

CROSS-IMMUNITY EXPERIMENTS BETWEEN DIFFERENT SPECIES OR STRAINS OF *LEISHMANIA* IN RHESUS MACAQUES (*MACACA MULATTA*)

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Abstract. This study evaluates cross-immunity in rhesus monkeys (*Macaca mulatta*) previously infected with one species of *Leishmania* and have had self-cured disease or were cured by antimony-based therapy upon development of full-blown disease. We found that a self-healing cutaneous leishmaniasis (CL) following experimental infection with *Leishmania (Leishmania) major* induces significant protection for *L. (L.) amazonensis* and *L. (Viannia) guyanensis*, and was dependent on time of re-challenge by *L. (L.) amazonensis* after animals had recovered from primary lesions, but lacked protection against *L. (V.) braziliensis*. In contrast, monkeys that recovered from *L. (V.) braziliensis* CL or *L. (L.) chagasi* visceral leishmaniasis following chemotherapeutic intervention were protected by challenge with *L. (V.) braziliensis* and *L. (L.) amazonensis*. These findings indicate the relative variability in protection after self-cure or drug-cured experimental leishmaniasis to challenge by heterologous leishmanial parasites. Further studying the immune response may provide information regarding relevant factors influencing cross-protective immunity.

INTRODUCTION

Leishmaniasis is one of the major parasitic diseases target by the World Health Organization. The genus *Leishmania* Ross, 1903 (Kinetoplastida: Trypanosomatidae) includes approximately 30 different species that are classified in two subgenera, *Leishmania* and *Viannia*,¹ and each of these parasites has a unique epidemiologic pattern. These data explain the limited success of current control strategies based on conventional measures (such as vector reduction and elimination of infected reservoirs) for American leishmaniasis. In addition, the containment of the disease is complicated by the fact that 1) many of the *Leishmania* species causing human illness readily acquire resistance to antimonial drugs and 2) response to treatment varies considerably depending on the parasite strain involved and the clinical form.²

The protective immunity observed following convalescence for cutaneous leishmaniasis (CL) or visceral leishmaniasis (VL)^{3,4} has suggested that vaccination may prove to be the most cost-effective intervention method for the control of the various leishmanial infections. Research into the development of safe and effective vaccines against the disease has been encouraged by reports of cross-protection between distinct, though by no means all, *Leishmania* species (Table 1). Cross-immunity experiments between different strains of *Leishmania* may give important clues as to the most suitable parasites for use in antigen production to protect against the disease. Vaccines consisting of viable or killed forms of the pathogen itself have been evaluated with CL.^{3–5}

While the use of murine models have improved our understanding of the factors involved in heterologous protection,^{6–9} the mechanisms behind this acquisition of immunity remain obscure. Whereas the taxonomic identity of the parasites plays a role in the degree of cross-reactivity, genetically determined susceptibility or resistance of the host may be equally influential in the development of a protective immunity.¹⁰ Nonhuman primates appear to have significant advantages over conventional laboratory animals in terms of modeling human leishmaniasis (in particular reference to infection induced with *L. braziliensis* complex parasites)^{11–13} for purposes of studies on cross-immunity between different species

or strains of *Leishmania*. A few experiments of this nature have been carried out in monkeys,^{14–17} but have not clarified this situation.

In our previous studies, the rhesus monkey (*Macaca mulatta*) was found to be readily infected with either *L. (Viannia) braziliensis*,¹³ *L. (L.) amazonensis*,¹⁸ or *L. (L.) major*.¹⁹ This primate model develops a human-like disease (namely, a self-healing chronic granulomatous CL and resistance to homologous challenges), exhibits antibodies to *Leishmania* and parasite-specific T cell-mediated immune responses, and can be protected effectively by *Leishmania* vaccination.^{20,21} We also found that primary *L. (L.) chagasi* infection of the rhesus monkey appears to parallel the human responses to infection, including the development of strong immunity to reinfection with the homologous parasite (Pereira MS and others, unpublished data).

The purpose of this study was to potentially examine cross-reacting immune responses and possible cross-protection between taxonomically distinct leishmanial parasites in this experimental model. This finding is important for vaccine development studies against leishmaniasis.⁵

MATERIALS AND METHODS

Animals. A total of 38 laboratory-bred and -reared young adult (3–10 years old, weighing between 4,100 and 14,650 grams) rhesus macaques of mixed sexes were used in this study. Thirty-four of the animals had been previously infected with either *L. (L.) major*,¹⁹ *L. (V.) braziliensis*,¹³ or *L. (L.) chagasi* (Pereira MS, unpublished data), but had recovered from clinical disease (following either self cure or therapeutic intervention) by the time of each re-challenge. The other four monkeys were naive controls that had never been exposed to *Leishmania* antigens. Their care and maintenance have been previously described.¹⁸ All procedures involving animals were performed according to the Brazilian Guide For Care and Use of Laboratory Animals (Projeto de lei 3.964/97-www.planalto.gov.br). The experimental protocols used in this study involving monkeys and hamsters were reviewed and approved by the Fundação Oswaldo Cruz Ethic Commit-

TABLE 1
Cross-immunity experiments between different *Leishmania* species in humans and other animals

