The relationship between parasitological status and humoral responses to *Loa loa* antigens in the *Mandrillus sphinx* model after immunization with irradiated L3 and infection with normal L3

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(*Received 25 May 2000; revised 28 November 2000; accepted 18 January 2001*)

SUMMARY

In order to identify antigens associated with protection and those associated with active infection, the humoral immune response of 6 *Mandrillus sphinx* immunized with 150 irradiated L3 and challenged with 100 normal L3 of *Loa loa* or 6 animals infected with 100 L3 were compared. The plasma of these animals was analysed by Western blot using adult, Mf and L3 antigens. Several antigens with molecular weights varying from 120 kDa to 13 kDa were recognized by the plasma of all animals. It was shown that early recognition of microfilarial antigens with molecular weights of 97, 68, 45 and 33 kDa correlated with the amicrofilaraemic state. A total of 83% of animals with circulating microfilariae had antibodies against the microfilariae 21 kDa antigen. Furthermore, the antibodies against the 21 kDa appeared 1 month before detection of microfilariae in the peripheral blood of 80% of these animals, and declined when animals became amicrofilaraemic. In contrast, when L3 antigen was used, a molecule with a relative molecular weight of 20 kDa was recognized by antibodies of the only animal which remained amicrofilaraemic for 1 year after immunization with irradiated L3. These results suggest that the microfilarial molecule of 21 kDa may be useful as a marker of *Loa loa* patent infection, whereas the 97, 68, 45 and 33 kDa molecules of microfilariae and the L3 molecule of 20 kDa may be associated with resistance against *Loa loa*.

Key words: *Loa loa*, antigen, parasitological status, *Mandrillus sphinx*, IgG.

INTRODUCTION

Loa loa is a human filarial parasite, which affects 13 million individuals in the West African forest block (Fain, 1978). Clinically, the disease has a large spectrum of signs varying from allergic symptoms to hydrocele, cardiopathy, and ocular passage of adult worms under conjunctiva. The parasite is transmitted by the bite of a *Chrysops* carrying 3rd-stage larvae (L3) within the head and mouthparts. The L3 develop to adults in the subcutaneous tissues and the female worm produces microfilariae (Mf) which circulate in the bloodstream. In humans, 70% of infected individuals remain amicrofilaraemic, suggesting the existence of a mechanism capable of clearing microfilariae from the peripheral blood of these individuals. One handicap in studying this immune mechanism in humans, in contrast to animal models, is the complexity of the spectrum of infection. For example, the 'endemic normal' and 'amicrofilaraemic' groups are heterogeneous, with some individuals harbouring cryptic infection or a single-sex infection (Eveland, Yermakov & Kenny, 1975). This spectrum can be affected by several external factors such as transmission intensity and age. At present it is difficult to distinguish between current and past infection. In human loiasis, previous reports have shown an association between the immune recognition of some antigens and the state of resistance or amicrofilaraemia (Pinder, Dupont & Egwang, 1988; Egwang *et al.* 1989; Akue, Hommel & Devaney, 1998). There is also some evidence of protective immunity in other human filarial infections (Day, Gregory & Maizels, 1991). However, the best evidence of protective immunity comes from animal models using irradiated L3 (Wong, Frederic & Ramachandran, 1969; Oothuman *et al.* 1979; Yates & Higashi, 1985), but, neither the mechanisms nor the antigens implicated in such immune responses are defined. In order to address these points, we used irradiated and normal larvae of *L. loa* in the Mandrill model of *L. loa* infection, with the goal to induce a different spectrum of infection, varying from patent microfilaraemic to the amicrofilaraemic state of infection, as encountered in human infection. Such a situation should allow the identification of important antigens in a well-controlled infection.

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Parasitology (2001), **123**, 71–76.

DOI: 10.1017/S0031182001007910 Printed in the United Kingdom
Cambridge University Press

MATERIALS AND METHODS

Mandrillus sphinx

A group of 12 *Mandrillus sphinx*, 3–7 years old were transported from a semi-free range colony in a natural rain forest enclosure to outdoor cages. The median weight was 9·03 kg varying between 5·3 and 18·1 kg. Clinical and biological examination showed no sign of filarial infection. These animals were split into 2 groups, one group for immunization, the other group of 6 for infection controls.

