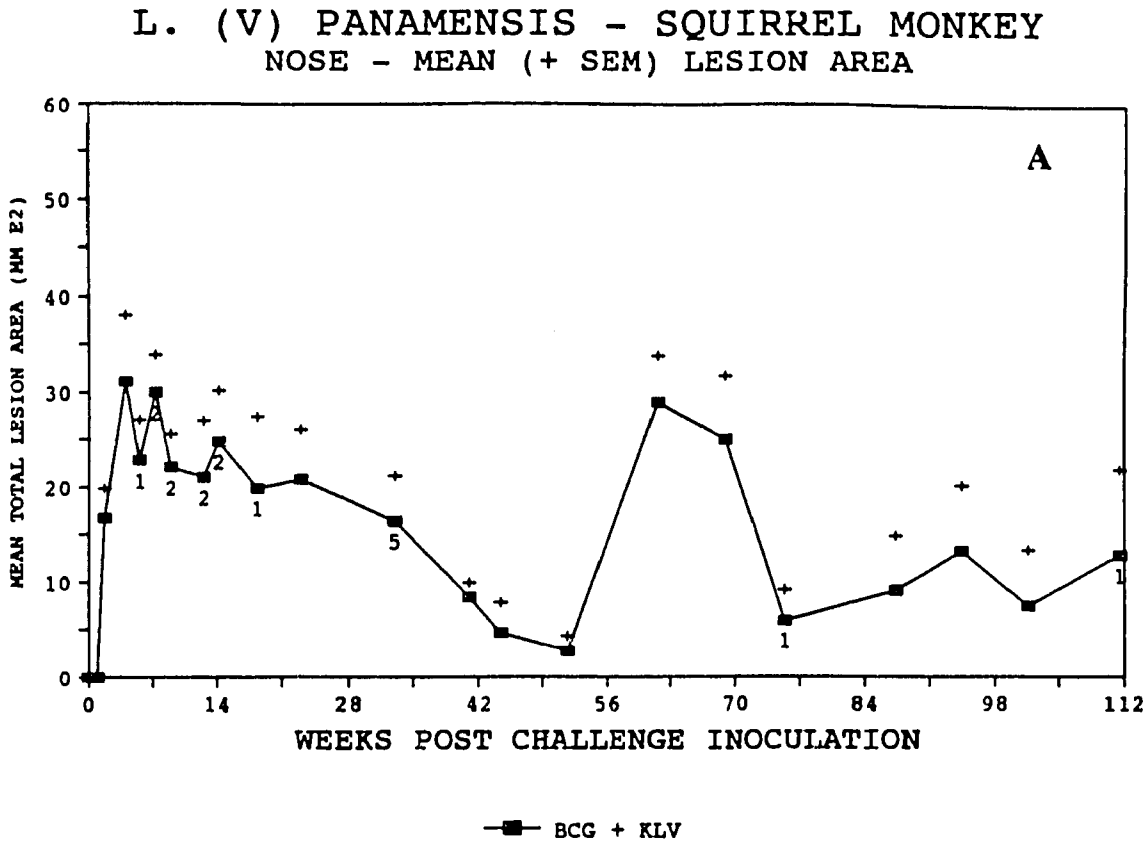


FIGURE I.7. Mean lesion area (mm^2) of cutaneous reaction at the site of primary vaccination (upper scapular crest) of squirrel monkeys (12 wk), after intradermal inoculation of BCG+KLV (KLV = "killed *Leishmania* vaccine") or BCG alone. Each inoculating dose consisted of BCG (200 ug) and/or KLV (400 ug) and lesions were measured at four week intervals. Each point represents the mean (± SEM) of six monkeys for each group.



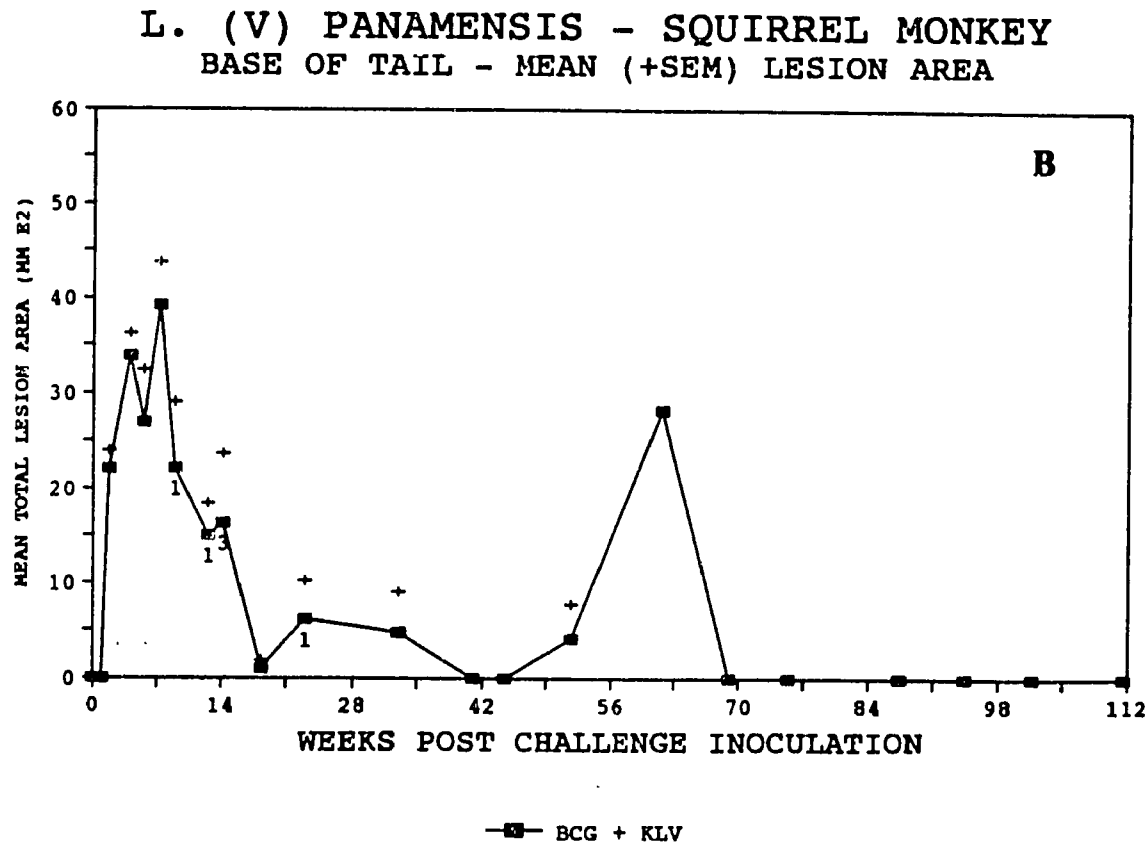
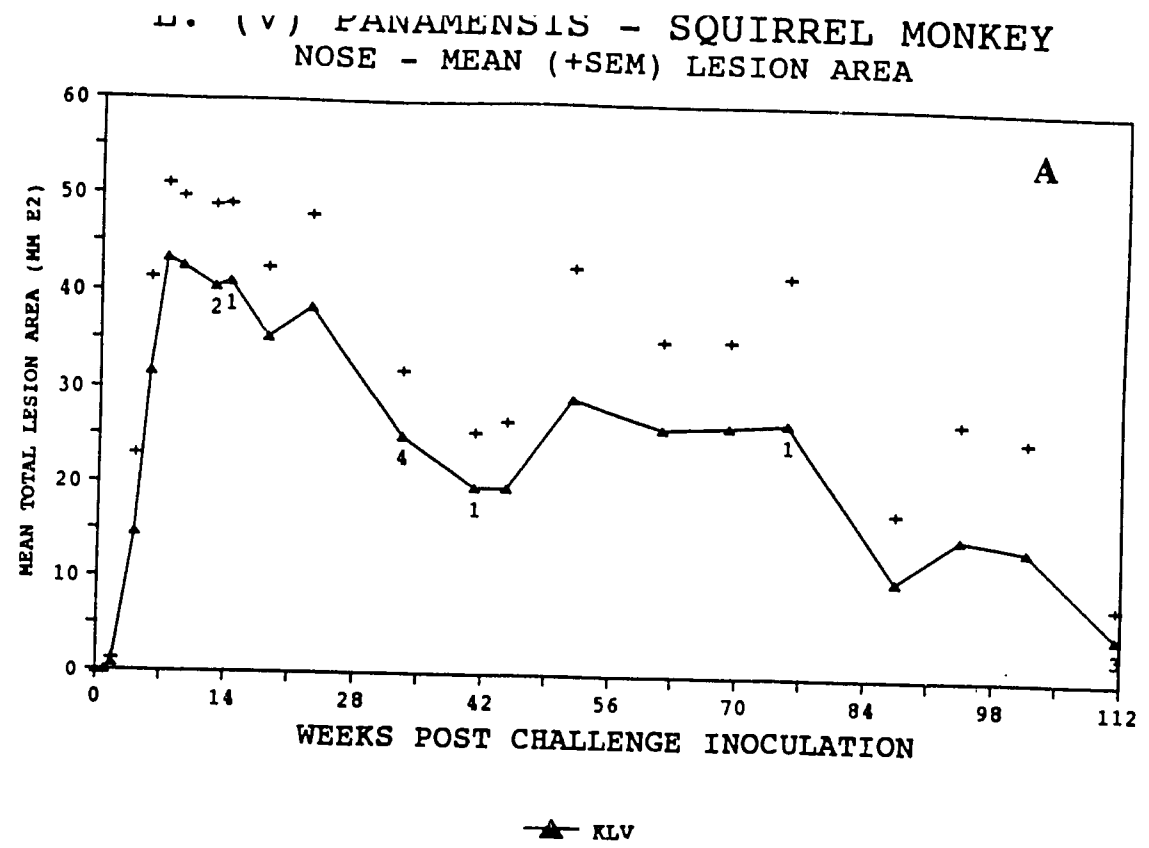


FIGURE I.8.1. Mean total lesion area (mm^2) of progression and regression of primary and satellite lesions of experimental cutaneous leishmanial infections in squirrel monkeys (112 wk) challenge-infected intradermally with $2x10^7$ promastigotes of *Leishmania (Viannia) panamensis*, four weeks after vaccination with BCG+KLV. (A) at the tip of the nose; (B) at the dorsal base of the tail. Each point represents the mean (+ SEM) of six animals and each subscript the number of satellite lesions.



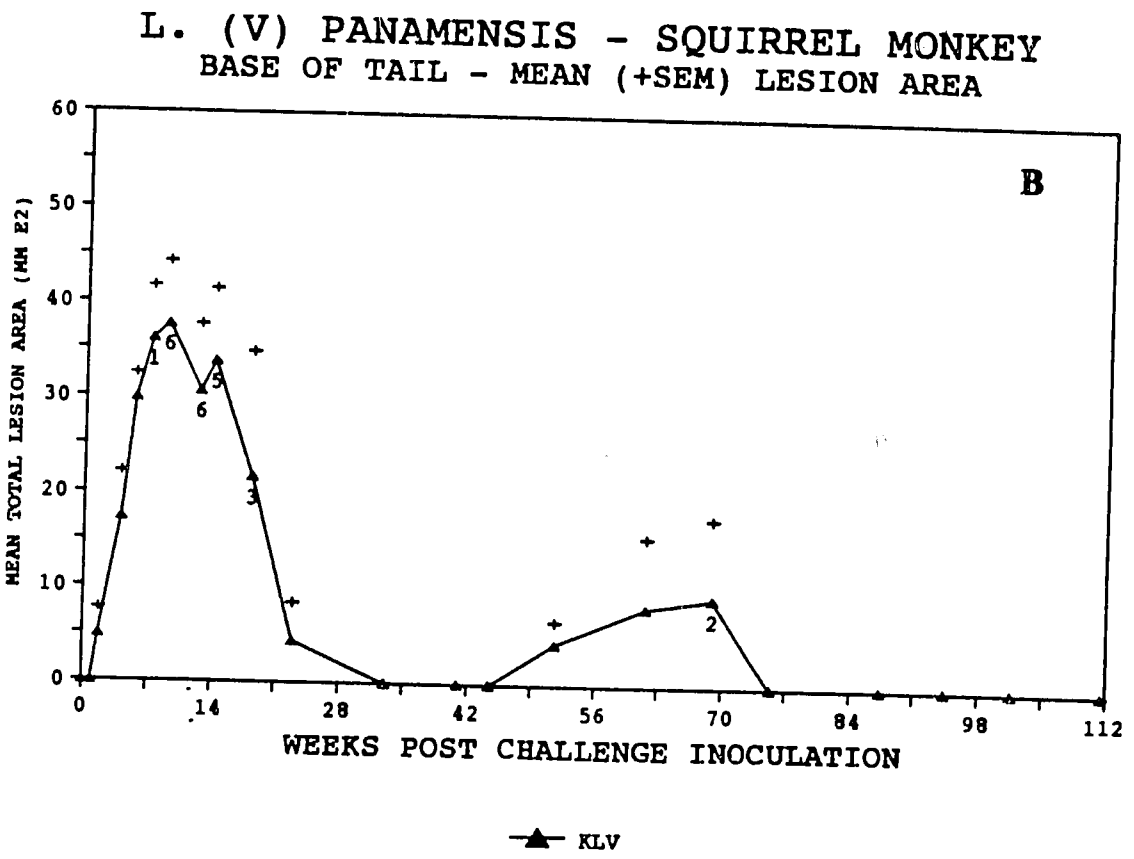
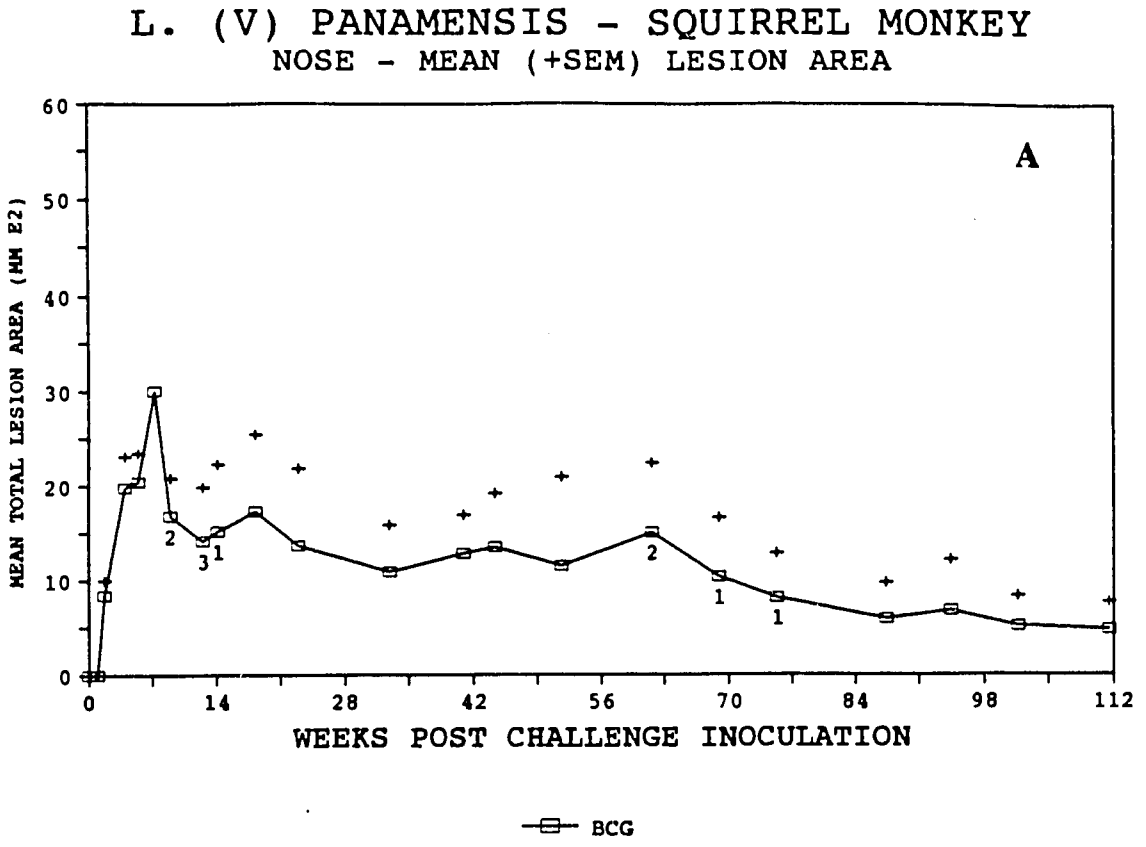