Host, number	Immunizing parasite, strain(s)	Rechallenge, parasite (strain)	Acquired immunity†	Reference
Human				
2	<i>L. tropica</i>	<i>L. donovani</i>	Complete	4
1	<i>L. tropica</i>	<i>L. braziliensis</i>	Complete	4, 41
2	<i>L. tropica</i>	<i>L. major</i>	Complete	42
2	<i>L. tropica</i>	<i>L. mexicana</i>	Complete	43
1	<i>L. panamensis</i> , L7‡	<i>L. mexicana</i> , L11	Complete	44
1	<i>L. mexicana</i> , H1‡	<i>L. mexicana</i> , L11	Complete	
1	<i>L. mexicana</i> , H1; L11	<i>L. panamensis</i> , L7	Lack of protection	
1	<i>L. amazonensis</i>	<i>L. guyanensis</i>	Lack of protection	15
1	<i>L. panamensis</i> ‡	<i>L. mexicana</i>	Complete	
1	<i>L. panamensis</i> ‡	<i>L. guyanensis</i>	Lack of protection	
1	<i>L. mexicana</i>			
1	<i>L. mexicana</i> ‡	<i>L. panamensis</i>	Lack of protection	
1	<i>L. mexicana</i> ‡	<i>L. braziliensis</i>	Lack of protection	
1	<i>L. panamensis</i>			
1	<i>L. braziliensis</i>	<i>L. major</i>	Lack of protection	23
Monkey				
<i>Macaca inuus</i> , 3	<i>L. tropica</i>	<i>L. donovani</i>	Complete (1), partial	33, 34
1	<i>L. infantum</i>	<i>L. donovani</i>	Complete	33
<i>M. mulatta</i> , 2	<i>L. braziliensis</i> , L1	<i>L. braziliensis</i> , L15	Complete (1), partial	14
6	<i>L. mexicana</i> , L11	<i>L. braziliensis</i> , L1	Complete	
2	<i>L. mexicana</i> , L11	<i>L. braziliensis</i> , L15	Partial	
2	<i>L. braziliensis</i> , L1	<i>L. mexicana</i> , L11	Partial	
3	<i>L. amazonensis</i>	<i>L. amazonensis</i>	Complete, partial (2)	18
10	<i>L. major</i>	<i>L. major</i>	Complete, partial (7)	19, 28
<i>Cebus a. apella</i> , 3	<i>L. mexicana</i> , L11‡	<i>L. amazonensis</i> , H6	Lack (2), partial	15
4	<i>L. mexicana</i> , L11‡	<i>L. braziliensis</i> , M1287	Lack (2), partial	
5	<i>L. amazonensis</i> , H6‡	<i>L. braziliensis</i> , M1287	Lack (1), partial	
2	<i>L. braziliensis</i> , M1287‡	<i>L. amazonensis</i> , H6	Complete	
3	<i>L. braziliensis</i> , M1287‡	<i>L. guyanensis</i> , M2061	Lack of protection	
1	<i>L. guyanensis</i> , M2061‡	<i>L. amazonensis</i> , H6	Lack of protection	
2	<i>L. guyanensis</i> , M2061‡	<i>L. braziliensis</i> , M1287	Complete	
1	<i>L. mexicana</i> , L11; <i>L. braziliensis</i> , M1287	<i>L. guyanensis</i> , M2061	Partial	
1	<i>L. amazonensis</i> , H6; <i>L. braziliensis</i> , M1287	<i>L. guyanensis</i> , M2061	Lack of protection	
2	<i>L. mexicana</i> , L11; <i>L. amazonensis</i> , H6	<i>L. guyanensis</i> , M2061	Lack of protection	
1	<i>L. braziliensis</i> , M1287; <i>L. amazonensis</i> , H6	<i>L. guyanensis</i> , M2061	Partial	
1	<i>L. braziliensis</i> , M1287; <i>L. guyanensis</i> , M2061	<i>L. amazonensis</i> , H6	Complete	
<i>Cercopithecus aethiops</i> , 5	<i>L. donovani</i>	<i>L. major</i>	Complete (4), partial	16
<i>Saimiri sciureus</i> , 3	<i>Trypanosoma cruzi</i>	<i>L. braziliensis</i>	Lack of protection	45
5	<i>L. donovani</i>	<i>L. panamensis</i>	Lack of protection	17
Mouse (strain)				
CBA, NIH, CFLP	<i>L. major</i>	<i>L. mexicana</i>	Complete	6, 8
	<i>L. mexicana</i>	<i>L. major</i>	Lack of protection	
DBA/2, male	<i>L. major</i>	<i>L. mexicana</i>	Complete	8
DBA/2, female	<i>L. mexicana</i>	<i>L. major</i>	Partial	
C57BL/6	<i>L. major</i>	<i>L. mexicana</i>	Complete	7
	<i>L. mexicana</i>	<i>L. major</i>	Complete	

* Number of individuals naturally or experimentally rechallenged after they had spontaneously recovered from a primary and/or secondary infection(s).

† As indicated by the level of clinical resistance to each rechallenge: complete (i.e., no lesion), partial (i.e., lesion size was smaller and healed faster than in the primary infection), or lack (failure) of protection; in this case, individuals that had recovered from previous infection(s) remained susceptible to the last rechallenge.

‡ Host infected twice with the same parasite.

tee (Comissão de Ética no Uso de Animais da Fiocruz, CEUA-FIOCRUZ, resolution # P0048-00).

Parasites. *Leishmania (L.) major*. Strain L1 (MRHO/SU/59/P) was originally isolated from a rodent in the Old World.

Leishmania (L.) amazonensis. Strain L2 (MHOM/BR/77/LTB0016) was isolated from a simple skin lesion of a patient infected in Bahia State, Brazil.

Leishmania (V.) braziliensis. Strain L3 (MHOM/BR/97/SIS) was isolated from a case with CL. Strain L4 (MHOM/BR/95/OSC) was isolated from a case with mucocutaneous disease in Rio de Janeiro State, Brazil. Strain L5, which originated from strain MHOM/BR/97/SIS, but represented a distinct genotype,¹³ was isolated from a nasal mucosal lesion of an infected monkey. Strain L6 (MHOM/BR/00/LTCP13396)

was isolated from a case with disseminated CL in the Bahia State of Brazil.

