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Immune Responses in Onchocerciasis Correlate with Microfilarial Destruction and Treatment History

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Immune Responses in Onchocerciasis Correlate with Microfilarial Destruction and Treatment History

By

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ABSTRACT

Immune Responses in Onchocerciasis Correlate with Microfilarial Destruction and Treatment History

By

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Onchocerciasis, or river blindness, is a parasitic disease that affects more than 20 million people in the world. It presents clinically with a wide spectrum of dermal and ocular manifestations, the basis of which is believed to involve the immune system. The induction of pathology is directly related to the presence of the microfilarial stages of this filarial nematode. The aim of this study is to gain better understanding of the role of immune response in the host reactions to *Onchocerca volvulus* parasite. Patients with either of two major forms of the clinical spectrum-mild/asymptomatic (n=12) and severe dermatopathology (n=16) were studied by assaying the antigen-driven proliferation of peripheral blood mononuclear cells and the ability of patients' serum antibodies to promote cytoadherence activity to microfilariae *in vitro*. Immune responses of those with severe disease were found to be stronger compared with the mild dermatopathology group. Mectizan® treatment was followed by an increase in immune responsiveness in those with initially poor responses. Thus the degree of dermatopathology is related to the host's immune response against microfilariae and immunocompetence may be necessary for Mectizan® to clear the infection efficiently.

To my loving wife Dr. Suzan

and

to my children

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Mf, microfilaria, OV, *Onchocerca volvulus;* L3, third Larval stage; pers.comm., personal communication; WHO, World health Organization; TH1/2, T helper 1, Th2; IL, Interleukin; TCR, T-cell receptor,IgE, Immunoglobulin E; PHA, Phytohemagglutinin; OVA, *Onchocerca volvulus* antigen; RPMI, Rosewell Park Memorial Institute; PBMC, peripheral Blood Mononuclear cells; HFCS, heat-inactivated fetal calf serum; cpm, count per minute; SI, stimulation index; CMI, cell-mediated immunity; AMI, Antibody-mediated immunity; OD, optical density.

Chapter 1.

Introduction

General Introduction

Filariae are a distinct group of nematode parasites. All those that infect humans are classified in the family Onchocercidae and the superfamily Filariodea. The name filaria is derived from the Latin word "filum" meaning thread, and refers to the thread-like morphology of the adult worm. Filariae share common features, including an adult stage that resides outside the digestive tract, the requirement of an obligate hematophagus arthropod vector as an intermediate host, and the release of motile offspring called microfilariae into the vertebrate host. Filarial worms continue to present a serious challenge to those concerned with public health and to control programs in many parts of the world. Seven principle species of filariae infect humans, and these are typically grouped into three categories. The lymphatic filariae, consisting of *Wuchereria bancrofti* and *Brugia malayi*, reside as adults in the lymphatics and infect some 120 million persons (Ottesen et al, 1997). Infection with these parasites may cause acute or chronic obstruction of the lymphatics that ultimately leads to elephantiasis. Onchocerciasis is caused by *Onchocerca volvulus*; the adults live in subcutaneous nodules or in deeper skeletal tissues associated with major bones. The remaining filariae including *Loa loa*, *Mansonella perstans*, *Mansonella ozzardi*, and *Mansonella streptocerca*, are less important as causes of disease and suffering, although *L.* loa can provoke temporary inflammatory swelling (calabar), hypereosinophilia and other allergic reactions. *Loa loa's* habit of

migrating widely throughout the body is especially disturbing if it crosses the eye and surrounding tissues.

Adult filariae are typically long, slender worms, with pronounced sexual dimorphism; females are characteristically much longer than males. Adult worms are observed rarely because of their location in the host, so the microfilariae are the usual diagnostic stage. Microfilariae are relatively undifferentiated when released by female worms. In some genera of filariae (e.g., *Wuchereria, Brugia*, and *Loa*), the microfilariae retain the egg membrane as a sheath, whereas the others release unsheathed microfilariae (e.g. Onchocerca). Once released by the female worms, microfilariae make their way into the circulation, or, in the case of *O. volvulus* and *M. streptocerca*, into the skin. They are generally not infective for non-human vertebrate hosts, undergo no further development in the humans, and survive for 1-2 years. Microfilarial periodicity is a phenomenon shown by some filarial species and it can pose a difficulty for their parasitological diagnosis (Mason-Bar and Aped, 1982).

Onchocerciasis or 'river blindness', one of the major filarial diseases of humans, is caused by *O. volvulus*, and transmitted by female black flies of the genus *Simulium.* Onchocerciasis is a leading cause of preventable blindness and a severe pruritic skin condition in endemic areas, and thus one of the most important public health and socio-economic problems faced by the rural populations in these areas (WHO, 1976). It is estimated that over 90 million people are at risk of infection, with more than 20 million being infected. The majority of patients suffer from variable skin lesions. One million people suffer

visual impairment as a result of onchocerciasis, with at least 340,000 cases of blindness attributable to the disease. More than 46,000 people lose their vision every year as a result of this devastating disease (WHO, 1995). The impact of blindness on a community is reflected in an increased mortality rate: the mortality amongst blind people is four times higher than that of non-blind persons of the same age in a community (WHO, 1994).

Onchocerciasis is endemic in large areas of Africa, on the Arabian Peninsula, as well as in central and South America. It mainly affects small isolated and more remote communities, with the result that some endemic areas have escaped detection and many thousands of villagers with onchocercal eye disease probably remained undiagnosed. In general, a favorable ecology for the intermediate host black fly determines the distribution of the disease. The disease is usually prevalent in rural areas around water sources where the vector prevails. The vector breeds in fast flowing rivers, hence the name "river blindness". The effect of the disease in communities includes the deterioration of living conditions and desertion of fertile riverside lands by the villagers (WHO, 1987). Fear of the disease has led to the depopulation of river valleys where the vector breeds. Therefore, the disease poses a significant obstacle to socioeconomic development. There are over 700 species of *Simulium* around the world. The black fly *S .damnosum* is the main vector of *O. volvulus* in Africa (Crosskey, 1969). Several subspecies of *S. damnosum* are distributed worldwide. The male black flies feed only on plant juices and have no role in the transmission of onchocerciasis.

The female flies feed on blood and can take up to 1 mg of blood at each meal. The eggs are laid 3-5 days after the meal. Within 24 hours of laying the eggs, the female fly takes another blood meal. The bite, which involves the creation of a blood pool under the epidermis, is not always noticed immediately, but may be followed by debilitating severe itching. *Simulium* can fly for up to 80 km in 24 hours along the water sources by the aid of their strong thoracic muscles. They may even go hundreds of kilometers with the help of wind from one river basin to another.

