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CELLULAR AND HUMORAL IMMUNE RESPONSES IN ONCHOCERCIASIS:
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ABSTRACT

CELLULAR AND HUMORAL IMMUNE RESPONSES IN ONCHOCERCIASIS: CORRELATIONS WITH CLINICAL SIGNS AND INFECTION INTENSITY

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Characterizations of circulating lymphocyte subset populations, *in vitro* lymphocyte responsiveness, serum-mediated effects on lymphocyte proliferation and anti-*Onchocerca volvulus* antigen antibody recognition patterns were performed on groups of onchocerciasis patients selected from over 200 examined parasitologically and clinically in Sudan and Sierra Leone. These patients manifested a very broad range of clinical signs and infection intensities; some were heavily infected and asymptomatic while at the other extreme were individuals with severe clinical signs, but few or no detectable microfilariae (mf) in their biopsies. Patients from Sudanese foci were worse afflicted than the W. Africans. Lymphocyte subsets in peripheral blood were not remarkably different from those in control samples. Lymphocyte proliferative responses to soluble *O. volvulus* antigen (sAg) were poor in infected persons; however, mitogen and PPD responses were in the normal range in one group of patients from southwestern Sudan, but were profoundly depressed in a group from Eastern Sudan. The latter were malnourished and in poor physical condition in a drought-stricken zone. Sera from

90 patients exhibiting a wide range of clinical signs and parasite burdens had no significant effects on *in vitro* lymphocyte responsiveness. Proliferative responses and INF-γ production by lymphocytes were very significantly depressed in the presence of live microfilariae and worm secretions/excretions (S/E).

Immunoblotting analyses revealed diverse antibody responses to a wide range of worm antigen bands, with up to 25 recognized by some sera. Subclass specific monoclonal probes revealed a predominance of IgG4 responses, often accounting for almost all the band spectrum seen with IgG reagents. There was, however, no obvious relationship between band number, location or intensity, and the clinical or parasitological status, except that there was an overall trend towards higher clinical severity scores in patients whose sera recognized more and more deeply staining bands. Specific patterns of band recognition with IgG3 probes were not associated with Sowda patient sera, unlike previous reports. Anti IgE probes revealed fewer bands with lesser intensity, compared to anti IgG, and they were also not peculiar to any patient subset.

Overall, *O. volvulus* infection and clinical consequences were not consistently associated with systemic deficits in cell mediated or humoral immunity. The demonstration of potent suppression of lymphocyte reactivity by mf and S/E *in vitro* suggests that direct parasite intervention in host cell responses could be taking place *in vivo*, perhaps at the local micro-environment level.

TO NADIA

WITH LOVE AND RESPECT

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TABLE OF CONTENTS

LIST OF TABLES

ARTICLE 1

LIST OF FIGURES

ARTICLE 1

ARTICLE 2

APPENDIX 1

FIGURE PAGE

INTRODUCTION

Onchocerciasis is a chronic parasitic infection caused by the filarial nematode *Onchocerca volvulus*. This disease afflicts some 20-40 million people world wide, and is considered a major cause of blindness in tropical Africa. Despite the morbidity and consequent economic losses, prevention, treatment and control of onchocerciasis lag behind other infectious conditions in the developing nations, where the disease is exclusively seen. *Onchocerca volvulus* occurs mostly in remote rural areas where the inhabitants have little political impact on the central decision making establishment.

The loss of manpower because of blindness coupled with the migration of people from fertile lands for fear of blindness has created severe economic difficulties in endemic areas. These factors initiated the implementation of a large control program in the Volta River Basin in West Africa. The program, now 10 years old, is directed towards eradication of the vector, the blackfly. This program has interrupted the transmission cycle, but at a very high price. Sustained financial support is needed, well over the 20 year plan projected initially, in order to insure continued success. In other endemic foci, rudimentary chemotherapeutic regimens from the 1940's are the only available means to combat the disease. Treatment with both the macrofilaricidal drug, Suramin and the microfilaricidal agent diethylcarbamazine (DEC) have serious, and on occasion, produce fatalities. This has limited their use by concerned medical personnel, and results in their rejection by infected individuals, for fear of the agonizing side effects. The recent introduction of ivermectin as a microfilaricidal drug is encouraging because fewer side effects are associated with it. However, caution is warranted until we have more experience with the drug and its side effects.

At present we are confronted with a disease that carries high morbidity, though seldom fatal, for which there is no suitable treatment for the adult parasites. There is no convincing evidence of acquired resistance and little hope of any vaccine being developed. Prevention of the infection through vector eradication in most areas is impractical, because of the enormous expenditure needed. Exploration of the different immunologic mechanisms involved in the pathology and pathogenesis of O. volvulus infection may bring about better understanding of the biology of the disease, ultimately leading to better treatment and preventive options. Experience from other infectious diseases for which we have developed better chemotherapeutic agents or vaccines, does not give reason for optimism in the fight against onchocerciasis. The logistical difficulties encountered in delivering "magic cures" to patients in remote endemic rural areas remains a challenge.

The work contained in this thesis involved studies on both cell mediated and humoral immunity in onchocerciasis in patients in Sudan and for comparative purposes, in Sierra Leone, W. Africa. The epidemiological and biological background to O. volvulus infection in these regions is reviewed below, along with an overview of the current state of knowledge of the pathogenesis and host-parasite relationship. A brief account is provided of the problems of chemotherapy and control, and of the most recent findings in the areas of cell-mediated and humoral responses to _O. volvulus_.

LITERATURE REVIEW

Geographical Distribution

Onchocerciasis occurs throughout the greater part of tropical Africa. It extends from Senegal on the west coast to Ethiopia in the east. Foci endemic for the disease are also seen as far south as Malawi in Africa, and in Central and South America. In the Middle East, the disease is confined to the Arabian peninsula. For the most part, onchocerciasis endemic foci are located in rain forest and Savannah regions. However, occurrence of the disease outside those two climatic regions is becoming recognized now as more careful epidemiological surveys are conducted. One unexpected focus for onchocerciasis is in Abu Hamad in northern Sudan. The disease was discovered in a dry arid region of the Nubian Desert in 1958[1]. It is not unlikely that additional foci will be discovered and the disease should be suspected wherever blackflies of the Simulium species are found.

a. Onchocerciasis in Sudan:

Probably the first clinical description of the disease in Sudan was in 1908 by Ensor in Bahr ElGhazal province[2]. Later the disease was termed Jur blindness since it was contracted along the banks of River Jur[3]. Subsequently, additional foci were discovered in Upper

Nile[4], Southern Darfur[5], in Kasala Province in Eastern Sudan[6,7] and in Abu Hamad in the Northern province[8].

Thus, the distribution of onchocerciasis in Sudan is wide, encompassing different climatic regions, from the rain forest in the south to the desert in the north. Each geographical area has its peculiarities regarding the infection intensity and clinical manifestations.

It is estimated that about 1 million Sudanese (5% of the population) are infected with O. volvulus. The disease is most prevalent near seasonal fast running waters which constitute optimal breeding sites for the vector, the blackfly.

The vector in the South and Southwest has been definitively identified as Simulium damnosum si[10]. There is a single report about infected S. damnosum from the Abu Hamad area[11], however, subsequent workers were unable to substantiate this. No entomological studies to identify the vector along the Atbara River area were carried out.

Clinically, the dermatological manifestations can be seen in all foci, but the Atbara River focus offers the most severe presentation[7]. It is believed that eye lesions, culminating in blindness, are more severe in the southern part of Sudan with exceptionally few cases of eye involvement or blindness in the Abu-Hamad and Atbara River foci. Factors which may contribute to differences in clinical presentations include genetic backgrounds, parasite strain variability, length of the transmission season and intensity of infection.

b. Onchocerciasis in Sierra Leone:

Two important benchmarks in the history of onchocerciasis were established in Sierra Leone. Onchocercal nodules[12], were first described there, and the life cycle of O. volvulus[13] was unravelled.

The ecological system in Sierra Leone was primarily rain forest, but because of the intense deforestation and clearance of land for farming, not much of the original primary forest remains unaltered. Numerous foci of onchocerciasis exist in the Northern, Eastern, and Southern provinces. Around the Bo area, in the South, onchocerciasis is prevalent in villages along the tributaries of the Tabe and Sewa Rivers[14]. Detailed entomological, clinical, and epidemiological surveys of this area by the British Medical Research Council staff and the Sierra Leonian Ministry of Health showed an overall prevalence of 68.6%[14]. Clinically, there are relatively mild skin and eye lesions, but there is an unusually high rate of blindness (7.5%), the explanation for which is not entirely clear. Four species of the *S. damnosum* complex are implicated as vectors for the disease in the Bo area.

Onchocerciasis transmission occurs year-round, with a peak during the rainy season (October to December), when 80% of the transmission takes place[14].

Life Cycle

Onchocerca volvulus is transmitted by blackflies of the genus *Simulium* which act as an intermediate host. Although closely related parasites are seen in mammals, humans are the only definitive hosts for *O. volvulus*. Several species of *Simulium* have been shown to transmit the infection, but , *S. damnosum* complex members are the main vectors, especially in Africa[15]. Others such as *S. ochraceum* and *S.*

metallicum play a major role in transmission of the disease in Central and South America[15].

Simulium are small, strongly built flies, the females of which bite preferentially on animals or birds in order to obtain a blood meal needed for oogenesis[16]. The male flies do not bite, and hence do not participate in the transmission of the parasite. When the female feeds on an infected individual she ingests skin microfilariae along with the blood. Upon reaching the stomach, microfilariae penetrate the abdominal tissues and go into the thoracic muscles where they undergo maturation and metamorphosis, transforming into the infective larvae. This may take 6-10 days, depending mainly on environmental temperatures[17]. The infective larvae then migrate to the proboscis. When the fly bites again, it deposits these infective larvae along with saliva, which contains an anticoagulant. The larvae then probably migrate in the subcutaneous tissue, and develop into mature male or female worms, usually within 12 months[18]. The female worms elicit a granulomatous inflammation around them, and with time they become encapsulated within a mesh of fibrous tissue and inflammatory cells forming what is known as the onchocercal nodule.

While the females have relatively restricted mobility within the human body, the male worms are believed to travel from one nodule to the other. Fertilization of eggs occurs following mating of a male and a female worm. The result of this is the periodic release of thousands of microfilariae by the female worms[9,19]. Microfilariae are found in the skin and subcutaneous tissue and they can travel to the eye. Microfilariae and the inflammatory responses directed against them, not the adult worms, are believed to be the major cause for the pathologic events seen in the host.

The life span of adult O. volvulus, although not known with certainty, is estimated to be in the range of 15 to 20 years[20]. The adult female is a white to tan, threadlike, worm, between 25 and 70 cm in length, and 1/3 mm in diameter[21]. The male, though similar in appearance, is much smaller in size, measuring up to 4 cm in length[21]. The microfilariae are 220-360 u long and 5-10 u in diameter[22], and their life span is estimated to be about 6 months[23].

Breeding of blackflies takes place in well aerated fresh running waters. The mature female deposits about 200-400 eggs at a time on aquatic plants or submerged rocks and vegetation[24]. Four to thirty days are needed for the larvae to hatch from eggs, temperature being the major influence[16]. The larval stage is the target for control measures against black flies. The larvae then develop into pupae within 10 days, and the pupae mature into adult flies within 2-4 days. The longevity of blackflies is not fully known, although some may live for up to 180 days[25]. It is suggested that flies infected with O. volvulus live for a much shorter time than non-infected flies. The flight range is variable and can reach >400 miles[26], though infected flies are thought to have a much reduced flight range, possibly because the parasite is located within the thoracic muscles. The large flight range has created problems of re-invasion of onchocerciasis control program regions from neighboring uncontrolled areas[26].

Besides being responsible for transmission of O. volvulus, blackflies inflict damage on man and animals by their vicious biting behavior. People easily become sensitized to blackfly bites and on subsequent exposure can develop both immediate and delayed hypersensitivity reactions[27]. This is thought to contribute to the skin pathology seen in onchocerciasis.

Animals may experience fatal anaphylaxis or bleed to death if bitten by large numbers of blackflies[27,28]. Thus, the idea of eradicating the vector as a control measure for the spread of onchocerciasis is appealing, since human morbidity and animal mortality caused by blackflies can also be eliminated. However, because of the nature of their breeding sites, often in small seasonal streams over wide geographical areas, control of blackflies has proven difficult and expensive.

Diagnosis

a. Detection of the Parasite:

The most reliable method for the diagnosis of *O. volvulus* is the demonstration of microfilariae in a bloodless skin snip. The simplest way is by using a needle and a sharp double edge razor to obtain the snip. Care should be taken not to have a bloody specimen since confusion may occur if the blood contains microfilariae of *D. perstans* or *Loa loa*. The snip can be incubated in normal saline or tissue culture medium, at ambient temperature, for 1/2 hour to 4 hours. Increasing the incubation period gives a better chance for microfilariae to emerge from the skin snips of patients with low microfilarial densities.[29] The use of a corneoscleral punch is recommended in research surveys, and in large medical centers because it provides relatively uniform samples. However, because of the expense and the need for frequent sharpening it may not be suitable for use by paramedical staff in remote endemic foci. For diagnostic purposes several skin biopsies from the pelvic and subscapular areas should be obtained. A negative skin snip does not necessarily exclude infection and additional snips should be obtained and tested.

The probability of obtaining a negative skin snip in patients with low microfilarial densities (less than 2.5 mf/mg) is about 50%[30]. In such patients repetition is recommended. In addition, DEC provocation test have been advocated to confirm clinical impressions in such patients. The test consists of administration of DEC orally or in the form of topical lotion[29,31]. The patient is then observed for development of the characteristic maculopapular eruption which is part of the Mazzotti reaction, described as a side effect of DEC therapy [32]. The latter procedure, since it entails risks, should be reserved for patients who consistently prove to be skin snip negative, but having the characteristic clinical stigmata of onchocerciasis.

Identification of subcutaneous onchocercal nodules constitutes an important part of the clinical and parasitological evaluation of patients. This can be achieved by visual inspection and palpation for the nodules, usually in the pelvic region. Demonstration of O. volvulus adult worms after surgical removal of nodules constitutes definitive diagnosis of O. volvulus infection. Recently, a non-invasive method for detection of O. volvulus nodules using ultrasound[33] has been described. However, the practical utility of such a method remains doubtful.

b. Immunodiagnosis:

It is obvious that detection of microfilariae, though it has a high specificity, is not a very sensitive method. False negatives are seen in prepatent individuals and in patients with low microfilarial densities, either due to low levels of infections or strong immunologic responses resulting in destruction of microfilariae. Scientists have thus explored more sensitive procedures, i.e. immunologic diagnosis. Different formulations of immunological assays have been listed for detection of anti-onchocercal antibodies. These include hemoglutination[34], complement fixation[35], radioimmunoassay[36], and enzyme linked immunosorbent assays[37], all of which utilize O. volvulus crude antigenic extracts for the detection of specific anti onchocercal antibodies. Obviously, one major problem of this approach is that it lacks specificity. The extensive cross reactivity between O. volvulus antigens and other filarial antigens makes it difficult to differentiate between patients infected with O. volvulus from those infected with other filariae or helminths[38]. In addition, the mere presence of anti-onchocercal antibodies does not necessarily indicate infection. For example, exposure to the infective stage, which may not develop into an adult worm, can give rise to an antibody response. Because of this, recently the trend has been towards detection of worm antigens in serum, urine and other body fluids[39,40,41]. This approach is attractive since the presence of worm antigens is indicative of active infection.

Theoretically, detection of O. volvulus antigens should have high specificity and sensitivity, especially when one utilizes monospecific antisera directed against unique worm antigens. The limited experience with O. volvulus antigen detection has shown that the sensitivity is interfered with by the presence of host antibodies directed against those unique antigens, thus giving false negatives[41]. The use of crude adult worm antigenic extracts[42] and microfilarial excretory and secretory products[43] for skin hypersensitivity reactions did not meet with much success. More work is obviously needed in the area of immunologic diagnosis to come up with an acceptable, reliable, and practical assay.

Recent advances in molecular biology which culminated in the identification of O. volvulus specific DNA probes are encouraging and may offer more sensitive and specific approaches to the diagnosis of O. volvulus in the future[44,45].

Clinical Presentation

Infection with O. volvulus results in a spectrum of clinical manifestations. Many variables can affect the outcome of the disease, including host immune response, exposure rate, age and strain variation. Although in most textbooks and publications the disease is often presented in its most severe form, a single visit to an endemic area would convince anyone of the concept of spectrum. Generally the clinical presentation ranges from completely asymptomatic individuals to those who have severe skin and eye manifestations. Different categories exist between these two extremes.

a. Dermal Changes:

Skin changes are best classified into two main categories[46]:

1. Acute changes:

One of the early skin changes is the development of a papule, an event signifying an inflammatory reaction around dead or dying microfilaria[47,49]. Papules can be discrete and their number and distribution may vary. Sometime, they may coalesce together. The development of papular eruption is usually accompanied by itching, and self inflicted injuries, from excoriation, is a common finding in onchocerciasis. The injured skin surface may get secondarily infected, thus aggravating the initial insult to the skin.

The papules may disappear completely without leaving any mark of their previous existence or they may become indurated, depigmented initially and hyperpigmented later[47,48]. Death of large numbers of microfilariae either naturally or secondary to chemotherapy, e.g. Mazzotti reaction[32], leads to the development of large numbers of papules accompanied by edema of the skin. The acute changes seen in onchocerciasis are readily reversible. However, with time, repeated insults to the skin lead to the more characteristic chronic changes.

2. Chronic changes:

These are thought to result from repeated local pathologic events in the skin evoked by dead or dying microfilariae. The chronic clinical stigmata of onchocerciasis include dermal atrophy with loss of elasticity and thinning of the epidermis[47]. The skin becomes wrinkled, assuming the appearance of old age in afflicted patients.

Pigment loss, especially on the skin overlying the tibia is characteristic, giving the name of leopard skin[50]. In some patients marked thickening of the skin (hypertrophy) is seen prior to the development of skin atrophy. The hypertrophied skin may also be edematous and darker in color. This may be an intermediate stage between the acute changes and the more permanent chronic changes. It may result from the continuous, repeated itching which can lead to lichenification of the epidermis. In addition, the inflammatory response in the dermis is often accompanied by increased fibroblastic activity[50]. Thus, thickening of the skin results from thickening of both the epidermal and dermal layers of the skin.

Patients differ in the degree of skin involvement. Both acute and chronic changes can be seen within the same patient, in distinct body areas[49].

Some patients may show predominantly acute changes while others may exhibit only chronic changes. Furthermore, some patients may show no evidence of disease, and can only be diagnosed as _O. volvulus_ infected during routine epidemiological surveys.

An interesting form of onchocercal dermatitis is known as Sowda (Soda), the Arabic word for black pigmentation. This was first described in Yemen[51], but, similar forms exist in virtually all onchocerciasis endemic foci. Sowda is defined as an asymmetric limb involvement, usually of the legs, where pronounced acute and chronic changes are seen. Most prominent changes are edema, papular eruption and hyperpigmentation together with hypertrophy of the skin[52]. This is associated with enlarged non-tender lymph nodes in the region draining the afflicted limb. Skin biopsies from such patients usually yield no microfilariae, and the diagnosis is often made on clinical grounds. In the author's opinion the definition of Sowda may need some modification from the exclusivity of single limb involvement to a localized severe reaction in one limb not precluding the presence of other milder skin changes in other parts of the body. Our experience with Sowda cases in Sudan shows that most patients demonstrate true papular eruptions in other parts of the body, especially the back, in addition to the presence of severe localized disease in one limb.

Another clinical presentation related to the skin is the hanging groin. This is a consequence of regional lymphedema and skin atrophy leading to loss of the elastic tone of the skin. Consequently the dependent structures in the femoral and inguinal regions gravitate downwards[53].

b. Ocular Changes:

O. volvulus microfilariae can invade the human eye, sometimes in large numbers. The ocular changes seen in patients can be divided into temporary and permanent changes. These changes are pathologic consequences to the presence and/or death of microfilariae within the eye. Both anterior and posterior chamber lesions can be seen. Punctate keratitis ("fluffy opacities") are seen in the cornea and are caused by an acute inflammatory reaction around dead or dying microfilariae[54,55]. This usually resolves within a few weeks with no permanent consequence. A serious and permanent form of eye damage is sclerosing keratitis[56]. This starts in the limbus in the form of haziness and extends into the corneal epithelium accompanied by pigmentary changes. Later it changes into opacification extending into the central cornea. Soon extensive scarring with neovascularization is seen all over the cornea. At this stage, the transparent cornea is essentially replaced by fibrous tissue which does not permit passage of light. Blindness is inevitable.

Other changes within the anterior chamber include anterior uveitis leading to development of synechiae and iris deformity, iritis and cataract.

