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presented by

SEYOUM TATICHEFF

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IMMUNE RESPONSES TO *SIMULIUM* BITES AND THEIR RELATIONSHIP WITH THE PATHOLOGY OF ONCHOCERCIASIS IN BEBEKA, ETHIOPIA

BY

SEYOUM TATICHEFF

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ABSTRACT

IMMUNE RESPONSES TO SIMULIUM BITES AND THEIR RELATIONSHIP WITH THE PATHOLOGY OF ONCHOCERCIASIS IN BEBEKA, ETHIOPIA

BY

SEYOUM TATICHEFF

Onchocerciasis is a helminth induced disease of major health and socio-economic problems in tropical Africa and Latin America where it is endemic. The clinical picture of the disease has a wide spectrum ranging from the absence of any obvious clinical manifestation in-spite of the presence of the infection agent to the occurrence of a varying degree of dermal lesions with blindness as the most severe sequel in progressive chronic conditions. The parasite, Onchocerca volvulus is transmitted by black flies (Simulium spp.). Although controversial opinions exist, repeated bites by the vectors have, on numerous occasions, been incriminated in independently inducing immunologically mediated inflammatory responses, possibly resulting in dermal lesions mimicking those caused by an onchocercal infection.

In this study, an attempt was made to determine whether or not black fly bites have, in their own right, the ability to cause lesions similar to onchodermatitis.

The approach taken in the study is the first of its kind. It essentially involves the use of physical, parasitological, immunological and histological procedures. These were carried out with samples

collected cross-sectionally, and as well as longitudinally during the course of periods defined as pre-rainy, rainy and post-rainy seasons in Bebeka, Ethiopia. The specific methods of laboratory examination included skin snip for microfilarial detection, intradermal challenges with antigenic material from black fly saliva and O. volvulus L3, histological observations of biopsies made from skin test reactions and immunoblot profiles using patients' sera and black fly and O. volvulus antigens probed with IgG and IgG1-4.

Results of the hypersensitivity study as well as the immunoblots and the histological observations indicate that there is an immunological basis for the reactions to Simulium bites and that these reactions are seasonally influenced. Evidence to support the notion that the responses to Simulium were causal in the morbidity changes seen over the course of onchocerciasis season was obtained. There was evidence of antibody formation in all IgG isotypes with increased prevalence of band recognition during the study period, and prominence of an antigenic protein with Mr. 60KD in the salivary antigen was observed.

On the basis of the in vivo and in vitro observations, the hypothesis that black fly bites contribute to the clinical manifestations of onchocerciasis is supported. Further follow-up studies are suggested.

TO HIRUT

For her patience, encouragement and love

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INTRODUCTION

This study entails an investigation into the possible contribution of host immune reactions to Simulium bites to the pathogenesis and clinical manifestations of onchocerciasis in endemic areas. Although skin reactions to bites of Simulium are commonly seen, very little information is available on this subject in the literature. An additional problem in approaching this research topic is that the sequence of pathologic changes and skin manifestations which appear in onchocerciasis, as well as the mechanisms involved in skin pathology, have not been fully characterized.

The differentiation of lesions due to Onchocerca volvulus and those specifically due to Simulium bites has been controversial. Gibbins and Loewenthal (1933) were the first to suggest that many of the onchocercal skin changes were, in fact, due to Simulium bites. Their hypothesis has been supported since then by Browne (1960), Edungbola et al. (1983) and Fuglsang (1983). More recently, however, Connor and Palmieri (1985) have challenged the proposal, and it remains unresolved whether or not the blackfly bite has, in its own right, the ability to cause dermal manifestations mimicking onchocercal lesions.

Very little experimental work to address this question has been attempted, despite recent advances in our understanding of immunological responses to bacterial, viral, protozoal, and helminthic infection This is in part because host reactions to arthropods have been rather

neglected. The limited amount of work done on arthropod infestations in general may be explained by the difficulty of obtaining manageable supplies of the insects, and especially of securing sufficient quantities of their antigenic secretions. Nevertheless, because of the improvements made in the laboratory colonization and the subsequent collection of secretions of some arthropods, our perception of the role played by the saliva of such ectoparasites in host hypersensitivity has improved in recent years. Comprehensive reviews of immune responses to arthropods and their products have been published by Feingold et al. (1968) and Wikel (1982).