Leishmania (V.) guyanensis. Strain L7 (MHOM/BR/97/212-P) was isolated from a case with CL in the Amazon State of Brazil.

Leishmania (V.) panamensis. Strain L8 (MHOM/CO/1986/1244M) was isolated from a mucosal lesion of an infected patient in the Department of Valle del Cauca in Colombia.

Leishmania (L.) chagasi. Strain L9 (MHOM/BR/98/1669) was isolated from a case of VL in the Bahia State of Brazil.

The strains were typed by multilocus enzyme electrophoresis²² in our laboratory before being used for infection. Parasites were maintained by hamster-to-hamster passage. The procedure for parasite derivation for experimental infections

was as previously described.¹⁸ Suspensions of promastigotes were used throughout, and the parasites were derived from portions of hamster skin lesions cultured initially in NNN blood agar medium. When promastigotes appeared, the parasites were inoculated into liquid Schneider's *Drosophila* medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 20% heat-inactivated fetal calf serum (FCS), and passaged no more than three times before use for needle inoculation. Culture metacyclics were harvested by centrifugation at $1,500 \times g$ for 10 minutes at 4°C. The pellets were washed three times in sterile phosphate-buffered saline (PBS) by centrifugation as reported. Stocks of infective metacyclics derived from each parasite species were stored as stabulates at -190°C in liquid nitrogen containers using 8% glycerol in Schneider's medium containing 30% FCS.

Experimental infections. The expression of disease and time course of primary infection and/or homologous re-challenges with *L. (L.) amazonensis*, *L. (L.) major*, and *L. (V.) braziliensis* were as previously described.^{13,18,19} Primates were infected intradermally with 10^7 virulent promastigotes of each parasite species. The skin lesions were short-lived and all were cured three months after infection with *L. (L.) major*. The disease persisted somewhat longer in either *L. (L.) amazonensis*- (4–6 months) or *L. (V.) braziliensis*-infected monkeys (from approximately six months to as long as three years) and eventually healed but with cryptic parasitism and/or relapses. Infection with *L. (L.) chagasi* in the rhesus monkey showed a sustained course, ranging from a mild disease to a severe fatal VL (Pereira MS, unpublished data). Therefore, we used meglumine antimoniate (20 mg/kg of body weight/day given intramuscularly for 28 days) for treatment of long-lasting *L. (V.) braziliensis* and *L. (L.) chagasi* infections in rhesus monkeys.

This study was divided into three experiments. An infection profile of the monkeys and number of animals used for each cross-immunity trial is shown in Table 2. Groups of animals

were challenged at different time points after they had recovered from either CL (groups A-F) or VL (group G). Primates were inoculated with 10^7 stationary phase promastigotes of each heterologous parasite either into the skin of the opposite eyebrow or forearm, depending on the site of the original inoculation. Groups of two naive controls were similarly infected with either *L. (V.) guyanensis* or *L. (V.) panamensis* for determining the progression and resolution of primary skin lesions induced by each parasite in rhesus monkeys. In addition, two control hamsters were in each case inoculated to check viability of the parasites. Skin lesions were measured with a vernier caliper and lesion size was estimated using the following formula: area (mm²) = $\pi \times$ greatest radius \times smallest radius.

Antigens. Promastigotes of *L. (L.) major* (L1), *L. (L.) amazonensis* (L2), *L. (V.) braziliensis* (L5), and *L. (L.) chagasi* (L9) were the source of antigens. A preparation of soluble leishmanial antigens (SLA) was made as previously described.¹⁹ The SLA was used at different concentrations for an enzyme-linked immunosorbent assay (ELISA) and *in vitro* blast transformation assays.

Assessment of delayed-type hypersensitivity (DTH) responses. Animals were assessed for DTH reactions according to the method used for humans (leishmanin skin test [LST]). The antigen (leishmanin), provided by the FIOCRUZ Biomanguinhos Unit (Rio de Janeiro, Brazil), consisted of pooled, heat-killed, cross-species promastigotes suspended in PBS containing 0.5% phenol. For the LST, 0.1 mL of antigen containing macromolecules from 5×10^6 parasites was inoculated intradermally into the forearm, and the induration produced was measured in millimeters 72 hours later. An induration diameter ≥ 5 mm was considered positive.

Assessment of recall proliferative and interferon- γ (IFN- γ) responses. The cellular immune responses were studied *in vitro* by lymphocyte proliferative assays with SLA (10 μ g of protein/well) and mitogen (phytohemagglutinin [PHA] from

TABLE 2

Infection profile of rhesus monkeys in this study and level of cross-protective immunity among different species or strains of *Leishmania*

Group (no.) [*]	Primary infection [†]	Rechallenge infection(s) [‡]			Immunity [§]
		Secondary	Tertiary	Quaternary	
Experiment 1					
A (4)	<i>L. major</i> , L1	<i>L. amazonensis</i> , L2¶			Complete
B (9)	<i>L. major</i> , L1	<i>L. amazonensis</i> , L2#			Lack of protection
C (4)	<i>L. major</i> , L1	<i>L. guyanensis</i> , L7			Complete, partial (1)
D (6)	<i>L. major</i> , L1	<i>L. braziliensis</i> , L5			Lack of protection
E (4)	<i>L. braziliensis</i> , L3	<i>L. braziliensis</i> , L5			Complete, partial (1)
F (3)	<i>L. braziliensis</i> , L4	<i>L. braziliensis</i> , L5			Complete
G (4)	<i>L. chagasi</i> , L9	<i>L. braziliensis</i> , L5			Complete
Experiment 2					
A (2)	<i>L. major</i> , L1	<i>L. amazonensis</i> , L2¶	<i>L. braziliensis</i> , L6		Lack of protection
C (3)	<i>L. major</i> , L1	<i>L. guyanensis</i> , L7	<i>L. braziliensis</i> , L6		Lack of protection
D (2)	<i>L. major</i> , L1	<i>L. braziliensis</i> , L5	<i>L. braziliensis</i> , L6		Lack of protection
E (3)	<i>L. braziliensis</i> , L3	<i>L. braziliensis</i> , L5	<i>L. guyanensis</i> , L7		Complete, partial (2)
F (3)	<i>L. braziliensis</i> , L4	<i>L. braziliensis</i> , L5	<i>L. amazonensis</i> , L2		Complete, partial (2)
G (3)	<i>L. chagasi</i> , L9	<i>L. braziliensis</i> , L5	<i>L. amazonensis</i> , L2		Complete
Experiment 3					
E (3)	<i>L. braziliensis</i> , L3	<i>L. braziliensis</i> , L5	<i>L. guyanensis</i> , L7	<i>L. panamensis</i> , L8	Complete
F (3)	<i>L. braziliensis</i> , L4	<i>L. braziliensis</i> , L5	<i>L. amazonensis</i> , L2	<i>L. guyanensis</i> , L7	Complete

