

PARASITOLOGICAL AND IMMUNOLOGICAL EFFECTS INDUCED BY IMMUNIZATION OF *MANDRILLUS SPHINX* AGAINST THE HUMAN FILARIAL *LOA LOA* USING INFECTIVE STAGE LARVAE IRRADIATED AT 40 KRAD

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Summary:

Six mandrills were immunized with 150 *Loa loa* infective stage larvae (L3) irradiated with 40 Krad, and challenged with 100 L3, 60 days after initial vaccination. The parasitological outcome of this immunization was compared to results from six mandrills infected with normal L3. No clear association was seen between vaccination and microfilaremia until day 245 when a significant drop in the level of microfilaria occurred in vaccinated compared to infected animals (5 vs 10 mf/ml; $p = 0.012$). A one-year follow-up of the humoral immune response showed a strong adult microfilariae (Mf) and L3 specific IgG response, with distinct profiles for each extract. In immunized animal a significant decrease in antibody level was systematically observed between days 90-145 for the anti-L3 and anti-adult IgG. However, in the same group anti-Mf antibody levels that peaked around 160-175 days post-challenge, were inversely correlated with the decrease in Mf density between day 200 and day 386. These results suggest that immunization with irradiated L3 using these specific conditions may affect the appearance of Mf.

KEY WORDS : irradiation, vaccine, *Loa loa* L3, IgG, mandrill, microfilaria.

Résumé : RÉSULTATS PARASITOLOGIQUES ET IMMUNOLOGIQUES INDUITS CHEZ *MANDRILLUS SPHINX* PAR UNE IMMUNISATION RÉALISÉE À L'AIDE DE LARVES INFESTANTES DE LA FILAIRE HUMAINE *LOA LOA*, IRRADIÉES À 40 KRAD

Six mandrills ont été immunisés avec 150 larves infestantes de *Loa loa* (L3) irradiées à 40 krad. Cette opération a été suivie 60 jours plus tard d'une infection d'épreuve à l'aide de 100 L3 normales. Parallèlement, six mandrills ont été infectés avec des larves normales. Les résultats parasitologiques ne montrent aucune association spécifique entre la vaccination et la microfilarémie jusqu'à J245. Toutefois, une chute significative de la microfilarémie apparaît chez les animaux immunisés par rapport aux animaux infectés (5 mf/ml vs 10 mf/ml; $p = 0,012$) après J245. Un suivi longitudinal de la réponse en IgG spécifique vis-à-vis des antigènes de vers adultes, de microfilaries (mf) et de larves L3 a montré des taux d'anticorps très élevés, avec des profils distincts pour chaque extrait antigénique. Dans le groupe des animaux immunisés, une chute importante du taux d'anticorps a été observée entre les jours 90-145 pour les antigènes de ver adulte et de L3. Toutefois, dans le même groupe, les anticorps anti-mf, qui atteignent leur maximum entre les jours 160-175 après l'infection d'épreuve, étaient inversement corrélés à la baisse de la densité de microfilaries circulant entre les jours 200 et 386. Ces résultats montrent que dans ces conditions l'immunisation pourrait affecter l'apparition de microfilaries.

MOTS CLÉS : irradiation, vaccin, *Loa loa* L3, IgG, mandrill, microfilaire.

INTRODUCTION

The human filarial *Loa loa* is endemic in the West African forest block, where it affects three million people (Fain, 1978). As in many other filariae, existing drugs are active on microfilariae only and have little effect on adult worms (Richard Lenoble *et al.*, 1988). Vector control is unrealistic and the existence of animal reservoir is suspected. Therefore, alternative means of controlling this infection must be considered. There is evidence from human infections