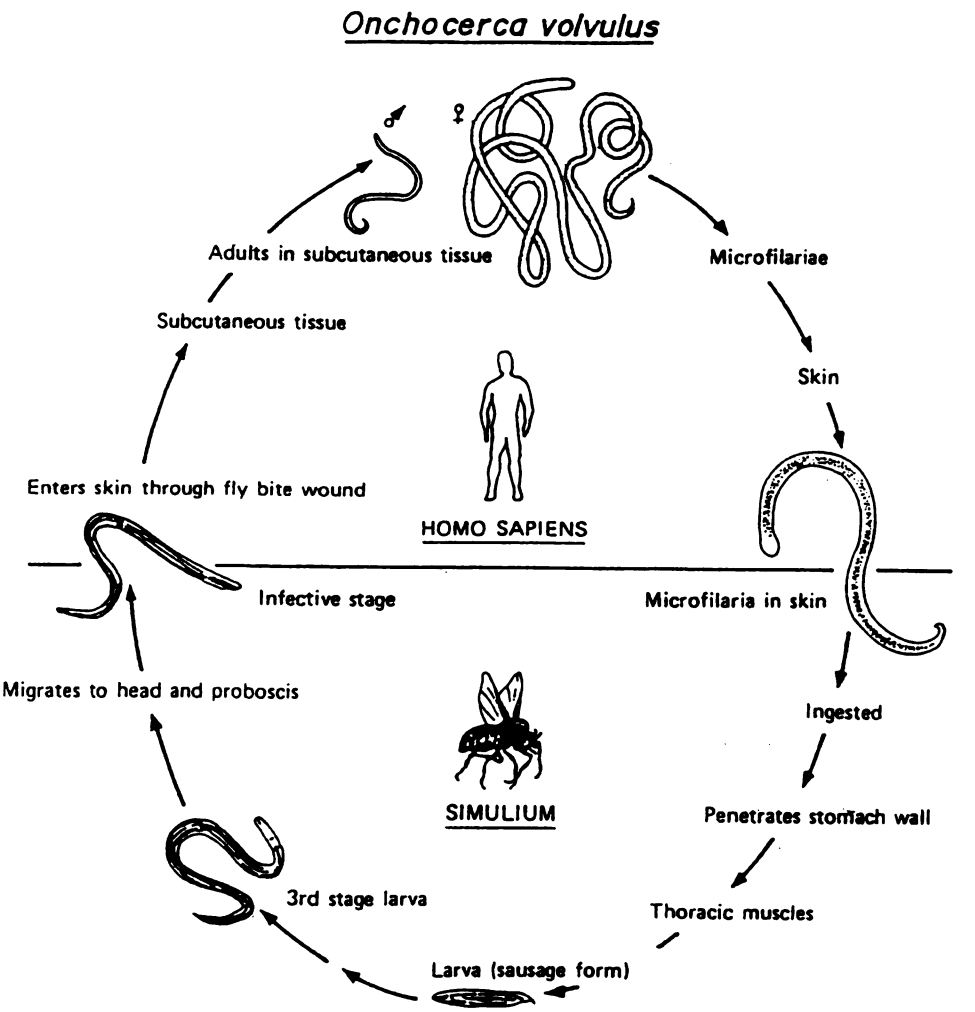
Life Cycle of *Onchocerca volvulus:*

The life cycle of the parasite in the host still holds some mysteries, due to the lack of a truly suitable comparable animal model and the dearth of postmortem information. The life cycle stages take place in two hosts, the human primary host and the black fly as the intermediate host. As an infected female black fly takes a blood meal from the human, it injects the infective parasitic larvae (known as L3) into the skin. It is proposed that the larvae penetrate the superficial layers of the skin, migrate and develop into adult worms elsewhere in the body over a period of 6-12 months. Within 1-3 years after infection, onchocercal nodules appear in subcutaneous tissues, usually at sites of bony prominences such as the hips and ribs. These nodules are formed around coiled mature worms and are encapsulated by host reactive tissues with a rim of vascularized fibrous tissue; they usually contain both male and female adult worms. The adult males are 2 –5 cm in length and 0.02 mm in diameter, while

the females are 50 –70 cm in length and 0.04-0.06 mm in diameter. Fertilized females give birth to live embryos, about 500,000 to 1,000,000 per year through out her sexually active life of 8-12 years. It has been estimated that the total number of microfilariae distributed throughout the dermis, the eyes, and other parts of the body, in heavily infected individuals may reach 200 million. They are not commonly found in blood, but may be detected in the urine. The microfilariae may live for > 2 years

O. volvulus develops through a number of stages in the female black fly. On biting an individual with dermal microfilariae, the fly ingests microfilariae, which pass to the stomach, where most of them are digested. However a few may survive and then pass to the thoracic muscle, where they moult twice to become infective filariform larvae measuring 650 microns in length. These latter forms migrate to the tip of the proboscis in the mouthparts and are transmitted to a human during a subsequent blood meal. The number of infective larvae in one black fly is usually < 10 (WHO, 1987). The cycle of development in the black fly takes about 7 days at 27 to 30 ^{0}C, but can last up to 10 or 12 days at lower temperatures. Development stops when temperature falls below 16^{0} C, as often happens in the night (Duke, 1968). *O.volvulus* can also be pathogenic to the black fly: on biting an individual with very heavy loads of microfilariae, the fly may ingest several hundred microfilariae which can destroy the fly's own tissues during migration and development, thus preventing flight and other functions (WHO, 1987).

Fig.1 Life Cycle of the parasite



Clinical Manifestations:

The pathogenesis of filarial diseases is characterized by acute and chronic inflammation. The parasite, the immune response, and/ or consequent opportunistic infections initiate these inflammatory responses.

The clinical changes seen in human onchocerciasis are believed to be associated with the destruction of the microfilarial stage of *O. volvulus* in skin and eyes. Clinical disease is caused by the microfilarial stages of *O.volvulus*, with inflammation and tissue damage mostly seen when the parasite is dying or being destroyed by drug and/or host responses. A papular eruption in the skin leads to varying degrees of dermal and epidermal pathology that is complicated by self-inflicted trauma due to the intense pruritic nature of the infection. Thus, the consequent pathology represents the cumulative tissue and functional outcomes of a long-standing interplay between host and parasite, often lasting many decades. Severe skin changes take place making it one of the most devastating infectious diseases of human skin. However, the disease is best known for its ocular pathology, which can affect many parts of the eye causing irreversible damage and blindness. The adult male and female worms are encapsulated in subcutaneous nodules, usually palpable on bony prominences such as iliac crest, hip, ribs knees and skull.

The period between infection with infective larvae and the production of microfilariae by fertilized adult worms (the prepatent period) varies from 7 month to more than 2 years. Generally, clinical symptoms are not present during this period. However, the immature worms may stimulate some immunological

responses (Duke, *et al* 1991). The time from invasion by infective larvae to the development of clinical signs (the incubation period) is usually longer than the prepatent period and may last for many years; it is much longer for the manifestation of the ocular disease than the dermal presentations (WHO, 1987).

Dermal Onchocerciasis

A broad spectrum of skin manifestations can result from inflammatory reactions associated with damage due to the disintegration of the microfilariae. During the early skin lesions, mf are found primarily in the dermis, and cause no observable reaction as long as they are alive. It is only after the death of mf that inflammatory responses appear. However, it is debatable as to which comes first, parasite death or the host's responses. Circulating antigens released from disintegrating mf may be deposited into blood vessels and connective tissue in the skin and result in pathology. The clinical manifestations vary according to the microfilarial load in the skin, the immune responses of the human host, and the duration of the infection (Mackenzie et al 1987; Murdoch, 1992). Eosinophils, neutrophils and macrophages aggregate around dead or dying microfilariae. The commonest early symptoms of the disease include irritation of the skin, pruritus that can be accompanied by oedema and a sensation of hypothermia. During the early stages, the signs are often confined to one part of the body. Chronic infection may present as depigmentation (leopard skin), a striking feature seen mostly in the shins and has been used for rapid assessment of the prevalence of the disease (Edungbola et al 1987). These lesions are white and interrupted by

foci of persistent pigmentation around the pores and hair follicles. The areas may be slightly depressed or atrophic. In later stages, the skin loses elasticity and may become wrinkled and hang in loose folds (hanging groin) (Salih, 1985); this is often associated with local underlying lymphadenopathy (Mackenzie, pers.comm.).

Dermal onchocerciasis has two forms: a "generalized form" which is more prevalent with high microfilarial load, and a "localized form" manifesting as a chronic hyper-reactive, onchodermatitis or sowda. Sowda is the Arabic word for black, and the condition was first described in Yemen (Fawdry, 1957) and then in West Africa (Bartlett *et al*, 1979) and in eastern Sudan (Ghalib *et al,* 1987). The affected area of skin is intensely pruritic, dark and thickened. There is an extensive inflammatory cell infiltration (plasma cell, eosinophils, occasional lymphocytes and histiocytes) that forms broad cuffs around dermal vessels, greatly thickens the dermis, and causes sclerosis, edema, hyperpigmentation and papular eruption. Moreover, the regional lymph nodes are soft and greatly enlarged but usually not tender. The condition is usually localized and typically involves only one lower extremity. The live microfilarial load is extremely low and even sometimes undetectable. Severe pruritic skin rashes with acute inflammatory reactions may also occur in individuals who are visitors and not from the endemic area when they became infected for the first time; they are immunologically hyper-responsive to the parasite and its antigens. This could be due to strain differences in the parasite or HLA differences in the patients. HLA-DQB10201 *has been* found to be associated with sowda (Danfack *et al* 1999).

Cooper *et al* (2001) confirmed the notion that early exposure and early parasite patency are associated with a vigorous cellular response, unlike chronic infections in which the cellular response becomes down regulated partly through an IL-10- dependent mechanism. Moreover, host differences in attractiveness for the vector might also affect the immunity to this disease. However Kruppa & Burchard (1999) showed that infected flies similarly bite infected and non-infected (putatively immune individuals).

A new candidate for the cause of the tissue reactions in filarial infections are the *Wolbachia* organisms carried by this parasite, which are proposed to be major stimulators of inflammation (Taylor et al 2000). The impact of pruritus is definitely reflected on the general health appearance of the infected individuals leading to noticeable weight loss in people in the hyperendemic areas (Burnham, 1991).

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Nodules (Onchocercomata)

Many patients develop fibrous subcutaneous masses containing the adult onchocercal worms know as "nodules". These nodules are painless in themselves (unless pressing on a vital organ), mobile and 0.5 –1.0 cm in diameter. They can group together to form conglomerates as big as 6-8 cm in diameter containing 10-15 individual worm masses. They are mostly seen over bony prominences of the trunk, thigh, shoulders, arms and cranium (Krupp & Chatton 1978). However, some nodules lie deeper and are impalpable. The sites of the nodules depend in part on the biting habits of the vector black fly (Duke,

1972). For example it is mostly seen in South America on the skull due to this biting habits.

The composition of the nodules encasing adult onchocercal worms includes an outer capsule of fibrous tissue, an inner dense inflammatory cell infiltrate surrounding the enclosed adult worm and a less dense layer of chronic inflammatory cells between the outer and inner layers. The character of the infiltrate varies in terms of the presence or absence of eosinophils but macrophages are almost always the predominant cell type. Lymphocytes are most abundant at the periphery of the nodule and surrounding the inner core of a dense inflammatory cell infiltration composed primarily of macrophages around the adult worm itself. Immunohistological and cytochemical staining of the various cell population in the nodules show that infiltrating macrophages closest to the worm stain strongly positive for complement receptor CR4 (CD11, CD18) and for MRP8/MRP14 (an intracellular calcium-binding protein involved in chemotaxis and inactivation of certain cells), while they are variably positive for CD68 (a marker for dendritic cells), Fc receptor FcR1 (CD64), and HLA class II (activation marker) (Gatrill *et al* 1987; Edgeworth *et al* 1993). Interestingly, macrophages seem to be the cell type most intimately involved with adult parasites, while the eosinophils are the cell type most prominently interacting with mf. Recently, strong neutrophil infiltrates adjacent to the live adult worms were seen in untreated onchocerciasis patients, unlike those who received doxycycline treatment, and showed drastically reduced accumulation. This finding may relate neutrophil chemotaxis and activation to the endobacterial

products of *Wolbachia* (Brattig et al, 2001). T cells are distributed in a manner suggesting that they play more of an overseer role than one of an effector cell population that is directly anti-parasitic.