It has been known for a long time that people in endemic areas of onchocerciasis and elsewhere complain of hypersensitivity due to bites by Simulium spp. Stokes (1914) wrote an extensive article with a complete bibliography on the early reactions to Simuliid insects. Winkler (1951) observed severe erythematous and edematous reactions persisting for one week in patients bitten by blackflies in Germany. There is therefore precedent for the approaches taken in this study in which attempts are made to answer the following specific questions: a) How much of the dermal pathology and clinical manifestations of onchocerciasis (eg. chronic pruritus, erythema and papules progressing to pigmentary changes, loss of skin elasticity and eventually atrophy) is mimicked or accentuated by the superimposed reactions to Simulium bites? b) What are the components of hypersensitivity/allergic reactions to Simulium bites i.e. what are the allergic constituents of the blackfly adult homogenate and what are the types of human antibody responses? c) Do these reactions change demonstrably over the course of a Simulium exposure season? d) What are the histopathological changes

induced by hypersensitivity to black flies? It is hoped that the results emanating from this study will help in the understanding of onchocerciasis in Ethiopia where, up to the present time, very little work has been done.

LITERATURE REVIEW

It is beyond the scope of this review to present a detailed account of all facets of human onchocerciasis. Nevertheless, the thesis deals with some aspects of this disease and with patients identified as being infected with O. volvulus, and therefore some highlights of a general nature are needed. A brief summary of onchocerciasis follows, describing the parasite, its vector and life cycle, geographic distribution, host-parasite relationships/immunology, diagnosis, clinical and pathological features, treatment, control and prevention. Onchocerciasis in Ethiopia is reviewed in part B.

A. General Aspects of *Onchocerca volvulus* Infection

1. The parasite, its vector and its life cycle

Onchocerciasis is caused by the ovoviviparous nematode *Onchocerca volvulus*. Taxonomically, this parasite belongs to the Phylum: Nematoda, Class: Phasmida, Order: Filariata, Family: Onchocercidae, Genus: *Onchocerca* and species: *volvulus* (Despommier and Karapelon 1987). The parasite is found only in man. With the exception of the chimpanzee which is susceptible to some strains, attempts to transmit this parasite to animals have failed.

Adult worms live free in the subcutaneous tissue or in fibrous nodules beneath the skin. The body of the adult is filiform, tapered at both ends and is white in color (Strong, 1934). The male adult measures from 18.2-32 mm in length by 0.13 to 0.21 mm at greatest width. The female is considerably longer and measures 355-500 mm by 0.27-0.4 mm. A detailed description of the morphology of the adult worms has been published by Neafie (1972). Adult worms can live for more than 12 years during which the females liberate large numbers of minute microfilariae (WHO, 1987). The microfilariae measure 220-360 by 5-9 microns (Faust et al., 1970) and migrate to the skin. The only known vectors of *O. volvulus* are the black flies (Diptera: Simuliidae). Two black fly species groups have been shown to act as vectors in the African region. Flies in the *Simulium damnosum* complex serve as the principal vectors in Africa and southern Arabia, and those in the *S.*

naevi group are a secondary vector in Eastern and Central Africa. In the endemic areas of Latin America a large number of anthropophilic flies have been incriminated.

Much remains to be learned about the life cycle of O. volvulus. It may be surmised that the parasite has five life cycle stages: the adults, the microfilaria (L1?), the microfilariae to infective larva transient stage (L2), the infective or third stage larva (L3), and the infective larva to adult worm transient stage (L4) (Figure 1).

In the process of feeding, female blackflies bite an infected person. The strong cutting mouthparts of the fly thus create a pool of blood just beneath the epidermis. The pool of blood remains uncoagulated, most probably assisted by the anticoagulatory property of the injected saliva (Hutcheon and Wilson 1953; Yang and Davies 1974). The parasite stages infective to the vector are imbibed along with the blood meal; they migrate to the thoracic muscles of the fly where they first develop to a rhabditiform and subsequently to a filariform larvae. The filariform larvae further migrate through the flight muscles to the proboscis and eventually are released at the fly's next meal of human blood, thus infecting a new host where adult males and females develop to maturity. About 6-10 days are required for the parasite to develop into an infective stage from the time it is picked up by the vector (Duke 1968,a). Parasite reproduction in humans is by mating of males with females, which become gravid. Microfilariae in the skin undergo no further development until they either die in the course of 6-30 months, or are ingested by a biting black fly (Duke 1981).