that the immune response may play a role in protection against filariae (Day *et al.*, 1991) and *Loa loa* offers an interesting spectrum of infections characterised by a high percentage of amicrofilaremic subjects in endemic areas (Van Hoegaerden *et al.*, 1987). Interestingly, it is through different animal models of filarial infection that the best evidence for protective immunity has been shown. For example, Wong *et al.* (1969), using irradiated larvae of the zoonotic species of *Brugia malayi* in rhesus monkeys, obtained clearance of microfilariae; Yates *et al.* (1985) using irradiated larvae of *Brugia pabangi* obtained protection in jird; similarly, Oothuman *et al.* (1979) observed the same effect in dogs using irradiated larvae of *Dirofilaria immitis*. One needs to look at the mechanisms implicated in the efficacy of an irradiated vaccine, since these are still unknown. However, despite this success, irradiated larvae cannot be administered to humans. *Mandrillus sphinx*, a non-human primate found in central Africa has been shown to be per-

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missive to human *Loa loa* (Pinder *et al.*, 1994). In the present study, we have injected irradiated infective stages of human *Loa loa* into mandrills to study the mechanisms implicated in a protective immune response against filarial parasites.

MATERIALS AND METHODS

MANDRILLUS SPHINX

A group of 12 *Mandrillus sphinx*, three to seven years old of both sexes from CIRMF's breeding colony were housed in outdoor group cages. The average weight was 9.03 kg, varying between 5.3 to 18.1 kg. Basal clinical and biological examinations were performed and showed no sign of filarial infection. These animals were split into two groups, one group was immunized, and the other group was treated as an infection control. Ethical approval was obtained from CIRMF's Primate ethical committee on the use of animals before any experimentation began.

INFECTIVE STAGE (L3) OF *LOA LOA*

L3 larvae were purified from naturally infected *Chrysops* flies by maceration, using the Baerman technique, as modified by Wahl *et al.* (1995). L3 were picked up individually using a Pasteur pipette and transferred to a fresh solution of phosphate-buffered saline (PBS).

IRRADIATION OF L3

Live, motile larvae were placed in tubes as groups of three hundred in PBS. The tube was placed into the irradiation chamber of a Gamma cell 1000 Elite apparatus (Norton International Inc, Kanaka, Ontario, Canada). The larvae were then exposed to 223 rad/minute from a Caesium 137 source. All larvae were motile after irradiation, and were used within an hour after irradiation.

IMMUNIZATION AND CHALLENGE OF MANDRILLS WITH L3 OF *LOA LOA*

Six mandrills were immunised three times on day - 60, - 30, - 15 with 150 L3 irradiated (50 L3 × 3) at 40 krad. Irradiated larvae, resuspended in sterile PBS solution, were pulled up into 1 ml syringes with a 21-gauge needle. These were inoculated subcutaneously on either the right or left side of the chest. After inoculation the syringe was refilled with PBS and this was inoculated again if some larvae were still visible in the syringe. Two weeks after the third immunization (Day 0), the six immunized mandrills were challenged with 100 fresh L3 each in one step, and the six naive mandrills were infected with the same number of fresh L3 per

animal in a similar manner, to that described for vaccination.

FOLLOW-UP OF MANDRILLS

Animals were systematically bled under anaesthesia at different time points, before, during and after immunization/challenge or infection, from day - 120 to day 386. Lymph nodes were palpated and body temperatures were measured on each occasion. The uncoagulated blood collected on EDTA was used for leucocytes, differential counts, and to separate peripheral blood mononuclear cells (PBMC) from plasma by Ficoll hypaque gradient.

1 ml of uncoagulated blood was used to determine the microfilaremia using a concentration technique described previously (Akue *et al.*, 1996). The pellet was examined under a microscope. *Loa loa* microfilariae were identified by their size, sheath and movements. The results were expressed as the number of microfilariae per ml.

PARASITE AND ANTIGEN PREPARATION

Adult worms of *Loa loa* were removed by an ophthalmologist during ocular passage. Microfilariae were obtained from heavily infected patients and purified on percoll gradients as described (Van Hoegaerden & Ivanoff, 1986). L3 were obtained by dissection of naturally infected *Chrysops* species caught in *Loa loa* - endemic village. Parasites were stored in liquid nitrogen until required.