Lymphatic nodes in onchocercisiasis

O. volvulus affects lymph nodes of infected individuals. There is a range of inflammatory responses. Acute responses are mast cell-based reactions to cuticular collagen (Gonzalez et al 1999) In chronic stages of this disease, fibrotic and degenerative reactions are seen. However, there is a possibility of autoimmune responses (Eggleton et al 1999). Two general forms of presentation exist. The first is seen in the most active, or hyperreactive onchodermatitis "Sowda", in which the lymph nodes are greatly enlarged with follicular hyperplasia, activation of germinal centers, and sinus histiocytosis with varying numbers of plasma cells, eosinophils, and neutrophils. The nodes are firm but not painful or tender; the inguinal and femoral nodes are the most commonly affected. Histologically, these lymph nodes are active in the cellular and humoral arms of the immune response. The second general form is as shrunken, hard feeling and fibrous lymph node or group of lymph nodes. These are common in older patients with a long history of infection and chronic degenerative dermal disease (atrophy, depigmentation, etc). Histologically, these lymph nodes are replaced by sclerosing changes and are immunologically quiescent. Sowda patients often present with swollen, edematous legs resembling elephantiasis, but these changes are mostly reversible (WHO, 1987).

Ocular onchocerciasis

The development of ocular lesions correlates with the degree and duration of infection (Fuglsang & Anderson, 1977). In patients with palpable nodules, serious pathology of the posterior segment of the eye was found twice as frequently as in onchocerciasis patients without nodules (Berghout et al, 1987). Both the anterior and the posterior chamber of the eyes are likely to be affected by the parasite, resulting in diminution of visual acuity or complete loss of vision. Corneal inflammation (keratitis) is a major cause of visual impairment in *O. volvulus* infection. Two distinct forms of corneal diseases are recognized: reversible punctate keratitis (snowflakes opacities) and the irreversible more severe sclerosing keratitis, which has a permanent effect (WHO, 1987). There are geographical differences in the prevalence of onchocercal blindness. Blindness is more common in the Savannah areas of West Africa (2-15% of the population of hyper-endemic areas) than in the rain forest areas of West Africa (blindness < 2%). There is evidence for genetic variations amongst *O. volvulus* (i.e. parasite variants) associated with variations in virulence, i.e. in the ability to experimentally infect rabbits (WHO, 1987). Furthermore, when using DNA classification techniques, it was found that there is a strong correlation between disease severity and strain of parasite i.e. pathogenicity is strain related (Zimmerman *et al,* 1992).

It has been postulated that host immunological responses could account for ocular pathology (Bryceson, 1976). Recently, evidence has become available that antigen-specific T cell and antibody responses are essential for the

development of *O. volvulus* keratitis and the sequence of molecular and cellular events leading to migration of inflammatory cells to the cornea, thus leading to the loss of corneal clarity (Pearlman & Hall, 2000). The ocular disease in this infection may have an autoimmune component, because patients continue to show chronic, low-level, progressive pathologic changes of the retina and retinal pigment epithelium, even after chemotherapy to reduce parasite load. In addition, progression of the disease of the retina and optic nerve, unlike that of the cornea, does not appear to be related to microfilarial worm burden (Semba *et al,* 1990). Molecular mimicry or immunologic cross-reactivities between host and bacterial or viral antigens have been suggested to have a role in the development of a number of autoimmune diseases and has been also suggested to play a role in the development of ocular onchocerciasis. The *O. volvulus* antigen Ov39 is cross-reactive with the retinal antigen hr44 and induces ocular inflammation in rats after immunization (McKechnie *et al,* 2002). It was also indicated that CD4 (+) cell lines specific to the antigen Ov39 can induce ocular inflammation in naive rats and suggested that recruitment of CD8 (+) T cells may play a regulatory role, the inflammation however, is milder than that produced by immunization. They concluded that the absence of antibody responses to hr44 in the animals receiving the T-cell lines might indicate a role for antibody in the development of ocular onchocerciasis. Other human factors, such as ecological, nutritional, and multiple infections with other parasites, may also influence the clinical appearance of onchocerciasis in different geographical areas.

Immunology and immunopathology:

The pathologic manifestations of *O. volvulus* infections result not only from the parasite but also from the magnitude and quality of the host immune response (Mackenzie *et al*, 1985). The existence of immunity to helminth infections in humans is a highly debatable issue. Protective immunity, acquired and specifically directed toward filarial parasites, can be demonstrated in a variety of experimental animal models (Grieve et al 1988; Hyashi *et al,* 1989). However, it is not easy to induce protective immune responses to *O. volvulus* in laboratory experimental animals and only limited success has been achieved in this area (Naduri and Kazura, 1989). The existence of immunity to *O. volvulus* infection in humans is inferred from the presence of a small number of uninfected individuals in hyperendemic areas. Immunological characterization of such individuals has been pursued (Nutman *et al* 1991) by determining antigens that might elicit antibody responses responsible for protection. Integrating clinical and epidemiological information with polymerase chain reaction (PCR) and ELISA data, the immune response in putatively immune individuals was found to correlate with a low titer of specific IgG, IgG subclasses, and Ig E compared to infected individuals (Elson *et al,* 1994). Nevertheless, the putatively immune individuals were found to produce significantly more IFN gamma to *O. volvulus* antigen than did those in the infected group, and less IL-10 spontaneously (Elson *et al,* 1995). Thus protective immunity to onchocerciasis may be mediated in part by an antigen-specific Th1 type response. However, some other reports relate the immunity to the production of IL-5 and the resulting increased eosinophilia

(Hogarth *et al,* 1998). T helper type 1 (TH1) and type 2 (TH2) cells represent terminally differentiated effector cells characterized by different cytokine production and homing capacity. The generation of either type of response can confer protection against pathogens or lead to immunopathology. TH1 and TH2 polarization is a stochastic process, which is promoted by interleukin 12 (IL-12) and IL-4 (Trinchieri 1995) Other factors that contribute to the TH1-TH2 balance are the dose of antigen, strength of antigenic stimulation, duration of T cell receptor (TCR) engagement and the nature of co-stimulatory molecules.(Bird *et al,* 1998; Gett & Hodgkin 1998; O'Garra, 1998). The balance between TH1-TH2 cytokines regulates human immune responses to infection. IL-4 and IL-10 regulate antibody production and can suppress cell-mediated immune responses.

Distinct type 1 and type 2 T helper cytokines cross regulate the expression and magnitude of *O. volvulus*-specific cellular responses in humans (Soboslay *et al,* 1999). However, recently Doetze *et al* (2000) suggested that the antigen-specific cellular hyporesponsiveness in a chronic human helminth infection is mediated by T helper 3/ T helper 1 –type cytokines but not by a T h1 to Th2 shift. Nevertheless, the TH1 pattern of cytokine production has long been associated with immunity or resistance to helminth infection. A Th2 or type 2-cytokine pattern has been associated with the progressive forms of *O. volvulus* infection in humans (Nutman & Modlin, 1993; Johnson *et al*,1998; Soboslay *et al,* 1999; Turaga *et al,* 2000). Recently, Graham *et al* 2001, using the bovine *O. ochengi* model, suggested that neither a classical Th2 response nor a simple Th1 to Th2

switch is sufficient to explain the immunomodulation associated with a patent *Onchocerca* infection.

Natural killer (NK) cells are viewed as an important component of innate resistance against a variety of pathogens. NK cells secrete IFN gamma and have the capacity to activate macrophages before the induction of the antigen -specific T- cell response. Therefore, NK cells might play a role in the balance of Th1 versus Th2 (Bancroft, 1993; Biron *et al,* 1999)

The immune attack on mf usually leads to onset of clinical symptoms (Piessens & Mackenzie, 1982; Mackenzie *et al,* 1986). The clinical changes seen in human onchocerciasis are believed to be associated with the destruction of the microfilarial stage of *O. volvulus* in skin and eyes. The interaction between the immune system and *O. volvulus* that on the one hand allows the survival of the parasites for long periods of time, at other times promotes the death and removal of parasite. The diversity in clinical presentation of onchocerciasis is considered to reflect the intensity and quality of immune responses to the parasite or its products. The immune responses of the host are suggested to play a crucial role in the pathology of onchocerciasis which is usually localized to the sites where microfilariae are attacked in the skin, eye, and subcutaneous tissues (Mackenzie et al, 1985). The demonstration of antibodies to retinal –S-antigen (S-Ag) in onchocerciasis patients (Chan *et al,* 1987; Mackechnie *et al,* 1993) suggests the possibility of auto-immunity in blinding disease.