Posterior segment changes are becoming more recognized as an important cause of blindness. They vary widely from a few depigmented retinal spots to severe choreoretinal changes. Initial changes secondary to acute inflammation are seen as pale edematous areas[55]. These are followed by focal areas of hypertrophy which precede choreoretinal atrophy and clumping of retinal pigment[57]. Eventually subretinal fibrosis occurs. Reduction in vision is related to the extent of retinal lesions. When the lesions become extensive and confluent total loss of vision occurs. The optic disc and nerve are not spared[57]. Optic neuritis and optic atrophy are commonly seen in onchocerciasis. Many literature reports suggest that optic nerve changes can be consequent to diethylcarbamazine therapy, and that the damage is induced by microfilarial death[57,58]. However, it is known that DEC induced damage is an acceleration of a biological process, induced by the occasional death of microfilariae. The natural process of optic neuritis and atrophy may already be occurring, but at a slower rate. This concept remains to be investigated.

c. Nodules:

These are firm, subcutaneous tumors composed of adult worms, entrapped and encapsulated within a fibrous stroma. Onchocercal nodules are firm in consistency, rounded and readily movable. They are often found on bony prominences of the pelvic girdle, ribs and cranium. Nodules by themselves do not cause any significant medical problem. However, their removal is clinically justifiable since it will reduce the overall worm burden, and consequently the number of microfilariae produced. Multiple worms can be found within each single nodule and there is a proportional increase in the number of worms within a nodule as the patient's age increases[47].

Although onchocercal nodules are commonly seen within the subcutaneous tissue in association with the musculo-skeletal system, unusual internal locations, such as in the wall of the aorta, have been reported[59].

d. Lymph Nodes:

Enlarged superficial lymph nodes which may be fibrotic are seen in onchocerciasis patients. These are usually regional lymph glands that drain an affected areas (e.g. limb). They are usually non-tender, except following treatment.

e. Other Manifestations:

Onchocerca volvulus microfilariae were found in many of the deep organs including the liver, kidney, spleen, pancreas, lung, peripheral nerves, and arteries[59]. The significance of such "deep organ invasion" is not clear, but there is little evidence that it contributes to the clinical picture of the disease. It probably follows DEC therapy.

Microfilariae can also be found in blood[60], urine[60,61], sputum[62], vaginal secretions[63], peritoneal fluid[64], and cerebrospinal fluid[65,66]. The latter finding, coupled with clinical observations of epilepsy and dwarfisim, have led to the belief, by some investigators, that microfilariae may invade the brain and pituitary gland[67,68]. These suspicions, however, remain to be substantiated scientifically.

Pathogenesis

Most host tissue damage is believed to be a consequence to an inflammatory response directed against the microfilariae[69,46]. The adult worms only elicit localized granulomatous reactions in the subcutaneous tissue resulting in onchocercal nodules. The female worm periodically releases thousands of microfilariae[9,19] which migrate to the skin and subcutaneous tissue as well as the ocular tissue. Mechanical destruction of host tissue by microfilariae, especially in the eye has been proposed; however, little evidence exists to that effect. Other possible mechanisms of direct tissue damage could be through the production of a collagenase-like substance secreted by the worms[70]. This may play a role in the pathogenesis of skin atrophy where one finds degenerating collagen bundles. The most important pathologic changes, however are due to the inflammatory responses directed against dead or dying microfilariae, and these are a function of the host immune system[46].

Two theories currently exist as to why live microfilariae are protected from the host immune system. The first deals with the masking of microfilarial surface antigens by host proteins[49,71]. Microfilariae are then recognized as self by the host immune system, and no inflammatory response would be evoked. However, it has been shown that live microfilariae _in vivo_ have IgE immunoglobulins in their surfaces indicating that host proteins may not mask all the antigenic sites. Interestingly, the preferential binding of IgE to live microfilariae may play a protective role in microfilarial avoidance of the immune system, since _in vitro_ studies have shown that eosinophil mediated, complement enhanced killing of microfilariae is dependent on IgG and not IgE[72]. The other mechanism of avoidance could be through active suppression of the host immune system. Microfilariae of _Brugia_ produce an immunoinhibitory substance(s) which suppresses lymphocyte responses to antigens and mitogens as well as causing marked reduction in the production of lymphokines[73].

DEC and ivermectin do not kill microfilariae _in vitro_, even at high non-physiologic concentrations[74,75]. However, _in vivo_, they lead to rapid killing and disappearance of microfilariae accompanied by immunologic reactions (e.g. Mazzotti reaction)[32,76]. The mechanism of microfilarial killing by these two agents, though poorly understood, is thought to be immunologically mediated. Possibly their mode of action could be through inhibition of suppressor production by microfilariae.

The inflammatory response directed against the microfilariae lead to destruction of microfilarae as well as surrounding host tissue. The killing is mostly by eosinophils where they first attach to the microfilarial surface and then degranulate, expelling their toxic granules into the surface of microfilariae as well as the surrounding host tissue[73,103]. The degree of pathologic changes seen is a function of the magnitude of the host inflammatory response. Thus, in a philosophical sense, it is better not to mount any inflammatory response. In such a case, both the host and the microfilaria remain viable and relatively healthy.

Another factor that contributes to the pathologic changes seen in the skin is the intense excoriation and itching seen in Onchocerca volvulus infected individuals. This, when repeated, can lead to skin damage. The self inflicted trauma is often followed by secondary bacterial infection which causes more skin damage. The role of blackfly bites in the pathogenesis of skin lesions is becoming more recognized. Blackfly bites cause intense hypersensitivity reactions which may lead to additional disfigurement of the skin[27,28]. The association of blackfly bites and leopard skin has been noted[78].

The involvement of immune complexes in the pathogenesis of onchocercal lesions remains unclear[49,79,80], as does the role of autoantibodies described recently as possible causes of the development of eye pathology[81,82].

Thus, to summarize, the most important cause of pathologic changes is the host inflammatory response directed against the microfilariae. Excoriation and blackfly bites play additional roles in development of skin damage. Mechanical damage caused by migrating microfilariae may cause tissue pathology in ocular tissues.

Pathology

a. Skin:

The histopathologic changes seen in onchocerciasis usually parallel the clinical picture. In asymptomatic patients microfilariae are seen in the upper dermis free of any inflammatory infiltrate. At times mild perivascular cuffing composed predominantly of lymphocytes, plasma cells and macrophages is seen around small blood vessels in the upper and deep dermises[83,84]. With acute clinical changes, one sees prominent perivascular cuffing and the cellular infiltrate would include in addition eosinophils and mast cells, some of which may be degranulating[47,77]. Dermal edema with separation of collagen bundles as well as hyperkeratosis, focal parakeratosis, and acanthosis have been described[83]. Microscopically the papule represents an intraepithelial microabscess containing fragments of microfilariae and varying numbers of lymphocytes, macrophages and eosinophils[77,85]. Eosinophils, either intact or degranulating, are seen in close proximity to the microfilariae[46].

In chronic disease there is thinning of the epidermis with flattening of the rete ridges. Collagen in the upper dermis is disrupted and later becomes fragmented. This is accompanied by increased deposition of mucinous ground substances[85]. Increased fibroblasts and fibroblastic activity may be seen in initial chronic changes; however, in later stages fibrosis may ensue[83]. Microfilariae then are seen mostly in the deep dermis. Macrophages, laden with melanin pigment may also be noted in the upper dermis[47,50].

The clinical entity of Sowda is characterized by an extensive diffuse inflammatory infiltrate involving both the upper and deep dermises. The cellular infiltrate is composed of lymphocytes, macrophages, eosinophils, and plasma cells[86]. The latter is seen predominantly surrounding dermal appendages and blood vessels. Microfilariae are rarely seen in tissue sections taken from Sowda patients[52]. Edema may also be prominent as well as dilatation of lymphatic vessels[86]. The epidermis shows hyperkeratosis and parakeratosis, and increased pigmentation of the basal layer of the epidermis is also seen[86].

b. The Nodule:

The onchocercal nodule is essentially a granulomatous reaction directed against adult worms, mainly females. The cellular infiltrate in the nodule is composed of mononuclear cells and eosinophils and with time, a thick fibrous capsule develops and the worm becomes entangled within fibrous septae[47]. Death of adult worms within nodules is accompanied by caseous necrosis and the center of the nodule may become liquified.

An intriguing question is how the adult worm derives its nutrients within the nodule and whether the nodule constitutes a barrier that offers protection to the worm from systematically administered chemotherapeutic agents. It was initially postulated that the worms derive their nutrient supply by causing microhemorrhages through erosion of blood vessels in the vicinity[87]. Smith et al.[88] elegantly demonstrated the fine capillary network surrounding worms within nodules. They found no evidence of extravasation of blood or erosion of blood vessels. Neuvascularization was evident in nodules, suggesting that the worm may be producing an angiogenesis stimulating factor[88]. It is apparent then that the worm gets its nutrients directly from the vascular network surrounding it, likely by passive diffusion. The same mechanism can be responsible for helping the worm excrete its waste products. This intimate contact with the host blood makes the worm vulnerable to chemotherapeutic agents. Chemotherapeutic agents such as chloroquine and ivermectin can readily be found in nodular tissue and worms (Williams, J.F., personal communication). At the same time secreted worm products and shed antigens may have access to the systemic circulation of the host.

c. The Eye:

Pathologic changes in the eye are generally similar to those seen in the skin. These changes are again thought to be a consequence of microfilarial death either naturally or by chemotherapy. Unlike the skin, there is a scarcity in our knowledge about eye pathology and the few scattered reports on eye pathology deal mostly in end stage disease. Two pathologic events can be seen in the cornea. The first is punctate keratitis, where there is focal collection of lymphocytes and eosinophils around dead or dying microfilariae[89]. This localized inflammatory reaction usually resolves without any sequelae within 3 weeks. The second corneal change is sclerosing keratitis. Here there is development of fibrovascular pannus between the Bowman's membrane and the corneal epithelium which may become hyperplastic[47,89]. The translucent cornea becomes opacified. Sclerosing keratitis, when advanced, can lead to blindness.

Inflammation of the uveal tract is also seen in onchocerciasis. It can occur as part of the natural disease or following microfilaricidal therapy. It is characterized by the presence of inflammatory cells in the iris and ciliary body accompanied by hyperpigmentation due to increased activity of the iris pigment epithelium[90]. Later depigmentation is seen as well as hyalinization of small arteries and arterioles[90].

A spectrum of choreoretinal changes can be seen. Focally, one may see collections of inflammatory cells, eosinophils, and plasma cells where the underlying epithelium becomes atrophic[91]. Pigmentary changes in the form of pigment migration and clumping may also be seen. In advanced disease, profound choreoretinal atrophy with loss of photoreceptors, bipolar cells and ganglion cells is evident[89]. The latter stages are often accompanied by optic atrophy and severe restriction in vision or total blindness is the usual outcome.

d. Lymph Nodes:

Lymphadenopathy is commonly seen in onchocerciasis patients. Two types of pathologic changes can be seen in lymph nodes. The first is mostly seen in patients with long standing chronic features and include various degrees of fibrosis. This usually begins at the capsular area and gradually extends into the interior of the lymph node[92]. The fibrosis is accompanied by atrophy of the germinal centers and in some series, up to 80% of the lymphoid tissue is replaced by tough scar tissue[92]. This gives lymph nodes their characteristic discrete rubbery feeling. Microfilariae are commonly seen in such lymph nodes (in 75% of patients), located mainly within the fibrous capsule or interstitial connective tissue.

The second histopathologic feature is an enlarged soft homogenous lymph node which microscopically shows marked follicular hyperplasia, and enlarged germinal centers. This is commonly seen in the acutely reactive patient, and is characteristic of Sowda patients[93]. Microfilariae are rarely seen in such nodes.

The pathologic events occurring in lymph nodes, though not well understood, are again thought to be precipitated by inflammatory responses, directed against dead or dying microfilariae. The consequence of this, in the long term, is probably fibrous replacement of the lymphoid tissue. This process is mostly seen in inguinal lymph nodes; however axiliary and occipital lymph nodes can show similar pathologic changes. The fibrosis can eventually lead to impediment of lymph drainage which may lead to hanging groin[53] and elephantiasis of the genitalia[94] and limbs[95], seen occasionally in onchocerciasis.

e. Deep Organs:

There is only a single case report documenting the presence of *Onchocerca volvulus* microfilariae in deep organs at autopsy in a patient who died after Diethylcarbamazine therapy[59]. Sections from the liver, kidney, pancreas and lungs all showed O. volvulus microfilariae. This is most likely secondary to the effect of DEC in mobilizing microfilariae.

Treatment and Management of Onchocerciasis

a. Symptomatic Treatment and Treatment of Superimposed Secondary Bacterial Infection[96]:

Severe pruritus caused by the disease can be relieved by the use of antihistamines. This kind of treatment can reduce self-inflicted injuries. Secondary pyogenic infections of the skin seen in onchocerciasis can be prevented by adequate treatment of infected lesions by antibiotics.

b. Specific Antiparasitic Drug Therapy:

At present, specific drugs available for the treatment of onchocerciasis are not satisfactory[97]. They are often associated with adverse effects, some of which are severe and can be fatal[98]. Ironically, these may be the result of parasite death caused by the effective anthelmintic drug. An important sequel to treatment is the possible deterioration of vision which may ultimately lead to blindness[58,99].

Anti-onchocercal drugs in general, and microfilaricidal drugs in particular are thought to accelerate the natural processes of parasite death[74,96]. Until recently, the anti-onchocercal drugs available had many undesirable side effects, which made them unsuitable for mass treatment in any chemotherapy based control measure.

The recent introduction of ivermectin, with its milder side effects has renewed the hope of combating the disease by specific chemotherapy.

Only 3 antiparasitic drugs are available now for clinical use in the treatment of onchocerciasis. These are diethylcarbamazine (DEC) and ivermectin which are microfilaricidal drugs, and suramin which is a macrofilaricidal drug with some microfilaricidal action.

1. <u>Diethylcarbamazine (DEC):</u>

DEC was discovered in 1947[100]. It has considerable activity in killing the microfilariae of <u>Onchocerca volvulus</u> but it has no action against the adult worm[101]. DEC is given orally and within a few days can lead to elimination of more than 80% of the microfilarial load, and patients often turn from positive to negative on skin snip tests[102]. However, the microfilariae start to reappear again within 2-3 months, since the adult worm is still alive and capable of producing new microfilariae[101,102].

The microfilaricidal mode of action of DEC in onchocerciasis is poorly understood. In lymphatic filariasis, DEC leads to mobilization and rapid disappearance of circulating microfilariae from the blood to the liver and spleen[101,102]. There, the microfilariae are phagocytised by the reticuloendothelial system cells. In onchocerciasis, where microfilariae are found predominantly in the skin and subcutaneous tissue, DEC leads to mobilization of microfilariae from the dermis to the epidermis, blood stream and urine[60,74]. <u>In vitro</u> studies have shown convincingly that DEC by itself does not kill microfilariae[74]. <u>In vivo</u> DEC may promote cell mediated attack on the parasites[77,103]. Addition of DEC to immune serum and white cells leads to increased cell adherence and killing of the microfilariae[104]. However, the concentrations of DEC used in these experiments were well above those which are pharmacologically active <u>in vivo</u>.

DEC is a rather safe drug when given to normal individuals. The side effects seen in onchocerciasis are directly related to its microfilaricidal action. In the characteristic Mazzotti reaction seen with DEC treatment[32], the rapid killing of microfilariae in the skin, subcutaneous tissue and the eyes, results in severe allergic-like reactions. Intense itching occurs shortly after drug administration, accompanied with watering of the eyes. Hours later a stronger reaction takes place in the form of urticaria, fine maculopapular rash, and edema of the skin, especially over the buttocks, thighs, and genitalia, together with swelling and tenderness of inguinal lymph nodes. Pyrexia, headache, muscle pains and joint aches, and tachycardia are all common. If onchocercal eye involvement is present it may become worse[58,99]. Death of microfilariae in the anterior segment can give rise to punctate keratitis, which is reversible; however, serious, irreversible posterior segment lesions demonstrable by fluorescein angiography can occur[58].

Different forms of topical application of DEC were tried in an attempt to minimize the side effects of treatment[105,106,107]. Lotions, creams and eye drops were used, but, unfortunately, the reactions observed in these experiments were worse than oral administration[106,108].

In spite of all these problems, DEC remains a potent, commonly used microfilaricidal drug. It is cheap and affordable. However, the occurrence of the Mazzotti reaction makes it less attractive and many of the patients abandon therapy because of the reaction.

2. Suramin:

Suramin is the only available approved macrofilaricidal drug. It is effective in killing adult O. volvulus. Suramin was first discovered in 1920[109]. It was then used for the treatment of African trypanosomiasis. It was first used in the treatment of onchocerciasis in 1947.

Suramin is poorly absorbed from the gastrointestinal tract and should only be given intravenously. Intramuscular and/or subcutaneous injection cause intense local irritation, severe pain and possible sloughing and abscess formation at the site of injection. It strongly binds to plasma proteins and is slowly metabolized leading to a long half life (30 days)[110]. The drug, bound to plasma proteins, is taken up by the reticuloendothelial system cells and proximal convoluted tubules of the kidney. The latter process might be responsible for the frequently observed renal damage in patients treated with suramin[109,110].

Suramin is a toxic drug. Reactions are more common in malnourished people, especially if they have hypoproteinemia. Since excretion of drug is slow, toxicity may be cumulative[110]. Toxic reactions during therapy are classified by Hawking[109] into:

I. Immediate reactions: These include nausea, vomiting, collapse, shock, sweating and loss of consciousness. These are best avoided by slowly injecting the drug and starting treatment with a small dose.

II. Late reactions: These occur at 3-24 hours. Signs include pyrexia, photophobia, uveitis, lacrimation, abdominal distension and cutaneous hypoesthesia, and are thought to be due to the nicotinic effect of the drug.

III. Delayed reactions: These are the most common. The most frequent is albuminuria and cellular casts in urine, due to kidney tubular damage. Because of this serious toxic effect, examination of renal function, at least in the form of a urine-general exam, is essential before administering each weekly dose. Other less common components in the delayed late reaction category include exfoliative dermatitis, stomatitis and rarely jaundice.

IV. Allergic reactions: Some of these are thought to be due to the microfilaricidal action of the drug, similar to those seen with DEC. They include urticaria and pruritus, accompanied by vesicular rashes. Other reactions, thought to be primarily due to the macrofilaricidal action of the drug include tenderness and swelling around nodules containing the adult worms.

With all these unwanted side effects of Suramin, the drug remains the only available compound that offers cure for onchocerciasis. Almost 40 years have elapsed since it was first used in onchocerciasis, but we still lack knowledge about the pharmacokinetics, tissue distribution and effective blood levels needed for its macrofilaricidal action.

3. Ivermectin:

This is a semisynthetic macrocyclic lactone produced by the actinomycetae, Streptomyces avermitilis. It is extremely active against a wide variety of parasitic nematodes[111,112]. Although it has no detrimental effects on adult filariae in general, it proved to be active against O. volvulus microfilariae[113]. When administered orally in a single small dose of 100-200 ug/kg body weight, it can lead to killing of microfilariae and patients can remain microfilariae-negative after treatment for up to 9 months[113]. It is claimed that the side effects of the drug are less severe than those of DEC, manifested in the form of a milder "Mazzotti" reaction[114]. However, recently, moderate to severe reactions were noted in patients from Sierra Leone[115]. In addition, a possible association between ivermectin therapy and coagulation disorders was suggested in a small number of Sudanese patients[116]. Further testing of the drug is needed in order to exclude potentially serious side effects and evaluate efficacy on a larger scale, and in different geographical locations.

The microfilaricidal action of the drug is not well understood. It has been proposed that it acts by potentiation of gamma-aminobutyric acid (GABA) action thus reducing muscle membrane potential of the worm[117]. In mammals, GABA is a neurotransmitter found only in the central nervous system, while in nematodes GABA is a neuromuscular transmitter. Hence, ivermectin may lead to inhibiton of excitatory and inhibitory post-synaptic potentials and paralyis. This action, however, does not seem to apply to O. volvulus microfilariae in vitro, since the drug, at physiological concentrations does not have readily detectable effects on the motility of microfilariae. Recently, microfilarial emergence from skin snips following ivermectin therapy was assessed[118]. No difference in emergence was found between biopsies taken from treated or untreated patients. Thus, the hypothesis of an ivermectin-paralyzing effect on microfilariae is weakened further. Ivermectin has stimulated a lot of enthusiasm since clinical trials started, and the overwhelming success of ivermectin therapy in West Africa prompted the World Health Organization to encourage community based treatment programs. It is expected that large scale use of the drug in the treatment of onchocerciasis will be commence in many endemic areas, with the hope that the use of ivermectin may aid in the interruption of the transmission cycle[121].

c. Nodulectomy:

Surgical removal of onchocercal nodules from patients is a justifiable procedure since it reduces the worm burden in the body. Nodulectomies do not lead to complete cure because nodules may be deeply buried in the muscles and behind big joints[119], where they are inaccessible for surgical removal. In endemic areas, for the procedure to have a measurable effect, new nodules have to be removed yearly. In South America, removal of head nodules is routinely practiced because it is thought to reduce the number of microfilariae invading the eyes.

d. Prophylaxis:

The subject of prophylaxis in onchocerciasis has not received much attention. Studies with *Litomosoides carinii*, *Loa loa*, and *Brugia malayi* have provided some indirect evidence that prophylactic use of DEC can prevent new infections. DEC was shown to be effective in preventing loaisis[120], but this might not be the case for onchocerciasis. Ivermectin was found to be effective in preventing infection of cattle by *O. gibsoni*. There was hope that it could serve as a prophylactic drug against *O. volvulus* infection, but this has not been borne out by recent experiments in chimpanzees.