Immunization and infection of Mandrills with L3 of Loa loa

Six Mandrills were immunized 3 times at 0, 30 and 45 days (day −60, −30 and −15 respectively) with a total of 150 L3 irradiated at 40 krad. using a Cesium 137 source (*Gamma cell 1000 Elite* apparatus, Nordion International Inc, Kanata, Ontario, Canada). Two weeks after the second boost, the 6 immunized Mandrills were challenged with 100 fresh L3, and the 6 naive Mandrills were given a primary infection with the same number of fresh L3 per animal. Larvae were inoculated subcutaneously on the right and left side of the chest alternatively.

Collection of plasma and examination for the presence of microfilariae

The 12 animals were bled at different time-points (all 12 animals were bled at each time-point) before, during and after immunization and infection from day −120 to day 435. The uncoagulated blood was used to separate plasma from peripheral blood mononuclear cells (PBMC) in a gradient of Ficoll-Hypaque. This plasma was used for the analysis of the humoral response.

For the detection of microfilaria, 1 ml of uncoagulated blood with 9 ml of PBS containing 2% saponin were mixed. This mixture was centrifuged at 3000 ***g*** for 10 min. The resulting pellet was examined under the microscope, and *L. loa* microfilariae were identified by their size, sheath and movement. The results were expressed as the number of microfilariae per ml.

Parasites and preparation of antigens

Adult worms of *L. loa* were removed by an opthalmologist during ocular passage while Mf were isolated from the blood of heavily infected individuals. Mf were purified on Percoll gradients as described previously (Van Hoegaerden & Ivanoff, 1986). L3 were obtained by dissection of naturally infected *Chrysops*. Adult parasites and L3 stages were disrupted by homogenization in PBS while Mf were sonicated in PBS and then extracted by boiling in SDS sample cocktail containing 2% SDS and 100 mM dithiothreitol in Tris–HCl buffer, pH 6·8. The protein content of each extract was estimated by titration on Coomassie blue-stained mini-gels.

SDS–PAGE and Western blotting

Loa loa antigens were separated by SDS–PAGE under reducing conditions on 12·5% acrylamide mini-gels, with a discontinuous buffer system (Laemmli, 1970). A total of 100 μg of protein was analysed in a continuous well. Separation was optimal over the M_r range 97–10 kDa. Following electrophoresis, proteins were transferred onto nitrocellulose paper (NCP) by electrotransfer in a buffer containing 0·25 M Tris–HCl, 0·192 M glycine, 20% methanol, pH 8·3 (Towbin, Staehelin & Gordon, 1979). The NCP was stained with Ponceau S (Sigma Chemical Co., St Louis, MO, USA) to visualize the bands, then the paper was blocked for 1 h in Tris-buffered saline, 0·05% Tween 20 (TBST) with 3% BSA. Following 3 washes in TBST, each strip of NCP was incubated overnight at 4 °C with plasma from individual Mandrills at different time-points of the follow-up diluted at 1/400 in TBST 1% BSA. The strips were washed and then incubated for 1 h with mouse anti-human IgG alkaline phosphatase diluted at 1/1000 in TBST 1% BSA. This was followed by another washing step and incubation in the substrate solution containing 5 bromo-4-chloro-3-indolyl (BCIP, 0·3 mg/ml) and nitroblue-tetrazolium (NBT, 0·15 mg/ml) in 1 M Tris–HCl, 500 mM $MgCl_2$ buffer, for visualization of bound antibodies. Blots were scored by visual inspection to identify particular components that were recognized by IgG from Mandrills of different parasitological status.