Eosinophilia and elevated serum IgE are immunological hallmarks of infection with parasitic helminths. Immediate hypersensitivity reactions are

characterized by the presence of IgE antibodies, eosinophils, mast cells and basophils, all of which have been implicated in resistance to infection as well as pathogenesis. Eosinophils are usually attracted to infection sites leading to macrophage activation and mf killing (David *et al,* 1982). The level of circulating IgE does not necessarily give complete information about the biological function of this immunoglobulin class and information gained from specific tests such as measuring the release of mediators from basophils and mast cells is usually more useful. Using histamine release measurement, it was found that sensitized basophils might play a role in the pathogenesis of onchocerciasis. The destruction of microfilariae was reported to involve eosinophils acting through antibody binding (Green 1980; Mackenzie *et al*, 1980), and is related to papular eruption *in vivo* and to punctate keratitis lesions in the eye (Mackenzie *et al* 1985). The antibodies involved in this killing phenomenon were thought to be specific IgG type (Ghalib *et al* 1985; Ngu *et al* 1989). Patients with low skin microfilarial loads have high IgG3 and low IgG4 titers, suggesting a protective role for IgG3 and a suppressive one for IgG4 (Dafa'Alla *et al* 1992). Moreover, serum concentrations of IgE and IgG are significantly elevated in sowda patients (Brattig *et al,* 1987).

IL-4 and IFN gamma play crucial roles in the regulation of IgE responses in onchocerciasis patients. IL-4 is associated with higher levels of IgE production, while IFN gamma has been found to down regulate the Ig E. The amount of IgE produced depends on the relative quantity of IL-4 and IFN gamma secreted by parasite- stimulated T cells (King *et al*, 1990). Furthermore, the induction of

Ig E by filarial antigens depends on the concentration of the antigens. Higher antigen concentrations suppress IgE production and this suppression can be reversed using anti IFN gamma antibody (King *et al,* 1990). The mechanism responsible for the induction of IgE by IL-4 appears to involve the regulation of isotype switching to IgE in uncommitted B cells (Bergstedt-Lindqvist *et al,* 1988). Eosinophilia has also shown to be regulated by T cells and human IL-5 is a potent stimulus for eosinophil proliferation *in vitro* (Limye *et al,* 1991). *In vivo* eosinophilia was found to be preceded by increased production of IL-5 following microfilaricidal therapy. IL-5 secretion is sustained during the periods of microfilarial disappearance (Limye *et al,* 1993). IL-5 producing cells depend upon IL-2 for proliferation (Steel & Nutman, 1993). IL-6 was found to be elevated in onchocerciasis patients and correlates with the occurrence and severity of post-treatment clinical symptoms. IL-6 levels appear to depend on the intensity of infection (Ali, unpublished). Serum TNF levels are also elevated after treatment, but did not correlate with the infection intensity or reaction severity (Turner, 1994).

Defective responsiveness of peripheral blood lymphocytes *in vitro* from onchocerciasis patients has been reported (Green *et al,* 1983, 1985; Gallin *et al,* 1988; El Khalifa *et al*, 1991; Soboslay *et al,* 1992). The major conclusion from these studies is that responses to parasite antigen in patients with the generalized form of onchocerciasis are minimal, compared to putatively immune persons living in (an)endemic areas without active infection. They were also found to have higher IL-2 and IFN gamma responses to onchocercal antigens

compared to patients with the generalized form of onchocerciasis (Ward *et al,* 1988; Soboslay *et al,* 1999). However, there is less published information on cytokines in onchocerciasis patients representing the varied clinical spectrum. In a murine model, CD4 (+) T cells mediate sustained *O. volvulus* keratitis by regulating eosinophil recruitment to the cornea (Hall et al, 2000). This may be due to the mobilization of blood and tissue eosinophils during clearance of infection. Previous work indicated that, CD4 (+) Th2 cell namely, IL-5 was found to be associated with clearance of infection (Hogarth & Bianco, 1999). However, it could also lead to pathology. Flow cytometric determination of lymphocyte subset distributions show improvement in the CD4 (+) T-cells status of patients after treatment to reduce microfilariae with ivermectin. Production of IL-2 and IL-4 by lymphocytes induced by phytohemagglutinin (PHA) increased one month after treatment (Freedman *et al*, 1991). Treatment with ivermectin seems to enhance cellular proliferative response 3-6 months post-treatment (Steel& Nutman, 1993). However, a generalized suppression in lymphocyte proliferation has been observed 2 weeks after treatment (Nutman, 1987), probably resulting from the release of suppressive microfilarial antigens (Lal *et al*, 1990). Several findings support the hypothesis that microfilaria-derived products are important in suppressing lymphocyte proliferative responses to *O.volvulus* antigens. Furthermore, ivermectin has immunostimulatory properties that are associated with altered functions of T-lymphocytes, particularly T-helper cells (Blackley *et al,* 1991). Parasite antigen-specific deficits in IL-2 and IL-4 production were not reversed by ivermectin treatment, leading to the suggestion that some

components of non-reactivity are long lasting and are not affected by diminished microfilariae in detectable numbers in skin tissues. Further work is needed to better understand the effect of ivermectin treatment on the host immune response. The interplay between infection status and the balance between immune responsiveness and immune modulation also remain to be addressed.

Diagnosis

Filariasis in general continues to present diagnostic challenges. The broad spectrum of clinical manifestations associated with this infection creates a diagnostic paradox. The accurate and specific diagnosis of filarial infection has become increasingly important as a mean of monitoring the efficacy of mass distribution of Mectizan. This goal has been hampered by our inability to distinguish between past and current infection.

1. Parasitological diagnosis of infection

The detection of unsheathed microfilariae in the skin involves removing small snips of skin (Consistent i.e. almost of the same size bloodless biopsy) and incubating them in an aqueous solution, preferably (RPMI 1640), to allow microfilariae to migrate out so that they can be observed microscopically. A bloodless skin snip weighing from one to several micrograms can be removed quickly and without much discomfort, is obtained using a razor blade, or Walser-type corneoscleral punch. This is more convenient and allows uniformity in snip size. Microfilariae will emerge from the sample within 4 hrs, and the intensity of

infection is reflected in the number of microfilariae emerged from the snip (Taylor *et al*, 1989). Sowda or severe reactive onchodermatitis patients have few or low microfilarial load of viable microfilariae, or may even show a negative test by this method. Skin from the pelvic girdle provides the best chances for detection (Williams *et al,* 1985 a, b).

2. Ultrasonography:

Onchocerciasis can also be diagnosed by detecting microfilariae in the eye by using the slit- lamp. Detection of subcutaneous nodules is also used for clinical diagnosis and the introduction of ultrasound facilitated the detection of these nodules (Homeida et al, 1986; Leichsenring et al, 1990). Onchocercal nodules must always however, be distinguished from other similarly presenting lesions such as lipomas, foreign bodies, granulomas, and sebaceous and dermoid cysts.

3. Immunological and Molecular Diagnosis:

The Mazzotti test, which is an allergy- based reaction, was frequently used as a diagnostic aid for patients with negative skin snips. It is (carried out done by administration of 0.5 mg DEC orally every 4-6 hours for three days and then 1 mg DEC every 4-5 hours. Dermal changes were seen, consisting mainly of papular responses (El Shiekh, 1985). New procedures have been developed for the immunodiagnosis of onchocerciasis and these will be of great significance since the detection of microfilariae by using skin snip is not feasible in prepatent infection or in those with strong immunological responses. A wide variety of antigens have been utilized in this procedure, including soluble *O.volvulus* crude

antigen. Also, recombinant polypeptides, a cocktail of three recombinants have been field tested for use in *O. volvulus* infection (Ramachandran, 1993). It was found to detect *O. volvulus* infection during the prepatent period. These antibody-based assays, however, cannot reliably distinguish past and current infection, nor have they been helpful in following individuals after chemotherapy. A PCR test for skin snip samples to detect the presence of *O. volvulus* DNA in order to rapidly assess the epidemiology of the disease in endemic areas (Zimmerman *et al,* 1994) was found to be more sensitive than standard skin snip examination. Circulating antibodies are detectable using an ELISA system with blood obtained from finger prick and collected onto filter paper. This test is useful in those under 15 years and could be integrated with other disease programs that monitor blood samples, like malaria (Botto *et al,*1999).

4. Epidemiological Diagnosis

Diagnostic procedures in onchocerciasis are used to determine the prevalence of infection, to identify individuals requiring treatment, to evaluate the success of treatment, and to assess the impact of control efforts. A non-invasive epidemiological technique is needed for the detection of disease to determine eligibility of mass treatment programs, as has been launched by the WHO in the African Program for Onchocerciasis Control (APOC). This technique is known as Rapid Epidemiological mapping for Onchocerciasis. It comprises a rapid search for nodules in individuals suspected to have the infection. It requires experience and knowledge for the personnel who perform the technique. It greatly helped to estimate endemicity in order to start the control program.