The period between the introduction of the L3 and the ability to

detect microfilariae in a new host, i.e., the prepatent period, ranges from 7 months to two years, while the clinical incubation period i.e., the time from the introduction of the L3 and the appearance of the first evident clinical symptoms and signs is more variable and may be longer (WHO 1987).

2. Geographic distribution

Onchocerciasis occurs in well defined areas throughout tropical Africa and Latin America. Because of the physiological requirements of the larvae and pupae, black fly endemic foci are usually near rivers or streams in hilly or mountainous regions.

In Africa, O. volvulus is endemic throughout the greater part of the tropical rain-forest regions and the savanna belt extending more than 6,500 km. from the Atlantic coast of Senegal to the Indian Ocean coast of Tanzania (WHO 1987). A small endemic focus was found relatively recently in Yemen by Fawary (1957).

Onchocerciasis in Central America has long been known to be endemic in the coffee-growing highlands of Guatemala and Mexico. The disease is also prevalent in Venezuela, Columbia, Ecuador and Brazil.

In the absence of data on the prevalence of onchocerciasis at global level, present assessments are extrapolations from relatively small population surveys. The World Health Organization (WHO 1987) estimates that over 17.5 million people in Africa, 20,000 in Yemen and about 100,000 people in Latin America are infected with O. volvulus. This same source indicates that over one third of a million people are victims of blindness attributed to onchocerciasis, and of the total