Adult worms or L3 were washed and then homogenized in 10 mM Tris-HCl, pH 8.3, containing protease inhibitors as described previously (Akue *et al.*, 1997). Mf were disrupted by sonication in the same buffer. Antigens were extracted with 1 % Sodium Deoxycholate for one hour at 4° C. The extracts were centrifuged at 10,000 g for 10 minutes, and the soluble supernatant was aliquoted and stored at - 70° C. The protein content was measured using the Bio-Rad (Richmond, CA) method (Bradford, 1976).

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Detergent soluble extracts of adult, Mf or L3 stages of *Loa loa* were diluted in carbonate-bicarbonate buffer, pH 9.6 and 100 µl volumes were distributed into the wells of microtiter plates (Immunolon II; Dynatech Laboratories, Chantilly, VA) at 10 µg/ml. After an overnight incubation at 4° C, the plates were washed three times for 10 min at room temperature with 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, and 0.05 % Tween 20 (TBST). The plates were then blocked for one hour with TBST-5 % bovine serum albumin, and the washing step was repeated. Mandrill plasma diluted 1/400 in TBST-1 % bovine serum albumin was added for one

hour, and the washing step was repeated and followed by incubation with mouse anti-human IgG Fc specific alkaline phosphatase conjugate (Sigma, St. Louis) for one hour. After washing the plates, the reaction was detected using p-nitro phenyl phosphate (Sigma) diluted in diethanolamine buffer, pH 9.8 and the optical density (OD) was read at 405 nm using a LP200 micro-ELISA reader (Diagnostic Pasteur).

STATISTICAL ANALYSIS

A Mann-Whitney U test was used to compare the median levels of IgG and microfilaria density between groups. P values < 0.05 were considered significant.

RESULTS

LEUCOCYTE COUNT

During the follow-up, leucocytes were analysed at different time points (Table I). None of the 12 animals showed any significant variations in the level of total leucocytes counts ($p > 0.05$). Differential counts of each cell type showed that eosinophils, lymphocytes and monocytes were similar in infected and immunised animal throughout the follow-up period. Neutrophils decreased in number in the circulation of infected animals during prepatency (median value: 4,208 *vs* 2,375; $p = 0.0058$) and returned to the original level during the patency. Although the level of neutrophils was similar in both infected and immunised groups before infection or immunization, it appeared that the level of neutrophils in immunized group become significantly lower during prepatency (median value: 2,196 *vs* 2,736; $p = 0.010$) and patency (median value: 2,220 *vs* 4,208; $p = 0.0005$) than in infected animals before infection or during the patency phase.

COMPARATIVE DENSITY OF MICROFILARIAE BETWEEN VACCINATED AND INFECTED ANIMALS

All 12 mandrills were followed-up parasitologically during 386-435 days, with 26 sampling points during this period. At each time point the overall density of microfilariae in vaccinated animals was similar to that in infected animals (difference not statistically significant: $p = 0.298$) (Fig. 1). However, the peak of microfilaremia in vaccinated animals (37 Mf/ml) was delayed by three months (day 230) after the onset of patency, compared to infected animals (37 Mf/ml) where the peak appeared one month (day 175) after the onset of patency. Following these peaks the density of microfilariae decreased in both vaccinated and infected animals. However, the drop in microfilaria between day 245 and 435 was deeper in vaccinated than in infected animals (5 Mf *vs* 10 Mf; $p = 0.0122$). Within the vaccinated group the density of microfilaria between days 145 and 230 was significantly different from that between days 245-435 ($p = 0.050$). This difference was not seen in infected animals during the same period ($p = 0.935$).

DYNAMIC OF THE HUMORAL IMMUNE RESPONSE AGAINST *LOA LOA* L3, ADULT, MICROFILARIAL ANTIGENS

In order to relate the outcome of the infection to immune responses, the levels of specific IgG against adult, microfilaria and L3 antigens were measured from day - 120 to day 386 in the plasma samples taken at different time points per animal. The results for each treatment group were averaged across all six animals. The mean level of each group for each time point was used to follow the kinetic of the humoral response (Fig. 2). All immunized animals showed three phases characterised by alternating increases and decreases in IgG levels.