Treatment

Treatment of onchocerciasis has been a problem in the past and is not yet satisfactory. Ideal treatment of infection with *O. volvulus* would include drugs that kill the adult worm (macrofilaricidal) and the microfilariae (microfilaricidal), with minimal side effects on those who receive this treatment. The history of treatment involves suramin, diethylcarbamazine (DEC) and recently ivermectin (Mectizan), which is currently the drug of choice. Suramin was administered intravenously in repeated doses, as a macro- and micro-filaricidal, but the drug had severe cumulative toxicity because of its very slow excretion. DEC was administered orally in a multiple dose regimen(s), but was associated with a very high increase of adverse reactions, resulting from the rapid killing of microfilariae in the skin, subcutaneous tissues and the eyes. These can lead to life threatening severe allergic-like clinical responses, including severe itching, lacrimation, urticarial oedma of the skin, swelling and tenderness of the lymph nodes, maculopapular eruption, pyrexia and sometimes severe postural hypotension (El khalifa *et al,* 1985; Awadzi *et al*, 1980). In the patients with ocular lesions, DEC aggravates the condition and can cause complete loss of vision (Bird *et. al*, 1980). Thus patients treated with Suramin or DEC should be hospitalized or treated under close medical supervision.

In view of these problems there was a desperate need for an alternative therapy for onchocerciasis. Ivermectin is a semi-synthetic macrocyclic lactone produced by the actinomycete *Streptomyces avermititis sp.* developed by (Merck

and CO., Inc.) it has been extensively used in veterinary medicine for treating internal and external parasites (Campbell, 1985). The drug was introduced for human onchocerciasis (WHO, 1987). It was a revolutionary breakthrough, as it has an effective microfilaricidal action and it clears microfilariae from the skin with minimum side effects (Aziz *et al*, 1982; Awadzi *et al,* 1980; Campbell 1991). The drug is given in a single dose and produces a prolonged reduction in microfilarial loads. It has provided for the first time a feasible chemotherapy for large-scale treatments. It has been used successfully in mass treatment programs (Baraka *et al,* 1995). It improves skin lesions except for depigmentation (Pacque *et al,* 1991), improves visual acuity, and reduces the source of infection (Newland *et al,* 1988). The drug was also found to be safe even in individuals with the severe ocular form of the disease. Recently, a model for human onchocerciasis (*O. ochengi* infection in cattle) demonstrated the effectiveness of administering the antibiotic tetracycline together with ivermectin, resulting in a macrofilaricidal effect. This response is hypothesized to be related to the action of tetracycline on *Wolbachia endobacteria*; abundant in *O. ochengi* (Trees *et al,* 2000) this could be a useful procedure that may increase options for the control of human onchocerciasis. Recent findings support a role for *Wolbachia* products in mediating the inflammatory responses seen following treatment of onchocerciasis and suggest new targets for modulating these reactions (Keiser et al, 2002)

Ivermectin uptake was thought to be mediated by specific high affinity receptor. The interaction between ivermectin and the receptor leads to the

release of gamma aminobutyric acid (GABA) from the nerve ending and enhance the binding of GABA to its receptor leading to direct increase in the membrane permeability to chloride ions (Campbell, 1985). Ivermectin is suggested to have a significant effect in the release of microfilariae from the female gravid uterus by reducing the number of the multicellular embryogenic stages in worms exposed to multiple does of ivermectin. This may be partially due to the reduction in the effectiveness of insemination in female worms and minor impairment of oogenesis (Chasse *et al,* 1993). Moreover, ivermectin may have chemoprophylactic effects, which might contribute to the maintenance of low microfilarial production in areas of on-going transmission (Klager *et al,* 1993). However, recently some recurrence was observed in a small proportion of treated patients with ivermectin after only one month of treatment, unlike previous reports (Ali, see chapter three, *Acta Tropica*, in press). This suggests that at least for the treatment of a small subset of individuals, Mectizan may need to be administered more frequently than once a year, perhaps as often as every three months. This also raises the possible involvement of the immune responses in microfilaricidal mechanism of action of ivermectin. Studies concerning immunological responses after repeated doses of ivermectin in patients with onchocerciasis emphasized the apparent long term safety of ivermectin through the demonstration of the absence of immunopathological responses induced by repeated ivermectin treatment (Steel et al, 1991). The immunosuppression due to the disease is reversible after ivermectin treatment (See chapter one; Ali submitted); there is a sustained production of IL-2 and IFN

gamma, which contribute *to O. volvulus* infection. Thus ivermectin may be needed to enhance immunity against onchocerciasis (Soboslay, 1994). It might also be involved in the mechanism of killing by the drug.

Recently, the African Program for Onchocerciasis Control (APOC), operated by W.H.O, adopted a control strategy for combating onchocerciasis, which relies mainly upon ivermectin treatment. This new approach employs a community directed treatment strategy to secure the issue of sustainability. As the drug will only kill the microfilarial stage, treatment should be repeated at least once a year for at least 12 years, the expected life span of the adult worm. The program started in nineteen African countries and has treated more ten million individuals.

The present contraindication criteria for ivermectin treatment include pregnancy; age <5 years or wt <15 kg and breast-feeding women up to one week after birth. Sickness in individuals or concurrent illness should also be borne in mind in the treatment campaign. The involvement of the central nervous system (CNS) in the pathogenesis of the infection needs to be considered especially the life threatening adverse reactions to ivermectin treatment of patients infected with the filarial parasite *Loa loa* (Ducorps et al 1995). Currently, with the introduction of the Mectizan-Albendazole program for lymphatic filariasis, the mechanisms for handling and minimizing the problems where the two diseases are co-endemic is considered essential part of control program management.

In conclusion, ivermectin alone has been extremely successful so far in the aim of eliminating onchocerciasis as a public health problem. However, elimination of transmission has proved to be more difficult.

It is worth noting that research is going on to identify a new drug that can kill adult worms without harmful side effects. Current studies are focused on the potential of moxidectin as a macrofilaricidal, the potential for existing antibiotics as alternative treatments for the elimination of *Wolbachia* as a target for drug development; and the development of methods for detection of ivermectin resistance.

Nodulectomy

Surgical removal of nodules from accessible parts of onchocerciasis patients helps in reducing the parasite burden in the body and hence decreases the severity of the disease. But many nodules are not easily detectable. This is not a feasible way of control strategy as it is very cost ineffective, and needs hospital support which is not available in most situations or cases of the remote areas where the disease prevails.

Rationale and Objective

Onchocerca species induce both cellular and humoral immune reactions. The intensity of these reactions varies considerably with the clinical manifestations of the infected hosts. Infections are chronic, and persistence of the parasites for several years argues for highly adapted mechanisms of immune evasion. Cellular responses to parasite antigens are suppressed in individuals with high parasitemia "generalized onchocerciasis", unlike in patients either free from infection, or those manifesting localized disease with very low live microfilarial loads if any. The mechanisms that control the levels of circulating microfilariae *in vivo* are not well understood. Speculations to the process of *in vivo* killing of the microfilariae have been made. Onchocerciasis persists in most of the treated patients who continue to live in endemic areas. A degree of resistance to the infection may exist. Many questions in onchocerciasis concerning the interplay between infection status and the balance between the immune responsiveness and immune modulation remain to be addressed.

Microfilaricidal drugs are thought to somehow involve the immune system in their killing mechanism. It has been proposed that their action involves unmasking previously hidden parasite antigens and thus stimulating antigen presentation to the effector cells.

This study is an attempt to gain better understanding to the role of the immune system in the development of pathology and varied clinical presentations seen in onchocerciasis patients. It is also intended to identify any role the immune system might play in the microfilaricidal action of ivermectin (Mectizan).

Objectives:

1/To characterize humoral and cellular immune responses in onchocerciasis patients with different clinical presentations

2/ To study the effect of ivermectin treatment in these patients and to see if the immune system mediates the outcome of treatment.

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Chapter Two

Immune Responses Against Microfilariae are Associated with the Severity of Onchodermatitis and Treatment History

This Chapter has been presented at the American Society of Tropical Medicine & Hygiene (ASTMH) 50th meeting

INTRODUCTION

Onchocerciasis is a filarial infection characterized by a wide variety of ocular and dermal pathology, the latter ranging from minor irritation to severe reactive dermatitis and in many cases includes visual loss. The basis for clinical variations in this disease is believed to primarily involve an intimate relationship between the parasite and the host's immune system (Mackenzie *et al,* 1980,1985, 1987). The interaction between the immune response, the major pathology-inducing event (microfilarial destruction), and other specific events that lead to this parasite-induced damage in all likelihood lies at the basis of all of the clinical presentations of this varied and complex disease.

Onchocerca volvulus microfilariae (Mf) have long been known to be involved in the acute papular dermal responses and the typical punctate keratitis seen in the corneas of affected people (Mackenzie *et al,*1985, Williams *et al,* 1987). It has also been assumed that the worsening clinical presentation of chronic papular dermatitis and other more severe changes seen in onchodermatitis involve inflammation related to microfilariae and their destruction. Although other theories have been suggested as contributory to the pathogenesis, autoimmunity and secondary infection to name two, the most likely hypothesis is that increasing cycles of inflammation involving microfilariae are central to all these clinical reactions (Mackenzie et al, 1985).