FIGURE I.8.2. Mean total lesion area (mm^2) of progression and regression of primary and satellite lesions of experimental cutaneous leishmanial infections in squirrel monkeys (112 wk) challenge-infected intradermally with $2x10^7$ promastigotes of *Leishmania (Viannia) panamensis*, four weeks after vaccination with KLV. (A) at the tip of the nose; (B) at the dorsal base of the tail. Each point represents the mean (+ SEM) of six animals and each subscript the number of satellite lesions.



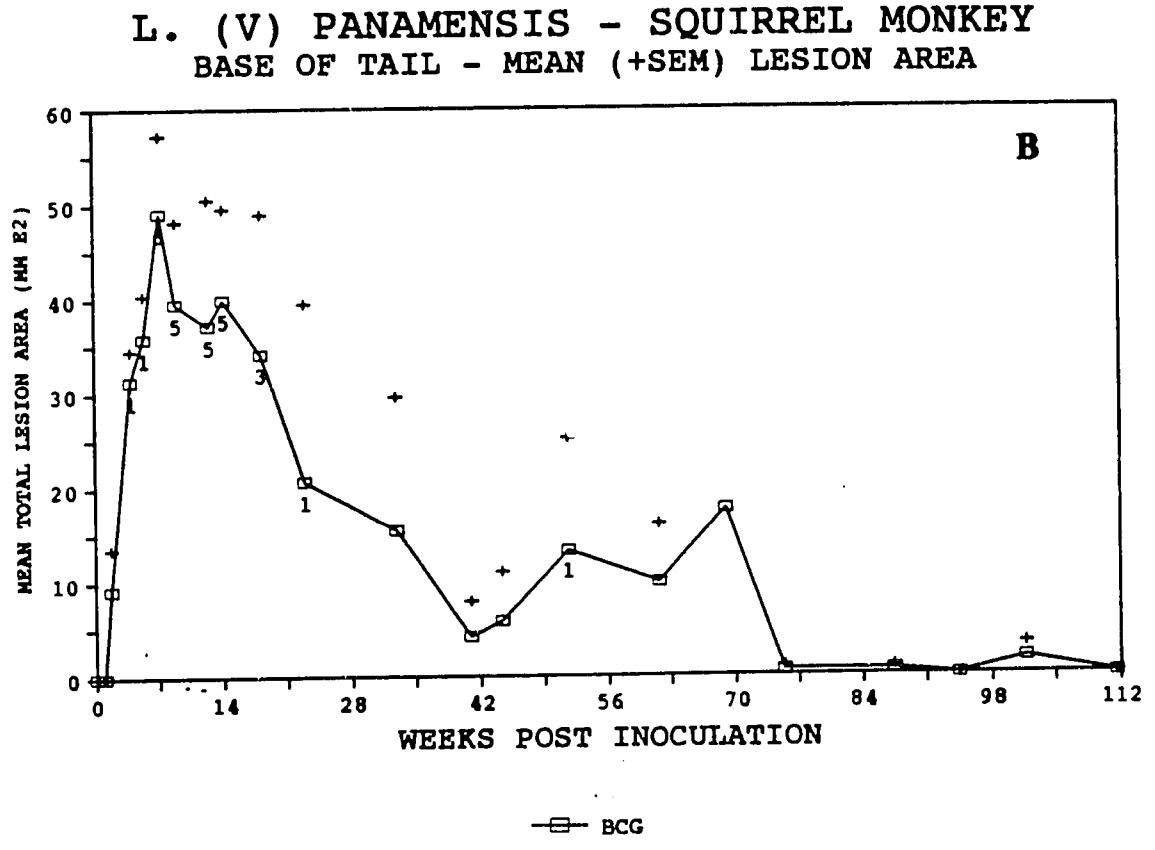
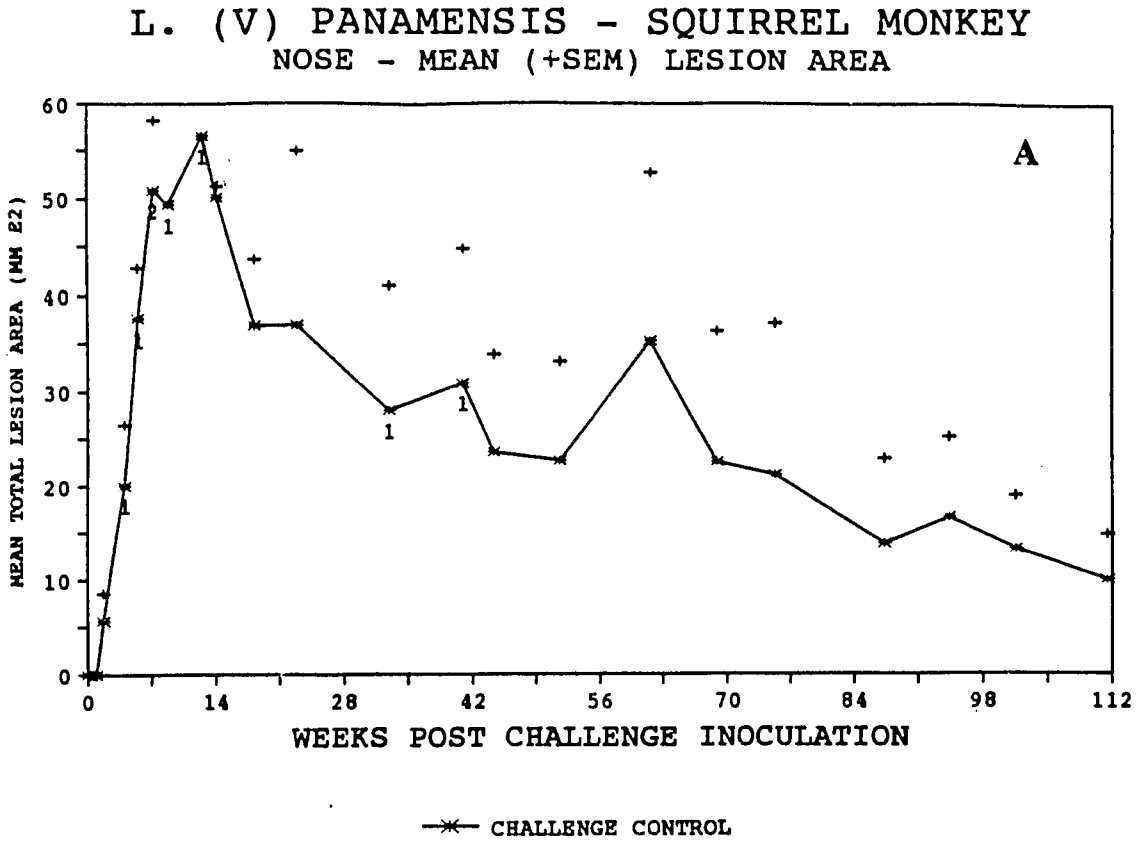


FIGURE I.8.3. Mean total lesion area (mm^2) of progression and regression of primary and satellite lesions of experimental cutaneous leishmanial infections in squirrel monkeys (112 wk) challenge-infected intradermally with $2x10^7$ promastigotes of *Leishmania (Viannia) panamensis*, four weeks after vaccination with BCG only. (A) at the tip of the nose; (B) at the dorsal base of the tail. Each point represents the mean (+ SEM) of six animals and each subscript the number of satellite lesions.



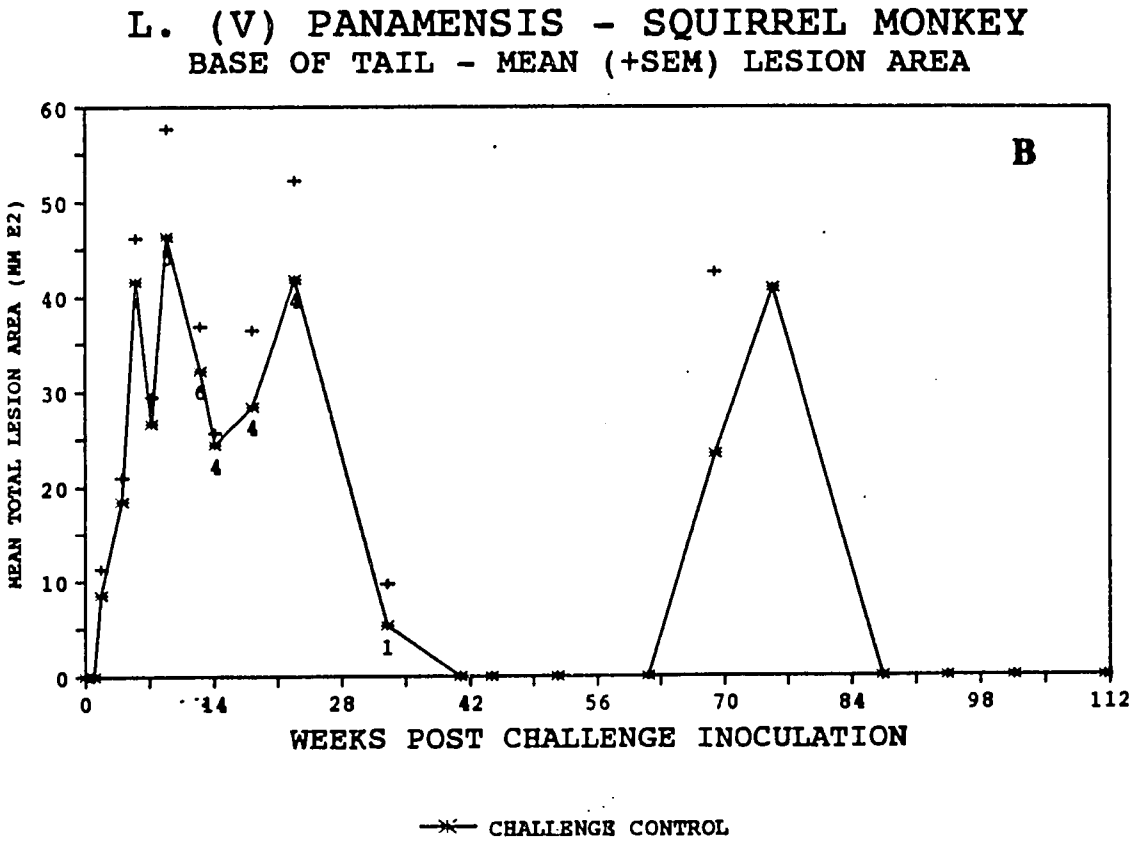


FIGURE I.8.4. Mean total lesion area (mm^2) of progression and regression of primary and satellite lesions of experimental cutaneous leishmanial infections in unvaccinated squirrel monkeys (112 wk) infected intradermally with $2x10^7$ promastigotes of *Leishmania (Viannia) panamensis* (normal control for challenge inoculation). (A) at the tip of the nose; (B) at the dorsal base of the tail. Each point represents the mean (+ SEM) of four animals and each subscript the number of satellite lesions.