* Number of animals used in each experiment.

† The stock codes of the strains used in this study are indicated and information on their origin is given elsewhere (Materials and Methods).

‡ Monkeys were rechallenge-infected after they had recovered from previous (primary, secondary, and/or tertiary) infection(s). In some experiments, animals were injected with the same parasite strain/dose, but at different time points as indicated (at 28¶ and 44# weeks postinfection).

§ Acquired immunity to *Leishmania* in tested monkeys was measured by the level of clinical resistance to each rechallenge: complete protection, animals were refractory to the disease (i.e., no lesion observed); partial protection (i.e., individuals developed a transient lesion with smaller size that healed faster than in the primary infection); or lack of protection (i.e., monkeys remained susceptible to re-infection).

Phaseolus vulgaris, 12.5 µg/mL; Sigma, St. Louis, MO).¹⁸ Cell proliferation was expressed as a stimulatory index (SI) and was considered positive if > 2.5. The IFN-γ in supernatants of cultures of stimulated cells were measured by an ELISA as previously described.¹³ A rhesus monkey IFN-γ ELISA kit was used following the manufacturer's instructions (Bio-source International, Camarillo, CA). The IFN-γ concentrations for unknown samples and controls were read from a standard curve.

Serologic analysis. Sera from the monkeys were analyzed by adapting a standard ELISA¹⁸ to detect parasite-specific antibodies (using a peroxidase-conjugated rabbit anti-monkey IgG; Sigma). A group of sera with previously known titers, as well as naive rhesus controls, were included in each test.

Statistical analysis. The Student's *t*-test was used for comparative analysis and a *P* value < 0.05 was considered significant. Concordance between *in vivo* and *in vitro* cellular immune responses was assessed as previously reported.¹⁹

RESULTS

Primates challenge infected with the different leishmanias (experiment 1) were kept under observation (we allowed a period of 3–6 months to elapse before judging the heterologous challenge result as negative). Subsequent challenges (experiments 2 and 3) were done after the complete disappearance of any resulting skin lesion (in this case, the time required for recovery varied from approximately one week to as

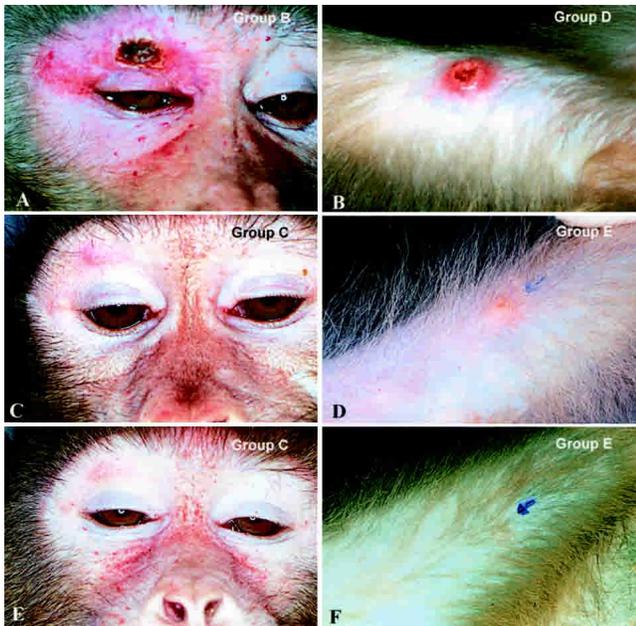


FIGURE 1. Level of cross-protective immunity among strains of *Leishmania* in the rhesus monkey model of cutaneous leishmaniasis. Shown are skin lesions visible at four weeks postinfection among groups (experiment 1). Acquired resistance was scored in terms of lesion size and development after challenge with the heterologous parasite. **A** and **B**, No protection: monkeys remained susceptible to re-infection, showing developing ulcerated skin lesions. **C** and **D**, Partial protection: animals developed a transient, small, nodular lesion with no ulcer (arrow in **D**). **E** and **F**, Complete protection: monkeys were refractory to the disease, i.e., no lesions were observed (arrow in **F**).

long as six months). Acquired immunity to *Leishmania* was then measured by the level of clinical resistance to each challenge, as indicated in Table 2 and Figure 1.

