Onchocerciasis Spectrum in South West Cameroon and Immune Responses of Endemic Normal Subjects to selected Recombinant Peptides

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ABSTRACT

An epidemiological survey of onchocerciasis was conducted in 13 remote villages of the rainforest of Cameroon. The aims of the study were to (a) determine the prevalence and spectrum of onchocerciasis (b) identity protected/resistant individuals (c) compare the humoral immure responses of protected and infected individuals to the recombinant antigens of O. volvulus (i.e. OI3, OV25, OV47 and OV62). Onchocerciasis prevalence rates among 1300 individuals examined averaged 52% and ranged from 15 to 95% in the studied villages, 6 of which were hyperendemic. Typical stigmata of onchocerciasis were observed to increase with age and did not differ significantly between the sexes. Though most residents, (>12years) of the hyperendemic villages were infected, 23 of them were free of microfilariae (mf) and stigmata of onchocerciasis despite life long residence in those high transmission villages. These subjects remained negative for skin microfilariae during follow-up survey period of 24 months and were considered to be protected from the disease. The protected subjects were compared with an equal number of onchocerciasis patients in the same age range and gender ratio. IgG3 and IgG4 responses were then compared in both groups. IgG3 responses to OI3 were spread with spotted high readings in a few individuals hence the overall response was not significantly different (P >0.05). However, IgG4 responses to OI3 were very low or negligible in both groups. Pronounced IgG3 responses to the recombinant antigens OV25 and OV47 were observed for the protected individuals against lower and statistically insignificant responses among those with skin mf. IgG3 responses to OV62 were roughly equal in both groups, through IgG4 levels to OV62 were more prominent in the infected group. These findings are consonant with the existence of protective immunity in onchocerciasis

Key words: Onchocerciasis, Onchocerca volvulus, recombinant antigens, immunoglobulin, endemic normal.

RESUME

Une enquête épidémiologique a été menée dans treize villages reculés de la région forestière du Cameroun. Cette étude visait essentiellement à: a) déterminer le taux de prévalence et le spectre de l'onchocercose; b) identifier les sujets immuns ou ayant développé une résistance contre la maladie c) comparer les réactions immunitaires humorales des sujets immunes et des personnes infectées avec l'aide des antigènes recombinants de (l'O. volvulus (notamment OI3, OV25, OV47 et OV 62). Sur un échantillon de 1300 sujets examinés l'on a obtenu un taux de prévalence moyen de l'onchocercose de 52% avec des taux oscillant entre 15 et 95% dans les villages cibles. Cette maladie s'est révélée hyperendémique dans 6 villages. Il apparaît que les lésions caractéristiques de l'onchocercose s'accentuent avec l'âge et ne varient pas de façon significative en fonction du sexe. La majorité des résidents (âgés de plus de 12 ans) de ces 6 villages sont infectés certes, mais 23 d'entre eux sont exempts de microfilaires cutanées et de signes de l'onchocercose, nonobstant leur séjour prolongé dans ces villages à haut risque de transmission. L'examen des biopsies cutanées a donné lieu à des examens négatifs chez ces sujets pendant les 24 mois de suivi. D'où la conclusion qu'ils jouissent d'une immunité protectrice contre cette maladie. Leurs réponses immunitaires ont été comparées à celles d'un nombre identique de personnes souffrant d'onchocercose, de la même tranche d'âge et avec les mêmes proportions par sexe. Les concentrations d'IgG3 et IgG4 ont alors été comparées dans les deux groupes. La répartition des réponses par rapport à l'antigène recombinant OI3 a révélé quelques titres élevés d'anticorps IgG3 chez certains sujets alors que dans l'ensemble, les réponses ne comportaient pas de différences significatives P>05). En revanche, les réponses d'IgG4 induit par OI3 sont relativement faibles, voire insignifiantes dans les deux groupes. L'on a relevé de forts titres des réponses d'IgG3 aux antigènes recombinants OV25 et OV47 chez les personnes immunes tandis que les chez sujets présentant des microfilaires cutanées, les taux se sont avérées plus faibles, mais statistiquement insignifiantes. En outre, les concentrations, d' IgG3 induit par OV62 sont pratiquement identiques, même si celles d'IgG4 contre OV62 s'avèrent plus élevées au sein du groupe des sujets infectés. Ces résultats attestent de l'existence d'une immunité protectrice contre l'onchocercose.

Mots clés : onchocercose, Onchocerca volvulus, antigènes recombinants, immunoglobuline.