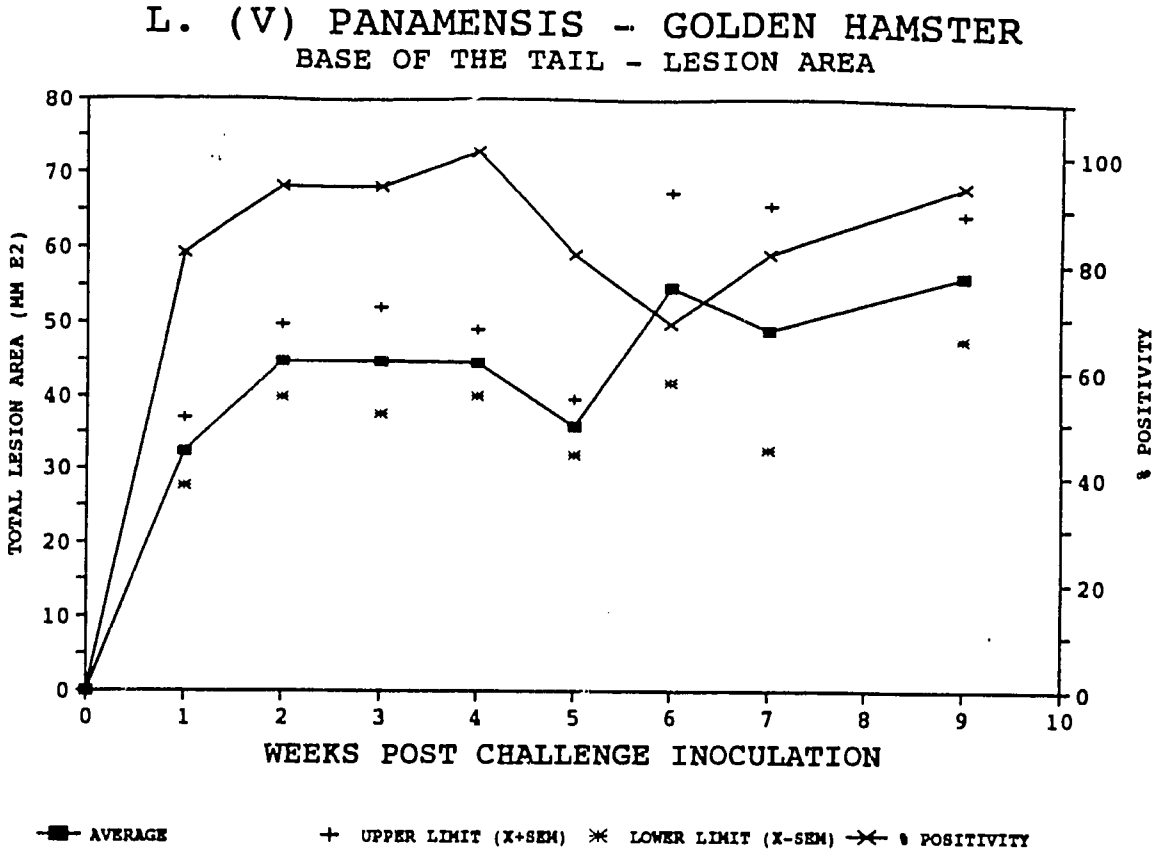


FIGURE I.9. Mean total area (mm^2) and percent positivity after experimental cutaneous leishmanial infections in hamsters (9 wk) inoculated intradermally with $2x10^7$ promastigotes of *Leishmania (Viannia) panamensis* at the dorsal base of the tail, parallel to the challenge infection induced in vaccinated and unvaccinated squirrel monkeys. Each point represents the mean (± SEM) of 22 hamsters.

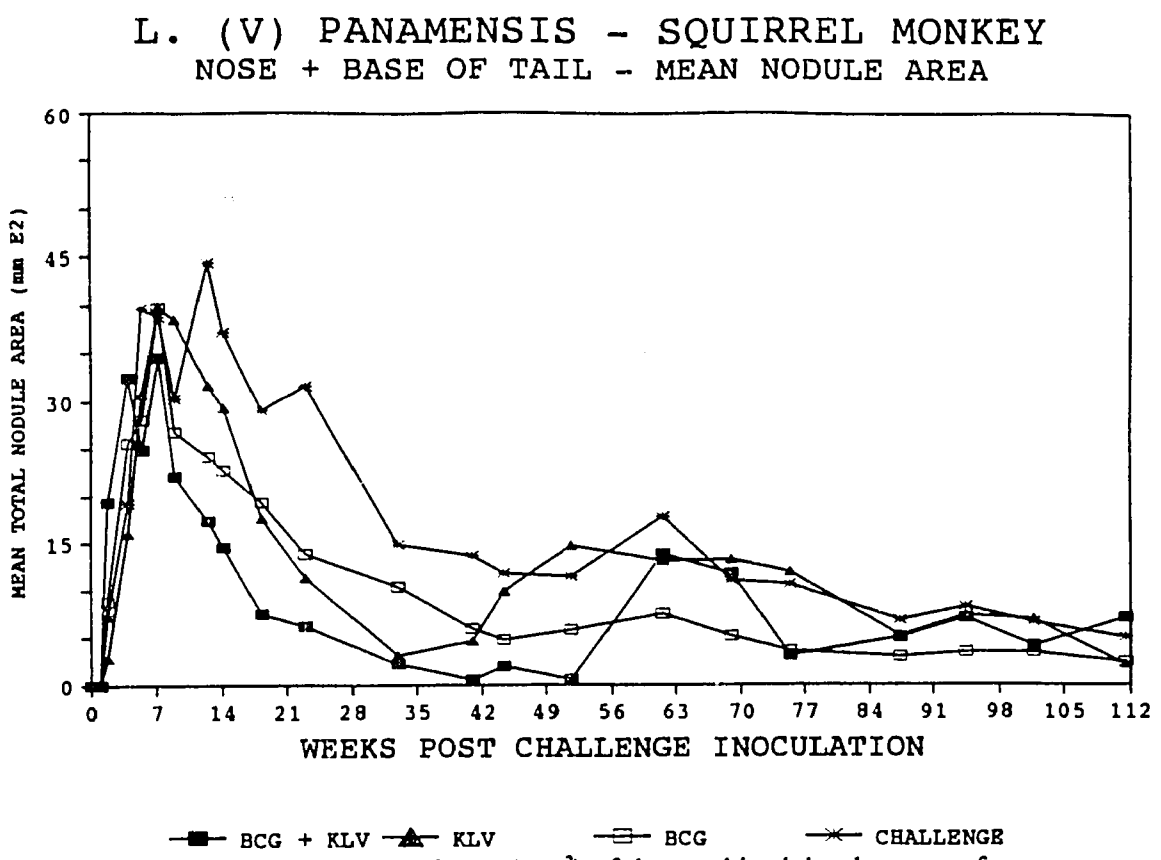


FIGURE I.10.1. Mean total area (mm^2) of the combined development of cutaneous nodules only at the tip of the nose and at the dorsal base of the tail in squirrel monkeys (112 wk) challenge-infected intradermally with $2x10^7$ promastigotes of *Leishmania (Viannia) panamensis*, four weeks after vaccination with BCG+KLV, KLV or BCG only and in unvaccinated (normal control for challenge inoculation) monkeys. Each point represents the mean of four to six monkeys.

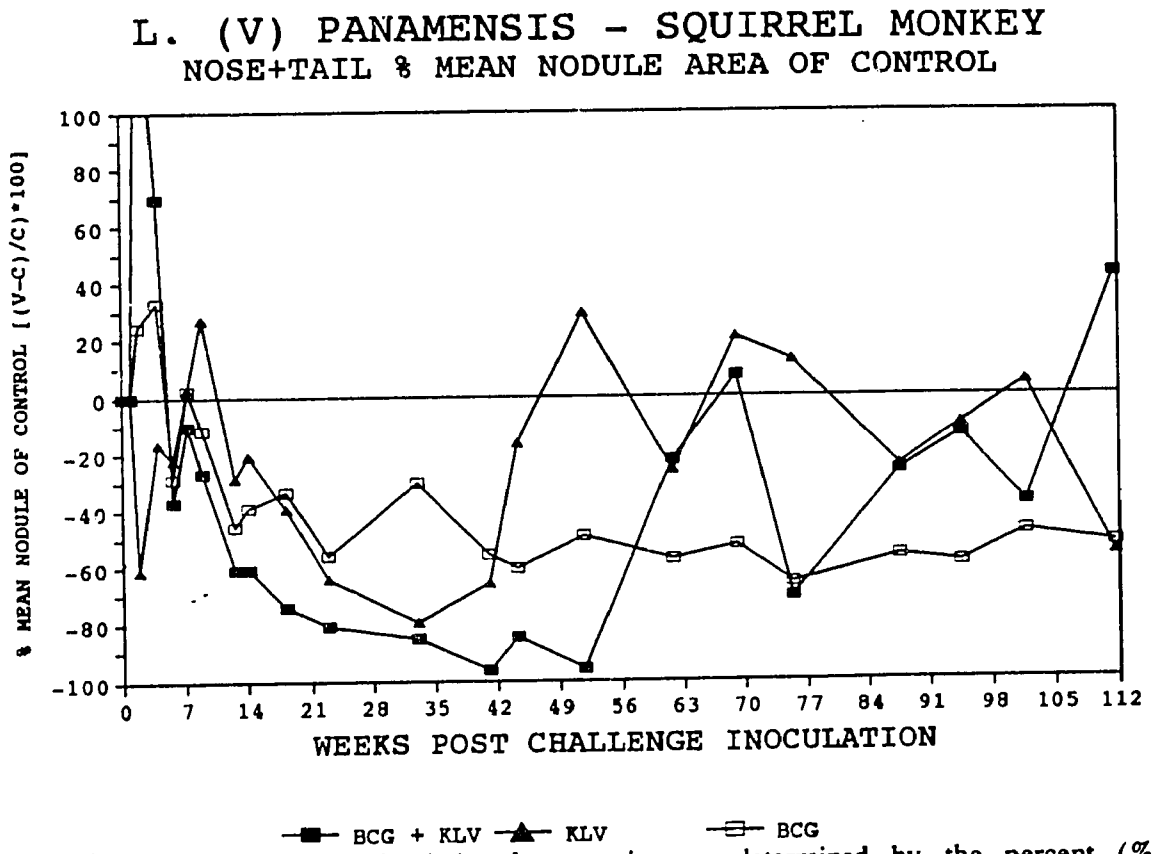


FIGURE I.10.2. Vaccine-induced protection as determined by the percent (%) of nodule development at the tip of the nose and at the dorsal base of the tail of squirrel monkeys (112 wk) vaccinated with BCG+KLV, KLV or BCG in relation to that of unvaccinated challenge-control (normal control for challenge inoculation) monkeys.

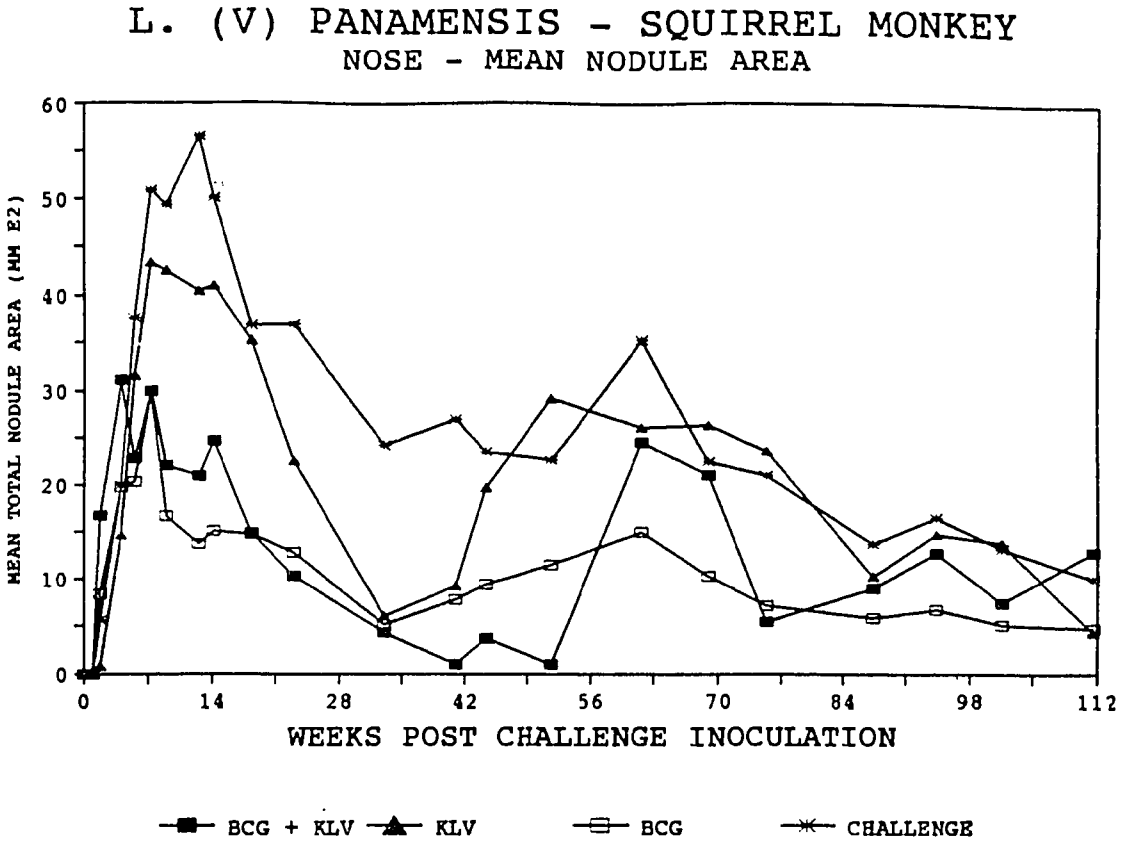


FIGURE I.10.1A. Mean total area (mm^2) of the development of cutaneous nodules at the tip of the nose in squirrel monkeys (112 wk) challenge-infected intradermally with $2x10^7$ promastigotes of *Leishmania (Viannia) panamensis*, four weeks after vaccination with BCG+KLV, KLV or BCG only and in unvaccinated (normal control for challenge inoculation) monkeys. Each point represents the mean of four to six monkeys.