Specific antibodies are generated against many *O.volvulus* antigens and various studies have been directed at describing them (Mackenzie et al, 1985; Green et al, 1981; Garate et al, 1996; Gallin et al, 1995; Wani, 1997). Antibodies specifically involved in the destruction of *O.volvulus* microfilariae through antibody-dependent cell cytotoxicity mechanisms have been described previously (Green,1981; Mackenzie et al, 1980; Williams et al, 1987). Cell mediated immunity (CMI) has also been described as being involved, and studies on certain clinical forms of the disease such as reactive onchocercal dermatitis ("sowdh") are known to involve CMI (Bartlett et al, 1978; Green et al, 1985; Elkhalifa et al, 1991; Mackenzie et al, 1985; Baraka et al, 1995; Darge et al, 1995). It is assumed that *in vitro* cellular responses to adult worm antigen cocktails also includes activities stimulated by microfilarial stage antigens, as microfilariae are a significant component of adult female worms.

In the current study we investigated the significance of these immune responses directed against microfilariae in onchocerciasis patients presenting with two major forms of dermal disease: mild or asymptomatic, and severe dermal manifestations. We examine these immune responses to determine the significance of reactions to *O.volvulus* microfilariae in the pathogenesis of disease in these two clinical groups.

Patients and Methods

Study populations:

The patients examined in this study became infected in either of two Sudanese onchocerciasis endemic areas; the south west (Bahr el Ghazal) or the Equatorial regions of Southern Sudan (Bryant et al., 1935; Mackenzie et al., 1987). They were displaced to the North due to civil unrest and settled in camps around the capital Khartoum.

The clinical classification of patients (n=28) in terms of onchocercal dermatitis was carried out using the schemes developed by Mackenzie et al. (1985) and Murdoch et al. (1993). The patients were grouped in two categories: Group A - mild (minor acute change) or asymptomatic (no obvious dermal change) onchodermatitis (n=12), and Group B – Severe (active and chronic skin changes) onchodermatitis (n=16).

A subgroup of nine patients (seven from group A and two from Group B) was studied after they had been treated with therapeutic doses of Mectizan® (150ug/Kg). Control sera and cells, obtained from a group of individuals who were resident outside the areas endemic for this disease and who had never been diagnosed as being infected with *O. volvulus*, were used in cytoadherence assays (n=2) and stimulation index (n=5).

Parasite antigen preparation:

Nodules from Sudanese patients were digested with collagenase (Schultz-Key et al. 1977), and the released adult worms homogenized using a tissue grinder; insoluble material was removed by ultracentrifugation. Soluble *O.volvulus* antigen (OvAg) was passed through a 0.2micrometer syringe filter and the protein concentration was adjusted to 1mg/ml (Lowry et al, 1951).

Microfilariae:

Two onchocercal nodules were surgically removed from patients and kept in RPMI-1640 media on ice for transport to the laboratory, cut into small pieces using sterile surgical blades and the Mf were allowed to emerge. The medium containing released Mf was collected and transferred to sterile petri dishes (Ngu et al, 1981). Equal volumes of 0.80% agarose dissolved in RPMI-1640 medium by heating and cooled to 40^0C were poured into the petri dishes and the plates then carefully rotated to mix the microfilariae suspension with the agarose. The solution was then allowed to solidify at room temperature. 10 ml of sterile RPMI-1640 medium were carefully layered on the agarose surface and the plates were incubated at 37^0C for 40 min. Live Mf that had migrated to the RPMI-1640 overlay were aspirated into 15 ml centrifuge tubes and centrifuged at 1000 rpm for 3 min, the media decanted and the Mf washed twice with RPMI-1640 by centrifugation as above. Mf were then finally suspended in RPMI-1640 medium containing 10% (v:v) heat inactivated fetal calf serum, counted and adjusted to 1000 mf/ml.

Cells:

Eosinophil Rich Population for Cytoadherence

Heparinized blood (20 ml) was collected from onchocerciasis patients whose white blood cell differential count was >43% eosinophils. 1/6 (v:v) of 4.2% dextran in phosphate buffered saline (PBS) was added to the blood in a centrifuge tube and mixed well and incubated at 37^0C for 40 min in an upright position. The superficial cellular layer was then carefully aspirated into RPMI 1640 and the cells pelleted by centrifugation at 1200 rpm for 10 min, washed twice in RPMI-1640 and finally resuspended for use at $4x10^6$ cells /ml.

PBM Cells for Transformation Cultures

Whole blood was obtained from each subject, and peripheral blood mononuclear cells (PBMC) were separated by centrifugation over a gradient of Ficoll-Hypaque (Histopaque 1077, Sigma Corp.) and adjusted after washing to the required cell concentration. PBMC were suspended in RPMI-1640 (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT), 2 m ML-glutamine, 1m M HEPES, 0.04 mM 2-mercaptomethanol, and 80 microgram of gentamycin, washed and counted.

Assays

Cyto-adherence Test:

Fifty microlitres of Mf suspension (containing 50 Mf) were pipetted into the wells of 96 well flat bottom tissue culture plates. The same volume of undiluted

serum from each patient or control was added to duplicate wells. The plate was then incubated at 37°C for 1 hour to allow the antibody to bind to Mf. 100 microlitres of eosinophil suspension ($4x10^5$ cells) were added to each well. Adherence was defined according to an estimation of the area of the parasite's surface covered with cells. The following scale was used as a guideline:

0 = none, or only a few cells adherent

1 = up to 25% surface covered with adherent cells

2 = 25-49% surface covered

3 = 50 – 74 % surface covered

4 = 75% to completely covered

Cultures were monitored, using an inverted microscope, after 3 hr incubation; and they were also checked at 24 hr to ensure no false negative assessments had occurred. At least 30 Mf were assessed before recording the adherence score for a particular culture.

***In - vito* blastogenesis**

Blastogenic responses of (PBMC) to mitogens and antigens were measured using a [H^3] thymidine incorporation technique. $1x10^5$ cells in 100ul RPMI 1640 were pipetted into the U-shaped wells of a 96 microtitre tissue culture plate (Corning, NY). Cells were stimulated by 10 ul of phytohemagglutinin (PHA) mitogen (Sigma) at 5 ug/ml or with Ov Ag at 20 ug/ml. The cells ($3x10^5$ /well) were distributed in triplicate into 96-well round-bottomed microtiter plates (Nunc A/S, Roskilde, Denmark). The cultures were incubated for 5 days at 37°C in a

humidified atmosphere of 5% CO_2 in air, with a final volume of 200 ul/well, in the presence of 20 ug/ml phytohemagluttinin (PHA - Pharmacia, Upsala,Sweden), or for specific stimulation, with 20 ug/ml of OvAg.

Eighteen hr before harvesting, 1 Ci of [^{3}H]-thymidine (Amersham International, Amersham,UK) with specific activity of 5 Ci/mmol, was added to all wells, including negative controls i.e. those without antigen or mitogen. Cells were harvested on fiber filters (Titertek, Flow Laboratories Inc.) Incorporation of [^{3}H] thymidine was measured in a beta-scintillation counter (Becton Dickenson Ltd). Results were expressed as the stimulation index (SI) derived from the counts per minute (cpm) of the average of three replicate wells containing antigen minus the background counts of non-stimulated wells; results are expressed as counts x 10^{-3}.

Statistics

Groups were compared using the SSPS program and significance determined using Student's t test, the paired "t" test and Pearson Correlation test

RESULTS

Clinical Presentation and Parasitology

Twelve patients (Group A) had a dermal presentation of essentially normal skin or with minor acute papular reactions. Sixteen individuals (Group B) had severe onchodermatitis typified by chronic indurated papules, extensive pigment variation, thickening of the epidermis, edema of the dermis and severe persistent pruritus. O.volvulus microfilarial loads were highest in the mild skin disease group (mean of those measured = 27.7 +/12.9), whereas markedly lower microfilarial loads were evident in the severe skin disease group (mean of those measured = 7.9 +/- 5.5). Thus there was an inverse relationship between the microfilarial count and the severity of clinical manifestation (Table 1). The microfilarial loads in Group A were significantly more than those of Group B ($p>000.1$) as determined by Student's t test.

Antibody Mediated Responses

The two groups differed in the ability of the serum to mediate eosinophil adherence to the surface of microfilariae also showed a difference between the two groups. Patients from the mild dermatitis group showed minimal positivity (average = +) in the cell adherence assay (Table 1), whereas sera from those with severe dermatitis mounted strong responses in the cell adherence assay (average = +++). The adherence system was optimal at 3 hr, and observation at 24 hours did not reveal any late developing adherence reaction.

Cell Immune Responses

Cell proliferation responses to O.volvulus parasite antigens (Ov Ag) - (Table 2) were higher (12.3 +/- 1.9) in patients with more severe skin manifestations (and lower Mf loads). The proliferative index in the mild dermatitis group was 2.9 +/- 0.6. This difference was statistically significant (p= >00.1). The responses to OvAg in control individuals (4.5 +/- 0.4) were similar to those in Group A (those with mild skin disease). PHA responses were measured with one group (Group A): these are within the range expected and indicate that the culture system being used was technically adequate.

Associations between Antibody and Cell Mediated Immune Responses:

A positive relationship between the presence of the ability to mount an active cell adherence response and an active cell mediated response was also seen. Using a Pearson Correlation this was seen to be a significant relationship.

Post Mectizan® Therapy

The cell-mediated responses of 9 patients (7 from Group A and 2 from Group B) were assayed after treatment with therapeutically with ivermectin and were increased in all cases, significantly in those with mild disease (Group A). This was more obvious in the mild/asymptomatic patients (average increase > 2.5 X) than in the two severe dermatitis patients, in which the increase was minimal (Figure 3).