RESULTS

Parasitological examination

The bloods of all animals were examined at different time-points, as described in the Materials and Methods section, for the presence of microfilariae. Table 1 summarizes the findings at the different times. Of the animals immunized with irradiated L3, three contained detectable Mf at only a single time-point (5i, 17E, 6B). In 1 of these animals (6B) the appearance of Mf was delayed, Mf being detected at day 435. Of the other 2 animals (5i, 17E), Mf were detected once at day 145 and not again. The remaining 3 animals which had received irradiated L3 (12H, 2D2, 17A2) first showed Mf around day 160, almost simultaneously as the animals that only received normal L3. Of the 6 animals which received normal L3, 3 developed a persisting microfilaremia

https://doi.org/10.1017/S0031182001007910 Published online by Cambridge University Press

Table 1. Evolution of microfilaraemia (Mf/ml) in Mandrill over 1 year

	Days																
Animals	−120	130	145	160	175	190	200	215	230	245	259	288	316	344	386	407	435
5i*	—	—	40	—	—	—	—	—	—	—	—	—	—	—	—	—	—
17E*	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6B*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	12
12H*	—	—	—	—	1	17	7	27	3	11	9	16	7	9	11	7	8
2D1*	—	—	—	—	—	11	33	59	13	16	15	12	13	5	3	0	1
17A2*	—	—	—	2	54	72	42	45	208	9	7	3	0	24	9	1	12
2H	—	—	—	37	138	143	27	36	36	50	57	57	31	11	15	18	12
12A2	—	—	1	1	70	8	0	14	47	1	16	0	16	33	74	12	18
12A3	—	—	—	3	11	49	5	8	5	19	17	3	10	2	5	0	23
12A5	—	—	6	—	—	—	6	—	—	—	—	—	—	—	—	—	—
5G	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—
2D2	—	—	—	7	—	—	—	—	—	—	—	—	—	—	—	—	—

*, Animal immunized with irradiated L3.
—, absence of Mf.

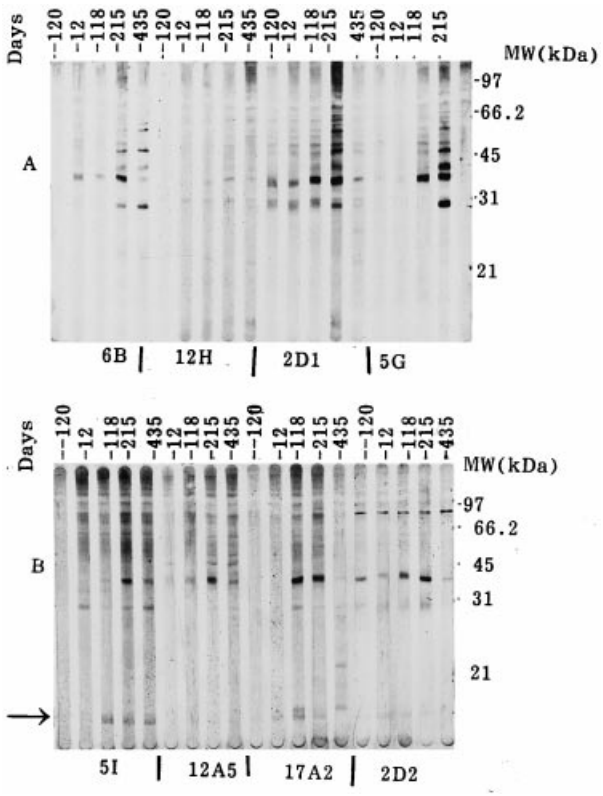

Fig. 1. Identification of adult antigens by IgG of Mandrills using Western blots. (A) Representative blot of 4 Mandrills (6B-12H-2D1-5G); (B) representative blot of the other 4 Mandrills (5i-12A5-17A2-2D2). All plasma samples were taken at days −120, 12, 118, 215 and 435, as indicated at the top (except for 12A5 which starts at day 12). Molecular weight standards are indicated at the right.

(2H, 12A2, 12A3), while the other 3 (12A5, 5G, 2D2) showed only intermittent Mf in the blood. This offered a large spectrum of infection which we therefore used in order to correlate each status with the pattern of antigen recognition.

Identification of adult antigen recognized by Mandrill IgG

In Western blots performed with adult extract, 21 major bands were detected by IgG. Most animals recognized molecules above 43 kDa, but only very few molecules below 29 kDa were recognized and their recognition was weak as shown in Fig. 1. This pattern followed a kinetic such that the recognition was maximum at day 215 for all animals. A few animals had very weak reactions with adult antigen at all time-points as indicated in Fig. 1A (Mandrill 12H is shown, and 17E is not shown). Only a few animals recognized the 15 kDa antigen (5i; 17A2; 2D2: Fig. 1B), and 1 animal (2D2: Fig. 1B), showed particularily strong recognition of a 90 kDa molecule at all time-points. However, the pattern of antigen recognition had no clear relationship with the parasitological status of the animal.