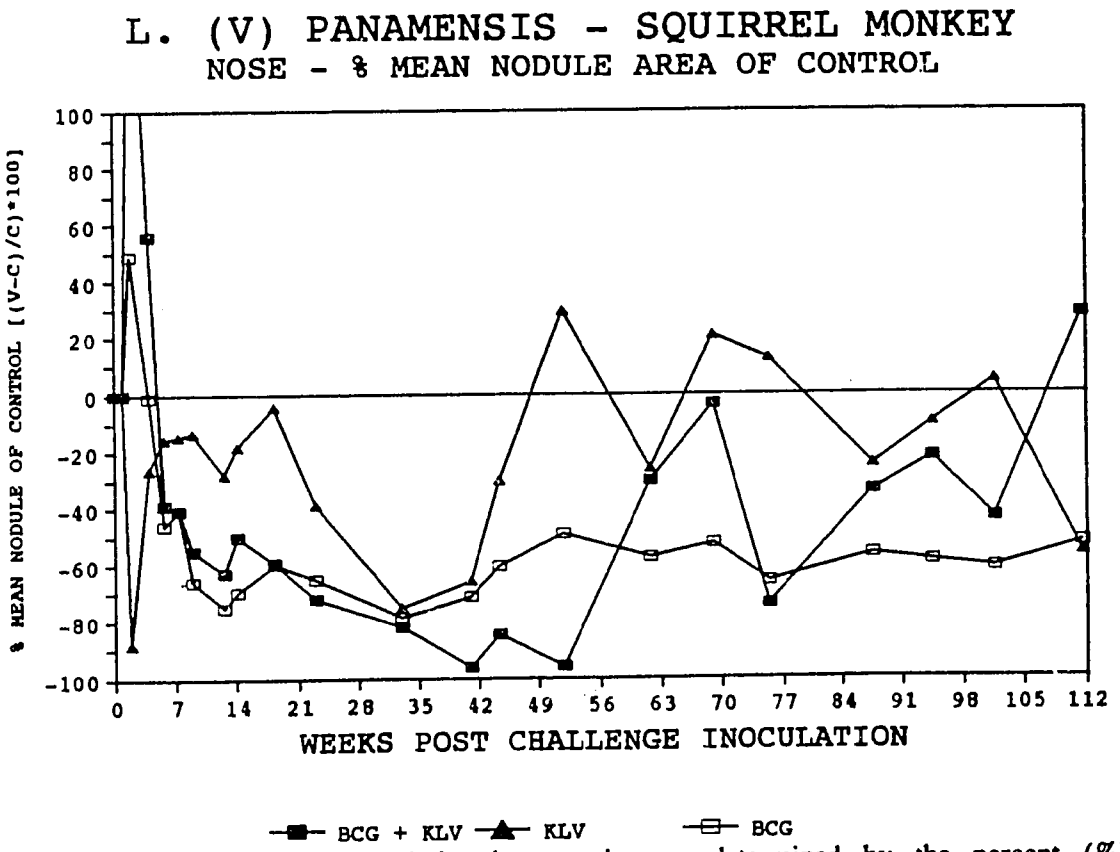


FIGURE I.10.2A. Vaccine-induced protection as determined by the percent (%) of nodule development at the tip of the nose of squirrel monkeys (112 wk) vaccinated with BCG+KLV, KLV or BCG in relation to that of unvaccinated challenge-control (normal control for challenge inoculation) monkeys.

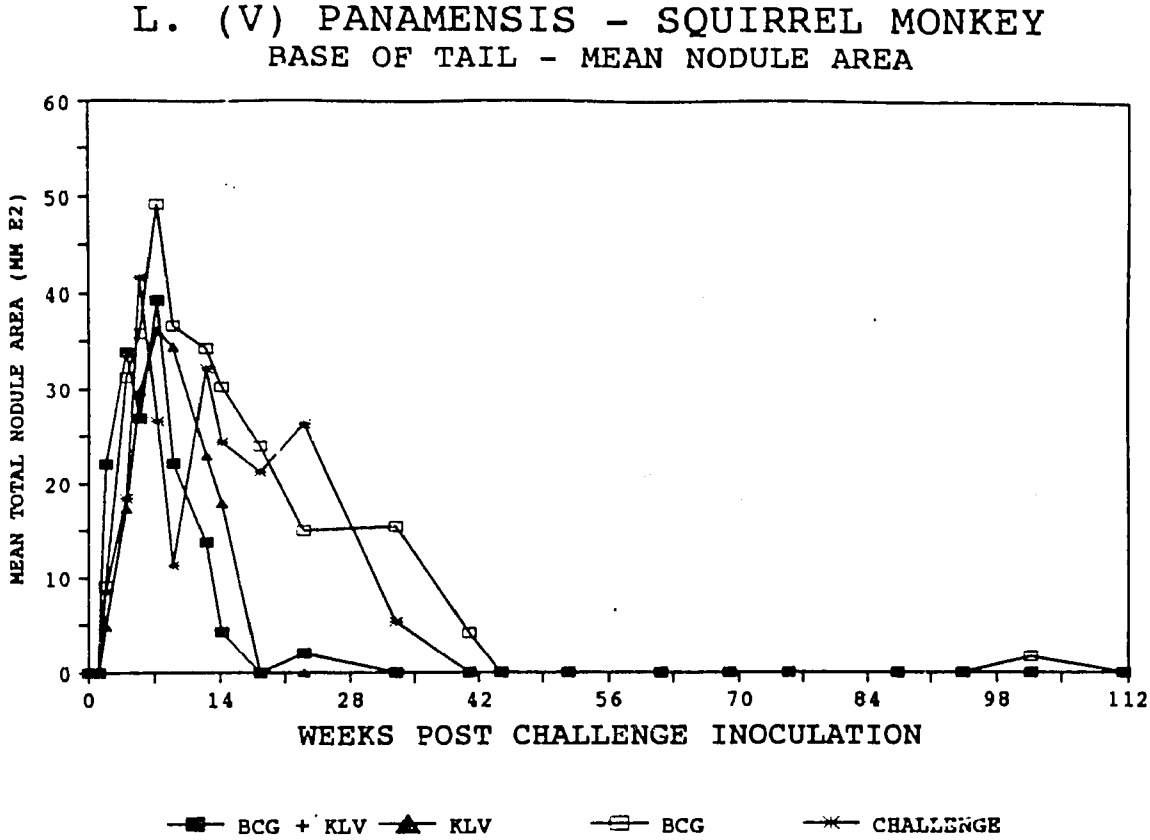


FIGURE I.10.1B. Mean total area (mm^2) of the development of cutaneous nodules at the dorsal base of the tail in squirrel monkeys (112 wk) challenge-infected intradermally with $2x10^7$ promastigotes of *Leishmania (Viannia) panamensis*, four weeks after vaccination with BCG+KLV, KLV or BCG only and in unvaccinated (normal control for challenge inoculation) monkeys. Each point represents the mean of four to six monkeys.

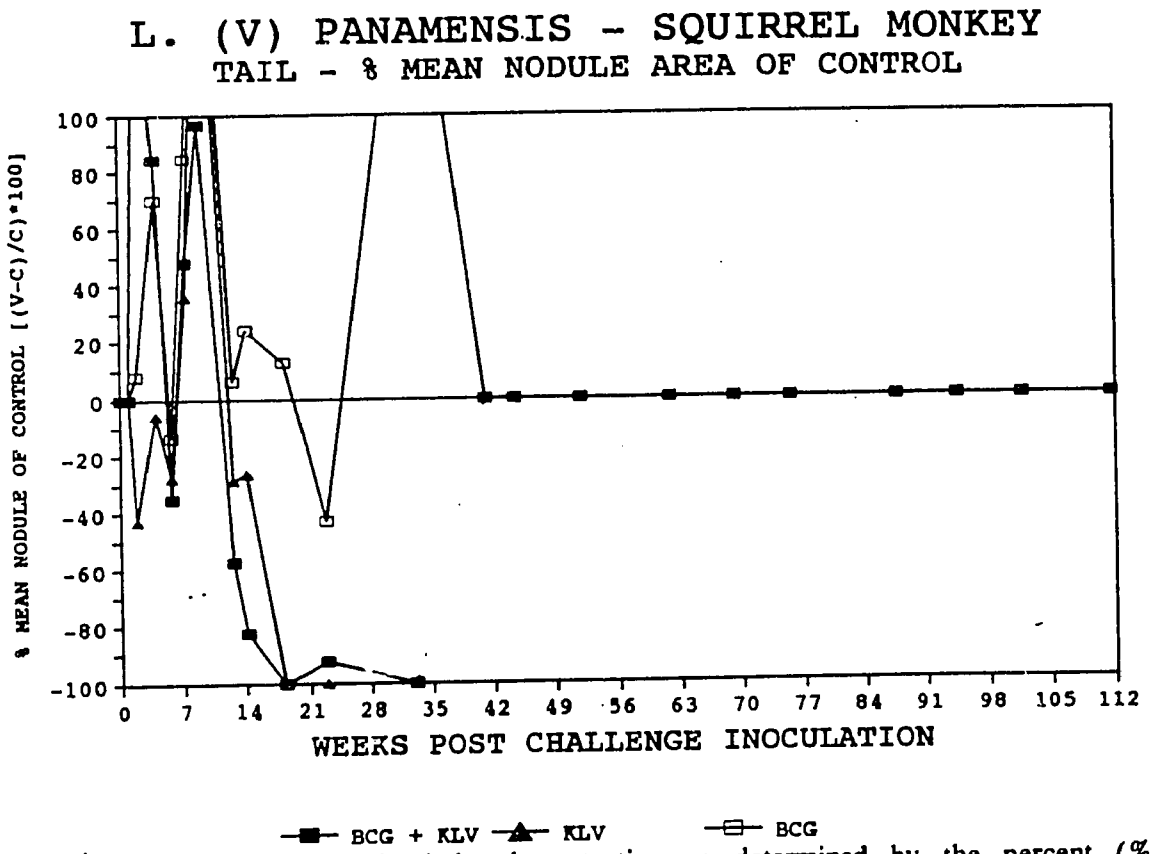


FIGURE I.10.2B. Vaccine-induced protection as determined by the percent (%) of nodule development at the dorsal base of the tail of squirrel monkeys (112 wk) vaccinated with BCG+KLV, KLV or BCG in relation to that of unvaccinated challenge-control (normal control for challenge inoculation) monkeys.

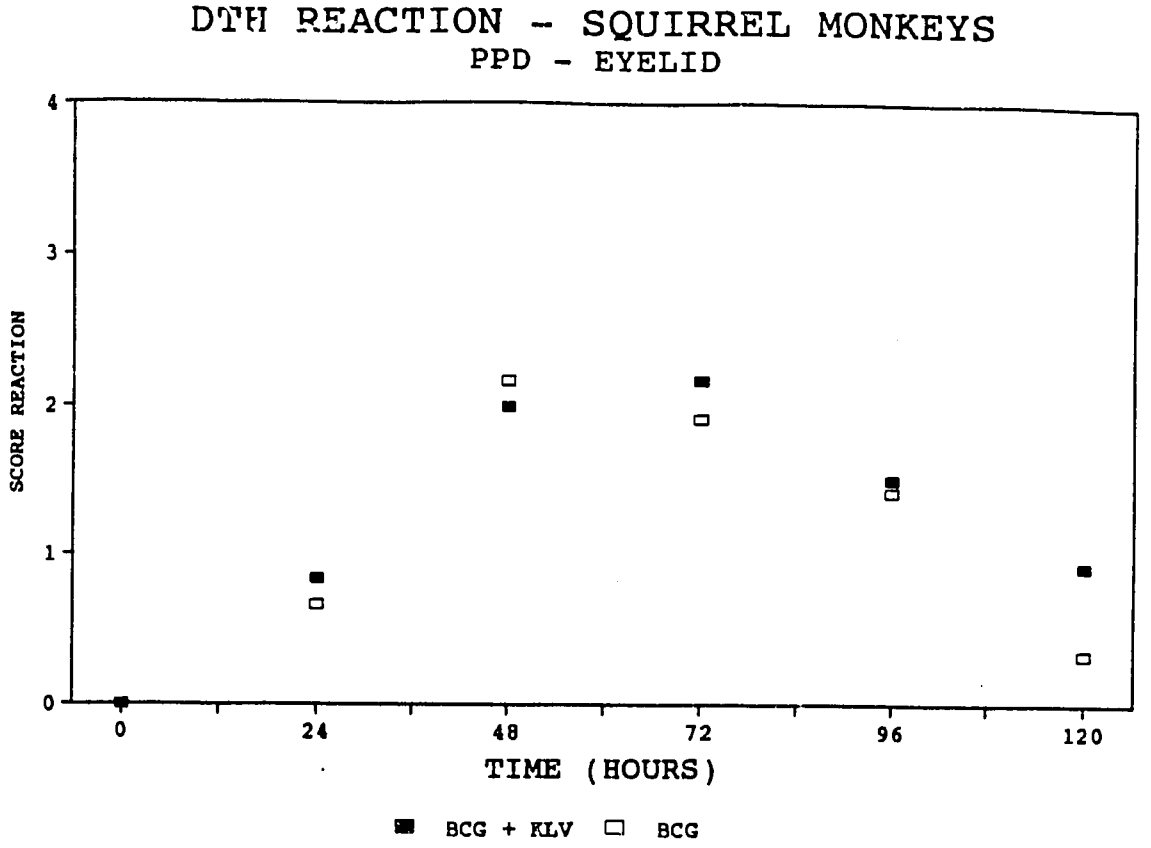


FIGURE I.11. Mean score reaction of delayed-type hypersensitivity response (120 h) to mammalian tuberculin in the eyelid of squirrel monkeys, 4 months after vaccination with BCG+KLV or BCG alone. No reactions were observed in KLV, unvaccinated challenge-infected and uninfected control monkeys. Individual score reactions were recorded as 0, 1, 2, 3, or 4. Each point represents the mean of six monkeys.