Immune Response in O. volvulus Infection

Immunological studies of *O. volvulus* infection are often hampered by the scarcity of parasite material. The lack of a suitable laboratory animal model makes it necessary to obtain the parasites from infected individuals residing in remote, often inaccessible endemic foci. Studies of cell mediated immune responses of patients have proven difficult, since most of the work has to be done on site, where field conditions dictate what can and cannot be performed. Simple tasks, such as *in vitro* lymphocyte cultures become a major challenge for problems of contamination and the lack of appropriate equipment. Despite all logistical difficulties, investigation of immune responses to *O. volvulus* infection is progressing at a good pace, and our knowledge about the biology and immunology of the disease has substantially increased during the last 10 years.

Most of the pathologic consequences seen in onchocerciasis are believed to be secondary to inflammatory reactions against microfilariae. Such inflammatory reactions are controlled by the immune system and require participation of both humoral and cellular elements to occur. Even the mode of action of the microfilaricidal drugs in current use is thought to be immunologically mediated. Thus, it is of importance to have a good understanding of the different immunologic mechanisms involved in onchocerciasis and its relationship to the pathogenesis of the disease.

It is often difficult to dissect immune responses to O. volvulus in individuals living in endemic foci, since concurrent parasitic and non-parasitic infections are commonly seen in the majority of patients. Therefore, it is imperative to include appropriate controls to minimize these variables, in any immunologic studies of patients.

a. Antigens of Onchocerca volvulus:

Several approaches to obtain O. volvulus antigenic preparation have been utilized. By far the most common is the preparation of crude somatic antigen extracts from whole worms obtained by surgical removal of nodules. O. volvulus worms may either be dissected or collagenase digested from host tissue[122] and then homogenized and extracted with buffer. The antigenic preparation obtained this way contains complex water soluble somatic and surface antigens, as well as contaminating host proteins. Another approach is the preparation of secretions/excretions of adult worms and microfilariae. This is achieved through in vitro maintenance of live intact adult O. volvulus or microfilariae in various culture media[122]. The culture supernatant should contain actively secreted, excreted or shed worm antigens. One of the problems encountered in this approach is that the preparation often contains host proteins. Also it is difficult to maintain the worms in serum-free media, for their survival time is greatly reduced, and consequently only a small amount of antigenic preparation can be obtained. Of course, the advantage of this approach is that the secreted/excreted antigens are likely to be the ones produced in vivo by live worms and which are usually encountered by the host immune system. A third approach is the use of live microfilariae for in vitro assay where the microfilarial surface acts as multiple antigenic epitopes which can be recognized by antibodies and cells[104,123].

Antigenic extracts from O. volvulus worms and microfilariae have been used in in vitro cell mediated responses as well as in testing of immediate and delayed hypersensitivity reactions[125]. They have also been used extensively for immunoblotting recognition patterns by sera from O. volvulus infected individuals.

Although the antigenic properties and the immunomodulatory effects of worm secretion and excretions were recognized in other filarial and helminth infections[73,124,126], they have received little attention in onchocerciasis. To date, few reports exist on the utility of O. volvulus microfilariae and adult worms secretions/excretions and in most instances the goal has been immunodiagnosis[127]. This lack of investigation of the immunologic role of secretions/excretions, is largely due to the extreme difficulty in obtaining live worms in numbers large enough to produce sufficient amounts of these products for research use.

b. Cell Mediated Immunity:

The data on cell mediated immunity in onchocerciasis are at best confusing. The earlier notions of generalized defects in cellular immunity similar to those seen in lymphatic filariasis are still being cited. Evidence for such defects stems from findings of depressed skin reactivity to the non-related antigens tuberculin and lepromin as well as to *O. volvulus* crude antigen[125,128].

The idea of generalized immunodepression was further supported by the *in vitro* work of Merino and Brand[129], where they found decreased reactivity to concanavalin A and by Greene et al. They demonstrated depressed reactivity to the bacterial antigen SKSD in a small number of patients and controls[130]. This has lead to acceptance of the concept of generalized immunosuppression among researchers, the clinical relevance of which was suggested by the appearance of a single report of an association between onchocerciasis and the development of lepromatous leprosy[131]. Haque and colleagues examined pooled sera from onchocerciasis patients and found that the addition of such sera suppressed lymphocyte responses to mitogens[132]. They postulated that serum inhibitory factors are responsible for "this state of generalized immunodepression in onchocerciasis".

Evidence to the contrary, i.e. lack of generalized immunosuppression is becoming more recognized in recent literature. Onchocerciasis patients living in endemic areas are not more susceptible to other infectious diseases when compared to non-infected endemic controls from the same ethnic and socioeconomic background. Bartlett et al.[125] have shown that *O. volvulus* infected subjects have normal delayed skin reactivity to the non-related antigen of *Necator americanus*.

Bryceson[133] and Greene[130] found normal lymphocyte blastogenic responses to mitogens in onchocerciasis patients. Lack of generalized immunosuppression is reported by Brattig et al.[134] where they were able to demonstrate normal peripheral blood lymphocyte phenotypes in onchocerciasis patients. In fact, the latter authors found an increase in NK activity among reactive (Sowda) patients. Studies from Venezuela in which the effect of individual patient's sera on lymphocyte blastogenic responses to mitogens was studied, showed mixed patterns where some sera augmented blastogenic responses while others suppressed it[135].

Two recent reports, however, clearly negate the idea of generalized immunodepression in onchocerciasis. In the first by Ward and co-workers[136] they found intact _in vitro_ lymphocyte blastogenic responses in Guatemalan onchocerciasis patients to mitogens and the non-related antigen PPD, when compared to endemic controls. The normal lymphocyte proliferative responses were accompanied by normal production of interleukin-2 (IL-2) and Gamma interferon (IFN-γ). Unexpectedly, they also found good lymphocyte blastogenic responses to crude _O. volvulus_ antigen among their patient population. However, they noted decreased IL-2 production by cells from _O. volvulus_ infected subjects when compared to non-infected, endemic controls. The second report comes from Gallin and her colleagues[137] where they found normal lymphocyte responses to mitogens and the bacterial antigen streptolysin O among Liberian onchocerciasis patients. They also found low reactivity to crude _O. volvulus_ soluble antigen among patients and endemic area controls. The _in vitro_ reactivity to _O. volvulus_ could be greatly increased in both groups by addition of

IL-2. In the above two studies, careful planning to include endemic and non-endemic controls was taken, as well as the utilization of pertinent clinical information to include or exclude participants in their studies. Such characteristics, which were mostly ignored by previous investigators, have proven useful in analysis of results.

However, in all of the studies sited here only crude O. volvulus antigenic extracts were used. These are composed of numerous antigens some of which may not be readily available for recognition by the host immune system before the worm death.

Thus, to summarize, our knowledge of cell mediated immunity does not consistently support the idea of generalized immunosuppression. Intact responsiveness to mitogens and to non-O. volvulus antigens is present. Clinically, O. volvulus patients do not exhibit signs or symptoms of immunodeficiency when compared to their respective endemic controls. Specific hyporesponsiveness to O. volvulus antigen is suggested in some studies by decreased lymphocyte responses to O. volvulus antigen and decreased production of IL-2.

c. Antibody Dependent Cellular Responses:

Sera from O. volvulus infected individuals were shown to promote leukocyte adhesion to and killing of microfilariae of O. volvulus and other animal onchocercal species[123,138]. Not all O. volvulus infected patients' sera have such capacity, and most of the activity is seen in sera obtained from patients with active acute skin and eye lesions. The cell type involved in the adhesion phenomenon is the eosinophil[104,138]; in the presence of sera it adheres to and degranulates onto the surface of live microfilariae. The adhesion is enhanced by the presence of complement. Purified eosinophils can adhere to microfilariae _in vitro_, but they are unable to degranulate and kill them, indicating that participation of other cell types and their secreted products is needed for killing to occur. The _in vitro_ finding of eosinophil adhesion is in accordance to what is seen _in vivo_, where eosinophils and their toxic products are seen in close proximity to dying microfilariae in tissue section[72]. Diethylcarbamazine was shown to enhance adhesion of granulocytes to microfilariae _in vitro_[74,139], however the concentrations used in those experiments were much higher than the physiologic therapeutic plasma level. Eosinophilia is a common feature in patients with onchocerciasis, sometimes reaching 70% of all leukocytes[104]. Administration of DEC to patients leads to rapid disappearance of eosinophils from peripheral blood to the tissues where they were considered to participate in killing of microfilariae[74]. All of these findings suggest an important role for eosinophils in the destruction of microfilariae, and they provide no support for the notion that neutrophils are the major players in the killing of microfilariae[123]. The paucity of neutrophils in the inflammatory infiltrate around microfilariae seen on histologic sections of the skin suggests that their role in killing of microfilariae and hence in the pathogenesis of this disease is rather limited.

Antibody dependent cellular cytotoxicity is most likely promoted by antibodies of the IgG class rather than IgE, since in histological sections, IgG can be seen on the surface of dying microfilariae, while IgE is seen on apparently health microfilariae, without any asociated cellular reaction[72]. No work has been done to determine the subclass of IgG responsible for microfilarial killing.

d. Humoral Immune Response

Until recently, most of the studies on humoral immune response in onchocerciasis were directed towards diagnosis. It has been mentioned in the "Diagnosis" section that several immunologic procedures were employed in pursuit of improving the specificity and sensitivity of these tests, but the failure to identify an Onchocerca volvulus specific antigen has severely curtailed efforts for development of a reliable and practical immunologic test. Recently, with the introduction of more sophisticated techniques, it has been shown that low molecular weight O. volvulus antigens may offer the greatest diagnostic specificity of the disease. These low MW antigens generally lack cross reactivity with other filarial antigens[140]. Of these antigens, two seem to offer the most promising potential; A 33 KD and its supposedly 21KD breakdown product[141]. These two antigens were shown to react specifically with sera from O. volvulus infected individuals with a specificity of 100% and sensitivity above 90%. However, more work is needed to refine the techniques for practical diagnostic use and more sera from patients with wider clinical presentations have to be tested to confirm their usefulness.

Detection of circulating O. volvulus specific antigen(s) is an attractive approach, since it can discriminate between patients harboring the infection from those who were exposed, but not infected or those who are cured from the infection, but still serologically positive. The two pieces of work dealing with this issue did not show greater advantage over the old fashioned method of demonstrating the parasite in skin biopsies. The sensitivity ranged from 31% to 75% in one report[40] and increased to about 80% in the other after an O. volvulus specific monoclonal antibody was used[39]. Nonetheless, antigen detection remains an attractive option which can be used to detect parasite products in various biological fluids, Thus minimizing the invasiveness of skin sampling and blood drawing both of which are sometimes opposed by patients.

High levels of total immunoglobulins, notably those of the IgG and IgE classes are usually seen in sera from onchocerciasis patients [142,143,144]. Serum levels of immunoglobulins do not usually correlate with the clinical status of patients, perhaps because of the frequent occurrance of other concomittant parasitic infections among onchocerciasis patients. Recently however, it has been shown that significantly higher levels of IgG and IgE are seen in patients with localized onchocerciasis (Sowda), when compared to others with the generalized form[134].

Studies aimed at detection of O. volvulus specific antibody responses have shown that parasite specific IgE can be seen in association with the infection[143] with up to 70% of patients manifesting high titres[144]. The highest levels of specific IgE anti O. volvulus antibodies seem to reflect clinical severity of the disease [145]. Unlike delayed intradermal skin testing where patients' reactivities differ, immediate hypersensitivity reactions to onchocercal antigens are invariably seen in onchocerciasis patients[122,127]. These findings coupled with findings in lymphatic filariasis [146,147,148] bolstered beliefs about the immunobiological significance of IgE in the pathogenesis of the disease. In 1982, Weiss and colleagues[149] showed intense IgE reactivities to more than 50 _O. volvulus_ antigenic bands using Western immunoblotting techniques. The above authors, however, used extremely concentrated patients' sera (dilution 1:2) which raises questions as to the specificity of their procedure. Since then no one has attempted to specifically look at IgE reactivities using the Western blotting technique. However, recently Cabrera et al.,[150] looking at IgG3 reactivity of Sowda patients to _O. volvulus_ antigens, also examined other immunoglobulin classes reactivities, including IgE. Although they did not comment on IgE recognition pattern to separated _O. volvulus_ antigens the results of their immunoblots clearly show minimal reactivity with IgE. The same authors have shown very restricted patterns of IgE recognition using _Onchocerca gibsoni_ as a source of antigen[151].

Reproducible patterns of _O. volvulus_ antigen recognition by other immunoglobulin classes using Western blotting have been demonstrated by a number of investigators[141,150,151,152,153]. Differential recognition of worm antigens by antibodies of the IgG immunoglobulin class and its relation to the clinical presentation of patients attracted much attention recently. The emerging pattern is that patients with severe disease tend to recognize more antigenic bands with greater intensity[152,153]. However, this is a generalization, since on occasion, asymptomatic patients and those with mild disease may recognize a similar or greater number of antigenic bands with stronger intensities[152,153]. This diversity and lack of consistent antigen recognition patterns in relation to the clinical signs is probably due to the dynamics of the disease, where the immunologic status of patients may change, without an immediate change in the clinical picture. Patients may revert from a reactive state to non-reactive or vice versa. Most of the immunological studies in onchocerciasis, including those concerned with immune responses, are cross-sectional ones. Better understanding of immunologic responses to the parasite should come from longitudinal studies, where the clinical and immunologic changes can be closely monitored.

In the most recent studies by Cabrera et al.[150] the role of IgG immunoglobulin subclasses in onchocerciasis was studied. The authors looked at IgG3 recognition patterns of O. volvulus antigens by sera from Sowda patients and those with generalized onchocerciasis. They claimed that IgG3 antibodies present in sera obtained from Sowda patients all specifically recognize 72 KD and 9 KD O. volvulus antigenic bands, while onchocerciasis patients with other forms of the disease failed to recognize either band. They proposed that this IgG3 recognition could serve as a diagnostic test for Sowda patients who are often negative parasitologically. However, their data were unusual in that these two proteins were not recognized when blots were processed with anti-total IgG. No explanation was offered for this unexpected finding. Cabrera et al.[150] also demonstrated the remarkable feature of high anti-onchocercal IgG4 antibodies in patients with different forms of the disease. This is similar to the findings in lymphatic filariasis, where IgG4 is being incriminated as a blocking antibody for IgE mediated killing of the parasite[154,155]. Whether this is true for onchocerciasis remains to be explored.

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SUPPRESSION OF HUMAN LYMPHOCYTE RESPONSES TO SPECIFIC AND NON-SPECIFIC STIMULI IN HUMAN ONCHOCERCIASIS

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ABSTRACT

Characterizations of circulating lymphocyte subset populations, in vitro lymphocyte responsiveness, and the serum-mediated effects on lymphocyte proliferation were performed on groups of onchocerciasis patients selected from over 170 examined parasitologically and clinically in Sudan and Sierra Leone. These patients manifested a very broad range of clinical signs and showed widely divergent parasite intensities; some were heavily infected and asymptomatic while at the other extreme were individuals with severe clinical signs, but few or no detectable microfilariae (mf) in their biopsies. Relationships between parasite burden and clinical severity varied; in Sierra Leone severity increased with detectable mf numbers, but the reverse was true in one group in Sudan. Patients from Sudanese foci were, on the whole, more badly afflicted than the W. Africans. Lymphocyte subsets in peripheral blood were not remarkably different from those in samples from endemic area and non endemic area controls, except in regard to a significant increase in NK cells in infected W. Africans. Lymphocyte proliferative responses to soluble Onchocerca volvulus antigen (sAg) were poor in infected persons, however, mitogen and PPD responses were maintained in the normal range in one group of patients from southwestern Sudan, but were profoundly depressed in a group from Eastern Sudan. The latter were malnourished and in poor physical condition in a drought-stricken zone. Sera from 90 Sudanese and W. African patients exhibiting a wide range of clinical signs and parasite burdens had no significant effects on responsiveness in vitro of lymphocytes from either normal or infected subjects. However, proliferative responses and IFN-γ production by lymphocytes were very significantly depressed

in the presence of live microfilariae of O. volvulus or secretions/excretions (S/E) from microfilariae or from females, but not male adult parasites. Lymphocyte responses were maintained near normal when exogenous IL-2 was added to the cultures. The results indicate that overall O. volvulus infection and clinical consequences are not consistently associated with systemic deficits in immune cell populations or responsiveness, or circulating suppressive factors; however, lowered lymphocyte responses can be a feature when health problems are perhaps exacerbated by prolonged malnourishment. Specific lymphocyte responsiveness to O. volvulus sAg was poorly manifested in all patients examined. The demonstration of potent suppression of lymphocyte reactivity by mf and S/E in vitro suggests that direct parasite intervention in host cell responses could be taking place in vivo, perhaps at the local micro-environment level, mediated by effects on IL-2 production. A hypothesis is presented concerning the consequences of anti-microfilarial therapy in vivo, and its potential effect on disruption of local immunosuppression.

INTRODUCTION

Modulation of host immune responses is considered a major factor in the disease course followed by patients infected with the filarial nematode *Onchocerca volvulus*[1,2]. Clinical manifestations may range from a complex of massive acute and chronic inflammatory changes in the skin and eyes of those infected, to mild pruritus or even asymptomatic infections at the other extreme. Acute exacerbations are generally associated with chemotherapy[3], and, increasingly, death of microfilarial forms in tissues is becoming recognized as a significant event in the pathogenesis of onchocercal lesions in skin and ocular tissues [4,5].

In spite of the clinicopathological and parasitological evidence to support this interpretation of onchocerciasis and its natural history, immunological determinants and correlates of disease stages have proven very difficult to identify. By analogy with findings in chronic lymphatic filariasis[6,7,8], specific and non-specific immunosuppressive mechanisms have been sought in onchocerciasis patients, but the evidence is conflicting about their contributions to the outcome of the host-parasite interaction. Both within and between patient groups there is a great deal of heterogeneity with regard to lymphocyte responses to *O. volvulus* antigens, mitogens and bacterial antigenic stimuli[9,10,11], and to the presence of circulating suppressors or enhancers in the sera of infected subjects[12,13]. Variables such as age, chemotherapeutic history, and geographic strain of parasite have recently been shown to have important bearing on immunological response patterns[14,15], and the need to include endemic area "normals" in such studies has been emphasized[25].

In developing a comparative account of onchocerciasis in several distinct geographic foci we have had the opportunity to examine the influence of patients' sera and parasite products and antigens on the *in vitro* lymphocyte responses of controls and of patients with differing clinical manifestations and *O. volvulus* infection intensities in a variety of epidemiological contexts. Our results confirm the occurrence of *in vivo* suppressive changes in some patient subgroups, and demonstrate the production of inhibitory factors for human lymphocyte proliferation by live adult females and microfilariae of *O. volvulus* *in vitro*. The effects of these inhibitors could be largely overcome by addition of exogenous IL-2, raising the possibility that direct intervention by the parasite into the immunoregulatory pathways of the host's immune system may be a component of suppression mechanisms *in vivo*.

MATERIALS AND METHODS

Study Areas. Patients from four different geographical areas endemic for onchocerciasis were studied. Three are in Sudan, and each shows some slightly different characteristics in the prevailing form of the disease: Abu-Hamad in the North[16]; Sundus in the East[4,17], and Bahr El Ghazal in the Southwest[18]. An additional group of patients was examined and sampled in Bo, Sierra Leone, West Africa [19].