Course of secondary infection. Primates that had recovered from *L. (L.) major* skin lesions after 11 weeks postinfection were subsequently re-challenge infected with 10^7 promastigotes of either *L. (L.) amazonensis*, *L. (V.) guyanensis*, or *L. (V.) braziliensis* (Table 2, experiment 1). Hamsters inoculated with the same number of promastigotes from the same suspension of each parasite species showed progressive lesion development and maintained a chronic, nonhealing infection. Monkeys in groups A and B were inoculated with the same *L. (L.) amazonensis* strain/dose, but at different time points (A = 28 weeks and B = 44 weeks) after the primary infection. While monkeys in group A were refractory to disease (i.e., no skin lesions developed at the injection site), those in group B were found to be completely susceptible to re-infection (Figure 1). The lesion resulting from re-inoculation of *L. (L.) amazonensis* shows the same state of development or regression (Figure 2) as the original infection induced with the same parasite strain in naive monkeys.¹⁸

As shown in Table 2 and Figure 1, prior infection with *L. (L.) major* in rhesus monkeys gave protection against re-infection with *L. (V.) guyanensis* (group C), but it failed to confer immunity to *L. (V.) braziliensis* (group D). Two control naive monkeys received a similar dose of *L. (V.) guyanensis* and both animals became infected (the nodular lesions that developed were up to 1 cm in diameter at the site of inoculation, ulcerated, and slowly disappeared during the six month after infection). In contrast, *L. (L.) chagasi* infection in the rhesus monkey induced a strong resistance to re-infection with *L. (V.) braziliensis* (group G). Similarly, monkeys previously exposed to infection with either the L3 (group E) or L4 (group F) strain of *L. (V.) braziliensis* were completely immunized against challenge with an homologous parasite (strain L5) that represented a distinct genotype.¹³

Course of tertiary and quaternary infections. As shown in Table 2 (experiment 2), previous infections with *L. (L.) major*

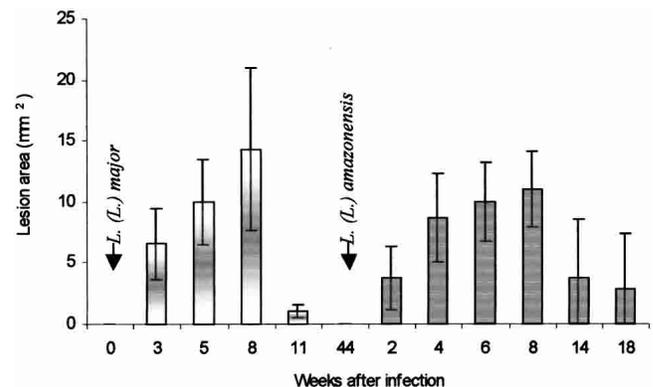


FIGURE 2. Time course of skin lesion development in *Macaca mulatta* monkeys following challenges with *Leishmania (Leishmania) major* and *L. (L.) amazonensis* (experiment 1, group B). The animals were initially injected with 10^7 *L. (L.) major* promastigotes into the skin of the left eyebrow. After recovery from the primary infection, the primates were challenged-infected in the opposite eyebrow with heterologous parasites. Animals were challenged at different time points as indicated (arrows). Lesions were scored by calculating the total lesion area as indicated (see Materials and Methods). Bars show the mean ± SD.

and either *L. (L.) amazonensis* (group A), *L. (V.) guyanensis* (group C), *L. (V.) braziliensis* (group D) did not protect monkeys against subsequent challenge with a distinct strain of *L. (V.) braziliensis* (L6) originating from a patient with disseminated CL, to which they are highly susceptible (Carvalho EM and others, unpublished data). Curiously enough, complete or partial resistance against tertiary challenge with either *L. (V.) guyanensis* (group E) or *L. (L.) amazonensis* (group F) was conferred by prior *L. (V.) braziliensis* infections. Moreover, animals that recovered from previous infections with *L. (L.) chagasi* and *L. (V.) braziliensis* also acquired resistance to challenge with *L. (L.) amazonensis* (group G). Recovery from combined infections of either *L. (V.) braziliensis*/*L. (V.) braziliensis* and *L. (V.) guyanensis* or *L. (L.) amazonensis* resulted in strong resistance to quaternary challenge with either *L. (V.) panamensis* (group E) or *L. (V.) guyanensis* (group F), respectively (Table 2, experiment 3). The two control naive monkeys inoculated in the forearm with the same dose of *L. (V.) panamensis* developed characteristic skin lesions at the site of inoculation that disappeared during the fifth month after infection. Again, hamsters inoculated with the same parasite suspensions as those used for challenging experimental monkeys showed chronic non-healing infections.

Cross-reacting immune responses. After inoculation with the different leishmanias, the antibody responses during active disease (at 8 weeks postinfection) were measured by an ELISA. As shown in Figure 3, animal groups had higher anti-*Leishmania* cross-reacting antibody levels when compared

with pre-challenge background levels (naive controls). The IgG response to *L. (L.) amazonensis*-derived antigens was apparently stronger when compared with other cross-species parasite antigens, but no statistically significant differences ($P > 0.05$) in antibody titers could be demonstrated between animals groups.

The cell-mediated immune responses detected in the rhesus monkey model following subsequent challenges with either distinct strains of *L. (V.) braziliensis* (group F) or *L. (L.) major* and *L. (V.) braziliensis* (group D) are shown in Figure 4. Peripheral blood leukocytes of animals were comparably responsive to the control mitogen PHA prior to and after challenges. The parasite-specific lymphocyte proliferative responses were negative ($SI < 2.5$) in primates prior to infection, and following primary and challenge infections, these animals exhibited proliferative and IFN- γ responses of peripheral blood leukocytes to leishmanial antigens. The findings indicated that in *L. (V.) braziliensis*-infected monkeys (group F), no significant difference was observed between the SI mean values throughout infection, but the levels of antigen-specific IFN- γ secreted were lower after the homologous challenge compared with the levels during the primary infection. In spite of wide variations from one infected animal to another (as shown by some large SD values), following the heterologous challenge, there was a statistically significant difference ($P < 0.05$) in the recall cell-mediated immune responses to the *L. major* and *L. braziliensis* antigens (Figure 4B). In addition, infected animals developed positive DTH responses; the pooled leishmanin antigen identified all monkeys with active