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INTRODUCTION

Despite the recent success of mass chemotherapy with ivermectin, onchocerciasis remains one of the major tropical parasitic diseases responsible for severe morbidity in tropical Africa, Latin America, and the Yemen. This debilitating disease caused by the filarial nematode *Onchocerca volvulus* is estimated to affect some 17 million people, leaving 85 million others at risk (WHO, 1995). Most unfortunately, several others ultimately become blind. The severity and variability of the clinical spectrum of this disease are dictated by the duration of stay in the endemic area, the intensity of infection and the degree and nature of the host immune response. However, certain individuals despite life-long and comparable exposure to infection remain microfilaria negative with neither clinical signs nor history of infection compared to their infected counterparts (Nutman, Kumaraswami & Ottesen, 1987; Ward et al, 1988; Ottesen, 1995). These apparently resistant or endemic normal individuals are indicators of possible existence of a protective immunity in onchocerciasis. The prime objectives of this study were to establish a clinical picture of the disease in the Upper River Meme basin of Cameroon and also identify individuals who despite life-long exposure to infection, remain disease-free in this rain forest onchocerciasis focus. These apparently immune subjects were matched by age and sex to a second group from the same population but with overt onchocercal lesions and detectable skin microfilariae. Immunoglobulin G3 and G4 responses of both groups to a selection of recombinant peptides were evaluated and compared.

MATERIALS AND METHODS

Study population: This comprised 1300 subjects from 13 randomly selected onchocerciasis endemic villages along the River Meme Basin in the Meme and Ndian Divisions of the South West Province of Cameroon. The purpose of the study, reasons for a regular follow-up by skin snipping and blood collection and other procedures involved were explained to the population through an interpreter and only those who granted informed consent were registered for the study. Ethical clearance was obtained from the Delegation of Public Health, SW. Province, and Cameroon.

We report herein, data from a cohort of 23 skin-snip negative individuals compared to another cohort of 23 onchocerciasis patients of the same age range, gender ratio and living in the same villages. These groups had comparable exposure. Sera from seven healthy, indigenous Africans resident in Buea, a non-endemic town of the region, as well as sera from four healthy Europeans who had never visited Africa served as controls in the serological assays.

Clinical examination: Physical examinations were performed on each individual at the start of the study, for the presence of onchocerca nodules, pruritus, hyperpigmentation, tiger skin, lymphatic involvement, visual impairment and blindness (W.H.O;1987). These examinations were repeated every six months for a total of 24 months that the follow-up survey lasted. Individuals who became mf positive were eliminated from the serological studies. A medical history based on the respondent report was also taken. Parasitological diagnosis was by two skin biopsies taken from the iliac crests and incubated individually in microtitre wells containing RPMI-1640 at room temperature and observed 24 hours later and the number of emergent microfilariae recorded. Infected subjects here are those with patent infections (i.e. were mf positive) while the endemic normal subjects were adult individuals with more than 5 years of residence in the endemic area, remained skin-snip mf negative without any signs or symptoms of onchocerciasis, during the entire 24 months survey period.

Recombinant antigens: The cDNAs coding for the recombinant *0. volvulus* antigen designated 0V47, (with accession no Y15993) and 0V62 (with accession no Y16017) were sub-cloned into pQE 30 or 50 and then mass expressed in *E. coli*. The recombinant peptides 013 and 0V25 were kindly donated by Dr. Perler, New England Biolabs, Beverly, USA, and Professor Richard Lucius of Humboldt University, Berlin, Germany, respectively. The antigen 013 is a fragment of a larger cuticular protein whose function has not been elucidated. As for OV25, it is an alpha crystallin analogue found in *0. volvulus*. Briefly, OV 47 and OV 62 were isolated in our laboratory using rabbit antibodies to *0. volvulus* female worms and shown to be novel antigens of the parasite (Titanji *et al*, 1998 a & b) . The roles of both antigens are not known, though OV62 is a proteoglycan analogue.