Identification of microfilarial antigens by Mandrill IgG

Ten bands were recognized, but 5 major bands of 100, 97; 68; 45; 33 kDa were differentially recognized by all animals (Fig. 2). In 100% of cases, early recognition of these bands before challenge was associated with the amicrofilaraemic state in immunized animals (Fig. 2A). In contrast, when there was no recognition or weak recognition of these bands prior to challenge (day 0), the immunized animals were microfilaraemic in 100% of cases (Fig. 2B). The amicrofilaraemic state was also associated with the maintenance of a strong reactivity against these antigens during the follow-up period. In animals which became microfilaraemic after infection or immunization, the recognition of an antigen of 21 kDa (Fig. 3) was correlated with the appearence of microfilariae in the peripheral blood in 10 out of

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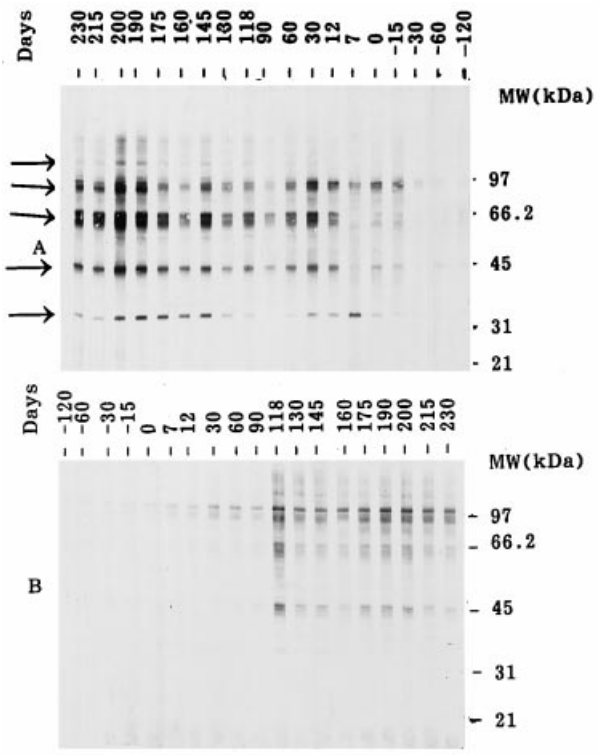

Fig. 2. Identification of microfilarial antigens by IgG of Mandrills using Western blot. (A) Representative pattern of recognition using plasma from an amicrofilaraemic Mandrill (6B), arrows on the left indicate the positions of the differentially recognized antigens. (B) Representative pattern obtained with plasma from a microfilaraemic Mandrill (17A2). The number at the top indicates time-point of bleeding from day −120 to 230. Molecular weight standards are indicated at the right.