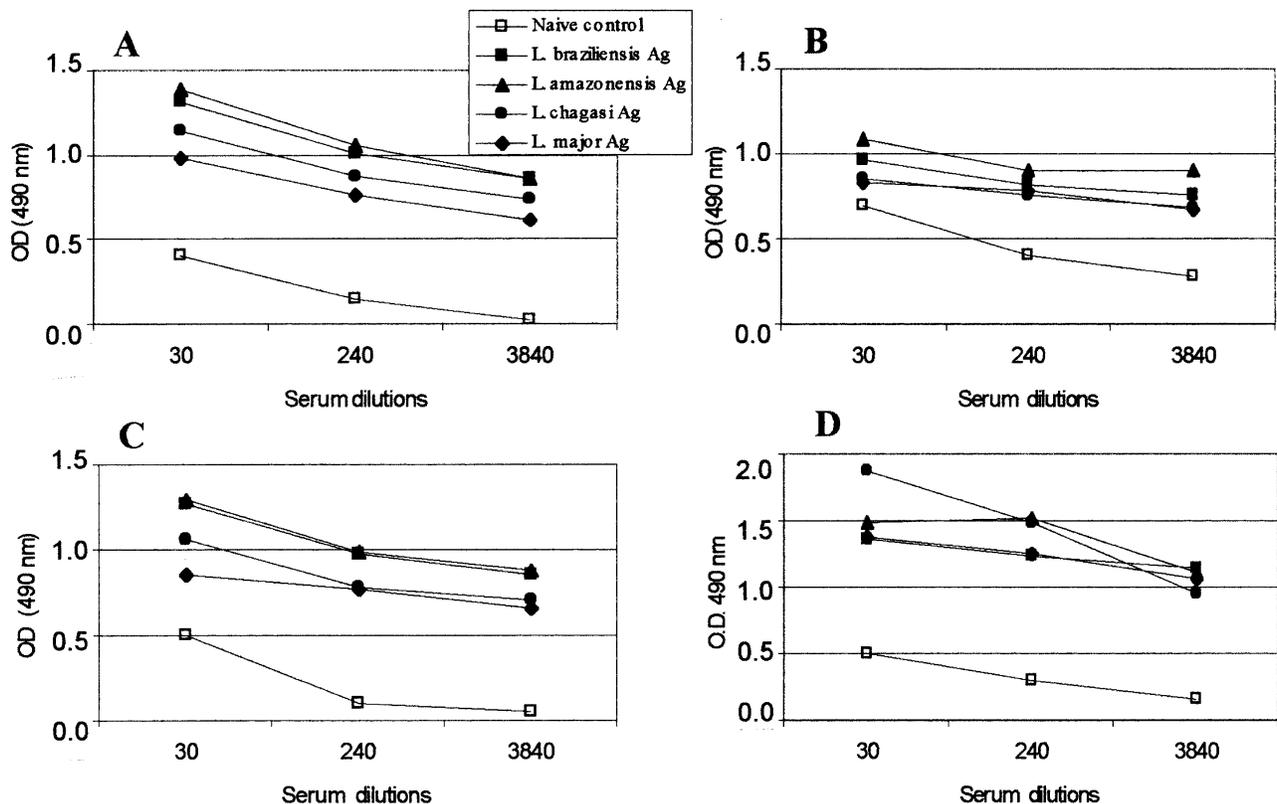


FIGURE 3. Comparative IgG antibody titers in A, *Leishmania (Leishmania.) major*-; B, *L. (L.) amazonensis*-; C, *L. (Viannia) braziliensis*-; and D, *L. (L.) chagasi*-infected rhesus monkeys to cross-species parasite antigens. Results are from pooled sera collected from monkeys at eight weeks postinfection (three animals per group) and naive controls, as determined by an enzyme-linked immunosorbent assay. All pooled sera were diluted, and results are expressed as the optical density (OD) at 490 nm. Ag = antigen.