Enzyme Linked Immunosorbent Assay: The titres of 1gG3 and 1gG4 in both study groups (the mf positive and negative groups) were compared employing the *0. volvulus* recombinant antigens OI3, OV25, OV47, and OV 62. For each antigen, anti-human lgG3 or 1gG4 specific monoclonal antibodies (Immunotech, France)

were used to quantify the IgG subclass in each individual's serum essentially as described by Mbacham *et al.* (1992) with modifications. Briefly, IOO uL of a 5ug/ml antigen in phosphate buffered saline (PBS) pH 7.4 was used to coat microtitre plates (Corning) overnight at 4°C. After washing, 3% bovine serum albumen (BSA) from Sigma chemicals in PBS with 0.05% Tween 20 (PBS-T) was added to block unbound sites on the microtitre plate wells for 1 hour at 370C. All sera were tested in duplicate at a previously established single dilution of 1:100 in PBS-T and incubated overnight at 40C. Monoclonal anti-human IgG subclasses (Immunotech, France) were applied at 1:1000 and left for 2 hours at room temperature. Following a 2 hours incubation at room temperature with alkaline phosphatase conjugated goat anti-mouse IgG at 1:2000 dilution, p-nitrophenylphosphate was applied and the plates read 30 minutes later at 405nm with an ELISA plate reader (Bio-Tek Instruments). All absorbance readings were corrected to a set reference positive serum pool that was applied to all the plates as described by Voller, Bidwell & Barlett (1979).

Statistical analysis: All comparisons between groups were tested for statistical significance with the Mann-Whitney test, assuming p = 0.05 or less as a significant difference.

RESULTS

Prevalence of and Onchocerciasis Spectrum in the study Villages:

The overall prevalence of onchocerciasis in the region was 52.3% varying significantly (P<0.05) between the villages studied (Table 1). Of the 13 villages studied, six viz: Ekombe Mofako, Ekombe Waterfalls, Kumukumu, Dora, Dienyi and Iriba Nyange were hyperendemic as they registered prevalences of onchocerciasis in excess of 60%. Ediki, Mbalangi,Baba, Ekombe Liongo, Metoko Bekondo and Mokono with prevalences between 20 and 60% were ranked mesoendemic while the remaining one, Iloani with an infection rate of barely 15.6% was clearly hypoendemic. There was no significant difference (P>0.05) in prevalence rates between males and females.

In Mesoendemic villages, prevalence rates rose steadily with age, attaining peak values after the 40-49 age group. A bell shaped profile was observer for the heperendemic and hypoendemic villages when the age-dependent accumulated prevalence of onchocerciasis was plotted as a function of age (Fig1A & table 2). In

Table 1: Overall Prevalence of Onchocerciasis in Communities Studied:

Villages	Males		Females		Total	
	N° EXD	N° (%) INF'D	N° EX'D	N°(%) InF'D	N° EX'D	N° (%) INF'D
EDIKI	81	23(28.4)	66	10(15.1)	147	33(22.4)
MBALANGI	87	31(35.6)	87	24(27.6)	174	55(31.6)
EKOMBE MOFAKO	17	13(76.5)	7	4(57.1)	24	17(70.8)
BABA	26	13(50)	12	4(33.3)	38	17(44.7)
EKOMBE LIONGO	69	40(58.0)	51	31(60.8)	120	71(59.2)
EKOMBE WATERFALLS	40	32(80)	26	23(88.5)	66	55(83.3)
ILOANI	38	4(10.5)	52	10(19.2)	90	14(15.6)
KUMUKUMU	39	36(92.3)	31	31(100)	70	67(95.7)
DORA	39	29(74.4)	55	43(78.2)	94	72(76.6)
METOKO BEKONDO	82	51(62.2)	59	25(42.4)	141	76(53.9)
DIENYI	54	44(81.5)	87	76(87.4)	141	120(85.1)
MOKONO	71	24(33.8)	58	15(25.9)	129	39(30.2)
IRIBA NYANGE	28	21(75.0)	25	19(76)	53	40(75.5)
TOTAL	**671**	**361(53.8)**	**616**	**315(51.1)**	**1287**	**676(52.5)**

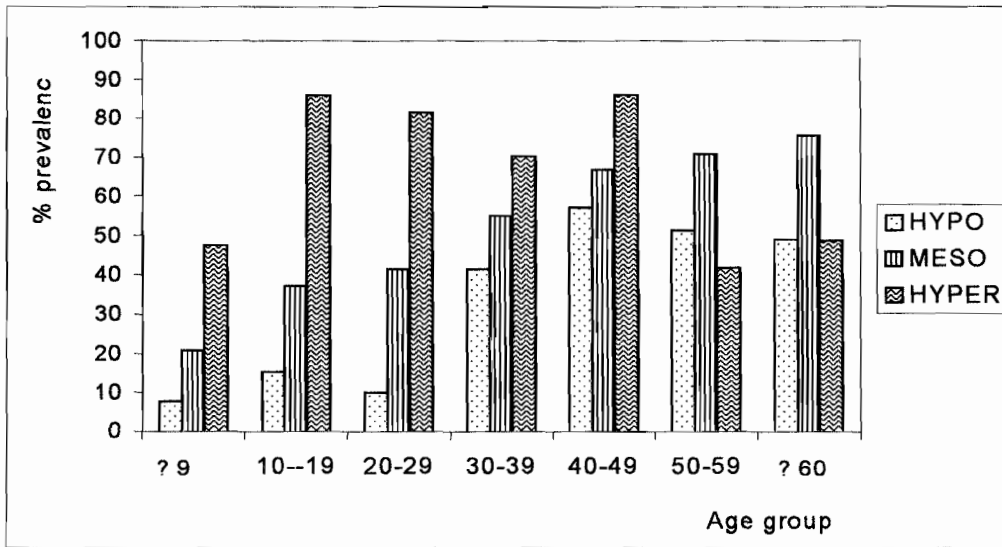N° EX'D = Number examined; N° INF'D = Number infected.