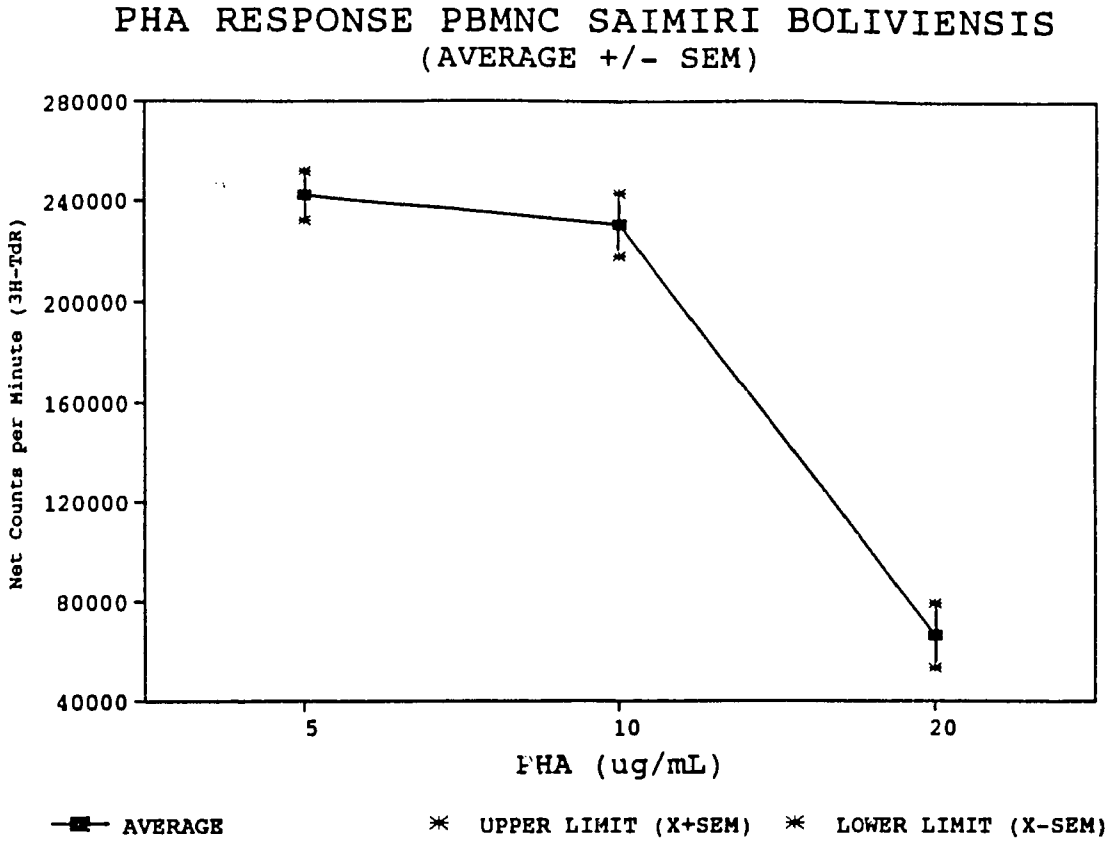


FIGURE I.12. Mean (± SEM) response of uninfected *Saimiri boliviensis* squirrel monkey peripheral blood mononuclear cells (PBMNC) to varying concentrations of phytohemagglutinin (PHA) ($2x10^6$ viable PBMNC/well, flat bottom wells, 4 day cultures, 1 μCi [^{3}H]-Thymidine). Net counts per minute (cpm stimulated - cpm unstimulated cells). Each point represents the mean of 25 monkeys.

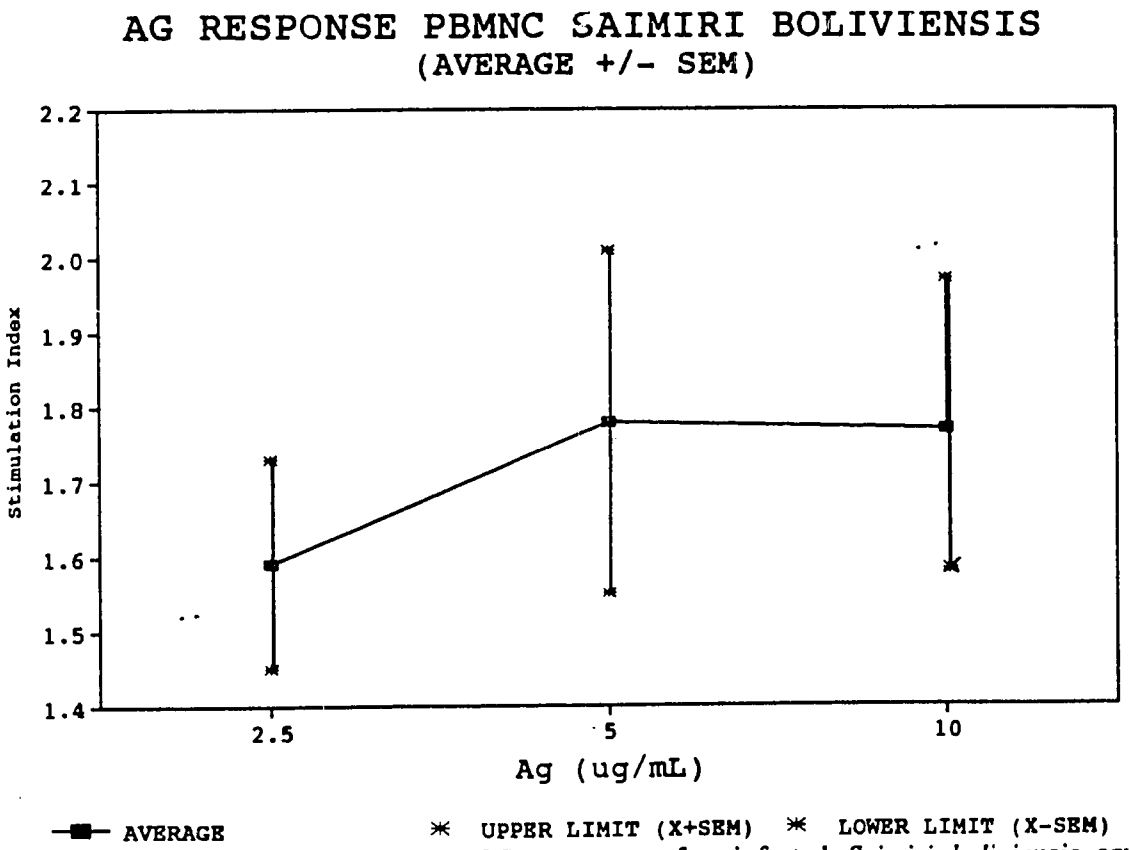


FIGURE I.13. Mean (± SEM) response of uninfected *Saimiri boliviensis* squirrel monkey peripheral blood mononuclear cells (PBMNC) to varying concentrations of soluble leishmanial antigen (SLA) ($2x10^6$ viable PBMNC/well, round bottom wells, 6 day cultures, 1 μCi [^{3}H]-Thymidine). Stimulation index (cpm stimulated / cpm unstimulated cells). Each point represents the mean of 25 monkeys.

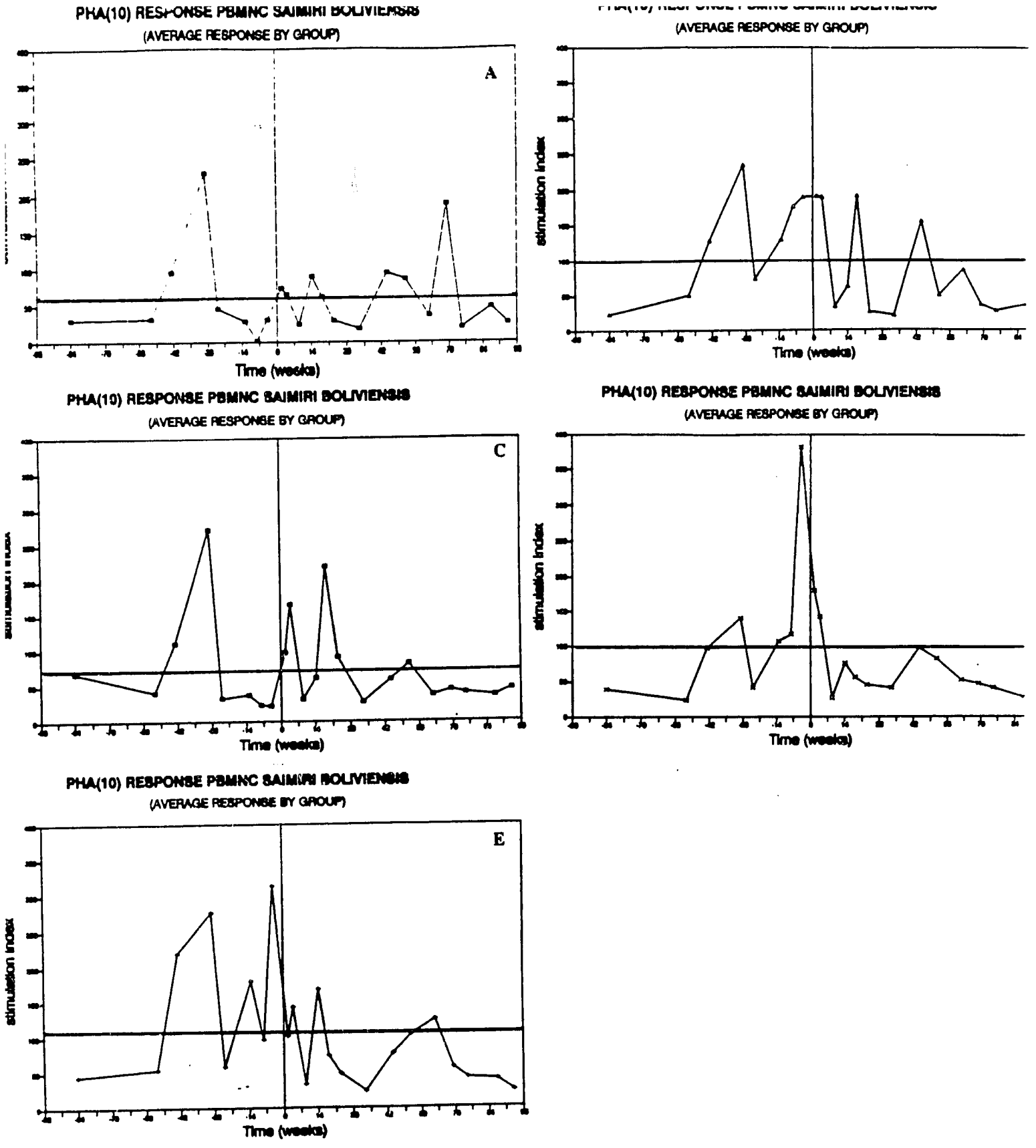


FIGURE I.14. Average response of squirrel monkey PBMNC to PHA (10 μg/mL), prior to, after vaccination and challenge-infection (112 wk) with $2x10^7$ promastigotes of *Leishmania (Viannia) panamensis*. (A) BCG+KLV; (B) KLV; (C) BCG; (D) unvaccinated challenge-control; (E) normal uninfected monkeys. Each point represents the mean of four to six monkeys and the horizontal line the group mean.