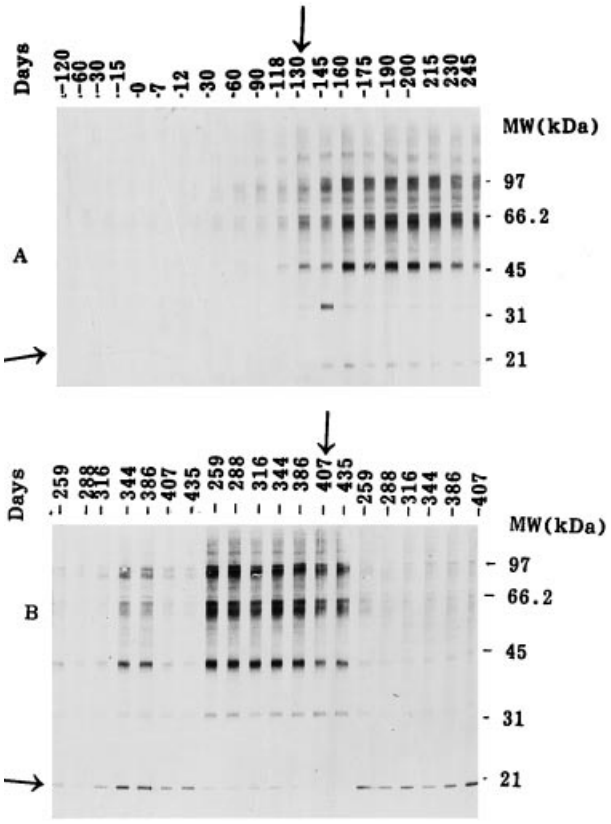

Fig. 3. Kinetics of anti-21 kDa recognition in a Western blot of *Loa loa* microfilarial antigens. (A) Representative blot of animal bleed (Mandrill 12A3) from day −120 to 245. The arrow at the top, day 130, indicates the time-point at which antibodies against the 21 kDa antigen appeared in most microfilaraemic Mandrills; arrow at the left, indicates the position of the 21 kDa. (B) Representative blot of conversion from amicrofilaraemic to microfilaraemic state and vice versa. Reactivity of plasma for Mandrill 6B; 5i; 12A2, taken at days 259, 288, 316, 344, 386, 407 and 435, respectively. The arrow at the top (day 407, Mandrill 5I) indicates the time-point at which anti-21 kDa antibodies began to decrease. Molecular weight standards are indicated at the right.

12 Mandrills (83%). This antigen was not recognized by animals which remained amicrofilaraemic until day 245 (Fig. 2A). During patency, we observed that antibodies against the 21 kDa antigen appeared at around the expected time of the onset of microfilaraemia (days 118–145) in 80% of the animals (8 out of 10 Mandrills) as shown in Fig. 3A, and at day 259, or day 288, for 2 animals: 6B, Fig. 3B and 17A2 (data not shown), respectively. The recognition of the 21 kDa antigen tended to change as Mandrill converted from the amicrofilaraemic state to the microfilaraemic state and vice versa. Thus, the appearance of microfilariae in Mandrill 6B at day 435 was preceded by recognition of the 21 kDa antigen on day 259, with high intensity until day 435, while the intensity of other bands decreased (Fig. 3B, Mandrill 6B). In contrast, with the demise of microfilariae, the intensity of recognition of the 21 kDa antigen declined (Mandrill 5i, days 407–435), while other bands were more strongly recognized (Fig. 3B). In animals which remained microfilaraemic, the intensity of the 21 kDa antigen remained strong, while other bands were weak (Fig. 3B, Mandrill 12A2).

Identification of infective larval antigens by Mandrill IgG

Eighteen bands were recognized by IgG. Antigens with relative molecular weight of 105, 103, 97, 95, 67, 45, 43, 32, 30, 23, 21, 20 kDa were recognized by the plasma of the animal which remains amicrofilaraemic for 1 year (6B) from day −30 throughout the follow-up (Fig. 4B), while the animal with a patent infection recognized the same set of antigens with the exception of the 20 kDa antigen, from day 30 with increased intensity until day 190. At this time-point antibodies against 30 kDa and 32 kDa were prominent (data not shown). In animals which became amicrofilaraemic after conversion (Mandrill 5i, 17E) the recognition of all the above bands was strong through the follow-up but these animals did not recognize the 20 kDa antigen (Fig. 4A).

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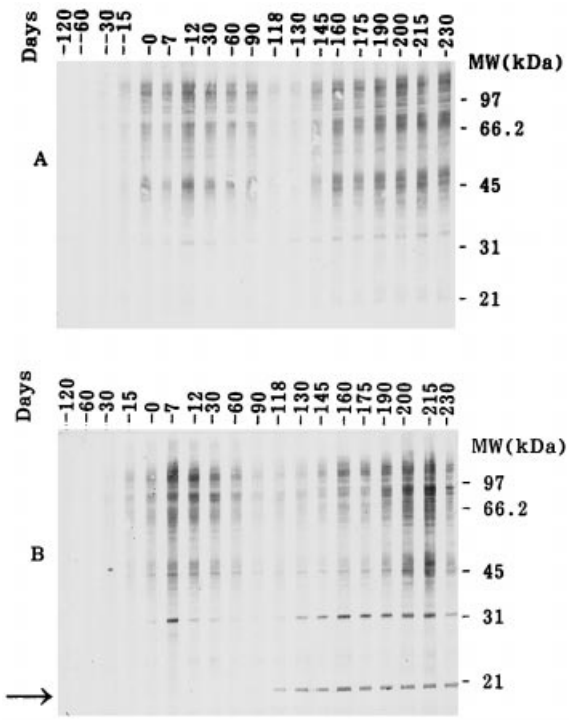