Patients and Control Selection. 174 patients were examined: 109 from Sudan and 65 from Sierra Leone. From each focus individuals representing the fullest possible range of clinical manifestations and parasite burdens were included. Subject ages ranged from 12-70 years, (mean, 32.8), with 120 males and 54 females. Two groups of non-infected controls were included: the endemic area controls were 13 (eight males and five females) individuals living in onchocerciasis endemic areas, but clinically and parasitologically not identifiable as onchocerciasis patients. The normal (non-endemic area) controls were 32 (22 males and 10 females) individuals living in Khartoum, Sudan, where the disease is not established. These people were similar in ethnic make-up and in their socioeconomic background to the Sudanese infected patients. Ages of both control groups ranged from 18 to 63 years (mean, 33.8).

Clinical Examination. All participants were given a complete physical examination with special attention given to the palpation of *O. volvulus* nodules, and to the evaluation of skin and eye changes consistent with *O. volvulus* infection. The clinical history taken included details of any antifilarial treatment received and of surgical removal of nodules. Details of the examination procedure and the assessment of lesions are described elsewhere[4,5]. As a result of physical findings, individuals were classified primarily on the basis of the type, distribution and severity of skin changes, using a point scale of 0-3, with 3 being most affected. For the purposes of comparison 3 groups of symptomatic patients emerged from this scheme; those with mild disease (score of 1), moderately affected people (score of 2) and those severely afflicted (score of 3). Asymptomatic patients with no signs or symptoms of onchocerciasis, but with microfilariae in their tissue biopsies were given a score of 0.

Parasitological Examination: To determine the microfilarial intensity of *O. volvulus* infection in each individual, bilateral skin biopsies (snips) were taken from the iliac crest using a Walser corneoscleral punch. Skin snips were then incubated in tissue culture medium RPMI 1640 (Sigma Chemical Co., St. Louis, MO) for 4 hours. Emerging microfilariae were counted and the wet weight of the snips was determined. Microfilarial intensity was expressed as the number of microfilariae per milligram of skin (mf/mg). Urine, stool and blood samples from patients in Abu-Hamad and Bo were examined for other parasites by conventional techniques.

Lymphocyte Subset Phenotyping of Patients and Controls. Anti-leu 2a, leu 3a, leu 4, leu 11b, and leu 12 mouse monoclonal antibodies were purchased from Becton Dickinson (Mountain View, CA). These probes recognize, respectively, human lymphocyte surface antigens, CD8, CD4, CD3, CD16, and CD19. Colloidal gold-conjugated goat anti-mouse antibody (Geometric Data, Wayne, PA) was used as detector for the primary mouse monoclonal antibody bound on lymphocyte surfaces.

The test procedure was similar to that described by the manufacturer. Briefly, from each individual a buffy coat suspension was obtained from EDTA-treated blood. Aliquots of buffy coats were first incubated 30 minutes with one of the primary monoclonal antibodies, then with the second, conjugated antibody for another 30 minutes. Cells were washed with phosphate buffered saline after each incubation. Finally, a thin film was made, fixed in methanol and counter-stained with modified Wright's stain. Enumeration of lymphocyte subtypes was done microscopically by counting the number of positive cells per 200 lymphocytes. Cells considered positive had at least four gold particles on their surfaces.

Preparation of O. volvulus Adult Worm Secretions/Excretions (S/E) and Soluble Antigen (sAg). Intact nodules were surgically excised from patients under local anesthesia. To obtain live female parasites, nodules were digested in collagenase using the method described by Schulz-Key[20]. Trimmed excised nodules were incubated in culture medium RPMI 1640 containing 10% pooled normal human serum, penicillin, 100 U/ml, streptomycin, 100 ug/ml (Gibco Laboratories, Grand Island, NY) and collagenase type IV (Sigma Chemical Co.) at a concentration of 2 mg/ml. Digestion was carried out at 37°C for 12 hours after which female parasites were recovered by gentle dissection. *O. volvulus* males were obtained either by collagenase digestion or from gently minced nodular tissue. Isolated adults were thoroughly washed in serum-free RPMI 1640 with antibiotics.

All *O. volvulus* female and male worms were maintained individually at 37°C in RPMI 1640 supplemented with antibiotics for 24-48 hours. For active males, the culture period was extended up to 96 hours. Culture supernatants were dialyzed at 4°C against three changes of 1,000 volumes of RPMI 1640 using Spectrapor membrane tubing (Spectrum Medical Industries Inc., Los Angeles, CA), with a molecular weight cut off of 10,000 Daltons. Supernatants were then filter-sterilized, aliquoted and stored at -70°C. The protein concentration in supernatants was measured using the Lowry method[21].

Isolated O. volvulus female worms were homogenized in phosphate buffered saline, pH 7.2 and extracted overnight at 4°C. Particulate material was pelleted by centrifugation at 5,000 x g/30 minutes and the supernatant was further clarified at 100,000 x g/1 hour. The final supernatant was dialyzed against RPMI 1640 and processed as for O. volvulus S/E products.

Preparation of O. volvulus Skin and Nodular Microfilariae (mf) and Their Secretions/Excretions. Live skin mf were obtained by incubating skin biopsies obtained from O. volvulus patients in RPMI 1640 and antibiotics for 4 hours. Nodular mf were obtained by teasing excised tissues containing O. volvulus female worms. The tissues were then incubated in RPMI 1640 with antibiotics for 1 hour. Emerging microfilariae from skin or nodular tissues were purified using the agar gel technique[22].

Preparation of O. volvulus mf S/E. Microfilariae obtained either from biopsies or nodular tissues were maintained at a concentration of 1×10^3 mf/ml in RPMI 1640 plus antibiotics at 37°C for 48 hours. Mf culture supernatants were then processed as for those of O. volvulus adult worm S/E.

Lymphocyte Stimulation. Peripheral blood was drawn from patients and controls into heparinized vacutainers (Becton Dickinson, Rutherford, NJ). Peripheral mononuclear cell (PMNC) separation was achieved by centrifugation on Ficol-Hypaque density gradients (Pharmacia, Piscataway, NJ)[23]. Unless otherwise specified, washed PMNC were cultured in triplicate at a concentration of 2 x 10^5 cells/well in U bottom 96 well microtitre plates (Corning Glass Works, Corning, NY), in 0.2 ml of RPMI 1640 supplemented with antibiotics and 10% human serum.

Lymphocyte responses to mitogens. To measure lymphocyte blastogenic responses, PMNC were cultured with either phytohemagglutinin (PHA) or concanavalin A (Con A) (Sigma Chemical Co.) at concentrations of 5 and 10 ug/ml, respectively. All cultures were maintained at 37^{o}C in a humidified atmosphere containing 5% Co_2. 18 hours prior to harvesting, cells were pulsed with 1 uCi of H^3 thymidine (New England Nuclear, Boston, MA) per well (specific activity of 6.7 mC/mol). Cells were then harvested onto glass fibre filters using a Bellco harvester (Bellco Glass Inc., Vineland, NJ). The incorporated radioactivity in counts per minute (cpm), was read in a Beta Scintillation Counter.

Lymphocyte responses to O. volvulus sAg and a non-related antigen. PMNC were cultured in the presence of O. volvulus sAg or purified protein derivative (PPD), each at a concentration of 20 ug/ml. These cultures were maintained for a total of 144 hours and harvested 18 hours after addition of 1 u Ci of H^3 thymidine.

Effects of O. volvulus adult worms S/E and mf S/E on lymphocyte blastogenic responses to mitogens. PMNC were cultured with either PHA or Con A, in the presence or absence of S/E from O. volvulus females, males or mf. Similar experiments were carried out in which O. volvulus S/E was replaced by either O. volvulus sAg or PPD. O. volvulus S/E, O. volvulus sAg and PPD were all used at concentrations of 20 ug/ml.

Serial dilutions of O. volvulus female S/E were also tested. Cultures were maintained for 72 hours, and harvested 18 hours after pulsing. The percentage inhibition caused by addition of O. volvulus derived materials was calculated using the following formula:

$$\text{Percent inhibition} = 1 - \frac{\text{cpm (mitogen + O.v. S/E) - cont. cpm}}{\text{cpm (mitogen alone) - cont. cpm}} \times 100$$

Effect of cocultivation of live skin and nodular O. volvulus mf on lymphocyte blastogenic responses to mitogens. PMNC were cultured with either PHA or Con A in the presence or absence of live mf. 30 skin mf/well and concentrations of 30, 50 and 120 nodular mf/well were tested. Cultures were harvested at 72 hours.

Effect of Interleukin-2 addition. To assess the effect of exogenous Interleukin-2 (IL-2) on the blastogenic response of PMNC cultured in the presence of mitogens and serial dilutions of O. volvulus S/E, IL-2 (Cellular Products Inc., Buffalo, NY) was added to the test cultures at a concentration of 20 units per ml.

Effects of patient sera on lymphocyte blastogenic responses to mitogens. PMNC obtained from a normal donor were cultured with 20% heat inactivated individual patient or normal control serum in the presence of either PHA, 5 ug/ml or Con A, 10 ug/ml. Cultures were harvested at 72 hours.

Gamma Interferon (IFN-γ) Production. The effect of O. volvulus female worms S/E and O. volvulus live mf on the production of IFN-γ, was measured by culturing 1 x 10^6 PMNC/ml with PHA in the presence or absence of *O. volvulus* female S/E, 20 ug/ml, or *O. volvulus* skin mf, 150 mf/ml. Cultures were incubated at 37°C for 24 hours and the resultant culture supernatants were stored at -196°C or -70°C. To some cultures IL-2 was added at the beginning of the incubation at a concentration of 20 units per ml. Concentrations of IFN-γ in the supernatants were measured in international units per ml (IU/ml) using an IMRX solid phase monoclonal antibody-based radio-immunoassay (Centocor Inc., Malvern, PA).

Statistical analysis. All statistical analyses were based on the unpaired Student's 't' test.

RESULTS

Clinical and Parasitological Results. The parasitological findings on the skin snip examinations of the four subgroups of *O. volvulus* infected individuals are shown in Table 1. Infection intensity was highest in Bo, Sierra Leone, whereas markedly lower numbers of parasites were evident in biopsies from patients from Abu-Hamad and Sundus, Sudan. Physical examinations revealed striking differences in disease manifestations, both between and within patient populations. In Sundus there was generally an inverse relationship between parasite intensity and the severity of clinical disease, but in Bo, Sierra Leone, there was a trend toward increasing severity with greater mf intensity (Table 1). The severely affected patients in Bo had the highest mf counts; in Sundus those severely affected had the lowest mf counts. None of the patients included in the study had a history of antifilarial chemotherapy; however, almost 50% of Bo patients had experienced surgical removal of onchocercal nodules within the past two years.

Overall, the clinical manifestations of onchocerciasis were milder in Bo, and only 14 (22%) of the patients fell into the categories of moderate to severe onchocerciasis (Figure 1), compared to 71 (65%) of the Sudanese (Figure 2). In Abu-Hamad, infection intensities were low, generally in the 1-2 mf/snip range, even though clinical manifestations were present and often severe. Four *O. volvulus*-infected individuals in Sudan had predominantly an asymmetric severe limb involvement (Figure 3), but none of the individuals seen in Sierra Leone showed this pattern. The overall clinical presentations and microfilarial

TABLE I

Clinical and parasitological status of *Onchocerca volvulus* infected subjects in four distinct foci endemic for onchocerciasis in Sudan and Sierra Leone.

Clinical Status of *O. volvulus* Infected Subjects	Score	BO, Sierra Leone (n=65)		Sundus, Sudan (n=58)		Abu-Hamad, Sudan (n=43)		Bahr Elghazal, Sudan (n=8)	
		No.	mf/mg	No.	mf/mg	No.	mf/mg	No.	mf/mg
No signs or symptoms	0	29	11.0	8	9.9	9	+[a]	0	—
Mild skin disease	1	22	4.6	13	3.4	6	+	0	—
Moderate skin disease	2	10	30.0	25	5.0	11	+	4	13.7
Severe skin disease	3	4	57.0	12	0.67	17	+	4	5.5

[a] In a previous survey of >200 patients[16] the average microfilarial intensity in Abu-Hamed was 2.7 mf/mg, and there was no evident relationship between intensity and disease severity.

Figure 1. An onchocerciasis patient from the Bo area. Note the mild fine papular erruption seen on the upper back. The clinical presentation of this patient is seen in the majority of Bo patients.


Bo56

Figure 2. Severe onchocercal dermatitis seen in a Sudanese patient. Note areas of hypo and hyperpigmentation, papular erruption and excoriation marks.

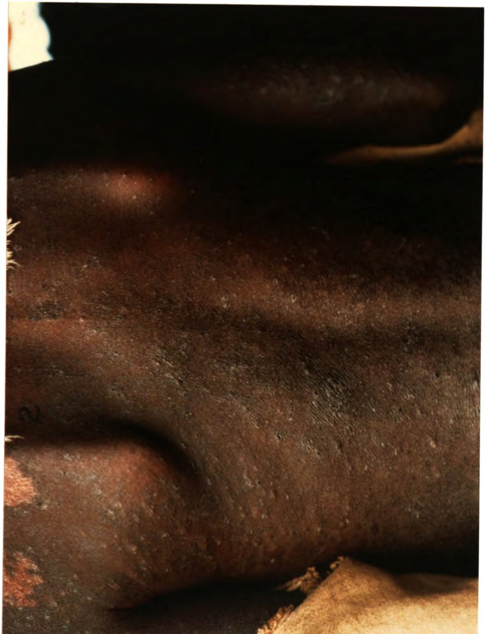


Figure 3. A Sudanese patient with localized limb involvement (Sowda).

3

intensities were not influenced by age or sex, however, in the Sundus group, younger patients had more pronounced acute changes[4].

No other filarial infections were diagnosed by examination of blood from patients in Bo, Sundus and Abu-Hamad, but one of the eight individuals from Bahr El Ghazal had Mansonella perstans. Malaria was most prevalent in patients from Sundus; despite being sampled in the dry season, 30% were blood film positive for either P. falciparum or P. vivax or both. In contrast, only one individual from Bo, Sierra Leone was positive for P. falciparum infection. Intestinal helminthiasis was prevalent among individuals examined in Bo, where 52% of onchocerciasis patients and 42% of the endemic area controls had intestinal nematode infection detected in a single fecal sample. Patients from Abu-Hamad were essentially free of parasitic infections other than O. volvulus. Endemic area controls were asymptomatic and had no demonstrable O. volvulus microfilariae in skin biopsies. However, they showed similar prevalence rates for other endemic parasitic infections (e.g. malaria, intestinal helminthiasis). Non-endemic area control subjects had no remarkable characteristics on physical examination, negative skin biopsies and were free of intercurrent parasitic infections.

Lymphocyte Subset Phenotypes. Peripheral lymphocyte subset phenotyping was done on 30 O. volvulus patients and 8 endemic area controls from Bo, Sierra Leone, and on 8 O. volvulus patients from Bahr El Ghazal and 17 non-endemic area controls from Khartoum, Sudan. The only significant differences seen (P = < 0.03) was an increase in natural killer (NK) cells in infected individuals from Sierra Leone when compared to endemic area controls (Table 2). The NK cells were not counted in samples from Sudanese subjects. No significant differ-

TABLE II

Peripheral blood lymphocyte subset distribution in onchocerciasis patients and normal controls from Bahr Elghazal, Sudan and Bo, Sierra Leone.

	B cells (CD19)	Total T cells (CD3)	Helper T cells (CD4)	Suppressor T cell (CD8)	CD4/CD8	NK cells (CD16)
Patients, Bo (n-30)	19.0 ± 8.4	61.5 ± 10.6	43.3 ± 9.2	31.1 ± 8.7	1.4 ± 0.2	18 ± 6.2[a]
Endemic area controls (n-8)	15.2 ± 2.3	64.8 ± 2.6	41.7 ± 2.7	28.2 ± 2.2	1.4 ± 0.15	12.1 ± 2.3
Patients, Bahr Elghazal (n-8)	27.5 ± 8.1	60.6 ± 10.6	43.7 ± 6.6	36.7 ± 11.8	1.33 ± 0.1	ND[b]
Normal controls (n-17)	23.9 ± 5.3	64.4 ± 10.3	43.6 ± 7.0	26.1 ± 4.5	1.73 ± 0.4	ND

[a]Statistically significant difference between Bo patients and controls ($p < 0.03$)
[b]NK cells analysis was not done (ND) in Bahr Elghazal patients.

ence in T-helper to T-suppressor ratio was seen between patients and controls and the absolute counts of both phenotypes were similar in the two groups.

Lymphocyte Responses to Mitogens. Lymphocytes from eight *O. volvulus* infected individuals from Bahr El Ghazal responded normally to PHA, Con A and PPD. There were no significant differences in lymphocyte blastogenic responses to PHA, Con A and PPD between the 15 non-endemic area controls and these *O. volvulus* infected individuals (Table 3). *O. volvulus* soluble antigen caused only slight stimulation of peripheral blood lymphocytes of *O. volvulus* infected subjects and controls; however, there was no significant difference in the blastogenic responses between the groups (Table 3). The *O. volvulus* infected patients showed infection intensities from 2.0 to 56.0 mf/mg; four had moderate (score of 2) and four had severe generalized (score of 3) dermal lesions. Although those eight *O. volvulus* infected subjects were originally from Bahr El Ghazal, they had been residing in Khartoum for at least one year. When similar experiments were carried out on twelve *O. volvulus* infected subjects from Sundus the results were different. These patients were generally in poorer physical condition than those from Bahr El Ghazal; they had been residing in a remote area and subsisting on an inadequate plane of nutrition. Their mf intensities ranged from 0.5 to 46 mf/mg, and the clinical scores assigned were as follows: seven had a score of 1; three had a score of 2; and two had a score of 3. These individuals showed much reduced responsiveness to PHA and PPD compared to controls, and they had the same very slight response to the presence of *O. volvulus* sAg as did patients from Bahr El Ghazal.

TABLE III

Blastogenic responses of peripheral blood lymphocytes of *O. volvulus* infected individuals and non-endemic area controls from Bahr Elghazal and Sundus, Sudan[a].

	Group 1		Group 2[c]	
Mitogen[b] or antigen	***O. volvulus* patients (Bahr Elghazal) n=8**	**Non-endemic area controls n=15**	***O. volvulus* patients (Sundus) n=12**	**Non-endemic area controls n=5**
PHA	146,215 ± 7,362	146,816 ± 16,050	9,918 ± 1,947	49,351 ± 18,300
Con A	93,645 ± 10,871	96,038 ± 9,538	ND[d]	ND
PPD	10,833 ± 4,977	9,703 ± 6,405	13,620 ± 13,398	33,510 ± 16,500
O.v. sAg	5,605 ± 1,361	4,438 ± 1,054	4,943 ± 9,103	3,165 ± 2,380

[a] Data is expressed as mean cpm ± SD.

[b] Different batches of mitogen and antigen were used for the 2 groups of patients and their respective controls.

[c] For Group 2, 5 x 10^{4} cells/well were used.

[d] ND Not done.

Effect of Patients' Sera on Lymphocyte Blastogenic Responses to Mitogens. Sera from 90 O. volvulus infected subjects and 30 endemic and non-endemic area controls were examined individually for their ability to modulate blastogenic responses of lymphocytes from a single donor to mitogens. Although sera differed slightly in their capacities to augment or reduce lymphocyte responses to PHA and Con A, this was not related to the presence, absence or intensity of O. volvulus infection, or to the severity of clinical manifestations of onchocerciasis (Table 4, Figure 4).

Sex, age and geographical location of the serum donors were not significant as variables influencing the effects of the sera in these experiments.

Effect of O. volvulus S/E on Blastogenic Responses of Lymphocytes from O. volvulus Infected Individuals and Normal Controls. Addition of pooled O. volvulus S/E to lymphocyte cultures virtually abrogated blastogenic responses to mitogens (Figure 5). Inhibition was no different with cells from either non-endemic area controls or O. volvulus-infected Sudanese patients. However, not all S/E preparations were equally effective, and when S/E from individual females were tested, some had no inhibitor effects. We examined S/E from a series of 83 O. volvulus females and 18 caused more than 50% inhibition at 20 ug/ml.

None of the 27 O. volvulus male S/E examined caused inhibition, nor did the addition of O. volvulus sAg or PPD influence blastogenic responses. O. volvulus mf S/E also caused inhibition of lymphocyte blastogenic responses to mitogens (Figure 6). At 20 ug/ml, skin mf S/E was more potent than nodular mf S/E. Co-cultivation of live O.