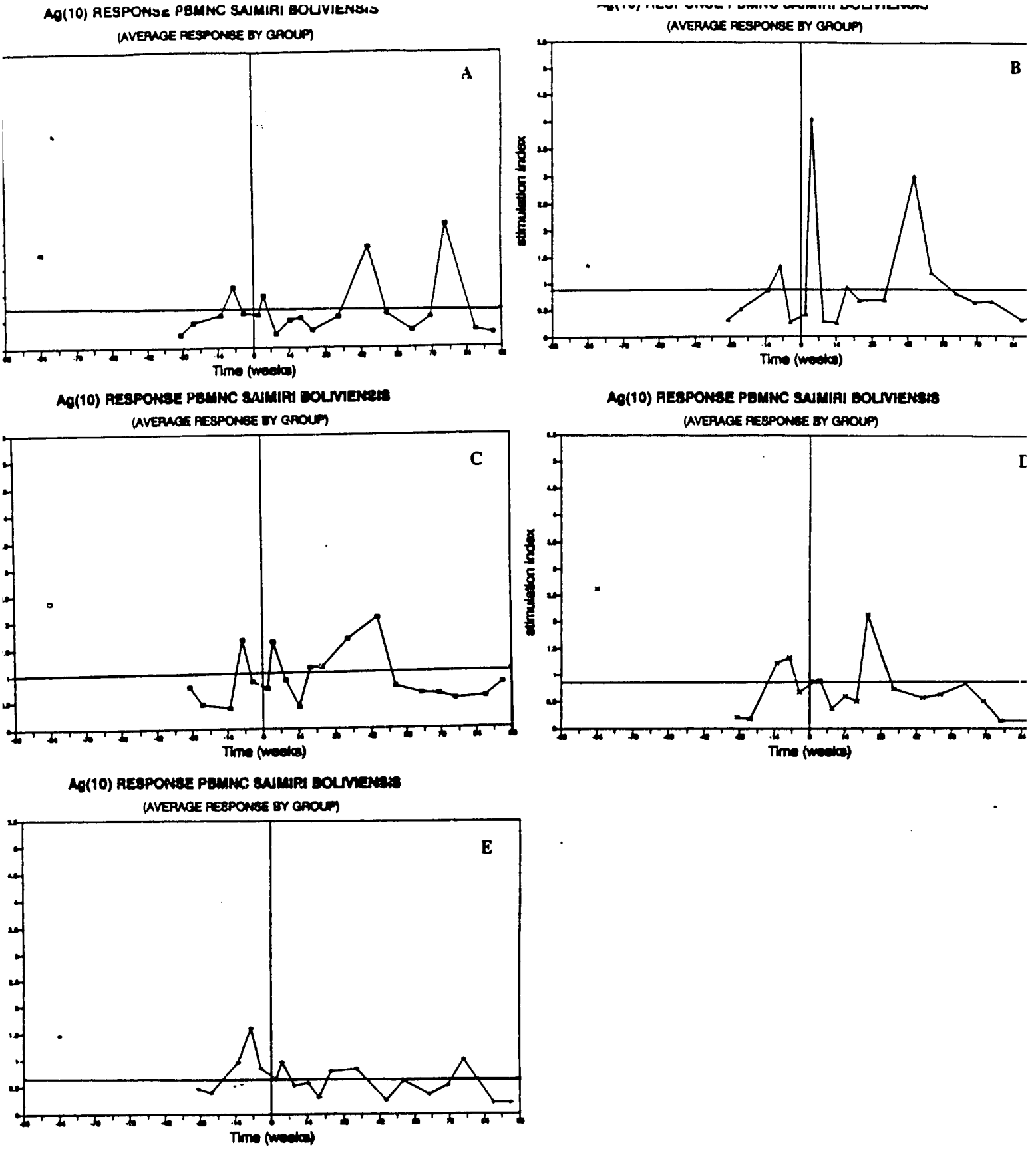
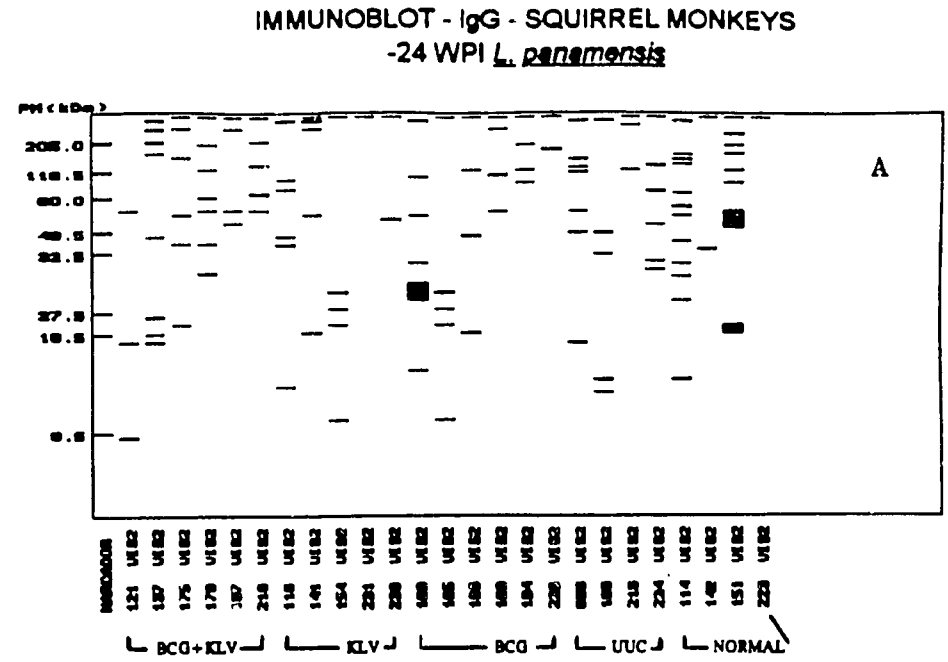


FIGURE I.15. Average response of squirrel monkey PBMNC to SLA (10 μg/mL), prior to, after vaccination and challenge-infection (112 wk) with 2×10^7 promastigotes of *Leishmania (Viannia) panamensis*. (A) BCG+KLV; (B) KLV; (C) BCG; (D) unvaccinated challenge-control; (E) normal uninfected monkeys. Each point represents the mean of four to six monkeys and the horizontal line the group mean.



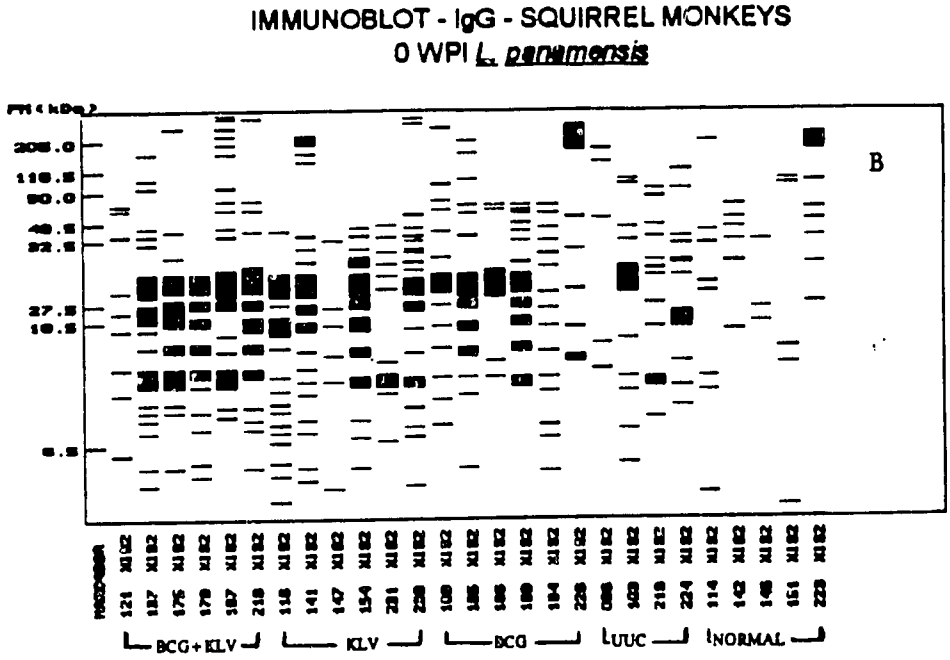
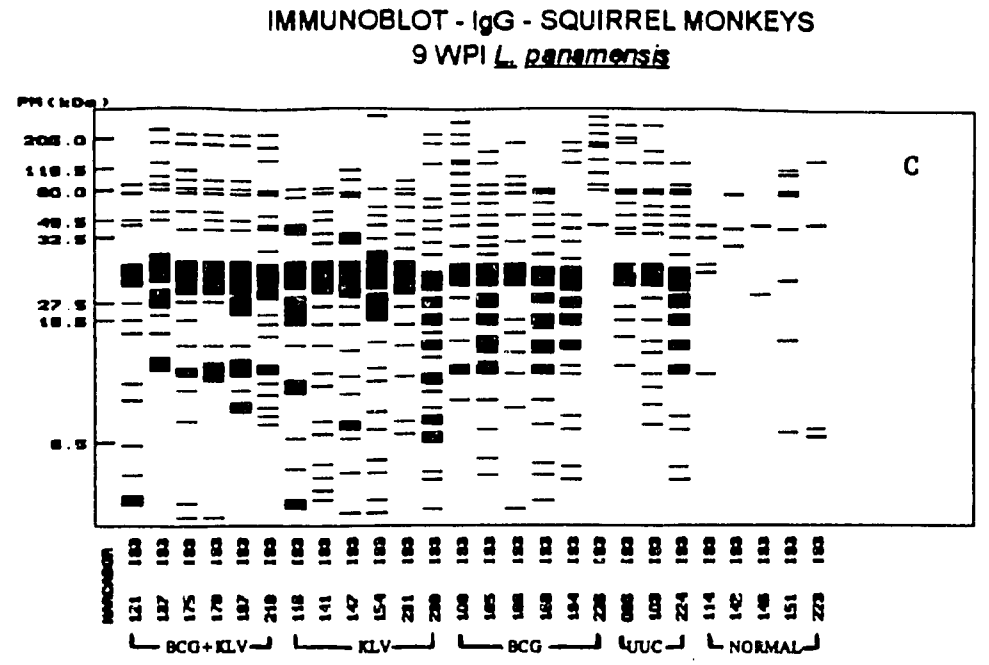


FIGURE I.16. Western blot analysis of IgG antibodies from *Saimiri boliviensis* squirrel monkeys [sera 1:50] to soluble leishmanial antigen (SLA), prior to, after vaccination and challenge infection (112 wk) with $2x10^7$ promastigotes of *Leishmania (Viannia) panamensis*. Each lane represents one monkey for each group: BCG+KLV, KLV, BCG, unvaccinated challenge-control (UUC) and normal uninfected monkeys. (A) Upper diagram corresponds to 24 weeks prior to vaccination and infection; (B) lower diagram corresponds to four weeks after last booster dose of vaccinated monkeys at time 0 prior to challenge infection.



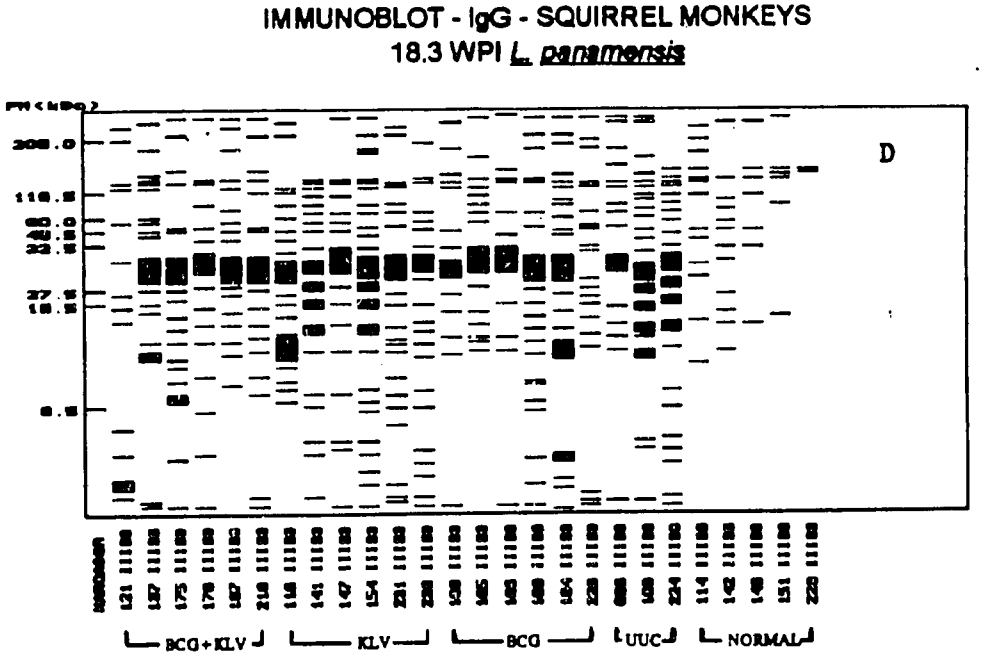
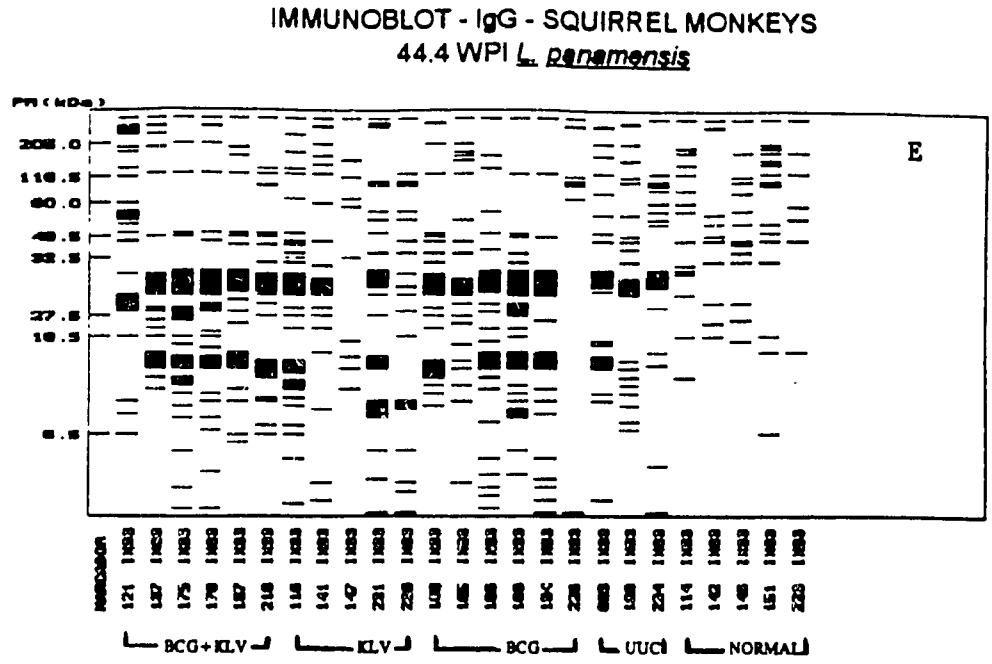


FIGURE I.16. Western blot analysis of IgG antibodies from *Saimiri boliviensis* squirrel monkeys [sera 1:50] to soluble leishmanial antigen (SLA), prior to, after vaccination and challenge infection (112 wk) with $2x10^7$ promastigotes of *Leishmania (Viannia) panamensis*. Each lane represents one monkey for each group: BCG+KLV, KLV, BCG, unvaccinated challenge-control (UUC) and normal uninfected monkeys. (C) Upper diagram corresponds to nine weeks after challenge infection (WPI); (D) lower diagram corresponds to 18.3 WPI.