Fig. 1A: Accumulated age-dependent prevalence of onchocerciasis
HYPER means to hyper-endemic, MESO refers to Mesoendemic whilst HYPO indicates hypoendemic villages as defined in
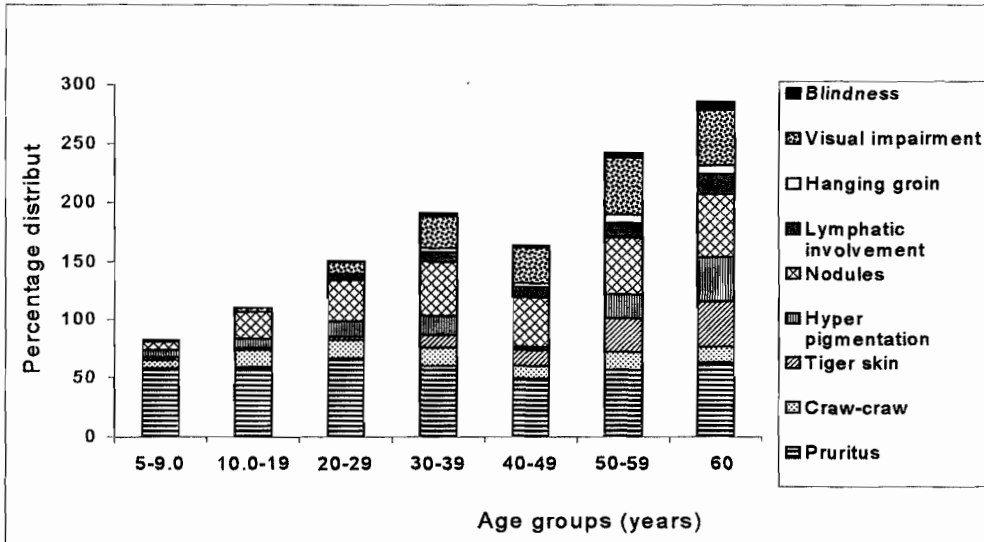Materials and Methods and in the legend of table 2.

Fig. 1B: Age-specific distribution of onchocercal lesions in the population. Stigmata of onchocerciasis were
determined after clinical examination as described in the Materials and Methods (see also WHO, 1987).

Table 2: Accumulated age dependent prevalence of onchocerciasis in different endemic zones.

Age Groups	Hypo-endemic			Meso-endemic			Hyper-endemic		
	Number Examined	In-fected	Prevalence %	Number Examined	In-fected	Prevalence %	Number Examined	In-fected	Prevalence %
0-9	78	6	7.7	24	5	20.9	21	10	47.6
10-19	130	20	15.4	75	28	37.3	128	110	85.9
20-29	80	8	10.0	38	16	42.1	98	80	81.6
30-39	41	17	41.5	40	22	55.0	61	43	70.5
40-49	63	36	57.1	63	42	66.7	58	50	86.2
50-59	37	19	51.4	24	17	70.8	48	20	41.7
> 60	49	24	49.0	41	31	75.6	43	21	48.4

The Hyperendemic villages (prevalence = 60%) comprised Ekombe Waterfalls, Kumukumu, Dora, Dienyi and Iriba Nyange.
Mesoendemic villages (20% = prevalence = 60%) included Ediki, Mbalanji, Baba, Ekombo Liongo, Metoko Bekondo and
Mokono. Iloani was hypoendemic (prevalence < 20%).