Fig. 4. Identification of L3 antigen by IgG of Mandrills by Western blotting. (A) Typical profile of amicrofilaraemic Mandrill (5i) from day −120 to 230. (B) Typical profile observed in a long-lasting amicrofilaraemic Mandrill (6B) from day −120 to 230. The arrow indicates the position of the 20 kDa antigen. Molecular weight standards are on the right.

DISCUSSION

Animal models offer all the advantages of controlled experimental conditions. This provides an excellent opportunity to investigate the relationship between the outcome of an infection and the immune response. In this experiment, irradiated *L. loa* larvae seem not to have induced a strong protection, however, it has been shown that protection may be dependent on the combined effect of irradiation dose and number of L3 in the *Brugia malayi*/rhesus monkey model (Wong *et al*. 1969). It is therefore possible that the 40 krad. combined with the 50 *L. loa* L3 was not appropriate to induce full protection against infection. Although there was no difference in the parasitological outcome of infection, Mandrills given normal or irradiated L3 still had a spectrum of infection which allowed correlation to be made between recognition of specific components and microfilaraemic state.

In this study an attempt was made to relate the antigen recognition by antibody to particular events during the course of the infection or immunization of *Mandrillus sphinx* of different parasitological status (microfilaraemic and amicrofilaraemic). It was observed that the pattern of antigen recognition by IgG differed with the parasitological status of the animal. This finding confirms previous observations in humans (Pinder *et al*. 1988; Egwang *et al*. 1989; Akue *et al*. 1998). Although the number of animals was limited, previous studies in different animal models have shown differential recognition of somatic antigen by IgG (Fletcher *et al*. 1986; Fletcher, Birch & Denham, 1992). Each developmental stage (adult, Mf, L3) had its own profile of antigens, suggesting that there is a stage-specific immune response. However, the particular pattern of recognition of microfilarial antigens from day −60 during immunization with L3 at the time when microfilariae were absent, suggests the existence of some cross-reactive antigens or epitopes shared by different developmental stages. This hypothesis is supported by the finding of Maizels *et al*. (1983) in lymphatic filariasis.

It is likely that the early recognition of these antigens affects the outcome of the infection. Although recognition of an antigen does not necessarily mean protection, the fact that the 97–68–45–33 kDa molecules were recognized prior to the appearance of microfilariae may be suggestive. In addition, during the follow-up the intensity of the reaction to these antigens was strong, and decrease in intensity of recognition coincided with the appearance of microfilariae. It has been shown also in human loiasis that antibodies may participate in clearance of microfilariae in an ADCC-like mechanism (Pinder, Leclerc & Everaere, 1992). All these observations, suggest that the clearance of microfilariae is a dynamic process which implies both an appropriate qualitative and quantitative response at the right time.

It was found that a microfilarial antigen of approximately 21 kDa was detected by IgG during patency. These antibodies tended to decline with the disappearance of microfilariae from the circulating blood. The recognition of this antigen may therefore be a marker of patency. Whether this antigen is excreted/secreted or is a somatic antigen, is not known. In contrast, it is clear that the 20 kDa antigen recognized in L3 was associated with a long-lasting state of amicrofilaraemia. No clear cut association between the recognition of adult antigens and the clearance of microfilariae was observed. One possible explanation is that the same antigen may be recognized in different life-cycle stages, but the epitopes recognized may not be the same in each developmental stage of the parasite. Also, the class of immunoglobulin involved in antigen recognition might be different. As we detected only total IgG in this study, such a difference in reactivity may not be apparent. A similar situation has been observed in human infection where the reactivity of IgG subclasses differs according to the subclass, the parasite stage, and the parasitological status (Akue *et al*. 1998). However, the lack of specific reagents for Mandrills precludes such analysis on subclasses. Anti-human IgGs were good for Mandrills, as preliminary experiments (Pinder, Evraere & Roelents,

https://doi.org/10.1017/S0031182001007910 Published online by Cambridge University Press

1994) have shown similarity between anti-human IgG and anti-Mandrill IgG reaction in an immunoenzymatic assay.