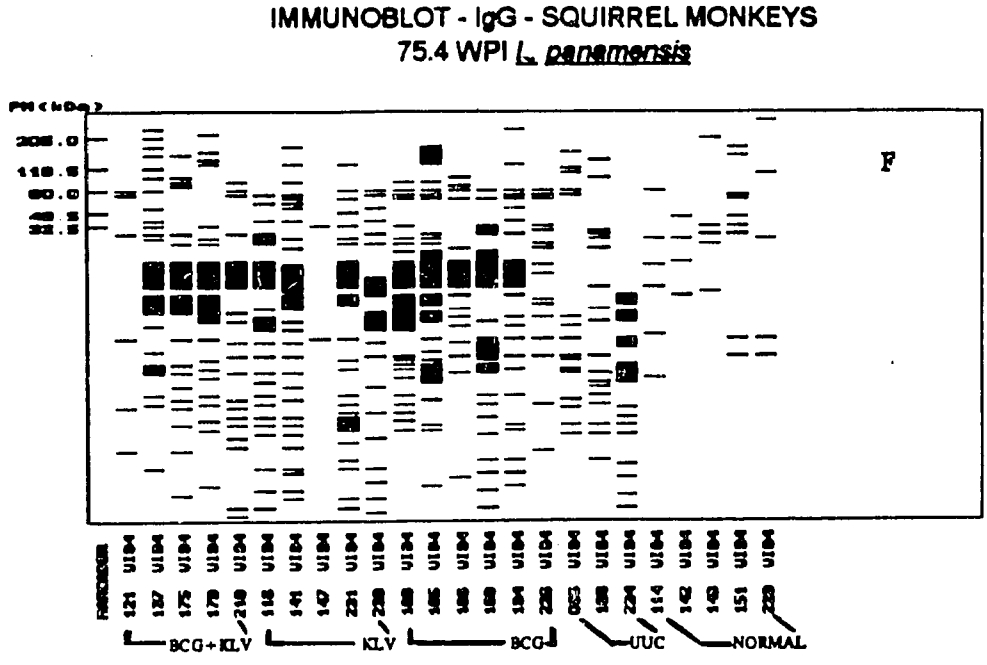
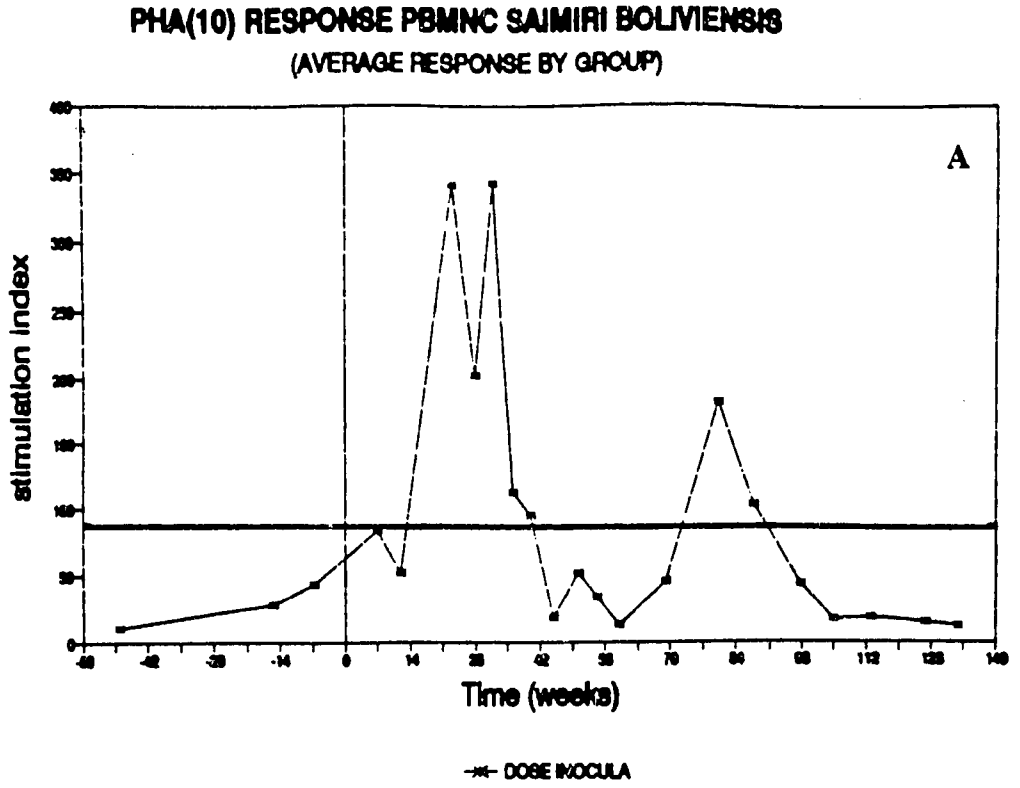


FIGURE I.16. Western blot analysis of IgG antibodies from *Saimiri boliviensis* squirrel monkeys [sera 1:50] to soluble leishmanial antigen (SLA), prior to, after vaccination and challenge infection (112 wk) with $2x10^7$ promastigotes of *Leishmania (Viannia) panamensis*. Each lane represents one monkey for each group: BCG+KLV, KLV, BCG, unvaccinated challenge-control (UUC) and normal uninfected monkeys. (E) Upper diagram corresponds to 44.4 weeks after challenge infection (WPI); (F) lower diagram corresponds to 75.4 WPI.



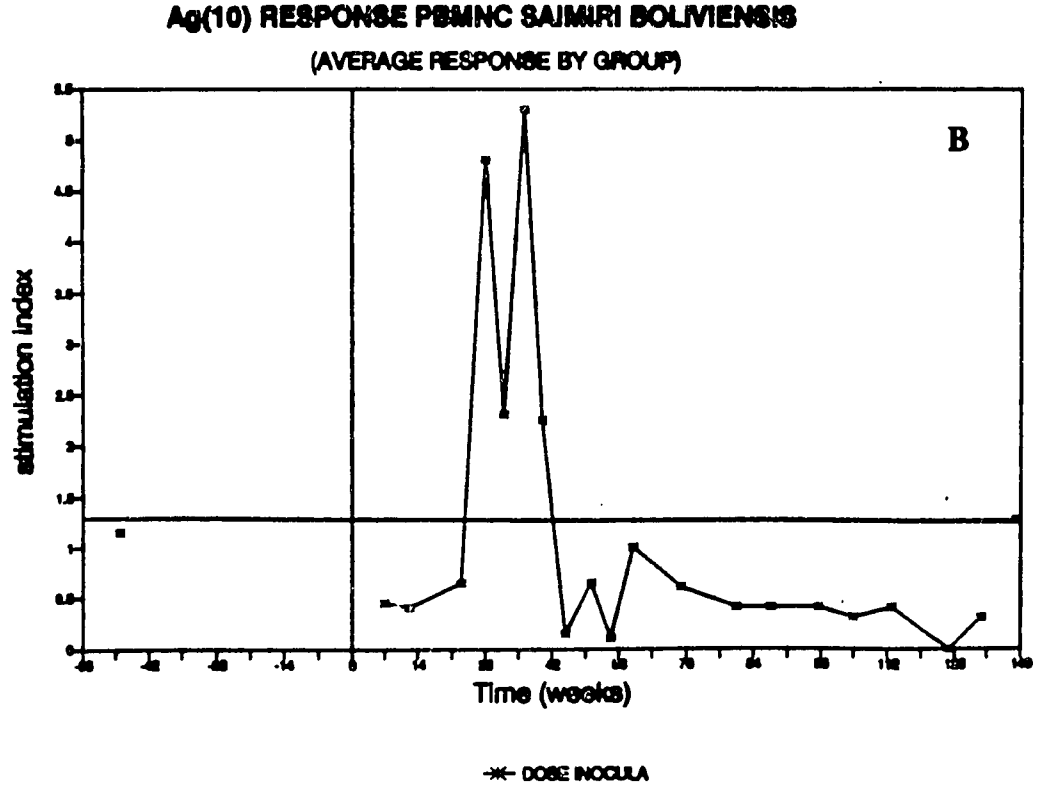
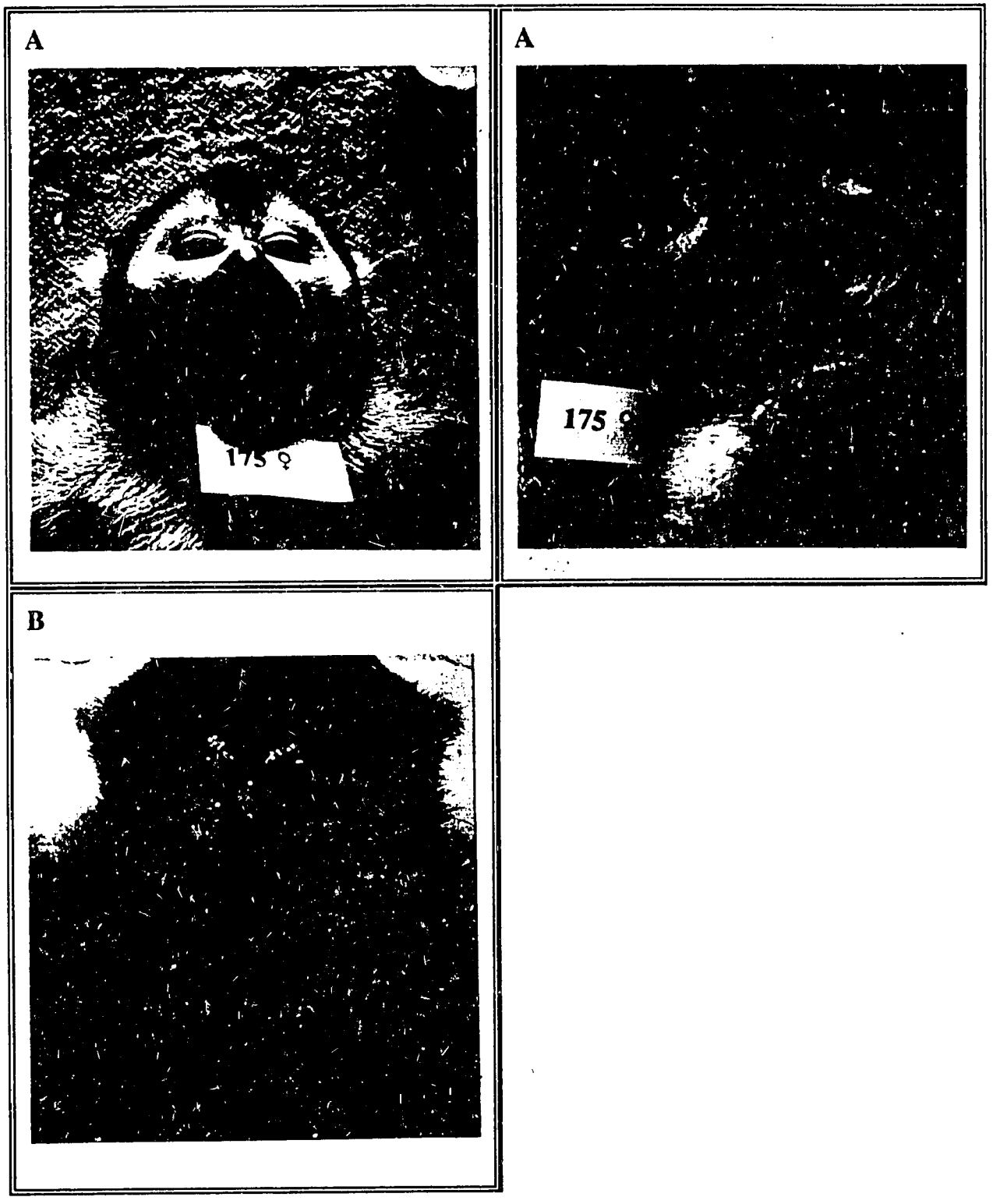
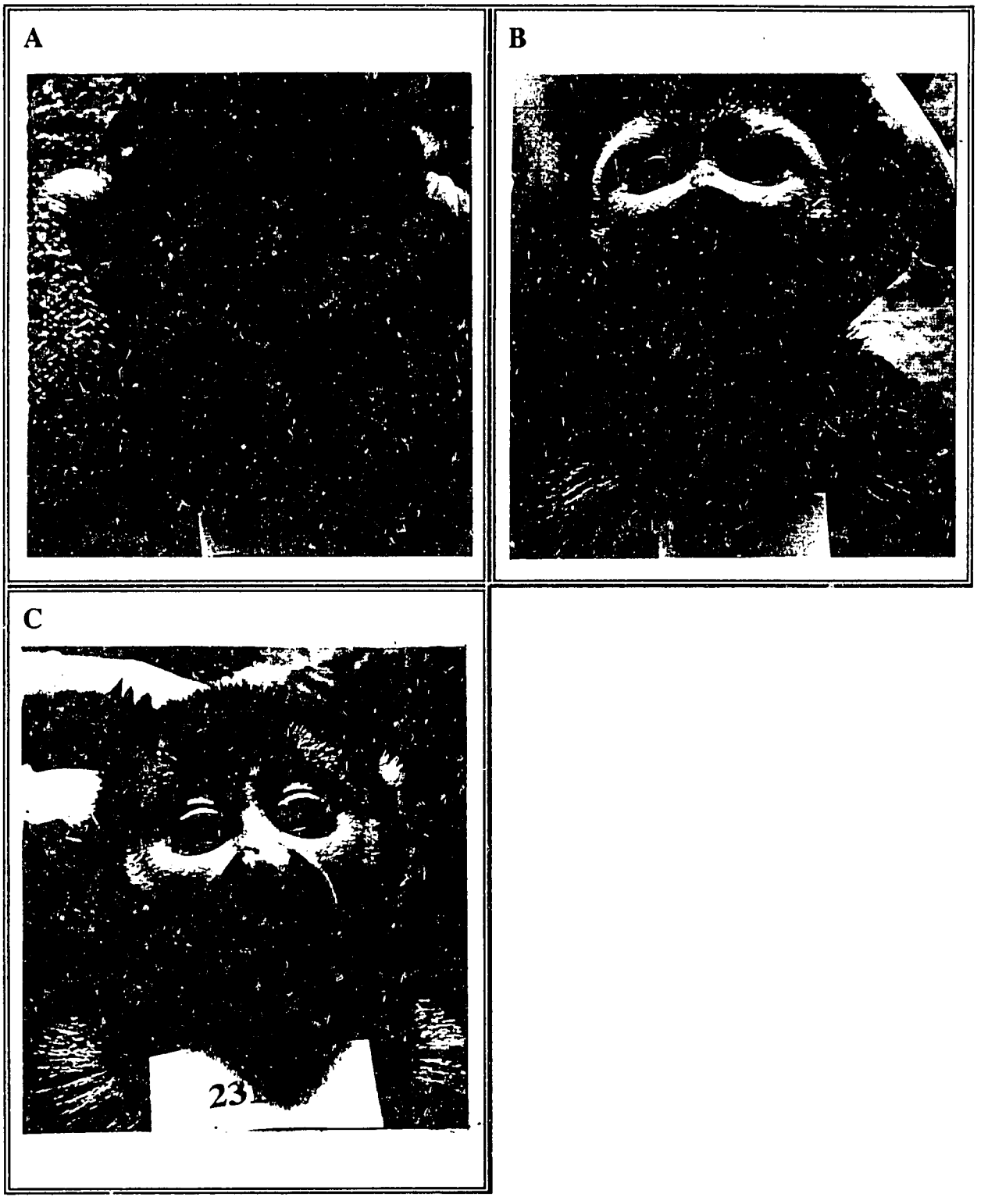