Table 3: Composition of Endemic Normal and Infected Groups

	ENDEMIC NORMALS			INFECTED			
SN	Subject code	Age	Sex	Duration of stay	Subject code	Age	Sex	Duration of stay
1	DRA49	70	F	From birth	DRA87	12	M	From birth
2	DRA82	12	M	From birth	DRA04	26	M	Frm birth
3	DRA50	26	M	From birth	DRA81	12	M	From birth
4	DRA64	12	M	From birth	DRA44	40	M	From birth
5	DRA51	40	M	From birth	DRA85	12	F	From birth
6	DRA78	12	F	From birth	DRA15	70	M	From birth
7	DRA25	70	M	From birth	DRA62	15	F	From birth
8	DRA73	10	F	From birth	DRA76	10	F	From birth
9	DYN86	26	F	From birth	DYN98	26	F	From birth
10	DYN44	39	M	10 years	DYN109	39	M	From birth
11	DYN32	54	F	From birth	DYN55	54	F	From birth
12	DYN23	50	F	26 years	DYN24	50	F	From birth
13	DYN17	50	F	10 years	DYN22	50	F	From birth
14	EL31	48	M	From birth	DYN130	14	F	From birth
15	EL71	12	F	From birth	EL38	45	M	33 years
16	EL120	10	M	From birth	EL79	17	M	From birth
17	EL124	14	F	From birth	EL108	18	F	From birth
18	EL125	10	F	6 years	EL25	68	F	From birth
19	IL82	67	M	15 years	EL84	40	F	From birth
20	IL67	36	M	8 years	WF41	25	M	From birth
21	IL77	39	F	9 years	WF29	23	M	From birth
22	IL21	56	M	35 years	WF32	20	M	From birth
23	IL97	60	F	From birth	IL01	52	M	8 years

The Endemic Normal subjects and Onchocerciasis patients were selected as described under Materials and Methods. The mean and standard deviation of the ages of the respective groups were 35.75 ± 21:49 for Endemic Normals; 32.09 ± 18.67 years for the patients (Infected subjects). The difference between these means was not statistically significant (p=0.558). The study villages were: Dora (DRA), Dienji (DYN), Ekombe Lyongo (EL), Iloani (IL), Ekombe Water falls (WF). Other details are given in the legend of fig. 2.

the study villages, a small number of adults, 46(3.5%) in the lot that had neither skin microfilariae nor clinical signs of the disease were retained for observations as endemic normals (Table 3). This number however narrowed down to 23, as 23 others either absconded or became mf positive on subsequent surveys within the 24 months period that the study lasted. They were thus eliminated from the serological studies.

A comprehensive picture of the onchocercal spectrum is depicted in fig. 1B. Pruritus was observed to be the most widespread skin manifestation of onchocerciasis while tiger skin and lymphatic involvement, principally hanging groin, were virtually absent in the younger age groups. These, however, remained the most noticeable skin signs associated with long-standing on-

chocerciasis as observed in the oldest patients. Impaired vision based on respondent reports and complete blindness assessed as the inability to count fingers at 3m and less were 19.4% and 1.7% respectively in the study population. Worthy of note are blindness rates of 10.0, 4.5 and 3.3% recorded in Kumukumu, Ekombe Waterfalls and Dienyi respectively that correlate closely with infections rates of 95.7, 83.3 and 84.9% respectively in these high transmission villages, that lie less than 1 km from the waterfalls. No case of visual impairment was recorded in Ediki, which was rather far removed from the hyperendemic zone.

Lymphatic complications, essentially hanging groins and a few isolated cases of scrotal elephantiasis, and hernia were observed in 24% of the population, with

the highest rates in Ekombe Waterfalls (10.6%) and Kumukumu (7.1%). Blindness and hanging groins were observed to increase closely with age as people below the 20-29 age group did not present with these complications.

Humoral immune responses:

The study of humoral immune responses were limited to two sub-classes of immunoglobulins i.e. 1gG3 which has been identified as a marker of putative immunity (Boyer et al, 1991) and 1gG4 which has been shown to correlate with filarial disease intensity (Ottesen, 1995). The results presented herein depict the IgG3 and IgG4 responses to the recombinant antigens of a cohort of 23 individuals who were free of skin microfilariae and signs of onchocerciasis after a six-monthly follow-up survey for a period of 24 months and 23 onchocerciasis patients matched to them by age and sex.