Discussion

It is well accepted that the pathological changes in onchocerciasis are related to the microfilarial stage of this nematode; however, it has not been clear as to the exact role of the immune system in this disease. Immune responses to *O.volvulus* are well long documented (Green et al, 1981; Mackenzie et al, 1980; 1985a; 1985b) but correlation of host responses with individual patient's clinical status has remained uncertain at best. The results of the present study show that two types of immune response mounted against *O.volvulus* antigens (including microfilariae) differ between patients with the two major forms of dermal presentation of onchocerciasis. This finding is in keeping with various published studies, but is the first study that clearly identifies different immune responses against the microfilarial stage as being directly related to the dermal clinical presentation.

A distinct positive correlation between immune response and clinical severity is seen. Corresponding to the increased immune response, and the expected increase in destruction of microfilariae, is a decrease in microfilarial load with severity of skin disease. Relatively low dermal Mf levels are seen in individuals with clinically obvious skin disease and active pathology (group B in this study). Very low levels of living microfilariae have been described in patients with the severe onchocercal skin disease known as "sowda" (or reactive onchodermatitis) (Bartlett *et al,* 1978; Baraka *et al,* 1995). This situation contrasts with the popular belief that microfilarial loads simply increase with duration of

infection, and that disease severity parallels these increased parasitic loads. The host's ability to destroy the microfilariae is at least as an important factor in the clinical outcome as is the duration of infection.

Individuals suffering from mild dermatopathology (group A) were less effective than severely affected patients in promoting eosinophil adherence to *O. volvulus* mf, and therefore probably less effective at destroying mf through an antibody mediated process. This finding that may explain in part their clinically quiescent state when compared to the severely affected group, whose serum mounted stronger responses against the worm surface. Adherence correlates with clinical manifestations associated with microfilarial destruction (Mackenzie et al, 1985). Likewise, observations on patients who were recently treated with ivermectin showed immunological signs of increased responses against these parasites.

The sequence of events and consequences associated with the death, breakup and removal of microfilariae are still open to conjecture. Immune, non-specific and drug-induced pathways can all occur at different times in this disease. This present study emphasizes the immune component and suggests that both antibody and cell mediated immunity are involved. Other studies have shown that the antibody response to microfilariae varies with time in an episodic manner (Mackenzie et al, unpublished). The death of microfilariae commonly induces a local inflammatory reaction (Mackenzie *et al,* 1985) and when killing is

induced by chemotherapeutic agents such as diethylcarbamazine severe anaphylactoid Mazzotti reactions can occur; these occur less with the now standard microfilariacidal agent for onchocerciasis, Mectizan®. Control of the associated pathogenic side reactions is important, especially in those individuals who react severely. Understanding the role of the immune response in the induction of these reactions, and the extend to which this response accounts for the drug effect is important.

Ivermectin appeared to enhance the cell-mediated responses in onchocerciasis patients when examined one to three months after treatment. This suggests that the immune system plays a significant role in chemotherapy if not in the initial induction of treatment, then at least in maintaining the ongoing mf destruction. The consequences of altered immune responses for the effectiveness of microfilariacidal regimes deserve examination, particularly in light of the suggestion that Mectizan® is sometimes minimally effective in certain individuals (Ali et al, in press). There are suggestions that ivermectin is less effective in small subset of infected individuals. This may involve a deficit in their immune response. Our results provide support for the involvement of the immune system in the acute and subsequent actions of ivermectin.

The finding of an inverse relationship between microfilarial load and severity of skin disease has important implications for control programs that are based on the assumption that microfilarial load is directly related to disease

status. Such programs should necessarily include those individuals with lower levels of infection if one wants to alleviate the pain and discomfort of those severely affected. Currently, mass drug administration programs often exclude hypo-endemic areas (APOC, WHO) and thus may miss areas where people suffer from severe onchodermatitis. This policy should be revisited to take account of those most severely affected with this disease, a policy that is incorporated into other filarial disease program strategies, such as that of morbidity control in the lymphatic filariasis elimination program. These findings also underline the importance of including clinical evaluations in epidemiological assessments of disease prevalence instead of relying on parasitological parameters alone.

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Table 1. In vitro cyto-adherence activity of serum from onchocerciasis patients with different clinical presentations and controls using eosinophils and Onchocerca volvulus microfilariae.

Patient status	Mf/mg	3 hour incubation
Asymp/Mild (n=9)		
	48.7	++
	37.9	+
	32.8	+
	25.7	-
	18.6	+
	Nd	++
	19.1	-
	Nd	-
	11.2	++
AVERAGE	***27.7 +/-3.9***	**+**
Severe (n=7)		
	0.9	+++
	3.7	++++
	12.8	+++
	8.7	++
	Nd	+++
	Nd	+
	13.4	++
AVERAGE	***7.9 +/- 2.8***	**+++**
Control (n=2)		
	0	-
	0	-
AVERAGE		**-**

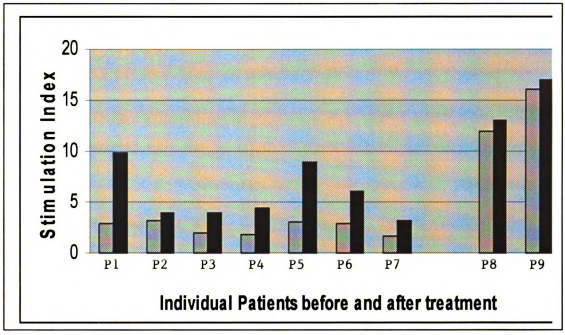
Table 2

Mitogen or Antigen	Mild skin group (SI) (n=12)	Severe skin group (SI) (n=16)	Negative controls (SI) (n=5)
PHA	121.15 +/- 7.5	21.5 +/- 3.7	132.6+/- 8.7
OV antigen	3.0 +/-0.6	12.3 +/-1.9	4.5 +/- 3.6
PHA+OV antigen	44.7+/- 9.7	ND	ND

Negative: never been diagnosed as onchocerciasis positive or suffered from any corresponding disease.

FIGURE 2.

CHANGE IN IMMUNE RESPONSE AFTER TREATMENT WITH MECTIZAN®



- Individual Patients:

  P1-7 = patients with asymptomatic/mild dermatitis

  P 8-9 = patients with severe dermatopathology

All tests were carried out 2 months after standard treatment with Mectizan®

Chapter Three

Immunocompetence may be important in the effectiveness of Mectizan® (ivermectin) in the treatment of human onchocerciasis

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The microfilariacidal agent Mectizan^{R} (ivermectin) has been donated since 1987 by Merck & Co. Inc., and has provided for the first time a feasible chemotherapy for treatment and control of onchocerciasis. This drug has been central to the ongoing efforts to combat this disease through self-sustainable community-based treatment. Original studies showed that annual doses of Mectizan^{R} reduced the microfilarial loads to, and maintained them at, very low levels for 9 months to a year after a single treatment (Awadzi et al, 1989; Brown & Neu, 1990; Ette et al, 1990); thus for mass drug administration a single annual dosing regime was recommended. As this drug does not kill adult worms it is believed that these annual dosing programs should continue for at least 12 years, the average of the adult parasite life span. The possibility of parasitological unresponsiveness to Mectizan treatment or selection for drug resistance in Onchocerca volvulus has been proposed (Grant, 2000; Boussinesq and Gardon, 1999), and here we report from Sudan our own findings concerning the possibility of reduced effectiveness of the drug in some onchocerciasis patients.

In our continuing effort to better understand the effects of Mectizan^{R} Treatment we have studied displaced people originally coming to Khartoum from Southern Sudan. Patients with onchocerciasis are usually expected to receive parasitological and clinical relief from Mectizan® (150 microgram/Kg-body weight) for at least 10 months after their initial

treatment; suppression of pruritus usually occurs for at least 12 months (Brieger et al, 1998). A group of 47 patients reported back to our clinic unexpectedly well before this time period complaining of a recurrence of the same intense pruritus that had affected them before the initial treatment. This pruritus was clearly distinct from the post treatment reactions often seen shortly after administration with this drug. A second group of 12 treated patients responding in the manner that is normally expected after treatment, i.e. suppression of pruritus for at least and usually longer than 10 months, were also studied for comparison.

Entry of individuals into the Mectizan ® treatment program required a baseline clinico-parasitological workup, including microfilariae (mf) loads in skin of the iliac crest region as assessed by the standard skin snip technique and the recording of any onchocercal nodules detected by palpation. Cellular immune responses to O*nchocerca* volvulus , namely antigen specific cell transformation of blood lymphocytes from these patients were carried out in standard lymphocyte - thymidine incorporation assays using parasite antigen derived from O. volvulus. Cells isolated on Ficol Hypaque gradients were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum at a final cell suspension of $3x10^5$/ml with O.volvulus parasite antigen (20ug/ml); control cultures with media only were run simultaneously. The results were expressed as the stimulation index -SI (counts per minute of stimulated cultures divided by those of unstimulated cultures).