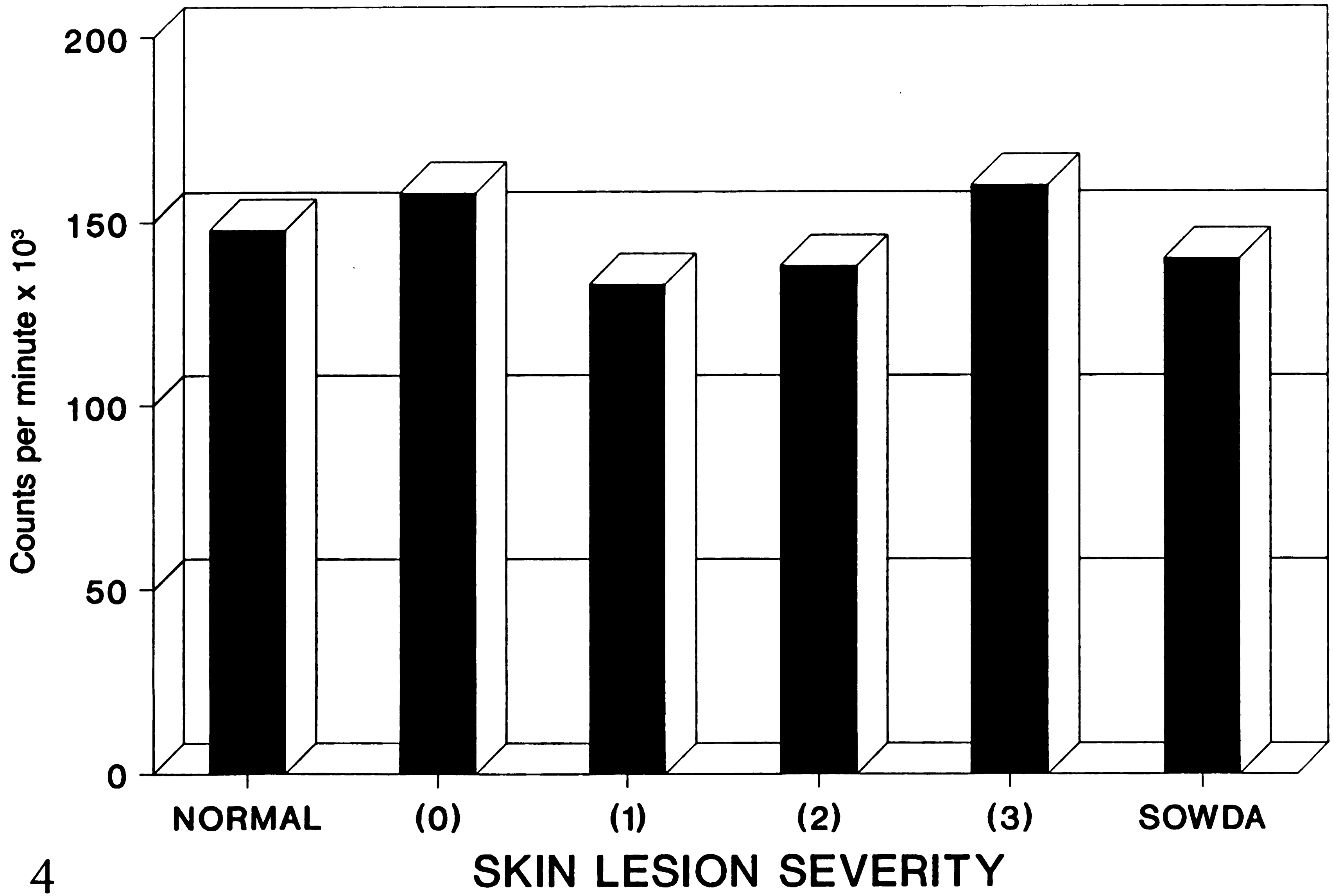
TABLE IV

Effects of sera from *O. volvulus*-infected individuals and controls from different geographical areas on lymphocyte blastogenic responses to phytohemagglutinin and concanavilin A.[a]

	Lymphocyte Blastogenic Responses to PHA		**Lymphocyte Blastogenic Responses to Con A**	
	In presence of sera from *O. volvulus* patients	**In presence of sera from controls**	**In presence of sera from *O. volvulus* patients**	**In presence of sera from controls**
Sundus	(n = 36) 142,963 ± 19,031	(n = 11) 147,726 ± 58,299	(n = 36) 91,296 ± 29,610	(n = 11) 74,053 ± 13,052
Abu-Hamad	(n = 42) 168,509 ± 18,770	(n = 12) 164,197 ± 6,988	(n = 42) 81,103 ± 33,554	(n = 12) 71,387 ± 20,921
Bo	(n = 12) 134,799 ± 14,272	(n = 7) 135,089 ± 18,089	(n = 12) 69,475 ± 20,248	(n = 7) 78,873 ± 36,407

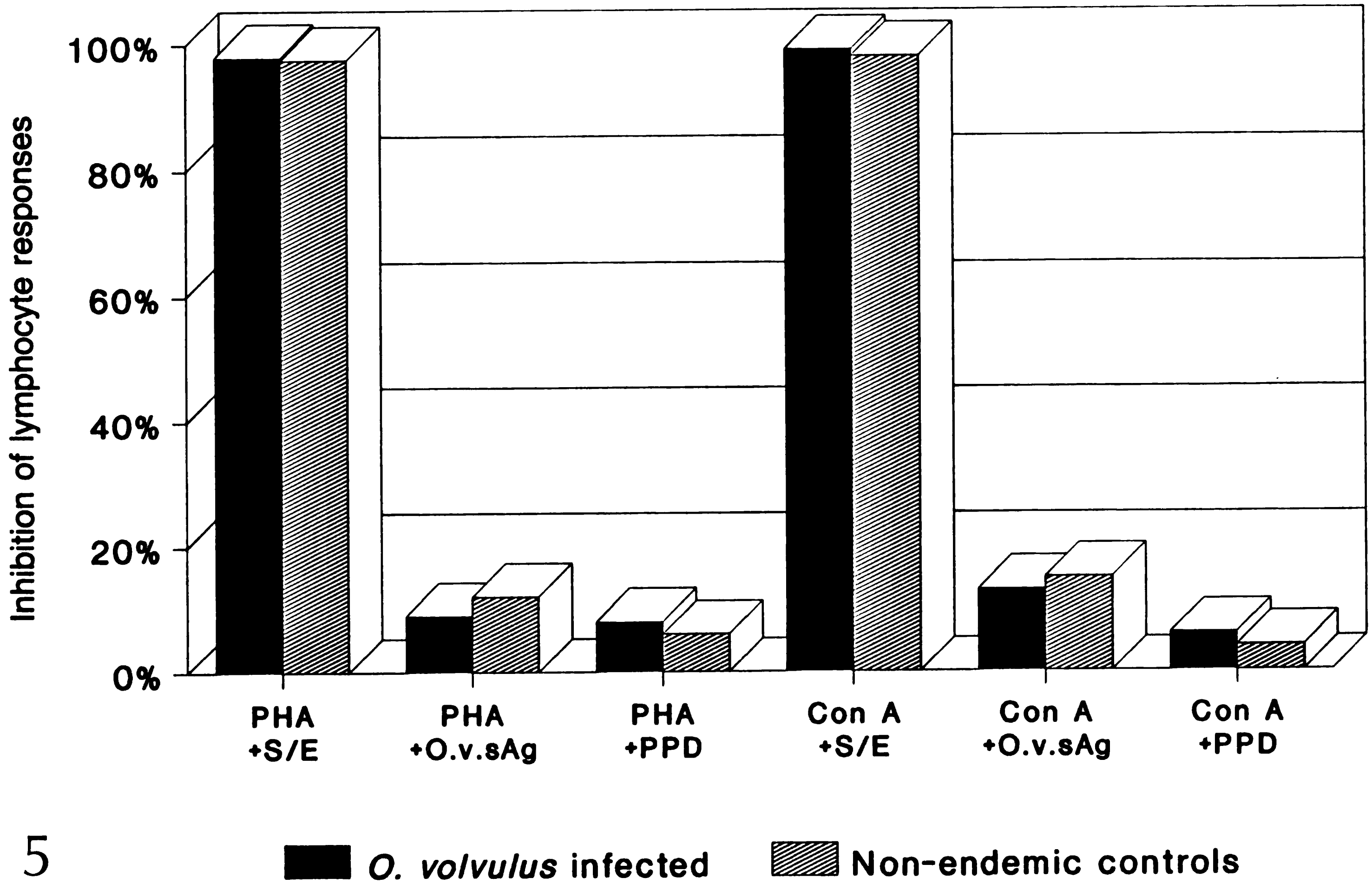
[a]Data represent mean cpm ± SD.

Figure 4. Effect of sera from Sudanese *O. volvulus* infected and non-endemic controls on lymphocyte blastogenic responses to mitogens.


200
150
100
50
0
Counts per minute x 10³
NORMAL
(0)
(1)
(2)
(3)
SOWDA
SKIN LESION SEVERITY

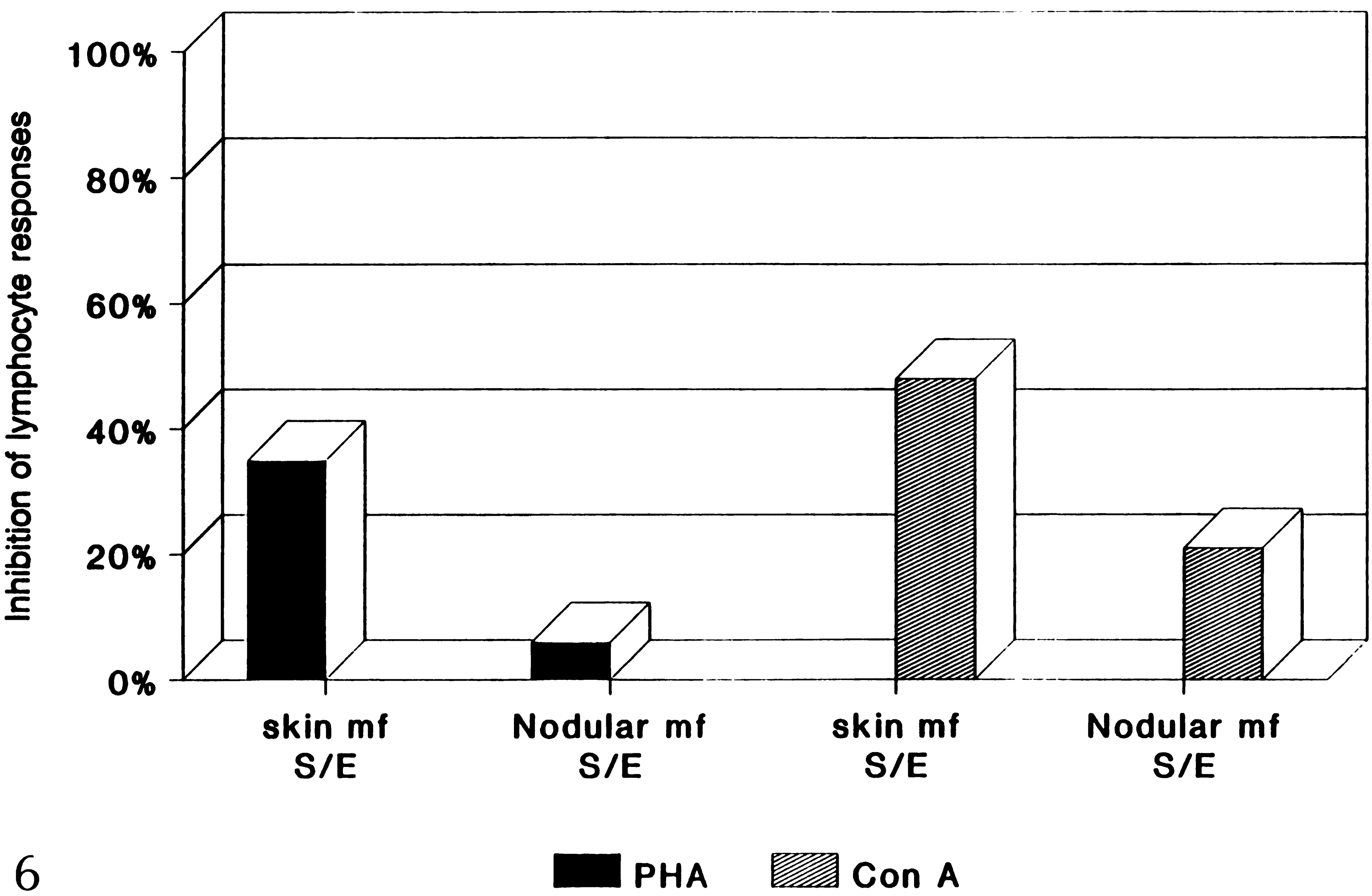
4

Figure 5. Inhibition of blastogenic responses of lymphocytes from *O. volvulus* infected subjects and non-endemic controls from Sudan in the presence of *O. volvulus* female Secretory/Excretory products. Concentrations of *O. volvulus* S/E, O.v. sAg and PPD were 20 ug/ml.


Inhibition of lymphocyte responses
100%
80%
60%
40%
20%
0%
PHA +S/E
PHA +O.v.sAg
PHA +PPD
Con A +S/E
Con A +O.v.sAg
Con A +PPD
O. volvulus infected
Non-endemic controls

5

Figure 6. Effect of *O. volvulus* microfilariae Secretory/Excretory products on lymphocyte blastogenic responses to mitogens.


Inhibition of lymphocyte responses
100%
80%
60%
40%
20%
0%
skin mf S/E
Nodular mf S/E
skin mf S/E
Nodular mf S/E
PHA
Con A

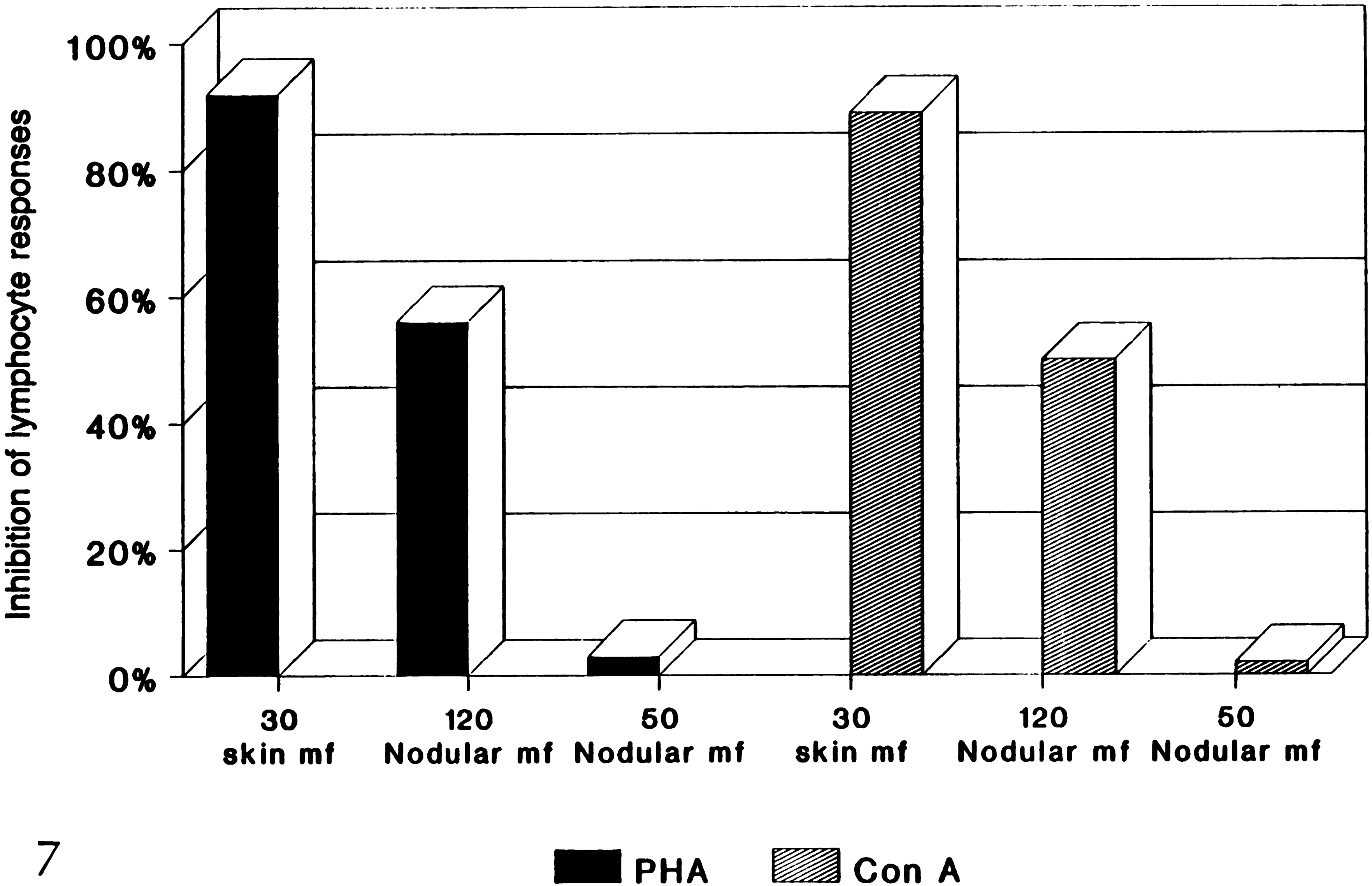
6

volvulus mf with PMNC, also caused inhibition of lymphocyte responses. When 30 skin mf were added to each well there was almost complete inhibition of PHA and Con A responses (Figure 7), but when the mf from nodules were used, higher numbers were needed to cause significant inhibition. Nodular mf at 50 mf per well had no effect. Inhibitory effects were not different when either O. volvulus infected individuals or non-endemic normal controls were the donors of PMNC.

Cell viability in the presence of all S/E preparations was maintained at greater than 90% for up to 6 days. The inhibitory effect of O. volvulus female S/E was concentration dependent, (Figure 8) declining to undetectable levels at 1.2 ug/ml. The activity of S/E from either source was not lost on dialysis (M.W. cut-off 10,000) and was stable on prolonged storage at -70^{o}C and on exposure to 56^{o}C for 30 minutes. Addition of exogenous IL-2 (20 iu/ml) partially overcame the O. volvulus S/E inhibitory effects at 20 ug/ml, while at lower S/E concentrations, the inhibitory effect was not detectable in the presence of IL-2 (Figure 8).

The amount of IFN-γ produced from mitogen stimulated cells was substantially reduced when PMNC were cultured with either O. volvulus female inhibitory S/E or O. volvulus skin mf. In the presence of 20 u/ml IL-2, IFN-γ production was improved, but not to control levels in either case (Figure 9).

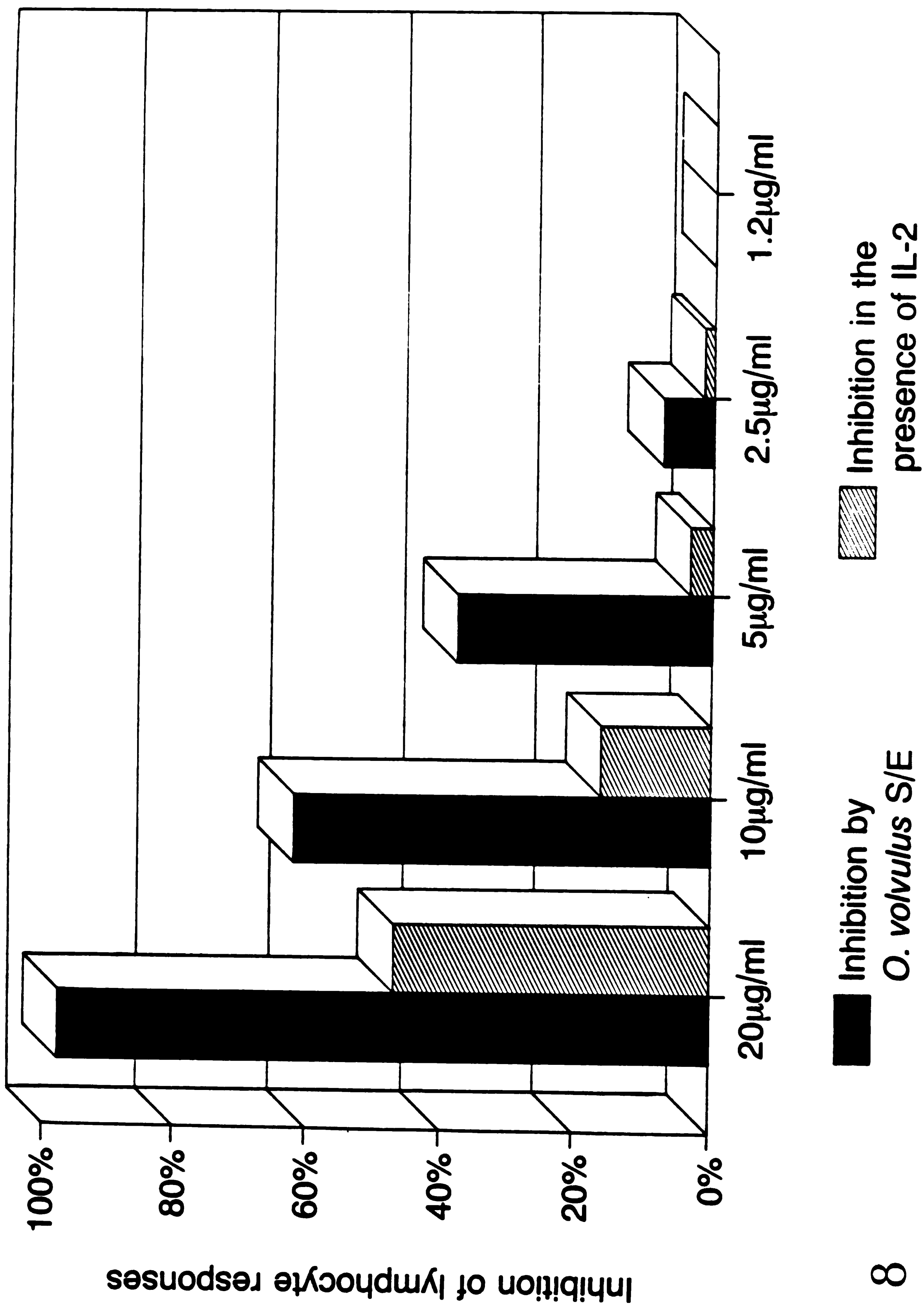
Figure 7. Effect of co-cultivation of O. volvulus microfilariae on Sudanese O. volvulus infected patients and controls lymphocyte blastogenic responses to mitogens.