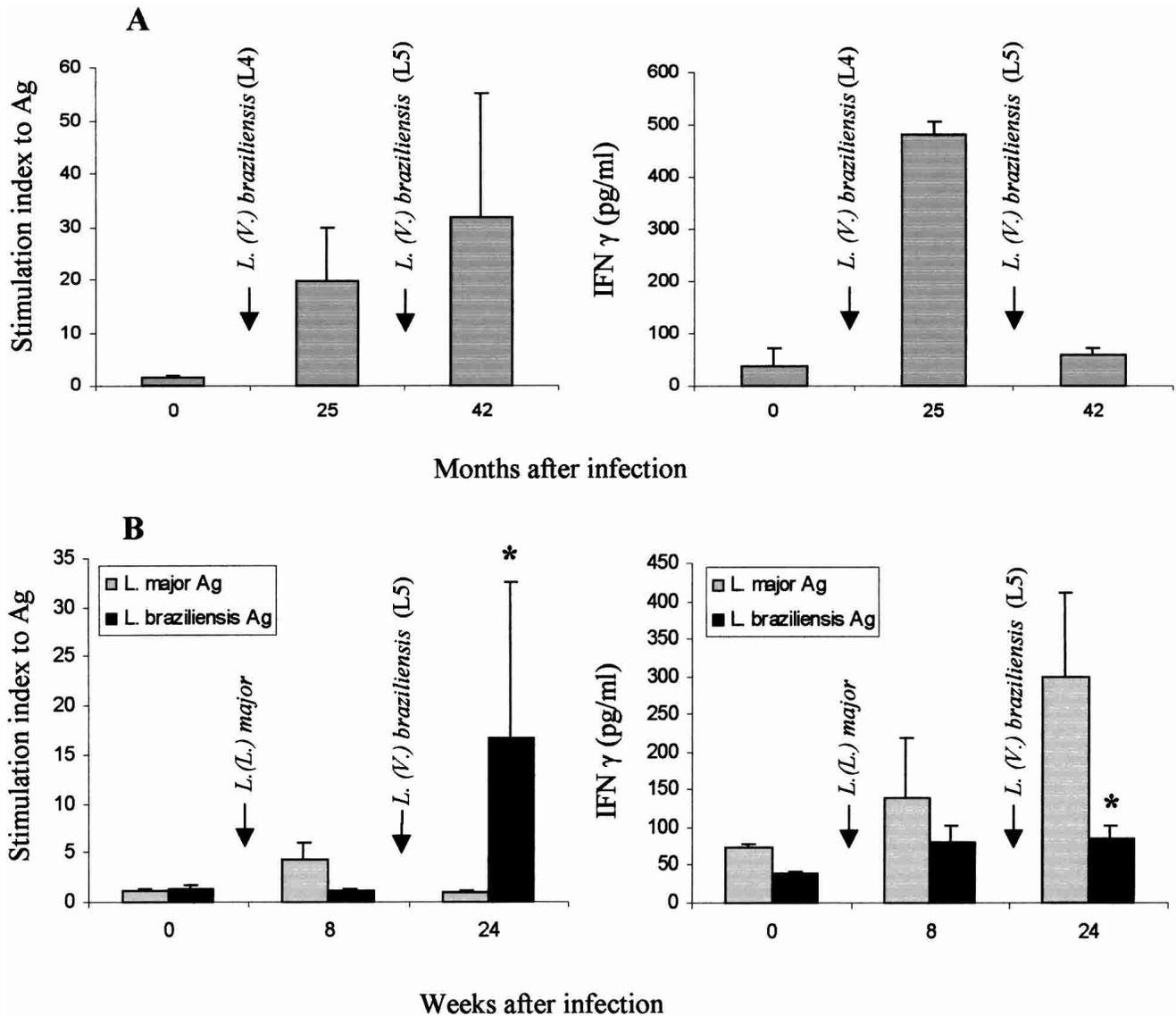


FIGURE 4. Parasite-specific recall proliferative and interferon- γ (IFN- γ) responses of T lymphocytes from rhesus monkeys infected prior to and following subsequent challenges with either **A**, strains L4 and L5 of *Leishmania (Viannia) braziliensis* (group F) or **B**, *L. (L.) major* and *L. (Viannia) braziliensis* (group D). After recovery from the primary infection, the primates were challenged at distinct time points (at either **A**, week 36 or **B**, week 15 postinfection as indicated [arrows]). Cell suspensions of purified peripheral blood leukocytes were restimulated *in vitro* in the presence of soluble leishmanial antigens prepared from *L. (L.) major* and *L. (V.) braziliensis* (strain 5). Cell proliferation was assessed by measuring ^3H -thymidine incorporation. Results are expressed as the stimulation index (mean counts per minute of stimulated cultures/mean counts per minute of unstimulated cultures). Cell culture medium samples pooled after 72 hours of stimulation were used for quantitative determination of IFN- γ in the supernatants. Bars show the mean \pm SD of three (group F) or six (group D) experimental monkeys tested. Ag = antigen. *Significant difference ($P < 0.05$) in the response to the two antigens.

and healed CL, but the reaction sizes (range = 5–28 mm) found among positive animals did not correlate with lesion development. Moreover, in *L. (V.) braziliensis*-infected monkeys (group F), there was no concordance between the positive DTH reaction size values and the levels of *in vitro* T cell proliferative responses ($r = 0.26$, $P = 0.53$) or IFN- γ ($r = 0.50$, $P = 0.26$) responses.

DISCUSSION

Recovery from *Leishmania* infection in humans is generally thought to give rise to a long-lasting clinical resistance against

homologous challenge.^{3,4} However, some observations suggest that acquired immunity to homologous parasites may not always be complete.^{23,24} Studies in animal models (Table 1) show that acquired immunity to *Leishmania* conferred by experimental infection can protect against homologous challenge. For example, monkeys challenge infected with *L. donovani* have a lower burden of parasites in their livers than can be found following primary infection with the same parasite strain.²⁵ Similar results were obtained in other primate models for CL.^{14,15,26–28} Unfortunately, in most studies of this nature it is difficult to accurately assess partial host immunity during infection since skin lesion size, a highly

variable parameter, is commonly used as indication of protection.

A self-healing phenomenon in experimental murine leishmaniasis is indicative of the development of acquired immunity. Protective immunity to *Leishmania* seems to require the development of an antigen-dependent Th1 immune response.²⁹ Studies with the vervet monkey model for *L. (L.) major* CL indicated that antigen-specific IFN- γ and DTH responses represent immunologic correlates of protection.²⁶ However, sensitization to the infection and/or the duration of the elicited immune responses, and their roles in acquired immunity, may vary in certain host-parasite combinations.³⁰ Our findings indicate that parasite-specific type 1 responses develop in either *L. (L.) major*- or *L. (V.) braziliensis*-infected monkeys, but the levels of T cell proliferative and IFN- γ responses and/or LST response do not always correlate with recovery from the skin lesions.^{3,19} This was also the situation in vaccinated monkeys, in which neither positive DTH reactions nor increased production of IFN- γ were predictive of protection.^{20,31} This fact raises a question on the current parameters that should be considered as a counterpart of expected protection induced by vaccine.