L3 and adult antigens (Figs 2B and C, respectively) showed a significant decrease in specific IgG levels bet-

Cells*	Infected mandrills			Immunized mandrills		
	Before infection	Prepatency	Patency	Before immunization	Prepatency	Patency
Total WBC	11,156 ± 3,970	9,567 ± 3,115	10,959 ± 4,116	10,633 ± 1,058	9,721 ± 3,690	9,743 ± 2,937
Eosinophils	275 ± 185	372 ± 261	478 ± 319	–	460 ± 333	504 ± 363
Lymphocytes	5,543 ± 2,719	5,368 ± 2,065	5,748 ± 2,163	6,954 ± 1,882	5,449 ± 2,085	6,141 ± 2,301
Neutrophils	4,405 ± 2,425**	2,692 ± 1,389	3,934 ± 2,375*	3,296 ± 2,691	2,824 ± 2,195	2,318 ± 1,335
Monocytes	1,253 ± 722	758 ± 593	776 ± 579	950 ± 328	789 ± 449	816 ± 521

* Significantly high compared to prepatency value in infected and immunized animals ($p = 0.035$; $p = 0.014$ respectively); also with patency in immunized ($p = 0.010$).

** Significantly high compared to prepatency value in infected and immunized animals ($p = 0.005$; $p = 0.010$ respectively); also with patency in immunized ($p = 0.0005$).

* cells are expressed as number of cells per ml of blood; WBC: White blood cells; coagulated blood; comparison are based on median value (Mann-whitney U test) rather than the arithmetic means presented in the Table.

Table I. – Comparison of the mean levels ± standard deviation of leucocytes in infected and immunized mandrills.

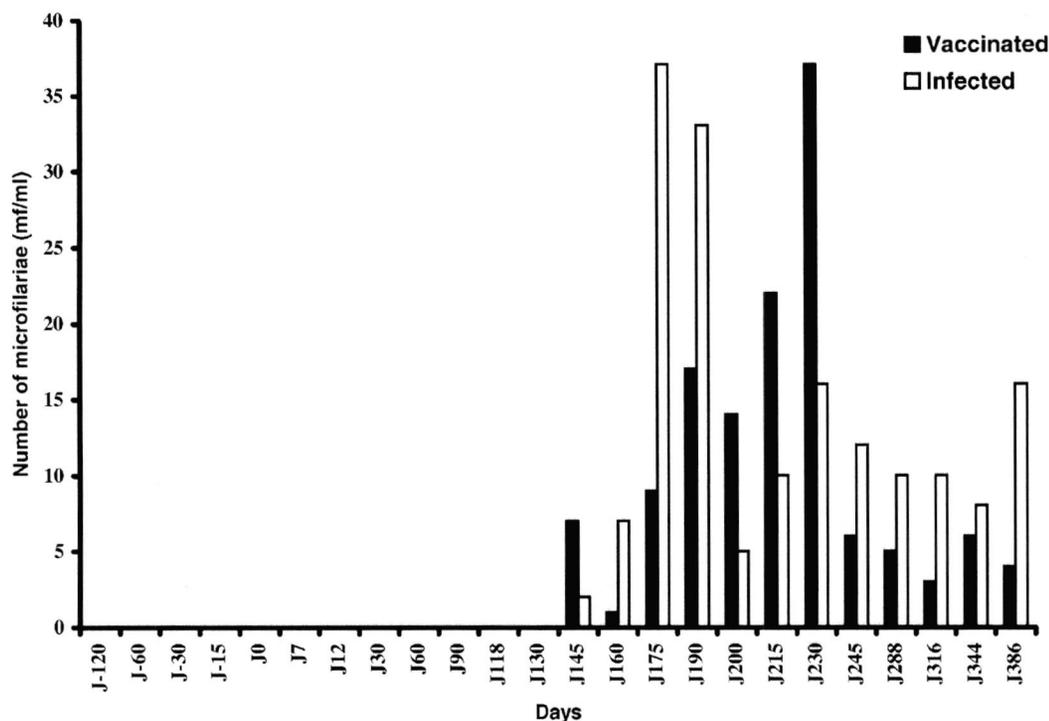


Fig. 1. – Comparative densities of microfilariae in infected versus vaccinated groups of mandrills. Microfilariaemia was determined at different time points before, during and after infection (unfilled histogram) or immunization (histogram in grey). The height of each histogram represents the mean level of microfilariae in the blood of the six animals of this group at each specific time point of the follow-up.