Inhibition of lymphocyte responses
100%
80%
60%
40%
20%
0%
30
skin mf
120
Nodular mf
50
Nodular mf
30
skin mf
120
Nodular mf
50
Nodular mf
PHA
Con A

7

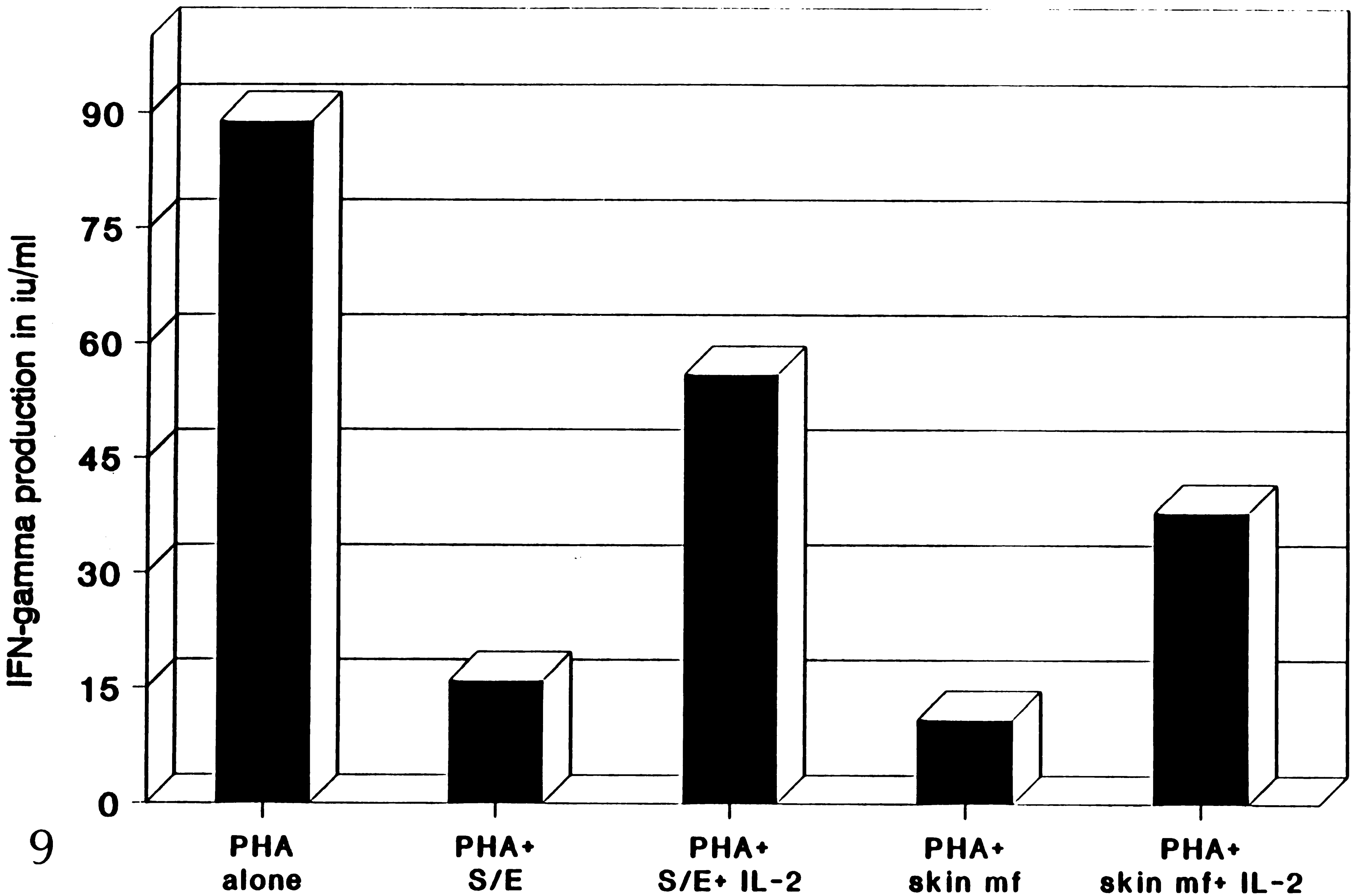
•

<u>Figure 8</u>. Effects of serial dilutions of *O. volvulus* female Secretory/Excretory products on lymphocyte blastogenic responses to mitogens. Addition of interleukin-2 at 20 U/ml partially overcame the inhibition.


Inhibition of lymphocyte responses
100%
80%
60%
40%
20%
0%
20µg/ml
10µg/ml
5µg/ml
2.5µg/ml
1.2µg/ml
Inhibition by O. volvulus S/E
Inhibition in the presence of IL-2

8

Figure 9. Effect of *O. volvulus* Secretory/Excretory products and skin microfilariae on the production of Interferon Gamma by mitogen stimulated lymphocytes.


IFN-gamma production in iu/ml
0
15
30
45
60
75
90
PHA alone
PHA+ S/E
PHA+ S/E+ IL-2
PHA+ skin mf
PHA+ skin mf+ IL-2

9

DISCUSSION

The relationships between the intensity of microfilarial infection and the extent of clinical manifestations in patients in our study groups illustrate very well the complexity of onchocerciasis. High numbers of live microfilariae in biopsy samples reflect high degrees of exposure, but they are not necessarily an indicator of the severity of pathological change. Of the subsets of patients we examined, those in Bo were the least afflicted clinically, even though their mf/mg counts were highest. Since the most disfiguring skin changes were in patients from Sundus whose skin snips yielded the lowest numbers of mf/mg, variables other than live parasite concentration in skin tissue must be important as determinants of infection outcome. Immunological responsiveness and its influence on the nature of host inflammatory reactions seems likely to be at least one contributing factor.

While our results have not identified any immunopathogenic processes that may account for the differences between patient groups, they do emphasize the heterogeneity that confounds extrapolation of findings from one endemic region to another, and from one subset of patients to another. For the most part patients in all clinical categories showed little evidence of a generalized immunological deficits, either in terms of disruption of peripheral lymphocyte phenotype populations, presence of circulating immune inhibitory or enhancing factors or deficient responsiveness to stimuli such as mitogens or PPD. The poor <u>in vitro</u> responses of lymphocytes from Sundus patients provide an exception to this finding. Unfortunately, because of the circumstances prevailing in the remote rural areas where most of these patients were examined, it was not possible to collect uniform sets of samples for a comprehensive comparison in all tests. Transportation conditions and patient cooperation, for example, were factors governing what could be collected for processing. The surprising difference we found between the mitogen and PPD responses in Sudanese patients from Bahr El Ghazal and those from Sundus illustrates how important this type of comparison can be.

Neither of those two groups, however, showed very remarkable responsiveness to O. volvulus soluble antigenic extract, and this observation is in accord with several recent reports from other groups [9,24,25]. The suggestion that O. volvulus specific cell-mediated mechanisms are subdued in this infection[9,11,24,26] is therefore corroborated, though again the relationship to clinical picture is not obvious. Patients from Sundus whose lymphocytes were examined in vitro were poorly nourished and not in good physical condition, yet, by virtue of their clinical signs and low parasite numbers, they might be interpreted as more reactive to the presence of microfilariae than other patient subsets. In contrast, Brattig et al.[27] recently reported that Sowda patients showed a high degree of immunologic responsiveness as evidenced by increased helper T to suppressor T-cell ratio, activated T-cells and increased antifilarial antibodies. They argued that these individuals, badly afflicted, but with low mf/mg counts, were indeed quite reactive to O. volvulus antigens. Obviously, further exploration of these relationships is necessary in our Sundus patients. Significant increases in responses to O. volvulus antigen and to PHA and PPD quickly followed ivermectin microfilaricidal treatment in these subjects (unpublished observations), again suggesting that parasite-mediated suppression may be operating in this infection.

A hypothesis to explain this phenomenon can be proposed based on our observation of the inhibitory effects of O. volvulus secretions/excretions on human lymphocyte reactivity towards mitogenic and antigenic (PPD) stimuli. The suppressive factors were not cytotoxic or dialyzable, suggesting that they may be macromolecular, rather than low molecular weight substances comparable to those described for O. gibsoni[28]. Since extracts of females were ineffective, the suppressor(s) may not be stored intact. S/E from males had no such effects, and females varied in their output, perhaps reflecting their reproductive status, known to be cyclical[29], and their output of microfilariae. Whether or not the suppressor substances in adult female O. volvulus are related to those released by microfilariae remains to be seen. The greater potency of skin microfilariae in causing inhibition compared to those derived from homogenized nodular and worm tissues, possibly results from the mixture of mf in the latter source, which probably contains many immature embryos. Microfilariae are known to acquire important biological traits in their migration to the skin [30], and potency in affecting local lymphocyte reactivity could be an additional characteristic. As few as 5 skin mf/well suppressed lymphocytes significantly in some cases (data not shown). Normal and patient lymphocytes were equally affected, so there does not seem to be a prerequisite for specific sensitization for this factor to be effective.

Microfilariae of Brugia malayi have been shown to produce a lymphocyte suppressive factor that inhibits proliferative responses and causes a significant decrease in IL-2 production[31]. Our results with O. volvulus are quite similar, because addition of exogenous IL-2 overcame suppression to mitogenic stimuli and partially restored IFN-γ production in our cultures. Depletion of adherent cells from PMNC (data not shown) did not alter significantly the suppressive effects of O. volvulus S/E on lymphocytes, suggesting that a direct effect on T-cells is involved. Because of the concentration of O. volvulus microfilariae in skin, their suppressive effects in vivo may be localized in that microenvironment, rather than systemically.

Our patients showed no obvious predisposition to intercurrent infection which might result from generalized immunological deficiency, and even though they were from areas renowned for leprosy, we did not see any obvious relationship between this infection and O. volvulus. Previous workers had reported a positive association, potentially attributable to defective immune responses[32]. Other reports on immunological suppression have provided conflicting sets of observations. Circulating non-specific suppressors were present in Cameroonian sera studied by Haque et al.[12], but were absent in plasma samples from Liberian patients examined by Greene et al.[9]. Potent inhibitors of lymphocyte proliferative responses were present in the majority of sera from Venezuelan Indians examined by Perez-Rojas et al. [13], but some of their sera contained substances that caused very significant enhancement of proliferative responses. These varied outcomes may be a function of infection intensity, parasite strain, exposure patterns or even of host capacity to neutralize parasite-derived inhibitory factors.

The latter might be an important element in the balance of host-parasite relationships. If neutralizing ability, whether humoral or cellular in origin, were an acquired trait, then hosts would vary in their ability to respond to the presence of microfilariae or even to attack and kill them. Neither of the two currently used microfilaricidal drugs in use for onchocerciasis, D.E.C. and ivermectin, has detrimental effects on the parasites *in vitro*. Yet, both result in microfilaricidal effects *in vivo*. Their modes of action are unknown, but if they were to disrupt microfilarial abilities to produce immune modulators, this could account for their chemotherapeutic efficacy *in vivo*.

Identification of determinants of host capacity to influence the effects or release of parasite suppressive substances can be proposed as an important goal in dissecting apart the dynamics of parasite survival or death *in vivo*. It may be a key element in the heterogeneity of host responses in this infection, contributing to the remarkable clinical spectrum. Immune responses in experimental filariasis have recently been shown to be influenced by maternal infection history [33], and prenatal and post-natal exposure to *O. volvulus* antigens are known to occur[34]. Such components of the host's immunological experiences with *O. volvulus* are likely to be valuable indicators of the prognosis and response to therapy in infected individuals.

ACKNOWLEDGEMENTS

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IMMUNOGLOBULIN RESPONSES IN ONCHOCERCIASIS

A COMPARATIVE STUDY OF IMMUNOBLOT PROFILES

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ABSTRACT

Immunoblotting analyses were done on 90 sera collected from onchocerciasis patients in several distinct endemic foci in the Sudan and in Sierra Leone. Human IgG and subclass responses were evaluated in relation to the clinical and parasitological profile of the patients and compared to endemic and non-endemic area controls. Patient lesion severity, type, and distribution were scored according to a previously reported system. Included were individuals ranging from heavily infected but asymptomatic, to those in whom microfilariae were few or undetectable but clinical signs were severe; those included seven with asymmetric lichenification. Total IgG and IgE concentrations were elevated in infected patients. Antibody responses to a wide range of soluble worm antigen bands (MW range 12 kD to >200 kD) were seen, with up to 25 bands recognized by some sera with IgG specific probes. Subclass specific monoclonal probes revealed a predominance of IgG4 responses, often accounting for almost all the band spectrum seen with IgG reagents. Many individuals had little or no evidence of reactivity in other subclasses. There was, however, no obvious relationship between band number, location or intensity, and clinical or parasitological status, except that there was overall more reactivity with Sudanese rather than Sierra Leonean sera. Within Sudanese sera subsets there was a trend towards higher clinical severity scores in patients whose sera recognized more and more deeply staining bands. Specific patterns of band recognition with IgG3 probes were not associated with Sowda patient sera, unlike previous reports. The most intense IgG3 reactivity was observed in an asymptomatic Sudanese patient. Anti IgE probes revealed fewer bands with lesser intensity, compared to anti

IgG, and they were also not peculiar to any patient subset. Antibodies were detected to up to 10 bands in secreted/excreted (S/E) antigens from adult females of _Onchocerca_ _volvulus_ maintained _in_ _vitro_, but these corresponded in position to bands in soluble worm extracts. Altogether 15 polypeptid S/E bands were labelled with ^{35}S methionine under these conditions, but the use of these materials does not seem to offer advantages in exploring antibody responses to _O._ _volvulus_ and their procurement is difficult and costly.

INTRODUCTION

Recent explorations of the relationships between the clinical manifestations of human filarial infections and patterns of immune responses have begun to identify potential correlates of pathogenesis [1]. In lymphatic filariasis, where categorisation of patient subsets has been based on well established parasitological and clinical criteria, distinct characteristics of immunoblot profiles have emerged in each group that provide insights into mechanisms of disease[2]. In human onchocerciasis immunoglobulin and antibody responses have not been as well examined so far, and the picture is less clear. This may be due in part to the spectral nature of pathologic responses and the complexities of evaluation of a wide range of changes in dermal and ocular tissues. It is also likely to be due in part to the geographic variation of the disease in endemic foci[3], perhaps attributable to recently identified genetically distinct "strains" within _O. volvulus_ [4]. Nevertheless, recent reports suggest associations between antibody responses in immunoglobulin subclasses and clinical signs that are of central importance to understanding the nature of onchocercal lesions, and the functions of immunoglobulin subclasses in disease causation[5].

We have been involved in the epidemiological characterization of onchocerciasis in a number of distinct foci in Sudan where, even within the country, the disease assumes very different forms in each of the provincial foci[6]. We have developed a comprehensive clinical scoring system based on the severity of acute and chronic changes that has proven valuable in classification of lesions and their changes over time in relation to parasitologic status[7]. We report here on immunoblot profiles in clinically distinct patient subgroups, from savannah and desert regions in Sudan, and some comparative aspects with regard to similarly examined samples from the forest foci of Sierra Leone[8].

MATERIALS AND METHODS

Study Areas. Patients from four different geographical areas endemic for onchocerciasis were studied. Three are in Sudan, and each shows some slightly different characteristics in the prevailing form of the disease: Abu-Hamad in the North[9]; Sundus in the East[7], and Bahr El Ghazal in the southwest[10]. An additional group of patients was examined and sampled in Bo, Sierra Leone, West Africa[8].

Individuals Studied. A total of 90 serum samples were collected from groups of 10-20 patients each in Abu Hamed, Bahr El Ghazal, Sundus, and Bo (Sierra Leone). The selection of patients for immunoblot studies was made to ensure representation of the range of prevailing clinical and parasitological features in each focus.

Control patients samples included those from individuals in the endemic areas who were parasitologically negative, and others from Central Sudan, who were resident in a non-endemic region.

Clinical Examination. All participants were given a complete physical examination with special attention given to the palpation of *O. volvulus* nodules, and to the evaluation of skin and eye changes consistent with *O. volvulus* infection. The history included details of any antifilarial treatment received and of surgical removal of nodules. Details of the examination procedure and the assessment of lesions are described elsewhere[7]. As a result of physical findings, individuals were classified primarily on the basis of the type, distribution, chronicity, and severity of skin changes, using a point scale of 0-3, with 3 being most affected. For the purposes of comparison 3 groups of symptomatic patients emerged from this scheme; those with mild disease (score of 1), moderately affected people (score of 2) and those severely afflicted (score of 3). Asymptomatic patients with no signs or symptoms of onchocerciasis, but with microfilariae in their tissue biopsies were given a score of 0.

Parasitological Examination. To determine the microfilarial intensity of *O. volvulus* infection in each individual, bilateral skin biopsies (snips) were taken from the iliac crest using a Walser corneoscleral punch. Skin snips were then incubated in tissue culture medium RPMI 1640 (Sigma Chemical Co., St. Louis, MO) for 4 hours. Emerging microfilariae were counted and the wet weight of the snips was determined. Microfilarial intensity was expressed as the number of microfilariae per milligram of skin (mf/mg). Urine, stool and blood samples from patients in Abu Hamad and Bo were examined for other parasites by conventional techniques.

Preparation of O. volvulus Soluble Antigen (sAg) and Secretions/Excretions (S/E). Intact nodules were surgically excised from patients under local anesthesia. To obtain live female parasites, nodules were digested in collagenase using the method described by Schulz-Key[11]. Trimmed excised nodules were incubated in culture medium RPMI 1640 containing 10% pooled normal human sera, penicillin, 100 u/ml, streptomycin 100 ug/ml (Gibco Laboratories, Grand Island, NY) and collagenase type IV (Sigma Chemical Co.) at a concentration of 2 mg/ml. Digestion was carried out at 37^{o}C for 12 hours after which female parasites were recovered by gentle dissection. Isolated adult females were thoroughly washed in serum-free RPMI 1640 with antibiotics.

O. volvulus female worms were maintained individually at 37^{o}C in RPMI 1640 supplemented with antibiotics for 48 hours. Culture supernatants were dialyzed at 4^{o}C against three changes of 1,000 volumes of phosphate buffered saline, pH 7.2 using Spectrapor membrane tubing (Spectrum Medical Industries Inc., Los Angeles, CA), with a molecular weight cut off of 10,000 Daltons. Supernatants were then filter-sterilized, aliquoted and stored at -70°C. The protein concentration in supernatants was measured using the Lowry method[12]. Additionally, to obtain radiolabeled S/E, live O. volvulus female worms were cultured in RPMI 1640 supplemented with antibiotics in the presence of S^{35} methionine (DuPont, Boston, MA), specific activity 1086 Ci/mmol, at a final concentration of 80 uCi/ml, for 24 hours. Proteins in the culture supernatants were then precipitated by 5% trichloroacetic acid (TCA).

For sAg preparation, isolated O. volvulus female worms were homogenized in phosphate buffered saline, pH 7.2 and extracted over-night at 4°C. Particulate material was pelleted by centrifugation at 5,000 x g/30 minutes and the supernatant was further clarified at 100,000 x g/1 hour.

Immunoblotting Procedure

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of O. volvulus sAg, and S/E was performed according to the method of Lammeli et al.[13], using a 7.5-15% gradient gel. The stacking gel was 3%. The separated proteins were electrophoretically transferred onto nitrocellulose sheets according to the method of Towbin et al.[14]. The portion of the nitrocellulose membrane containing molecular wt. standards and O. volvulus sAg was then stained with amido black. The rest of the membrane was cut longitudinally into 0.5 cm strips.