The patients who returned to the clinic with recurrent pruritus represented less than 10% of the total number of individuals treated in this particular program. These returnees where categorized according to the time at which they reported back after their treatment with Mectizan® (either 0-1 month, 2-3 months, or 4-6 months). They were found to have significantly higher loads of dermal microfilariae than the control group (Table 1; Figure 1); e.g. 68.4% of pretreatment loads at 4-6 months. Those who responded to treatment as expected, and did not complain of recurrent pruritus in this early period after receiving Mectizan®, maintained very low levels of dermal microfilariae for an extended period of time (rising from only 1.8% at 1 month to10.1% of pretreatment levels at 4-6 months after treatment).

There was a reduced proliferative response to Onchocercal antigens in the early returning groups. The average stimulation index (SI) for the group that came back after one month was significantly less (2.9+\-0.6) than the control group (12 normally responding onchocerciasis patients - 12.3+\-1.9; $p<0.005$). Those who came after 2-3 months had average SI of 3.2+/- 0.7 and the last returning group an average SI of 5.6+/-0.8 (Figure 2).

The reason for this reduced ability of Mectizan® to maintain low levels of dermal microfilariae, and its inability to reduce the major clinical

feature of dermal onchocerciasis - pruritus, can only be speculated upon. All these patients were displaced due to civil unrest and had remained outside the onchocerciasis endemic zone for more than 15 months, and thus there was no likelihood of re-infection being the cause of the recurrent pruritus. Many of them carried onchocercal nodules and all may have had non-palpable adult containing lesions. Such nodules may serve as sources of new dermal microfilariae.

The recurrence of pruritus, and apparent resurgence in dermal microfilariae, may involve a weakening of the paralytic effect of this drug on the release of uterine microfilariae in the adult worms, or may, as supported by our findings, reflect an inability of the host's immune system to contribute to drug-initiated microfilarial destruction. Such immune mechanisms thus may be less effective in the patients who show poor parasitological responses to ivermectin, and lead to the poor clinical response, i.e. the persistence of pruritus.

Our findings differ from previous reports where one annual dose appeared to be sufficient to curtail microfilarial loads for much longer periods before any resurgence in parasites occurred (Awadzi *et al.,* 1989; Brown & Neu 1990; Ette *et al.,* 1990; Brieger et al., 1998). This phenomenon, which although occurring only in a small proportion of patients, could have a negative effect on general patient participation in a control program: disappointment with the effectiveness of the treatment

could discourage the population at large. It also suggests that, at least in the treatment of some individuals, Mectizan® may need to be administered more frequently than once a year as suggested. Such a necessity also could have important implications to control programs as has been discussed previously by Richards *et al.* (2000). Lastly, our observations support the intriguing possibility that there is an active involvement of the immune system in the mechanism of action of ivermectin, a concept that needs further investigation.

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Table 3.

Dermal microfilarial loads of the Onchocerciasis Patients Before and After Treatment with Mectizan®

TIME OF OBSERVATION AFTER TREATMENT (WEEKS)	POOR REPONDER GROUP 1 [1] (N=16)	POOR RESPONDER GROUP 2[1] (N=13)	POOR RESPONDER GROUP 3[1] (N=18)	NORMAL RESPONDER CONTROL GROUP[2] (N=12)	SIGNIF[3]
O	113.5 +/- 11.9	128.2 +/-10.7	139.9 +/- 11.9	99.1 +/- 21.5	NS
<4	28.8 +/-3.6	NA[4]	NA	1.8 +/-1.1	<0.005
4 – 12	NA	45.8 +/- 6.1	NA	3.6 +/- 2.3	<0.005
13 – 24	NA	NA	95.7 +/- 9.7	10.0 +/- 4.1	<0.005

1. *Poor responders are grouped into three time periods after treatment depending on the actual time at which they sought re-treatment for recurrent pruritus.*
2. *Patients who had the expected response to treatment and whose pruritus had been continually suppressed since after treatment.*
3. *Significance of the difference between the mf loads of the recurrent pruritus and normal responding groups at each of the time points; Student's t test where NS (not significant) is > 0.05.*
4. *NA = not applicable.*

FIGURE 3.

Recovery of Dermal Microfilariae After Treatment with Mectizan®.

% of Pretreatment Dermal Microfilarial Load

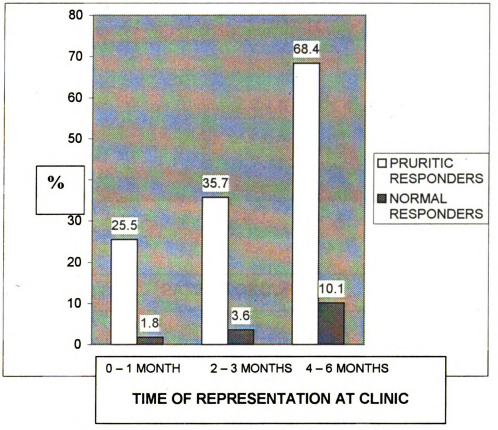
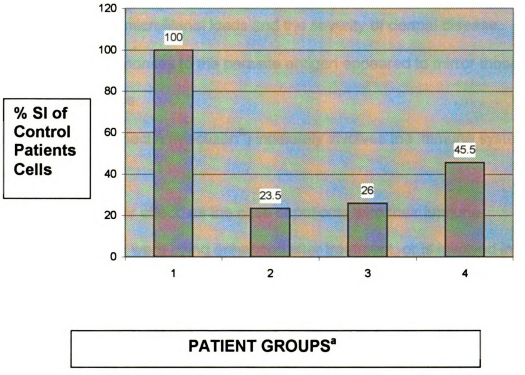


FIGURE 4.

Lymphocyte Transformation in Response to Culture with *Onchocerca volvulus* Specific Antigen.



a. Patient groups

1. Control patients
2. Those returning in less than I month
3. Those returning at 2-3 months after treatment
4. Those returning at 4-6 months after treatment

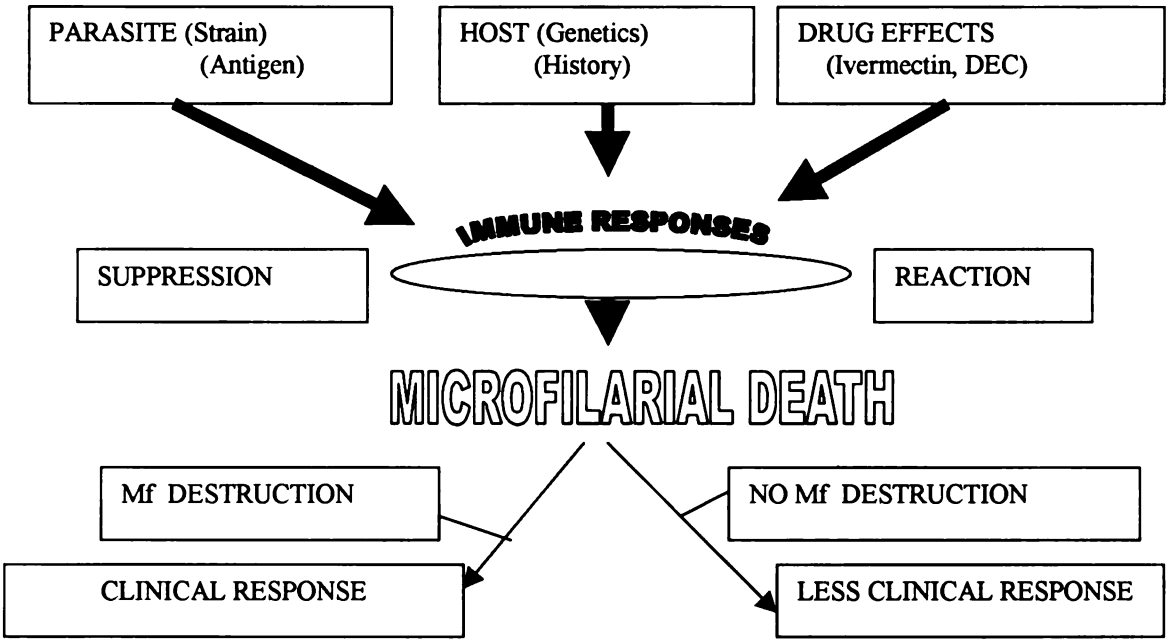
Summary

- Both Cell Mediated Immunity (CMI) and Antibody Mediated Immunity (AMI) are involved in the development of dermal pathology in onchocerciasis
- There is a positive relationship between cell cyto-adherence, active cell mediated immunity, microfilarial loads and the severity of dermal disease.
- Cellular immune responses to the parasite antigen appeared to mirror those of humoral responses.
- Treatment with ivermectin (Mectizan^{R}) intimately involves the immune system, either:

    As an "after effect" as the case of patients who their immune responses were being enhanced after treatment, or is involved in the drug's action as suggested for the returnee group of patients. Immunologically compromised individuals may not respond efficiently in treatment.

- The importance of including clinical evaluation in the epidemiological assessments of disease prevalence rather than relying only on parasitological parameters.
- The mass drug administration programs should not exclude hypoendemic areas in order not to miss areas where people suffer the most from the severe onchodermatitis.

Figure 5.


PARASITE (Strain)
(Antigen)
HOST (Genetics)
(History)
DRUG EFFECTS
(Ivermectin, DEC)
IMMUNE RESPONSES
SUPPRESSION
REACTION
MICROFILARIAL DEATH
Mf DESTRUCTION
NO Mf DESTRUCTION
CLINICAL RESPONSE
LESS CLINICAL RESPONSE