FIGURE I.17. Mean group response (stimulation index) of squirrel monkey PBMNC over time, before and after primary inoculation with $1x10^6$ or $1x10^7$ promastigotes of *Leishmania (Viannia) panamensis*. (A) phytohaemagglutinin (PHA) (10 μg/mL); (B) soluble leishmanial antigen (SLA) (10 μg/mL). Stimulation index (cpm stimulated / cpm unstimulated cells). The horizontal line corresponds to the group mean.



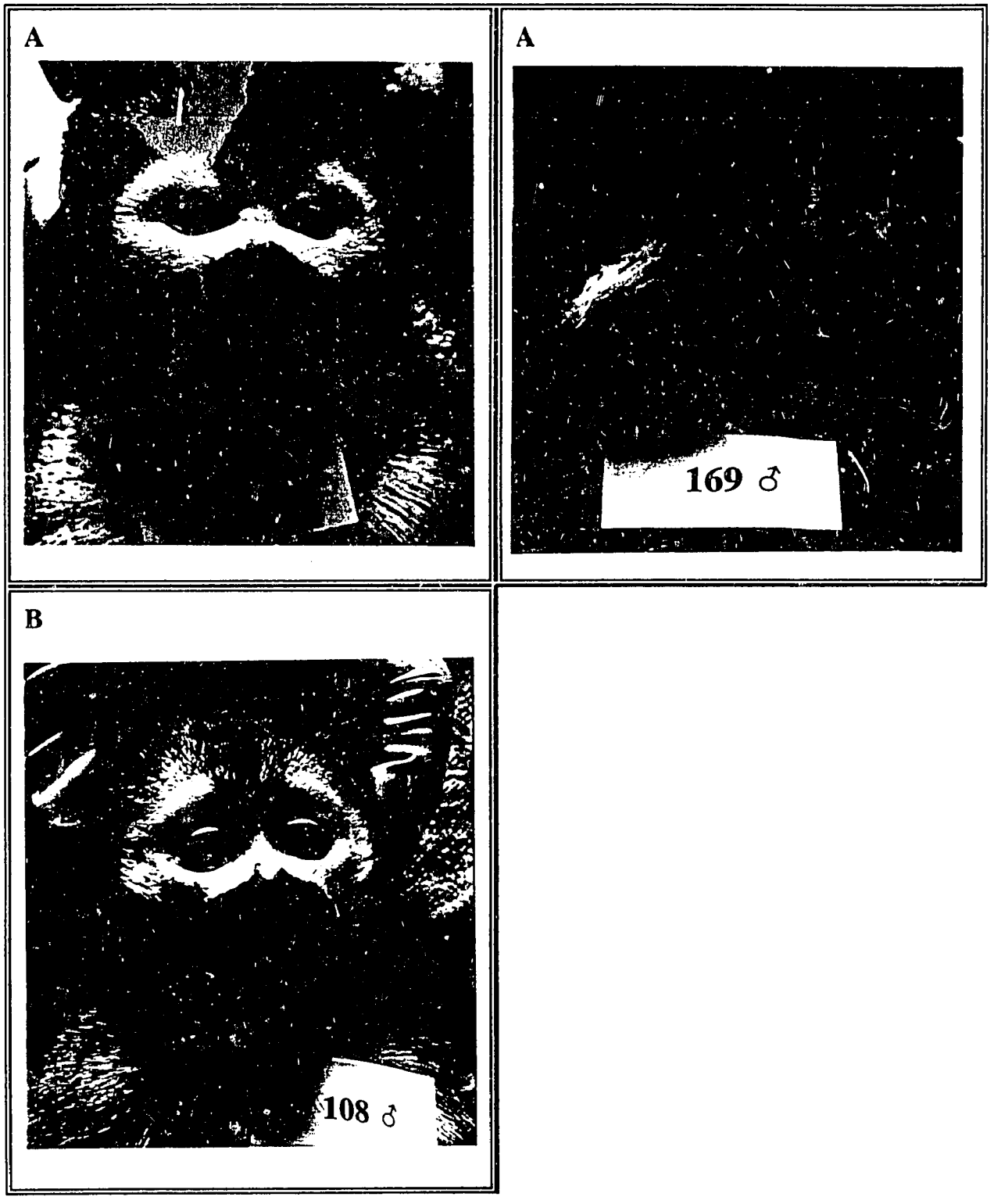
APPENDIX I.

FIGURE 1.A1. Cutaneous leishmanial lesions in squirrel monkeys vaccinated with BCG+KLV at 112 weeks after challenge-infection with $2x10^7$ promastigotes of *Leishmania (Viannia) panamensis* at the tip of the nose: (A) frontal and lateral view of monkey # 175 with a 52.43 mm^2 central raised nodule [tapir nose ?]; (B) frontal view of monkey # 218 with a 9.05 mm^2 central ulcerated nodule.



APPENDIX I.

FIGURE 1.A2. Cutaneous leishmanial lesions in squirrel monkeys vaccinated with KLV at 112 weeks after challenge-infection with $2x10^7$ promastigotes of *Leishmania (Viannia) panamensis* at the tip of the nose: (A) frontal view of monkey # 116 with a 11.58 mm^2 central raised nodule; (B) frontal view of monkey # 141 with a 2.14 mm^2 ulcerated satellite nodule; frontal view of monkey # 231 with a 3.57 mm^2 central raised nodule.

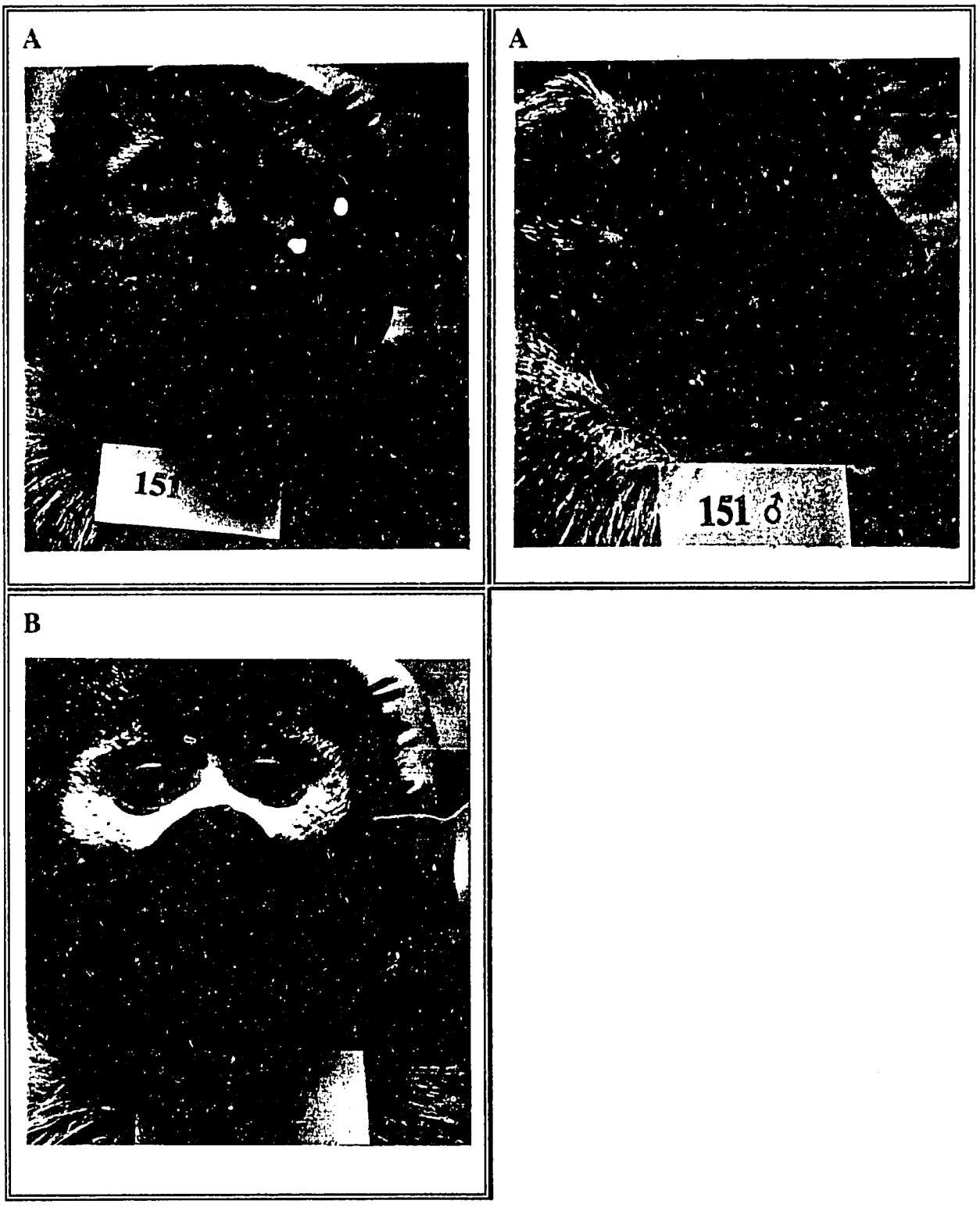


APPENDIX I.

FIGURE 1.A3. Cutaneous leishmanial lesions in squirrel monkeys vaccinated with BCG at 112 weeks after challenge-infection with $2x10^7$ promastigotes of *Leishmania (Viannia) panamensis* at the tip of the nose: (A) frontal and lateral view of monkey # 169 with a 18.43 mm^2 central raised erythematous nodule; (B) frontal view of monkey # 108 with a 10.05 mm^2 central nodule.

APPENDIX I.

FIGURE 1.A4. Cutaneous leishmanial lesions in unvaccinated challenge control squirrel monkeys at 112 weeks after infection with $2x10^7$ promastigotes of *Leishmania (Viannia) panamensis* at the tip of the nose: (A) frontal and lateral view of monkey # 224 with a 19.96 mm^2 central raised erythematous nodule with scar formation [tapir nose ?]; (B) frontal view of monkey # 096 with a 9.98 mm^2 satellite erythematous nodule.



APPENDIX I.
FIGURE 1.A5. Normal unvaccinated unchalleged squirrel monkeys: (A) frontal and lateral view of monkey # 151; (B) frontal view of monkey # 142.