The strips were then reacted with individual patient's sera (90 sera for O. volvulus sAg and 10 Sera for S/E) diluted 1:200 following an overnight blocking with 1% BSA and 0.5% tween 20. After washing with Tris buffer, pH 7.2, the strips were incubated with anti-human IgG, IgG1, IgG2, IgG3, IgG4 or IgE mouse monoclonal antibodies (ICN Immunobiologicals, Lisle, IL, USA), diluted 1:2000. Following washing, the strips were incubated with horse radish peroxidase enzyme conjugated goat antimouse antibody at a dilution of 1:2000. These concentrations were arrived at by bracketing those recommended by the manufacturer in preliminary tests.

Additionally, peroxidase conjugated, affinity purified polyclonal, antihuman IgE (ICN Immunobiologicals) was used. The substrate solution consisted of diaminobenzidine (0.5 mg/ml) and hydrogen peroxide (o.15%) in phosphate buffered saline, pH 7.2. The reaction was stopped after 15 minutes by washing with cold water. Visualization of different protein bands and estimation of band intensity was determined visually.

Analysis of S^{35} Labeled O. volvulus S/E:

S^{35} methionine labeled S/E, precipitated by TCA were solubilized in SDS sample buffer and separated by SDS PAGE using the protocol described above. Autoradiographs of dried gels were exposed for 7 days.

Immunoglobulin Quantitation:

Total IgG, IgM, and IgA levels were determined in patient's sera using commercially available radial immunodiffusion plates (Kallestad, Austin, Texas). Total IgE levels were measured by an enzyme immunoassay (Abbot Laboratories, North Chicago, IL).

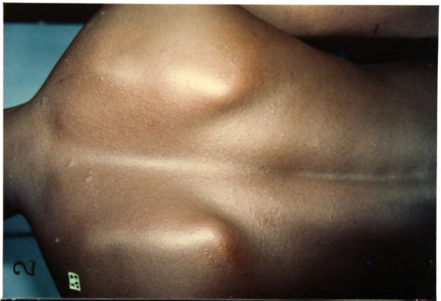
RESULTS

The parasitological and clinical features of patients in each region were markedly different. Details of the regional characteristics have been described elsewhere, (ElKhalifa et al., submitted) but the patient samples used in this study covered the range, in each area, from infected but clinically unaffected to severely afflicted with onchocercal lesions but with no demonstrable parasites in skin biopsies (Figs. 1-4). There were six affected with the localized asymmetric form of the disease in Sundus patient groups (Figs. 5-6), although this syndrome was seen in all Sudanese regions. Altogether 90 samples from infected individuals including 7 with Sowda syndrome, were examined in the immunoblot system.

Most patient sera showed high concentrations of total IgG, and extremely high IgE titers (Fig. 7). Separation of O. volvulus antigen extracts on SDS-PAGE produced a reproducible pattern of about 25 bands with molecular weights ranging from above 200 kD to 12 kD (Fig. 8 B1). The patterns of IgG responses of the patients varied within the subgroups, both with regard to the numbers and positions of bands recognized and their relative intensity. Sera from normal non-endemic area controls tended to show IgG reactivity with a band in the 50 kD region, and occasionally there were diffuse responses at higher MW levels which were poorly resolved. Reactivity to bands representing virtually the whole spectrum of Mw's visible on the SDS-PAGE profiles was present within each patient sample subgroup. Although, in some instances a trend towards increase IgG reactivity with increased clincal scores was noted in some groups, but for the most part this was lacking in other groups (Fig. 9b). Bands with MW lower than 50kd were consistently more

Figure 1. An asymptomatic O. volvulus infected individual with microfilarial intensity greater than 100 mf/mg. Note the presence of an onchocercal nodule while the adjacent skin is essentially free of any acute or chronic changes except for a single small papule.

Figure 2. Discrete papular eruption seen in this patient, confined to the upper back, giving him a clinical score of 1. The microfilarial intensity in the skin of this patient was 20 mf/mg.


2


1

<u>Figure 3</u>. Severe onchocercal dermatitis is seen in this patient (clinical score of 3). Note the macropapular eruption with lichenfication of the skin. Hypo and hyperpigmented, indurated papules are apparent. Some of these papules are excoriated, others are healing, while few have secondary bacterial infection.

<u>Figure 4</u>. Acute severe onchocercal dermatitis (score of 3) is seen in this patient mostly in the form of acute papular eruption and excoriation marks. Note areas of hypo and hyperpigmentation (left upper back).

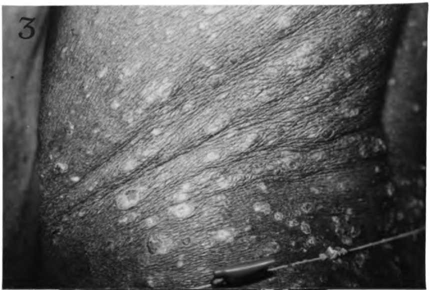

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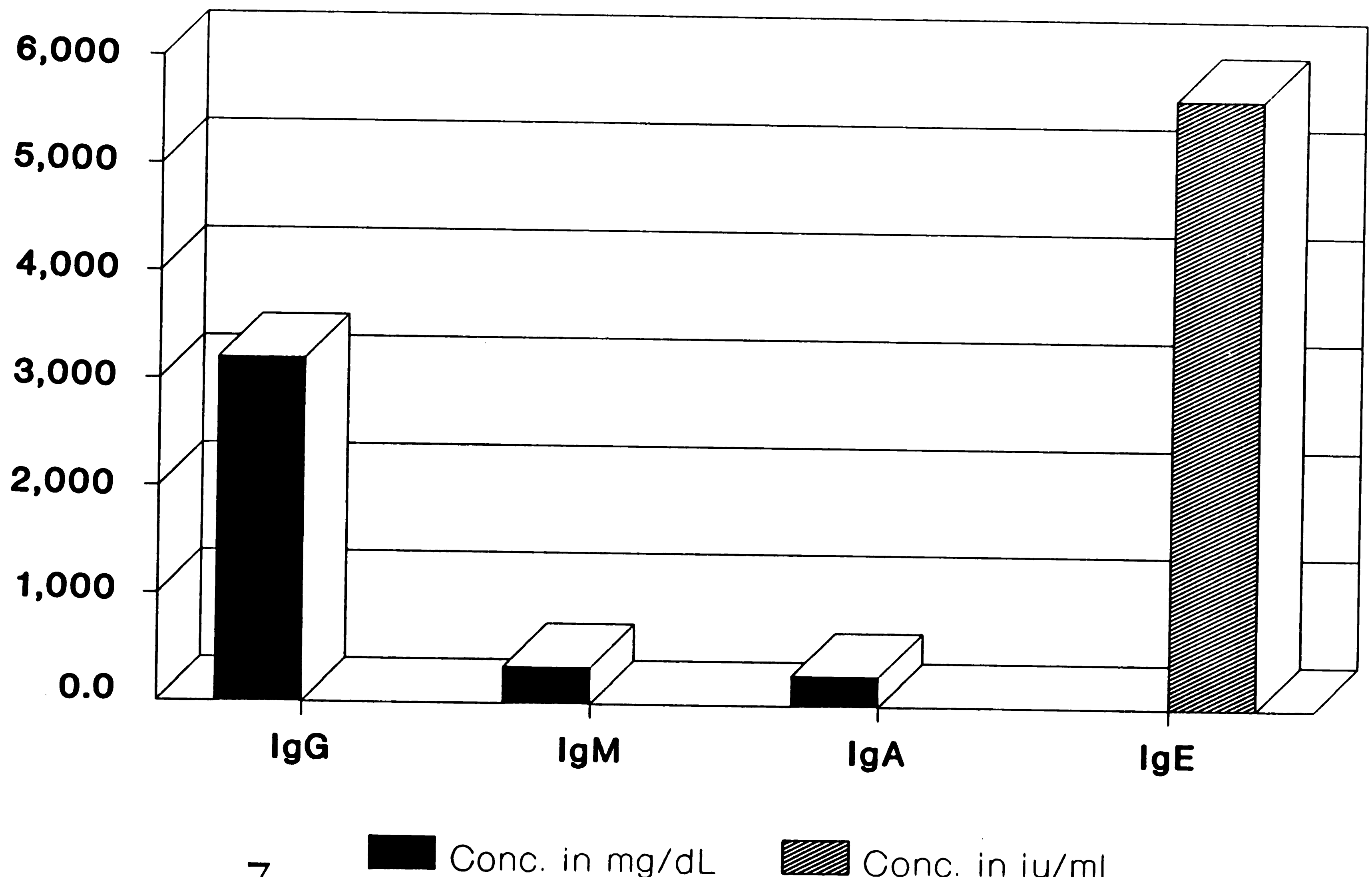
Figure 5. A "Sowda" patient with extensive dermatitis of the left leg while the right leg is spared. Note the hyperpigmented maculopapular eruption as well as the edema of skin in the left leg. It is worth noting that examination of this patient, one year after taking this photograph, showed remarkable improvement of the dermatitis in her afflicted limb, without any anti-onchocercal therapy.

Figure 6. Localized onchocercal dermatitis affecting the right arm of this patient. Note the maculopapular eruption as well as the thickening and hypertrophy of the skin. The features seen here fits the category of Sowda syndrome, despite the unusual upper arm location.


6


5

Figure 7. Total Immunoglobulin levels in onchocerciasis patients studied. Total level of serum IgG is elevated (mean 3202 $\pm$ 1288 mg/dL). More notable is the extreme elevation of IgE (5668 $\pm$ 5739 iu/ml). These high IgE levels are not associated with any intense recognition of *Onchocerca volvulus* soluble antigens by Western blotting technique, as shown in Figure 10.


6,000
5,000
4,000
3,000
2,000
1,000
0.0
IgG
IgM
IgA
IgE
Conc. in mg/dL
Conc. in iu/ml

7

Figure 8. SDS-PAGE of *O. volvulus* antigens and secretions/excretions.

A. An autoradiograph of SDS-PAGE, S^{35} methionine labeled, TCA precipitated secretions/excretions of *O. volvulus* female worms, showing at least 15 distinct protein bands with various molecular weights.

B.1 SDS-PAGE of adult *Onchocerca volvulus* PBS soluble extracts stained with Coumassie blue.

B.2 SDS-PAGE of adult *Onchocerca volvulus* secretions/excretions stained with Coumassie blue.

Molecular weight markers are seen on the right.

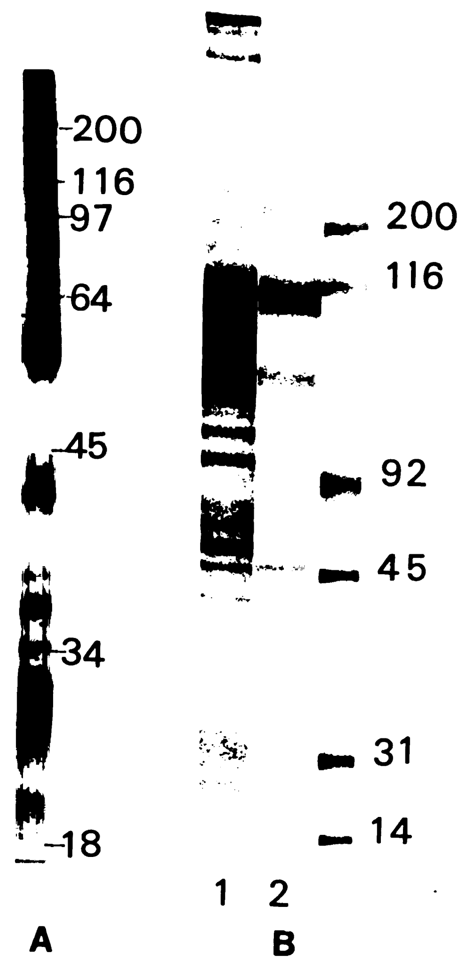


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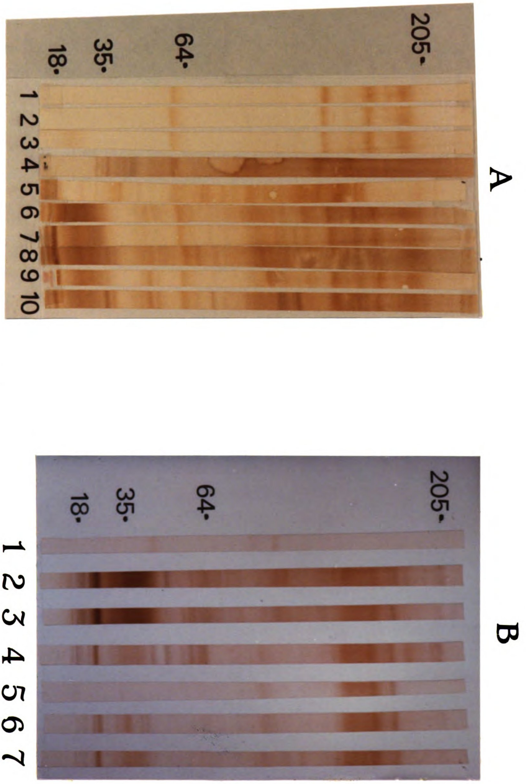
Figure 9. IgG recognition pattern of *O. volvulus* antigens separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose filters.

A. Sera from patients with different clinical presentations as well as controls were used. Lane 1, Non-endemic control; Lanes 2 and 3, endemic controls; Lane 4, asymptomatic; Lanes 5 and 6, mild disease (score of 1); Lane 7, moderate (score of 2); Lane 8, severe disease (score of 3); Lanes 9 and 10, Sowda patients. Note the increase in the number and intensity of the bands recognized with the increase in the severity of the disease. The endemic and non-endemic controls show non specific reactivity at the level of 50 KD as well as high MW bands.

B. IgG reactivity in another set of *O. volvulus* patients with a spectrum of clinical manifestations. Lane 1, normal individual; Lanes 2 and 3, patients with a score of 3; Lanes 4 and 5, patients with a score of 2; Lane 6, a patient with a score of 1; Lane 7, an asymptomatic patient.

It is apparent here that the IgG reactivity of those patients does not necessarily correlate with their clinical presentation.



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sharply separated. Most prominent among bands commonly recognized were those at MW 43 kD, 33 kD and 21 kD (Fig. 9a and b) where 80% of patients, but none of the endemic controls reacted to them.

Reactivities with anti IgE probes were generally more limited in scope and intensity (Fig 10). These reactions were not enhanced by altering the protocol to include a second (enzyme-labelled) antibody directed against the unlabelled class specific anti IgE probes. Generally less number of bands spanning the MW of 12-220 kD were detected, but with no obvious association with particular patient subgroups or their infection or disease status.

On further analysis of IgG responses with immunoglobulin subclass-specific probes the predominance of IgG4 in the antibody reactivity of sera from patients in all categories was most striking. In many cases bands recognized by IgG1, IgG2, and IgG3 were barely visible (Fig. 11), whereas reactions with the IgG4 probe covered the entire range of those seen with the IgG class-specific second antibody. Repeated absorption of patient's sera with IgG4 specific antibody failed to enhance reactions with other subclass specific probes (data not shown). The IgG4 responses, mirrored those of total IgG regardless of the clinical or prasitological status (Fig. 12), revealing the same marked heterogeneity as with IgG reactivity. No clear evidence emerged of a relationship between the degree of infection or clinical severity and blot profiles with any of the anti IgG reagents; however, there was an overall trend towards greater intensity in blots from Sudanese patients from all foci when compared to sera from forest zone patients in Sierra Leone. There was also a tendency towards more bands with a higher

Figure 10. IgE reactivities of the same group of patients in Figure 9A. Although some of those patients have total IgE levels in excess of 18,000 iu/ml, there is an apparent overall reduction in the number and intensity of bands recognized by those patients. Neither the clinical status, nor the level of parasite burdens were related to the recognition patterns.

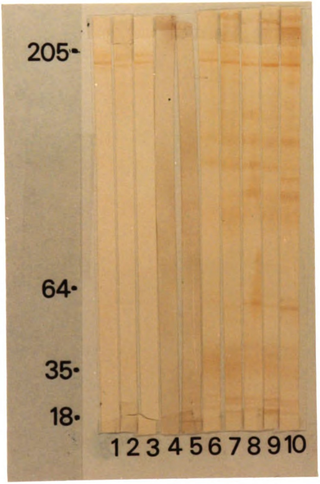

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Figure 11. *O. volvulus* antigen recognition patterns by different IgG subclasses. IgG1 (1); IgG2 (2); IgG3 (3); IgG4 (4).

A. IgG subclass recognition pattern by a serum from an asymptomatic infected patient.

B. IgG subclass recognition pattern by a Sowda patient.

Note that most of *O. volvulus* antigens are recognized by IgG4 with minimal contribution from the other IgG subclasses. No differences as to the recognition by IgG3 or any other IgG subclass can be identified in those two patients representing extremes of clinical presentations.

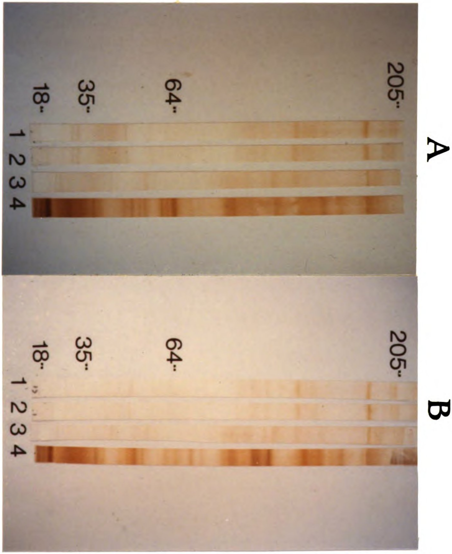

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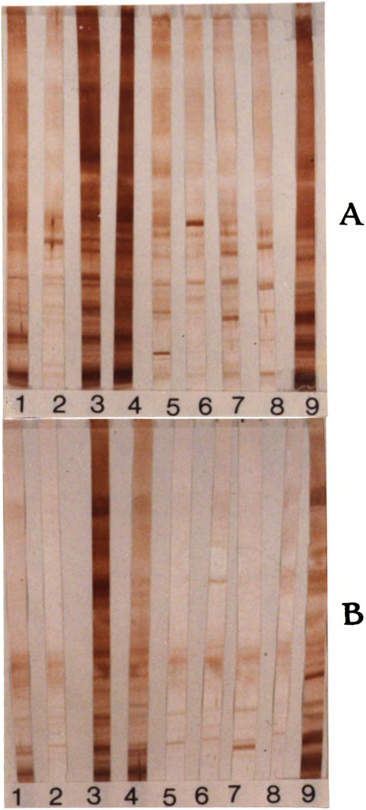
Figure 12. Total IgG and IgG4 recognition pattern of *O. volvulus* antigens.

A. Total IgG recognition pattern by a group of patients with different clinical manifestations.

B. IgG4 recognition by the same group of patients, reflecting almost identical pattern of the antigenic bands recognized. In most of our patients, the reactivities seen with total IgG can be attributed to IgG4.



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intensity of staining with sera of patients with moderate to severe disease rather than with sera from asymptomatic individuals.

Reactivity of IgG antibodies was pursued with particular attention to IgG3 responses in cases showing the asymmetry and lymphadenopathy associated with the Sowda syndrome. In several patients with severe Sowda or early Sowda-like afflictions blot patterns were not distinguishable from those of other patients in their respective subgroups (Fig. 11). Even where IgG3 positivity was present, reactions of the same sera with the anti IgG4 probe were much more intense and directed to a wider range of antigens. Several of the more pronounced IgG3 response patterns, especially to lower molecular weight bands, were seen with sera of patients who were asymptomatic.

SDS-PAGE of _O. volvulus_ S/E showed the presence of 10 protein bands with varying molecular weights (Fig. 8B2). All of the S/E protein bands had corresponding bands of the same MW in the _O. volvulus_ sAg SDS-PAGE preparation. Experiments with S^{35} labelled methionine incorporation demonstrated that up to 15 labelled polypeptides were produced by adult females maintained _in vitro_ (Fig 8A).

When 10 patient sera were tested against these S/E antigens, 12 bands were recognized. Again, different sera recognized different spectra of antigenic bands and there was no clear influence of the clinical presentation on the band recognition pattern. All of the protein bands appeared to be present in antigenic extracts of the entire worms, so that there did not appear to be any advantage to using the S/E fluids, especially since they were much more difficult to collect in quantity.