This work was supported by an INCO-DC grant no. IC 18-CT95-0026. CIRMF is supported by the State of Gabon, Ministère français de la cooperation and Elf Gabon. We thank Dr E. Devaney for critical reading of the manuscript and Leonard Nguiadoungou for the photography.

REFERENCES

AKUE, J. P., HOMMEL, M. & DEVANEY, E. (1998). IgG subclass recognition of *Loa loa* antigens and their correlation with clinical status in individuals from Gabon. *Parasite Immunology* **20**, 387–393.

DAY, K. P., GREGORY, W. F. & MAIZELS, R. M. (1991). Age-specific acquisition of immunity to infective larvae in a bancroftian filariasis endemic area of Papua New Guinea. *Parasite Immunology* **3**, 277–290.

EGWANG, T. G., DUPONT, A., LECLERC, A., AKUE, J. P. & PINDER, M. (1989). Differential recognition of *Loa loa* antigens by sera of human subjects from a loiasis endemic zone. *American Journal of Tropical Medicine and Hygiene* **41**, 664–673.

EVELAND, L. K., YERMAKOV, V. & KENNY, M. (1975). *Loa loa* without microfilaremia. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **69**, 354–355.

FAIN, A. (1978). Les problèmes actuels de la loase. *Bulletin of the World Health Organization* **56**, 155–167.

FLETCHER, C., BIRCH, D. W. & DENHAM, D. A. (1992). Cats with a single *Brugia pahangi* infection: relationship between parasitological status and humoral responses to somatic and surface parasite antigens. *Parasite Immunology* **14**, 339–350.

FLETCHER, C., BIRCH, D. W., SAMAD, R. & DENHAM, D. A. (1986). *Brugia pahangi* infections in cats: antibody responses which correlate with the change from the microfilaremic to amicrofilaremic state. *Parasite Immunology* **8**, 345–357.

OOTHUMAN, P., DENHAM, D. A., MCGREEVY, P. B., NELSON, G. S. & ROGERS, R. (1979). Successful vaccination of cats against *Brugia pahangi* with larvae attenuated by irradiation with 10 Krad cobalt 60. *Parasite Immunology* **1**, 209–216.

PINDER, M., DUPONT, A. & EGWANG, T. G. (1988). Identification of a surface antigen on *Loa loa* microfilariae, the recognition of which correlates with the amicrofilaremic state in man. *Journal of Immunology* **141**, 2480–2486.

PINDER, M., LECLERC, A. & EVERAERE, S. (1992). Antibody-dependent cell-mediated immune reactions to *L. loa* microfilariae in amicrofilaremic subjects. *Parasite Immunology* **14**, 541–556.

PINDER, M., EVERAERE, S. & ROELANTS, G. E. (1994). *Loa loa*: immunological responses during experimental infections in mandrills (*Mandrillus sphinx*). *Experimental Parasitology* **79**, 126–136.

MAIZELS, R. M., PARTONO, F., SRI OEMIJATI, DENHAM, D. A. & OGILVIE, B. M. (1983). Cross-reactive surface antigens on three stages of *Brugia malayi*, *B. pahangi* and *B. timori*. *Parasitology* **87**, 249.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembling of the head of bacteriophage T4. *Nature*, *London* **227**, 680–685.

TOWBIN, H. Z., STAEHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences*, *USA* **76**, 4350–4354.

VAN HOEGAERDEN, M. & IVANOFF, B. (1986). A rapid, simple method for isolation of viable microfilariae. *American Journal of Tropical Medicine and Hygiene* **35**, 148–151.

WONG, M. M., FREDERIC, H. J. & RAMACHANDRAN, C. P. (1969). Studies on immunization against *Brugia malayi* infection in the rhesus monkey. *Bulletin of the World Health Organization* **40**, 493–501.

YATES, J. A. & HIGASHI, G. I. (1985). *Brugia malayi*: vaccination with 60 cobalt-attenuated infective stage larvae protects against homologous challenge. *American Journal of Tropical Medicine and Hygiene* **34**, 1132–1137.