ween days 90 and 145. However, the overall level of specific IgG against L3 was similar in both vaccinated and infected (0.651 vs 0.446; $p = 0.0851$), while IgG levels against adult antigen were significantly higher in vaccinated compared to infected animals (1.1370 vs 0.859; $p = 0.0049$).

When the kinetics of microfilaria specific antibodies (Fig. 2A) were computed, it appeared that there was a steady increase in the infected group from day 12, to reach a maximum at day 160 followed by a drastic drop at day 215, when levels remained low until day 435. In contrast, vaccinated animals started to increase quickly from day -15 to reach a maximum at day 175. Despite a slight drop, specific anti-microfilaria IgG remained high. Furthermore, the overall level of specific IgG in vaccinated animals was significantly higher than in infected animals (0.368 vs 0.159; $p = 0.0033$).

DISCUSSION

This is the first attempt at immunization using irradiated human *Loa loa* L3. We used the absence of microfilaria as index of protection. However, as the number of microfilaria is not necessarily correlated to the number of adult worms, the use of absence or presence of Mf as an index does not fully reflect the overall degree of protection. A reduction in the adult worm burden would have been a better criteria, but for ethical reasons mandrills were not sacrificed for this purpose.

The irradiation dose of 40 krad retained for this experiment was thought to be sufficient for several reasons: first, this dose was within the range of irradiation that has been commonly used with success in other models (10-45 krad); secondly, when animals are inoculated with *Loa loa*, incubation periods for normal infective larvae to patency is ~ 145 days (Duke, 1960; Pinder *et al.*, 1994). Thus failure of larvae to be irradiated would have resulted in a much earlier appearance of Mf during the immunization process. In this study, Mf appeared only five months (day 210) after challenge in immunized animals.

Eosinophils were not correlated with the parasitological status of the animal. This result is in agreement with observations in the human population from endemic areas of *Loa loa* (Akue *et al.*, 1996). Interestingly, a fluctuation of neutrophils number was observed. The significance of this event is unknown. However, neutrophils are implicated in host defense against bacteria and it has been shown that apoptosis of neutrophils can be enhanced or delayed by various signals (Park *et al.*, 2002).

Two distinct patterns of IgG response were seen, one generated to L3 and adults, the other in response to Mf extract. For L3 and adult extracts, the levels of antibody had increased by days 7-12 and decreased significantly between days 90-145 then increased again after day 145. It is likely that adult maturation, which appears, between day 90-145 induces the state of energy which down regulates the immune response. This situation may favour parasite survival. The state