DISCUSSION

Patients infected with O. volvulus in endemic areas seem to react in widely disparate ways to the presence of the parasite. In the absence of accurate longitudinal assessment of clinical and pathological changes, it is difficult to construct a picture of the course of the disease. In our groups some individuals were both heavily infected and severely affected, whereas others were heavily infected and asymptomatic; yet others were clinically afflicted with many changes consistent with onchocerciasis, but without demonstrable parasites in their skin tissues. The relationship between infection intensity and disease was different between groups, with a positive correlation between these two factors established in the forest zone patients from Bo, and the reverse being true for the patients examined in Sundus[7]. In the face of this diversity, we were unable to identify any clear traits with respect to band specificity, scope of reactivity, or intensity that could be implicated in pathologic differentiations of subgroups. There was a tendency for those from the forest zone to recognize fewer bands and with less intensity than sera from Savannah regions in Sudan, but this was not marked. Previous workers have noted such differences between groups of patient samples collected from savanna and forest zones of W. Africa[3].

Brattig, et al.[1] also examined patients with the asymmetrically localized hyper-reactive form of the disease, referred to loosely as "Sowda" syndrome. They reported that such individuals have higher titers of IgG antibodies in Elisa tests. Our qualitative immunoblot profiles, although showing a trend towards greater IgG reactivity of symptomatic patients' sera, did not reflect this characteristic consistently. On occasion, strong IgG responses similar to those described by Lucius[31] were noted by asymptomatic patients. IgG profiles of Sowda patients and others were marked by one extraordinarily consistent trait: the large contribution of antibodies of the IgG4 subclass to the reactivity seen. In many individuals it appeared that most of the antibodies they had made, regardless of their clinical or parasitological status were of this type. Observations such as these have been made recently in other helminthiases in man,[27,23], and IgG4 responses form one of the hallmarks of antibodies detected in human lymphatic filariasis[2]. There, the hypothesis has been constructed that IgG4 may function in a protective or blocking role, ameliorating reactions potentially generated by the high levels of IgE antibodies to Brugia, or Wuchereria antigen in these patients[2,15]. Concentrations of IgE are elevated in human onchocerciasis,[16,22] but the blot profiles in our studies showed a rather restricted pattern of reactivities compared to those seen in lymphatic filarial infections[17]. The parallel antigenic recognition by IgE and IgG4 reported in lymphatic filariasis[20] was not observed in our study. Moreover, the IgE reactions in our patient groups showed no discernable trends related to clinical signs or parasite burdens. The relatively low number of bands detected in our IgE specific blots, regardless of whether or not direct enzyme-labelled probes were used or an IgE specific enzyme-labelled second antibody was involved, contrasts sharply with the results reported by Weiss et al.[18]. They found up to 50 bands detectable with anti IgE in their onchocerciasis serum pools from sub-Saharan regions of W. Africa. It should be mentioned here that the authors' experimental protocol involved high concentrations of patients' sera (sera were diluted 1:2), while we used sera diluted 1:200. It is possible that the majority of parasite specific IgE are present, but at low concentrations and with high dilution they are essentially titrated out. Our results, however, reflect similar findings to those of Nutman et al.[30], where high levels of non-specific IgE were prevalent among their loiasis patients population.

We were unable to demonstrate any association between IgG3 reactivity to any bands and the presence of Sowda-like lesions. Recently Cabrera et al.[5] have implicated IgG3 in the pathogenesis of this syndrome, showing that high levels of IgG3 antibodies were exclusively associated with Sowda patients and were directed preferentially against a 72 kD MW antigen and a low MW antigen in the 9 kD region, the latter being most specific. In our immunoblotting system, the lowest identifiable Mw O. volvulus antigenic band was in the 12 KD region, similar to other published reports[31]. Thus, we are unable to comment on the recognition of the 9 KD band and its relation to Sowda. This discrepancy might be due to our use of PBS soluble worm antigenic preparations rather than the detergent solubilized extracts used by the above authors. As for the 72 KD band, sera from Sowda patients in our study were not differentiable by this means. In the Cabrera et al. report, antibody recognition profiles by Sowda patients' serum pool, both the 9KD and the 72 KD bands were strongly recognized by IgG3, but neither band was recognized in the total IgG blots on the same patients' serum; no explanation for this difference was offered. In our series, IgG3 antibody reactivities were weak and when present they were seen equally in individuals with no dermal clinical changes suggestive of onchocerciasis. The reasons for these differences remain to be established, but for the moment generalizations about the involvement of IgG3 responses in this syndrome do not seem justified. Parasite genetic factors, patterns of exposure and host genetic traits may all play a role in these phenomena.

Some of our patients were infected with Mansonella perstans, and those in W. Africa had a higher rate of infection with gastrointestinal helminths than did the Sudanese groups, but these intercurrent parasitic problems did not detectably influence the antibody profile. Possibly some of the antibodies demonstrated in blots may have been against antigens shared with gastrointestinal nematodes because filariae are known to have many antigens in common with major human helminths[19].

Interestingly, some of the most consistent and best resolved areas of reactivity on blots were in the lower molecular weight regions where antigens of greatest O. volvulus specificity have been identified[20,21]. Of special interest to us were the 33 KD and 21 KD antigenic bands, the recognition of which has recently been shown to be of greatest specificity and sensitivity (>90%) in the diagnosis of O. volvulus infection[21]. Although these two bands were recognized by the majority of our patients and by none of the endemic controls, their utility as a specific diagnostic test for O. volvulus infection in our patients series is not evident because of the lower sensitivity (80%) of detection by this means. The prospects for discriminating between subsets of patients with specific diagnostic assay configurations seem dim in the face of the heterogeneity displayed here. Further progress on this and other aspects of the immunology of the host-parasite relationships in this infection probably will require a focus on purified or rDNA-derived defined antigens for more meaningful interpretation.

Our analysis of S/E showed that the antigenic bands seen are similar to those present in O. volvulus soluble antigenic extracts, at least in terms of their molecular weights. These antigens are clearly synthesized by the worm as shown by the ^{35}S methionine incorporation experiments. Although there is evidence from other helminths of advantages in using secreted or excreted antigens in serological studies[24,25,26], this was not apparent in our limited analysis. In view of the extreme difficulty of procuring live worms for this purpose and the scanty harvest of material for study it does not appear to offer worthwhile advantages.

ACKNOWLEDGEMENTS

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APPENDIX 1

CLINICAL EVALUATION OF ONCHOCERCIASIS PATIENTS

Clinical evaluation of patients included detailed past and present medical history. Special attention was paid to past history of skin problems. History related to previous diagnosis of onchocerciasis and whether treatment was given was also inquired about. Occupation, life style and possible exposure to black fly bites was also investigated.

General physical examination was then performed on all participants. This included examination of head and neck, cardiovascular and respiratory systems, abdomen and limbs. The presence or absence of onchocercal nodules was determined by visual inspection and palpation. Enlargement of superficial lymph nodes was also noted. Special attention was given to examination of the skin and the eyes.

Assessment of Skin Lesions:

The skin of all individuals studied was carefully examined for visible changes and a detailed record of results was kept. Skin changes were divided into acute and chronic lesions:

a. Acute skin changes:

   1. Pruritus and itching marks
   2. Papules, either discrete or containing microabscess
   3. Skin edema

b. Chronic skin changes:

   1. indurated papules which may be hyper or hypopigmented.
   2. Hyperkeratosis and lichenification of skin
   3. loss of turgor (atrophy)
   4. Confluent depigmentation.

Each acute and chronic change was quantitated using a scoring system. The body is divided into head and neck, arms, torso, pelvic girdle and legs. The absence of acute changes was given a score of 0. Presence of an acute lesion in one or two body areas was given a 1. The presence of a lesion in more than two was given a score of 2. Total scores for acute changes, chronic changes and the overall combined score for skin lesion severity can then be quantitated. Accordingly, 4 groups of patients can be identified. These are:

a. Asymptomatic patients
b. Patients with mild skin disease
c. Patients with moderate skin disease
d. Patients with severe skin disease

Ocular Changes:

Ocular examination included assessment of visual acuity using an illiterate E. chart. Anterior segment changes i.e. punctate keratitis, uveitis, iritis, and sclerosing keratitis were looked for and in some patients a slit lamp examination was performed. Posterior segment changes (chorioretinal and optic nerve changes) were determined using an ophthalmoscope.

<u>Figure 1</u>. An asymptomatic patient with an onchocercal nodule in the pelvic region.

<u>Figure 2</u>. A patient with localized severe onchocercal dermatitis and femoral lymphadenopathy. This patient fits the classical description of Sowda.


2


1

Figure 3. Severe acute onchocercal dermatitis with papular erruption, excoriation marks and pigmentory changes (score of 3).


3

Figure 4. Discrete papular erruption localized to the upper back (score of 1).

Figure 5. Moderate acute changes (score of 2). Excoriated papules, and early pigmentory changes can be seen.

5

4

Figure 6. Another Sowda patient in which the left limb is severely afflicted.

Figure 7. A patient with generalized severe onchocercal dermatitis. Note the indurated hypo and hyper pigmented papules. Some of the skin changes seen on this patient are similar to those seen in the Sowda patient in Figure 6.

Figure 8. Marked atrophy of the skin, a consequence of chronic onchocerciasis.

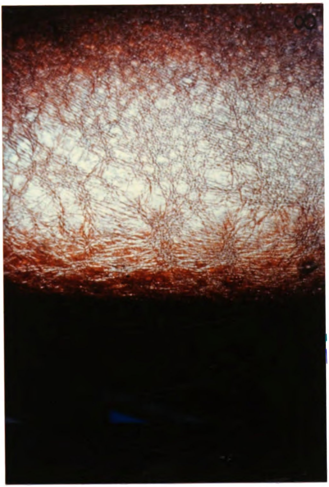

Figure 9. Severe secondary bacterial infection in a young boy with *O. volvulus* infection. This is not an uncommon consequence of pruritus, itching, and excoriation.

Figure 10. Classical depigmentory changes seen in both lower legs of this patient.


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Figure 11. Choreoretinal atrophy with focal pigmentation. These eye changes are not uncommon in onchocerciasis.

Figure 12. Optic nerve atrophy in an onchocerciasis patient.


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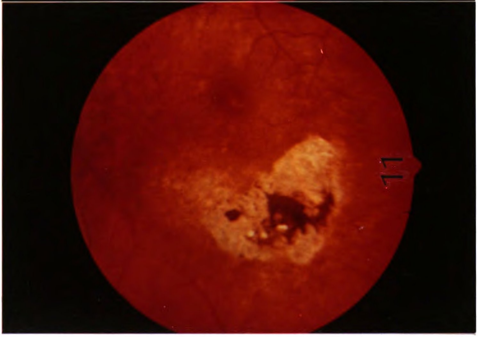
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APPENDIX 2

PATIENTS' DATA RECORD FORM

MICHIGAN STATE UNIVERSITY MEDICAL PARASITOLOGY RESEARCH PROJECT

NAME: ______________________ DATE: __________ NO. __________

AGE: ______ SEX: M ☐ F ☐ OCCUPATION: ______________________

PRESENT RESIDENCE: ______________________

PREVIOUS RESIDENCE: ______________________

HISTORY OF TRAVEL? Y ☐ N ☐ WHERE? ______________ WHEN? __________

COMPLAINTS: SCRATCHING Y ☐ N ☐ OTHERS Y ☐ N ☐ SPECIFY: __________

FOR HOW LONG? __________ ______________________

ANY SEASONAL VARIATIONS? ALL YEAR AROUND Y ☐ N ☐ SUMMER Y ☐ N ☐

WINTER ☐ ☐

IS IT RELATED TO BLACK FLY BITE? Y ☐ N ☐

DOES SCRATCHING INCREASE BY SWEATING? Y ☐ N ☐

DOES SCRATCHING PREVENT YOU FROM SLEEPING? Y ☐ N ☐

SITE OF SCRATCHING: HEAD ☐ NECK ☐ UPPER LIMBS ☐

THORAX ☐ ABDOMEN ☐ BACK ☐

BUTTOCKS ☐ GROIN ☐ HIPS ☐

HANDS ☐ FEET ☐ LEGS ☐

DID YOU NOTICE ANY NODULE OR SWELLING ON YOUR BODY? Y ☐ N ☐

WHERE? ______________________

ANY FAMILY MEMBER WITH THE SAME CONDITION? Y ☐ N ☐

NO. ______________

HAS YOUR VISION CHANGED SINCE YOU EXPERIENCED THIS CONDITION? Y ☐ N ☐

HAVE YOU BEEN DIAGNOSED AS HAVING FILARIASIS? Y ☐ N ☐

HAVE YOU HAD TREATMENT FOR IT? Y ☐ N ☐

IF YES, WHAT DRUG? SURAMIN Y ☐ N ☐ HOW MANY INJECTIONS? ______ WHEN? ________

DEC Y ☐ N ☐ HOW MANT TABLETS? ______ WHEN? ________

OTHERS Y ☐ N ☐ SPECIFY: ______________________________

DID YOU DEVELOP ANY HYPERSENSITIVITY REACTION? Y ☐ N ☐

TO WHAT EXTENT? ____

ANY ACCOMPANYING IMPAIRMENT OF VISION? Y ☐ N ☐

HAVE YOU BEEN DIAGNOSED AS HAVING SCHISTOSOMIASIS? Y ☐ N ☐ WHEN? __________

DID YOU RECEIVE TREATMENT? Y ☐ N ☐ WHEN? ______________________

IS THE COLOR OF YOUR URINE ABNORMAL? Y ☐ N ☐ IF YES, SPECIFY: __________

IS YOUR STOOL NORMAL? Y ☐ N ☐ IF YES, IS THERE: DIARRHEA ☐ MUCUS ☐ BLOOD ☐

OTHERS ☐ SPECIFY: ________

HAVE YOU HAD MALARIA? Y ☐ N ☐

PHYSICAL EXAMINATION:

PULSE: ________ B.P.: ________

PALE: ☐ ☐ CYANOSED: ☐ ☐

STATURE: ANOREXIC ☐ UNDERWEIGHT ☐ WD/WN ☐ OVERWEIGHT ☐ OBESE ☐

NORMAL ☐ COMMENTS ON ABNORMALITY: ______________________

NO. ____________

NECK:

THYROID: NORMAL ☐ COMMENTS ON ABNORMALITY: ____________

CHEST: NORMAL ☐ COMMENTS ON ABNORMALITY: ____________

HEART: NORMAL ☐ COMMENTS ON ABNORMALITY: ____________

ARTERIES: NORMAL ☐ COMMENTS ON ABNORMALITY: ____________

VEINS: NORMAL ☐ COMMENTS ON ABNORMALITY: ____________

ABDOMEN: NORMAL ☐ COMMENTS ON ABNORMALITY: ____________

MUS-SKEL: NORMAL ☐ COMMENTS ON ABNORMALITY: ____________

NAILS:

COLOR: PALE ☐ PINK ☐ CYANOTIC ☐ OTHER ☐ ____________

SHAPE/LESIONS: WNL ☐ CLUBBING ☐ OTHER ☐ ____________

LYMPH NODES:

CERVICAL: NORMAL ☐ COMMENTS ON ABNORMALITY: ____________

R. AXILLARY: NORMAL ☐ COMMENTS ON ABNORMALITY: ____________

L. AXILLARY: NORMAL ☐ COMMENTS ON ABNORMALITY: ____________

R. INGUINAL: NORMAL ☐ COMMENTS ON ABNORMALITY: ____________

L. INGUINAL: NORMAL ☐ COMMENTS ON ABNORMALITY: ____________

R. POPLITEAL: NORMAL ☐ COMMENTS ON ABNORMALITY: ____________

L. POPLITEAL: NORMAL ☐ COMMENTS ON ABNORMALITY: ____________

GROIN EXAM NORMAL ☐ COMMENTS ON ABNORMALITY: ____________

NO. ________

SKIN LESIONS / BODY PARTS	ATROPHY	PAPULES	ACUTE EXCORIATION	CHRONIC EXCORIATION	DEPIGMENTATION	HYPERPIGMENTATION	HYPERKERATOSIS	LICHEN	VESICLES	ULCER	EDEMA	KELOID	SCAR	TURGOR	APPEARANCE	OTHER
HEAD																
NECK																
RIGHT ARM																
LEFT ARM																
RIGHT FOREARM																
LEFT FOREARM																
CHEST																
ABD																
UPPER BACK																
LOWER BACK																
RIGHT BUTTOCKS																
LEFT BUTTOCKS																
GROIN																
RIGHT THIGH																
LEFT THIGH																
RIGHT LEG																
LEFT LEG																
HAND																
FEET																

NO. ___________

SCORING:

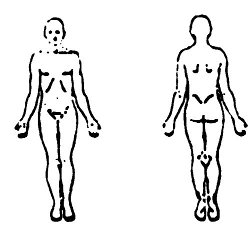
ACUTE		CHRONIC	
P Rx		CP	
PPRx		ATR	
PRU		HYK	
OED		PC	
			TOTALS

PROVISIONAL DIAGNOSIS?

NODULES, NUMBERS

NONE	HEAD	NECK	SHOULDER L	SHOULDER R	TRUNK ANT	TRUNK POST	HIP L	HIP R	COCCYX
☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

ARM R	ARM L	LEG R	LEG L	OTHER
☐	☐	☐	☐	☐



OPHTHALMOLOGICAL EXAMINATION:

VISUAL ACUITY CODE:

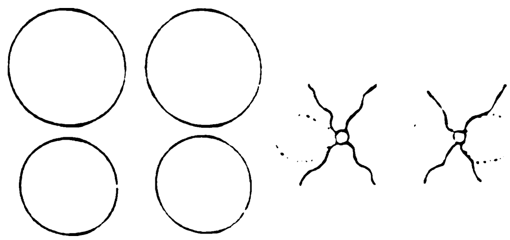
0	1	2	3	4	5	6	7	8	9
6/6	6/9	6/18	6/36	6/60	COUNT FINGERS AT 3 METERS	PERCEPTION OF LIGHT OR HAND MOVEMENT	NO PERCEP. OF LIGHT	UNABLE TO UNDERSTAND TEST, UNCOOPERATIVE	NOT DONE

	RIGHT	LEFT
VISUAL ACUITY:	0 1 2 3 4 5 6 7 8 9	0 1 2 3 4 5 6 7 8 9

OCULAR	R	L		R	L		R	L		R	L
Mf in AC:						Pupil:			Opt. disc:		
Live mf:			Trach:			Iritis:			Ret. ves:		
Dead mf:						Pig. disp:			Pig. epith:		
Coin les:						Lens:			C.r.a.:		
Fl. op cor:						Vitreous:			Ret. dot:		
Scl. ker:			IOP:								

NO. ____________

R L R L



BLINDNESS:

CAUSE:

LOCATION:

PHOTO:

VISUAL ACUITY:

LABORATORY RESULTS:

SKIN SNIP: SITE	RIGHT NUMBER	RIGHT WEIGHT	LEFT NUMBER	LEFT WEIGHT
1				
2				
3				
4				

SKIN SCRAPING:

RESULTS: ____

SKIN BIOPSY: TAKEN ☐ NOT TAKEN ☐ FROM WHERE: ____________

BLOOD:

MF. PRESENT	NO. ESTIMATE			
ONCHO. V.	3	2	1	0
WUCH. B.	3	2	1	0
LOA LOA	3	2	1	0
TET. P.	3	2	1	0
OTHER	3	2	1	0

DIFFERENTIAL

L	M	EO	N	B
___	___	___	___	___

WBC ____________ RBC ____________

PCB ____________

HB ____________

NO. __________

SERUM:

TOTAL PROTEIN	__________	VITA	__________
IGE	__________	ELISA	__________
IGA	__________	I/C	__________
IGM	__________	ADHER. AB.	__________
IGG	__________	MALARIA AB.	__________
IGD	__________	OTHER	__________

BLOOD TRANSFORMATION ASSAY USING PATIENT'S SERUM:

INHIBITION __________ ACTIVATION __________ NO EFFECT __________

IMMUNOBLOTTING RESULTS: ______________________________

____

____

LYMPHOCYTE STIMULATION ASSAY:

USING FILARIAL AGS: ______________________________

USING PHYTOHAEMOAGGLUTININ ☐ CON A ☐ OTHERS: __________

CELL TYPING OF PERIPHERAL BLOOD LYMPHOCYTES:

T - HELPER:

T - SUPPRESSOR:

B - CELL:

TOTAL LYMPHOCYTES:

RATIO OF T-HELPER TO T-SUPPRESSOR:

COMMENTS: ______________________________

____

CELL TYPING OF SKIN BIOPSY:

T - HELPER:

T - SUPPRESSOR:

B - CELL

TOTAL T AND B CELLS:

RATIO OF T-HELPER TO T-SUPPRESSOR:

NO. OF LANGERHANS CELLS:

COMMENTS: ______________________________

____

NO. ____________

URINE:

COLOR: ____________ SPECIFIC GRAVITY: ____________

SEDIMENT: ____________

BIOCHEMICAL ABNORMALITY (SPECIFY): ________________________________

STEROID LEVELS: ________________

PARASITES: ________________________________

FECES:

PARASITES: ________________________________

ADDITIONAL COMMENTS: ________________________________

____

____

____