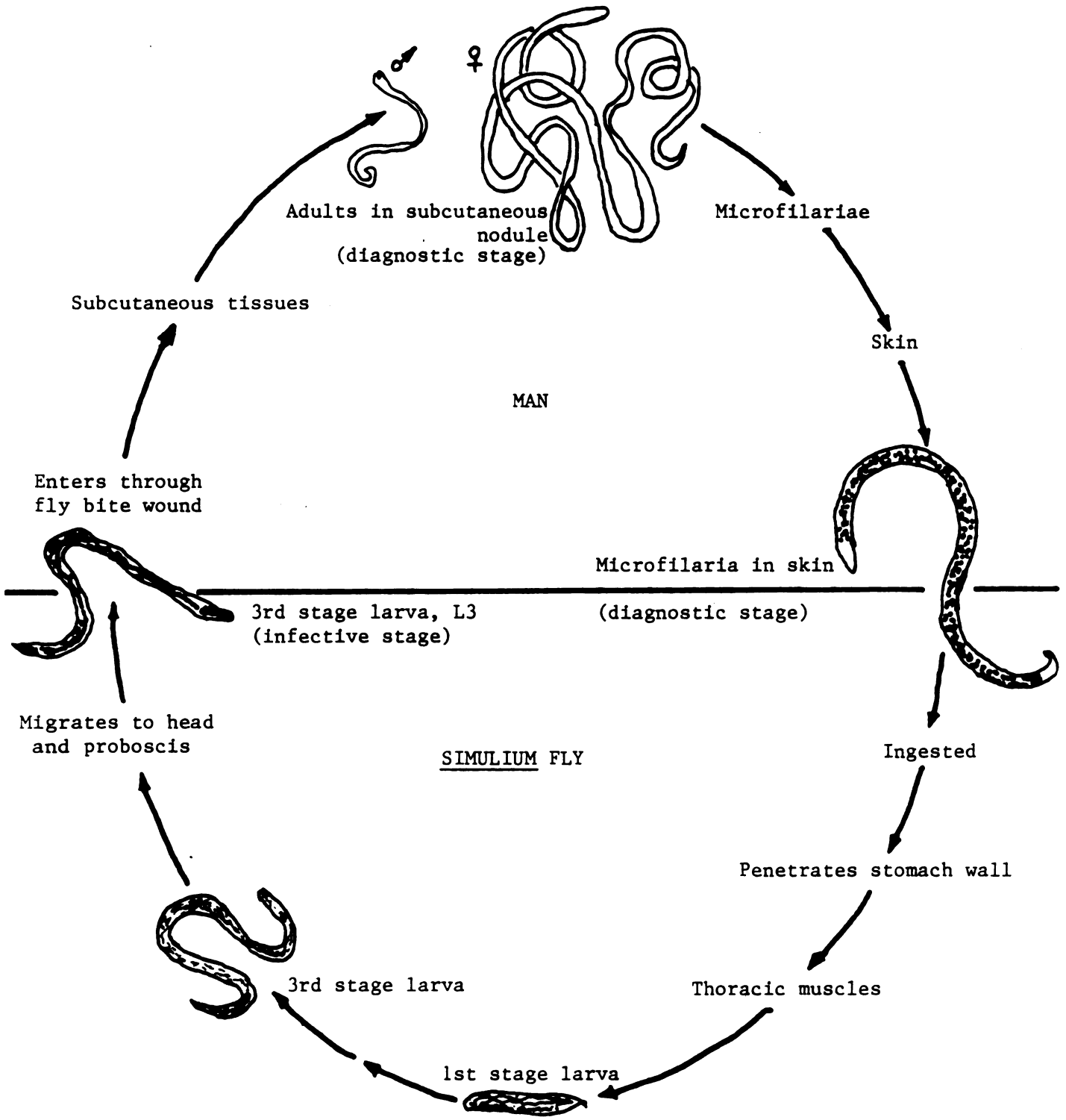

Figure 1. Life cycle of Onchocerca volvulus

population living in the endemic zones, 85.5 million are at risk of acquiring the disease.

The extent of propagation and the level of morbidity as well as the impact of the associated social and economic effects of onchocerciasis vary widely in different geographical areas where it is endemic. Nevertheless, infection has the potential to cause a severe decline in the quality of life, as a result of a series of intolerable skin changes (not rarely leading to suicide). There is a stigma associated with bodily disfiguration, and most importantly the disease can lead to blindness of a large proportion of the productive force in a community. Indeed, certain localities in West Africa have experienced disastrous economic losses because of onchocerciasis (Bradley 1975).

3. Host-parasite relationships/immunology

Host responses to the skin dwelling microfilariae of Onchocerca volvulus in man are still poorly understood. Factors contributing to the lack of information on host-parasite relationships are shortage of parasite material, the ethical prohibition of experimentation on human and other primates, and the lack of susceptible laboratory animals for infection with O. volvulus. Closely related veterinary parasites, however, have provided us with a means to study the immune response to microfilariae in some detail. Clinical observations made in the human host lead to two important questions: a) Does the host response contribute to the aggravation of the disease? b) Does the host response have any protective role?

A point which can not be overemphasized in an onchocerciasis population is the phenomenon of the negative correlation between

parasite load and clinical manifestations. It is not unusual to find patients with a sizable number of microfilariae in tissues, as well as the cornea, with virtually no visible response either in the skin or the eye (Mackenzie et al. 1985). The underlying reason for this phenomenon is not clearly understood yet. Conversely, some patients with a low number of demonstrable parasites are severely affected by the disease (Ghalib et al. 1987). On the basis of these kinds of data, it may be conjectured that variations in clinical pattern of onchocerciasis are very likely to result from host-parasite interaction where the host's immune defense system plays a pivotal role.

Information pertaining to the host's immune system vis-a-vis the chronological development and the prognosis of the disease, is scarce. Equally scanty information is available on the morbidity of the disease which is almost certainly attributable to the death of the microfilariae. Specific immune responses have a putative role in this event. One of the major barriers to understanding the immunology of onchocerciasis lies in the difficulties of characterization of the array of parasite antigens and the many types of host responses to these antigens which can come about.

Potentially, the number of antigens presented to the host at various stages of the parasite's life cycle is large. Some originate from the intact living worms, in particular, the secretory/excretory antigens (Schiller et al. 1980, Ngu et al. 1981) and, some from the cuticle region of microfilariae or the uterine components of adults (des Moutis et al. 1983). Others arise from degenerating microfilariae and/or other stages of the nematode (Mackenzie 1987). A third source

of antigens may arise during moulting in the process of parasite maturation in the human host (Anderson and Fuglsang 1977).

The problem of immunologic characterization of O. volvulus antigens is further compounded by a possible antigenic diversity between individual worms, and this also may lead to a qualitative or quantitative difference in immune response (Bryceson et al. 1976); by the cross-reactions of O. volvulus antigens with those from animal Onchocerca spp. (Ambroise-Thomas 1974); and by cross reactions with heterologous antigens derived from other nematodes (Phillip et al. 1982).