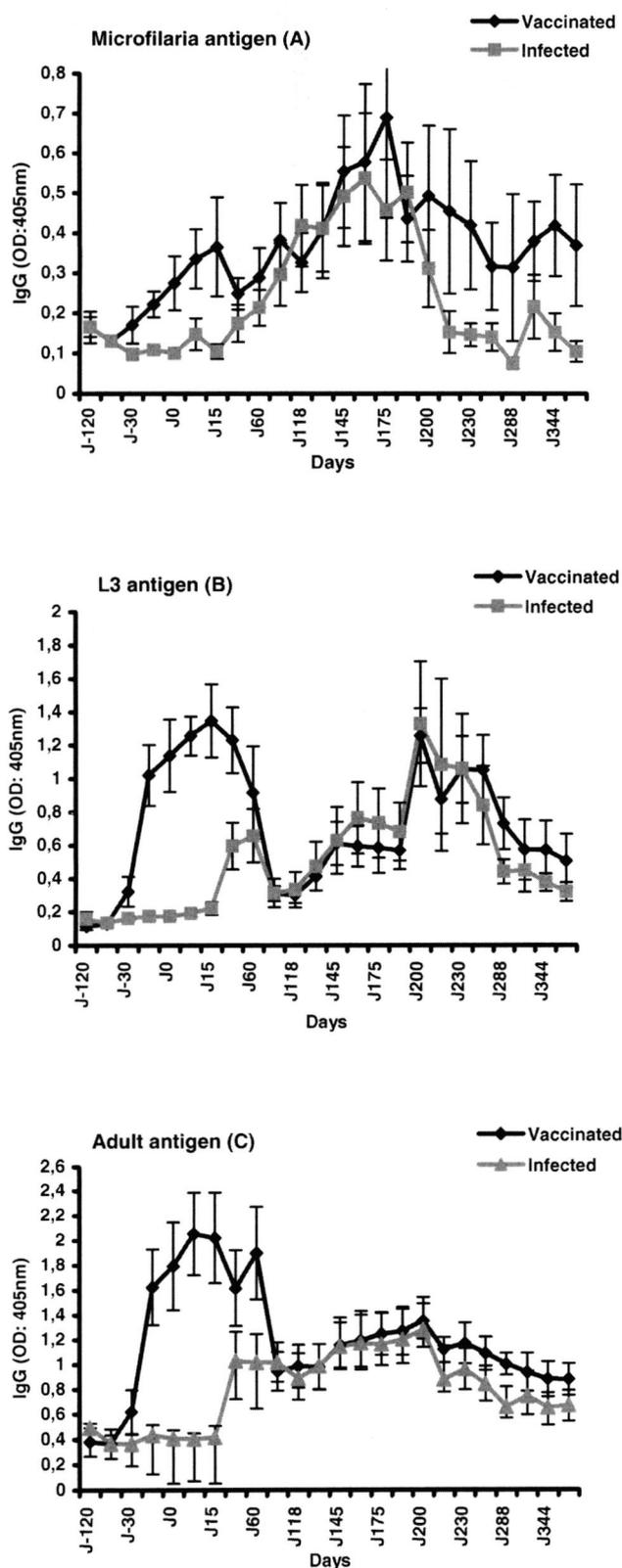


Fig. 2. – The dynamics of specific IgG responses between vaccinated and infected against *Loa loa* Mf, L3 and adult antigen from day – 120 to 386, as measured by ELISA. Each point in the curve represents the mean level of antibody expressed in optical density units (OD) \pm SD (standard deviation) for the six animals of the group (vaccinated/infected).

of anergy and tolerance has been shown in filariae and is usually attributed to Mf (Maizels & Lawrence, 1991; Nutman *et al.*, 1987; Piessens *et al.*, 1980; Yazdanbakhsh *et al.*, 1993). However, the fact that the immune response increased again after Mf appearance suggests that the young adult is involved. Indeed, Ungeheuer *et al.* (2000) have shown that Mf induced more proliferative response than L3.

Antibodies to the Mf extract started to increase quickly from day 0 in vaccinated animals but not in infected animals, with both groups reaching their maximum level around day 160-175; maximum levels were higher in vaccinated animals. This level dropped significantly thereafter in infected but not in vaccinated animals. Although the level of anti microfilaria antibody remains high in vaccinated group, it is not clear from our experience whether anti Mf plays a role in the observed reduction of Mf. It is likely that the clearance of Mf is a result of multifactorial component. This is substantiated by the fact that analysis of cytokine profile of the same animals (Ungeheuer *et al.*, 2000), showed that Th2 cells and some Th1 cytokine (IL2) against L3 and Mf antigen were elevated in animal microfilariae negative or with delayed peak of microfilaremia. Taken together, these results indicate that immunity induced by irradiated L3 under these specific conditions of immunization may act predominantly on microfilariae. Rather than regarding this type of protection as a failure, immunity which clears Mf instead of preventing infection is interesting since clearing Mf would result in a suppression of the transmission reservoir.

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