There were no differences in IgG3 responses to OI3 in both groups (fig. 2a), though IgG4 response to OI3 remained very low and more or less negligible (fig.2b). For both IgG3 and IgG4 responses to OV25, endemic normal subjects reacted more than their infected counterparts though differences were not statistically significant (P>0.05) as depicted in fig. 3a & b. IgG3 responses to OV47 were higher in the endemic normal individuals than in their age and sex matched infected counterparts (fig. 4a). IgG4 responses to OV47 were however significantly higher (P<0.05) in the infected group than in their endemic normal counterparts (fig. 4b). IgG3 responses to OV62 were very similar in both groups (fig. 5a), while IgG4 responses were higher in infected than endemic normal subjects (fig. 5b).
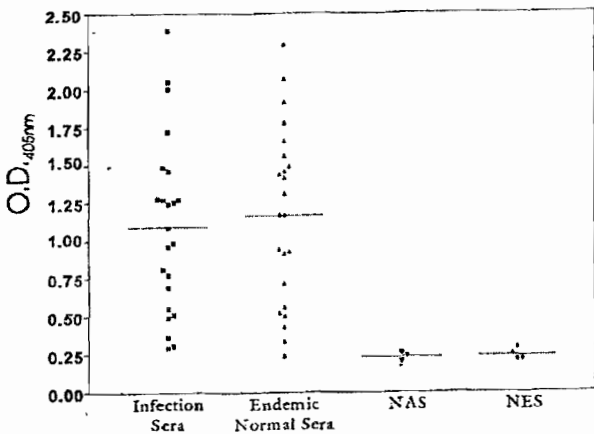

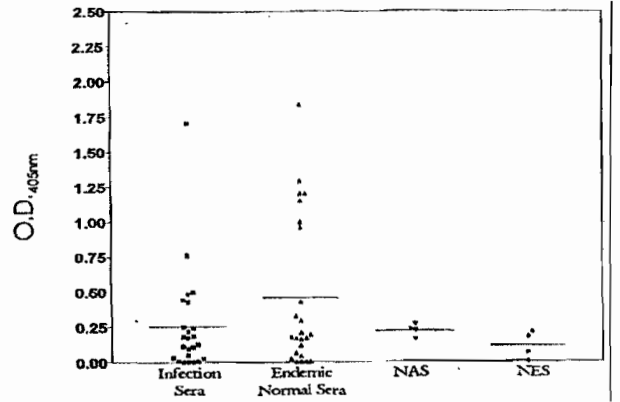**Fig 2 (a)** Immunoglobulin G3 levels to recombinant O13 antigen in infected and endemic normal subjects

Fig. 3 (a) Immunoglobulin G3 levels to recombinant OV25 antigen in infected and endemic normal subjects
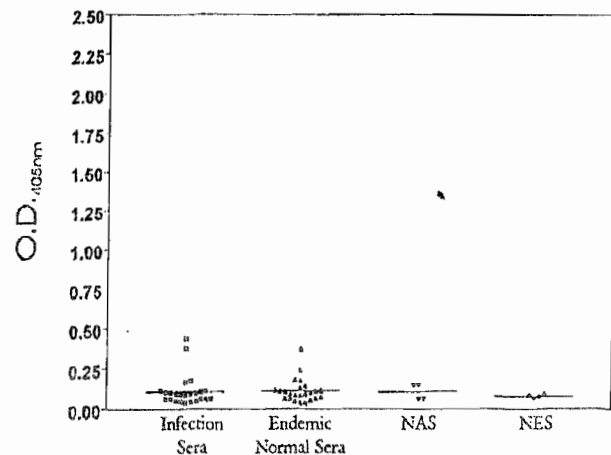