Whether the intensity of duration of the elicited responses and their roles in immunity may vary according to the nature of the infecting parasite strain or the genetic variability of the host remains to be established in the rhesus model. The experiments reported here show that following the resolution of an induced *L. (V.) braziliensis* CL,¹³ the rhesus monkey consistently develops immunity against homologous parasites (experiment 1, groups E and F), although the level of acquired resistance may be variable depending on the infecting strain (experiment 2, 2, group D). In contrast, monkeys that recovered from either *L. (L.) amazonensis* or *L. (L.) major* CL developed distinct levels of resistance (as reflected by either an absence of skin lesions or a smaller size and faster resolution of the lesions compared with the initial infection) to each homologous re-challenge.^{18,19} Therefore, the nature (i.e., etiologic agent) and the course of primary infection or disease tempo (i.e., the progression and resolution of leishmanial lesions) might influence protection. Similarly, vector factors also influence the evolution and outcome of CL. Infections from sand fly bites are more aggressive and require fewer parasites to initiate. The enhancing effect of sand fly saliva on CL has been demonstrated in rodents.² Moreover, culture metacyclics (the developmental stage of the parasite that is infective) are not as virulent as metacyclics isolated from sand flies. When infected with *L. (L.) major* transmitted by *Phlebotomus papatasi*,²⁸ rhesus macaques developed skin lesions that lasted longer (12–28 weeks) than can be found following infection (11 weeks) induced by needle inoculation with large numbers of *L. (L.) major* culture metacyclics.¹⁹

Cross-immunity between different strains of *Leishmania* parasites ranges from non-existent to full protection (Table 1). The study of cross-protective immune between leishmanial parasites presents a major practical implication because vaccination procedures could be based on the use of vaccine antigenic molecules from selected parasites to protect against many other parasite species. For instance, parasites of the subgenus *Viannia* offer considerable protection against those of the *L. mexicana* complex (subgenus *Leishmania*). Thus, *L. (V.) panamensis* protects against *L. (L.) mexicana* but not vice

versa in the rhesus monkey.¹⁴ Likewise, unilateral cross-immunity to either *L. (L.) amazonensis* by *L. (V.) braziliensis* or to *L. (V.) braziliensis* by *L. (V.) guyanensis* was observed in *Cebus* monkeys.¹⁵ Strangely, in the latter study, *L. (V.) guyanensis* did not immunize monkeys that recovered against *L. (L.) amazonensis* and lack of cross-protection was found as well to either *L. (L.) amazonensis* or *L. (V.) braziliensis* by *L. (L.) mexicana*.

In this study, we have analyzed potentially cross-reacting immune responses between taxonomically distinct leishmanias and possible cross-species protection in the rhesus monkey model. The experiments indicate that *L. (L.) major* cross-protects against either *L. (L.) amazonensis* or *L. (V.) guyanensis* in rhesus macaques, but recovery from subsequent challenges with either *L. (L.) major* and *L. (L.) amazonensis* or *L. (L.) major* and *L. (V.) guyanensis* did not protect against *L. (V.) braziliensis* infection. In contrast, the latter species appears to be a good immunizing agent, not only against *Viannia* parasites, but also against other distinct leishmanias (experiments 2 and 3). Conversely, monkeys immune to *L. (L.) chagasi* infection were found to be completely resistant to challenge with *L. (V.) braziliensis* (experiment 1, group G). This finding is not consistent with the results observed in a patient with concurrent infection with *L. (L.) chagasi* and *L. (V.) braziliensis*.³² Also, protection by primary *L. donovani* infection for *L. (L.) major* challenges occurred in the vervet monkey (*Cercopithecus aethiops*) model of the disease.¹⁶ In contrast, *L. (L.) donovani* infection does not appear to offer protection against challenge with either *L. (V.) panamensis* in *Saimiri sciureus*¹⁷ or *L. tropica* sp. in *M. inuus*.^{33,34}

All infected animals responded with increased production of immunoglobulins capable of binding to cross-reacting parasite antigens. This is not surprising since the various species of *Leishmania* share a number of antigens.³⁵ The current study also indicates cross-reactivity at cellular levels between *L. (L.) major* and *L. (V.) braziliensis* in rhesus monkeys, but recall T cell type 1 responses did not correlate with heterologous protection (Figure 4B). These data suggest that much remains to be learned about what is required to develop and maintain cell-mediated immunity to leishmanial re-infections. Nevertheless, a high level of cellular cross-reactivity paralleled by cross-protection of *L. (L.) donovani* against *L. (L.) major* was shown in vervet monkeys.¹⁶ More often, cross-reactivity at cellular levels among leishmanias has been positively correlated with cross-immunity in mice.^{36,37}

The relative variability in level of protection induced by experimental infection against subsequent challenges is probably due to several factors influencing the nature of immunity attained. One factor that can influence acquired protective immunity is the time between recovery from primary infection and re-challenge. For example, cross-immunity between *L. (L.) major* and *L. (L.) amazonensis* parasites in *M. mulatta* does not give rise to a long-lasting immunity against challenge. Groups of monkeys challenged with the same parasite strain/dose but at different time points (A = 28 weeks and B = 44 weeks) after the primary infection exhibited distinct level of protection. The biologic significance of this decrease in acquired resistance to subsequent challenge(s) is not clear, although those animals completely recovered and parasite-free would possibly develop lower resistance to re-infection. The long-term persistence of parasites in a host that is also able to maintain strong resistance to re-infection, referred to

as premunition or concomitant immunity, may be the mechanism by which a sustained immunity to *Leishmania* is achieved in the infected host.^{38,39}

Recent findings indicate that CD4⁺CD25⁺ regulatory T cells control *L. (L.) major* persistence and immunity in murine experimental leishmaniasis.³⁹ The significance of these observations may be related to a leishmanial vaccine. Those living in endemic areas and therefore continuously exposed to the parasite would acquire life-long protective immunity to re-infection. Alternatively, a vaccine that can prevent the disease (but not the infection) would produce a strong priming so that continuous immunity would be achieved by repeated exposure to leishmanial parasites through the bites of infected sand flies.⁴⁰

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