Fig. 2 (b) Immunoglobulin G4 levels to recombinant 013 antigen in infected and endemic normal subjects
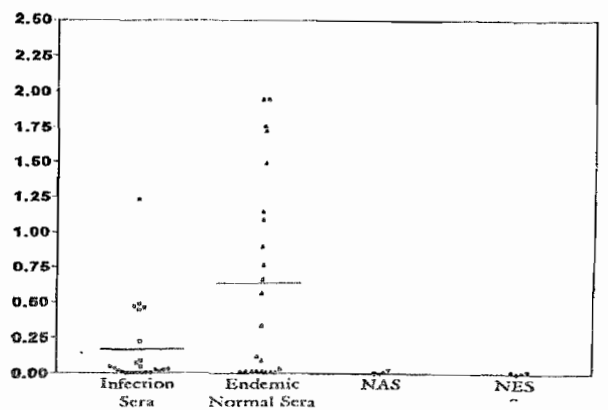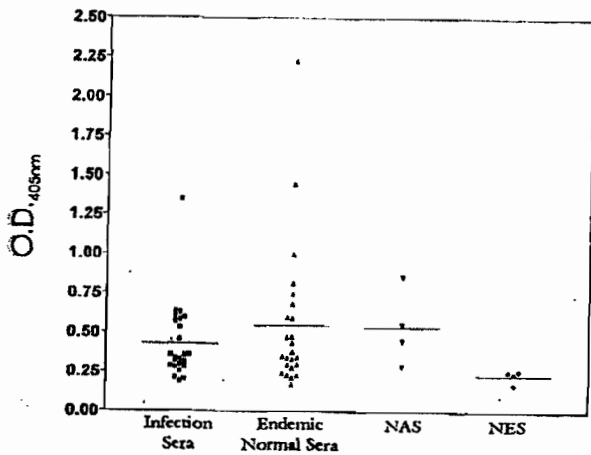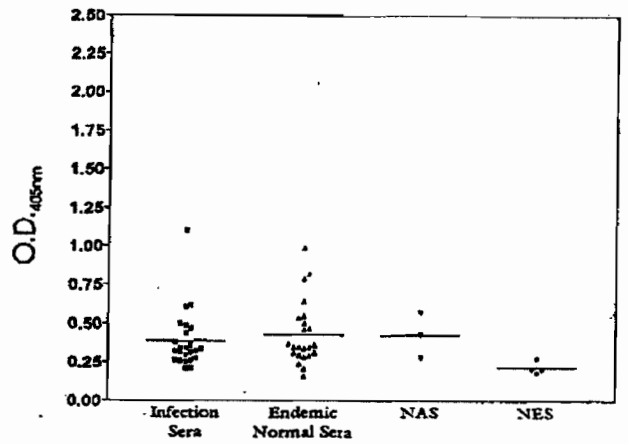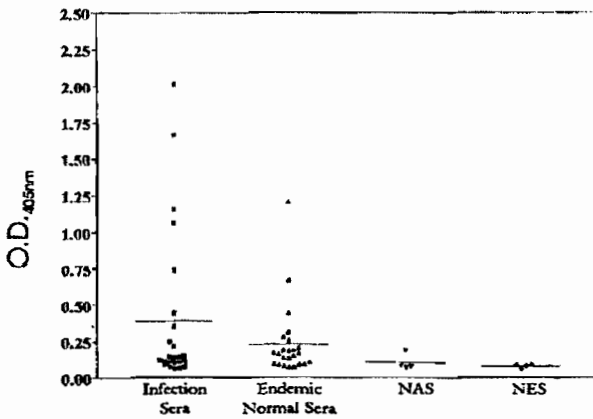

Fig. 3 (b) Immunoglobulin G4 levels to recombinant OV25 antigen in infected and endemic normal subjects

Fig. 4 (a) Immunoglobulin G3 levels to recombinant OV47 antigen in infected and endemic normal subjects

Fig. 5 (a) Immunoglobulin G3 levels to recombinant OV62 antigen in infected and endemic normal subjects

Fig. 4 (b) Immunoglobulin G4 levels to recombinant OV 47 antigen in infected and endemic normal subjects
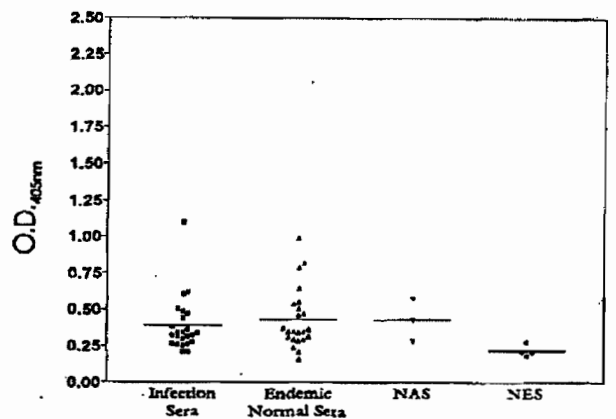

Fig. 5 (b) Immunoglobulin G4 levels to recombinant OV 62 antigen in infected and endemic normal subjects

In these figures CAS means Control African Sera and NES refers to Normal European sera. All other abbreviations are explained in the text.

DISCUSSION

The present study covers a remote area of the Cameroon rainforeset that had not been adequately studied in recent epidemiological surveys (Ngoumou & Walsh, 1993; Somo et al, 1993). The prevalence trends observed with age are typical with an infection like onchocerciasis where there is a close relationship between infection and occupation. The majority of the study subjects are involved in subsistence farming, an occupation that brings them into intimacy with the flies biting habits. The steady rise in prevalence with age is, therefore, expected while the fall in older age groups in the hypo- and hyper-endemic areas is probably related to less exposure, a direct consequence of lesser involvement in out door activities or perhaps a slow development of protective immunity (Fig 1 and table 2).

Pruritus is invariably a clinical sequel of onchocerciasis as shown by its high distribution in all age groups. However, where the practice of hygiene is poor, pruritus and itching could be caused by arthropod bites, streptococcal infection, scabies or may just be an immediate hypersensitivity reaction to other products and vegetation, and likely to be misinterpreted for onchocercal pruritus.

Tiger skin and lymphatic involvement were virtually absent in the younger age groups and rose steadily with age. They were the most noticeable signs associated with long-standing onchocerciasis. Tiger skin was observed to be intense and extensive, generally affecting both limbs. Impaired vision and complete blindness were age related and most common in the heavily infected villages and especially in those close to the waterfall, a potential *Simulium* breeding site the region.

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In an earlier study in the South West Province, Cameroon, Somo et al (1993) recorded a 10.5% total blindness rate, linking forest onchocerciasis to ocular pathology including blindness. Their value though comparable to the blindness rate of 10.0% observed in Kumukumu, is much higher than the overall rate of 1.7% in the present study. This is probably due to the large-scale distribution and use of ivermectin to control onchocerciasis over the past years in many parts of the tropics including Cameroon.

Lymphatic complications were diverse with hanging groins, enlargement of lower limbs and hernia being the most common and a few isolated cases of scrotal elephantiasis observed in the population.

Blindness and hanging groins were observed to tie closely with age and indicate that onchocerciasis is an important public health problem in this area especially as they affect the active and productive ages. Apart from the 5-9 years group, the prevalence of nodules did not differ significantly between the different age groups. The presence of nodules is directly linked to the vector biting habits and skin microfilaria densities, two factors that affected all exposed age groups equally. Parasitological and skin lesions assessment generally revealed a steady increase with age suggesting probably a little effective immunity within the population. It is evident that infection accumulated with increasing years of exposure and that the clinical disease reflected the severity of infection.

The presence in hyper-endemic regions of persons, who despite life-long exposure to high transmission rates remain infection-free is indicative of protective immunity (Ottesen, 1995). However, the identification of such individuals is usually compounded by the existence of low level microfiladermia and cryptic infections generally, which are not detectable by routine parasitological and clinical techniques. Such individuals were identified from this initial survey and became the subjects of a followed-up study.

A serological assay of lgG3 and 1gG4 subclass responses to some recombinant filarial antigens revealed some remarkable differences. A widely applicable approach to study different molecules for their protective or diagnostic potentials today, is to evaluate them on exposed but apparently disease free persons residing in a hyper-endemic area in comparison with their infected counterparts. We hypothesized that these antigens may play significant roles in onchocerciasis and then tested responses to them to determine if these would differ in the two study groups.

Previous authors (Dafa' Alla et al, 1991) observed a positive correlation between the microfilaria load and IgG4 antibody levels, suggesting therefore, that the high IgG4 levels may be indicative of a role in blocking mf clearance or destruction. Contrary to the observation in the infected individuals, the endemic normals showed higher IgG3 levels to 0I3, 0V25, and 0V47 though the differences were not statistically significant (P>O.O5). Boyer et al, (1991) and Stewarts et al, (1995) concluded from a study of isotype specific responses in putatively immune individuals that IgG3 may have a role in protection against *0. volvulus* infection. Though some endemic normal subjects exhibited elevated IgG4 responses, it is worthwhile noting that if such 1gG4 high responders that may represent individuals with cryptic infection are excluded from the endemic normal group, the differences in 1gG3 responses between infected and uninfected individuals become more pronounced (Stewarts et al, 1995).

It should be noted that the endemic normal group is a very heterogenous group possibly comprising individuals with no infection at all, those with cryptic infection, aborted or light infections that could not be detected by skin snip observations per se. These observations indicate the possible involvement of IgG3 and IgG4 antibodies in the variable spectrum of the disease in the different individuals in this region. Though our criteria for selecting infection free subjects did not involve the provocative Mazzotti test and the PCR, the fact that subjects remained skin snip negative, without any onchocerciasis lesions despite life-long residence in hyperendemic villages without using any protective measures is suggestive of some degree of resistance to infection. These findings point to the possible presence of putative immune subjects in this hyperendemic onchocerciasis zone, and that the recombinant antigens OV25 and OV47 could be possible markers of protective immunity while OV62 is a marker of disease severity. Therefore, the relevance of the selected peptides as potential protective candidate molecules deserve being investigated further.

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