Despite the above problems, some immunological foundations have been laid. To start with, there is now ample evidence that the presence of O. volvulus in an infected individual is recognized early on by the host's immune system (Bartlett et al. 1978; Steward et al. 1982; Mackenzie 1987). The immune responses which follow, both humoral and cell-mediated, thereafter show considerable variation and it is therefore difficult to make generalizations. Infected individuals develop a high level of antibodies of various immunoglobulin isotypes including IgG, IgM, IgE and IgA (Buck et al. 1973; Ngu and Blacket 1976; Akiyama et al. 1981; Greene et al. 1983a). Kawabata et al. (1983), using ELISA, have demonstrated the presence of specific IgG, IgM, IgA and IgE immunoglobulins to O. volvulus adult antigen in Guatemalan patients. These observations indicate that this filarial infection leads to polyclonal and Onchocerca specific B-cell activation.

Some persons infected with O. volvulus have been shown to have a generalized decrease in cell-mediated immunity to onchocercal (Greene

et al. 1983b) and to non-onchocercal antigens such as PPD (Rougemont et al. 1977) and tetanus (Prost et al. 1983). Such an immune unresponsiveness has additionally been inferred from an increased prevalence of lepromatous leprosy in areas endemic for onchocerciasis (Prost et al. 1979). The basis for the hyporesponsiveness is unclear. However, recent work by Gallin et al. (1988) suggests that diminished CMI to parasite-related antigens might be attributed to a defect in the production of Interleukin-2 (IL-2) accompanied with a possible defect in T-cell activation.

The natural acquisition of protective immunity to O. volvulus infections in human is still questionable. Observations that after middle age, intensity of infections decreases with increasing age (WHO 1976), and that sera from infected individuals promote in vitro opsonization of infective larvae (Mackenzie 1980) and microfilariae (Greene et al. 1981), suggests that protective mechanisms may develop. However, Duke (1968b) emphasized that previous infection does not appear to result in protection in the field.

A recent report by Ward et al. (1988) where differences in Interleukin-2 production between "putatively immune" and infected individuals was found, has led to the suggestion that resistance is T-cell mediated.

4. Diagnosis

A good number of clinical signs and symptoms, dermatologic as well as ocular, are fairly specific to onchocerciasis. These may be used for provisional diagnosis in endemic areas without recourse to laboratory procedures.

The most appropriate method of demonstrating a cause and effect relationship in the diagnosis of any disease would be to establish the presence of the etiologic agent. In onchocerciasis, as in other helminthic infections, the adult or its progeny (microfilariae) could be used to ascertain the presence of the disease. This, however, may be problematical, either due to the adults being lodged in deep seated and unpalpable nodules, or due to the microfilariae being too few to detect. As a consequence, in spite of vigorous attempts, both nodules and/or microfilariae may not be observable even in the presence of an actively fulminating disease. It is also common to find people with onchocerciasis without any nodules. Most people in the present and other (Iwamoto et al. 1973) studies done in Ethiopia did not have nodules. Therefore, the absence of either nodules or microfilariae cannot be used as a definitive tool for differential diagnosis.

The alternative to diagnosis based on the detection of the adult worm or the microfilariae is through immunological methods. Immunodiagnosis in onchocerciasis is based on the detection of the antigens of the parasite or their antibodies in various biological samples. Again, the problem in this alternative is to find a method specific enough and at the same time able to differentiate active infections from those which have died out. Developments in the immunodiagnosis of onchocerciasis have been reviewed by Mackenzie et al. (1986) and it is evident that more rigorous evaluation is still necessary before any of them could be adopted for routine work. Methods presently available for positive diagnosis of onchocerciasis in the field are therefore based on the detection of either the adult worm or the microfilariae in the context of an appropriate clinical picture in an endemic area. This

is the basis on which diagnoses were made in the studies reported in this dissertation.

i. The adult worm

One of the sequela of onchocerciasis is the formation of nodules. Recognizing onchocercal nodules usually presents no problem to the experienced worker and was a routine feature of examination of Ethiopian patients in this study. Typical nodules measure 0.5-2 cm in diameter, and form a firm, round or elongate mass; they may or may not be lobulated, and generally cause no pain to the patient (WHO, 1987). Clinically, nodules may be confused with lymph nodes and should also be distinguished from lipomata, foreign body granulomas, sebaceous and dermoid cysts, ganglia and nodules from other causes, particularly cysticercosis and histoplasmosis. Reliable diagnosis can be made by surgical removal of the nodule followed by identification of the adult within. Excised worms in this study were used as an antigen source for immunoblot experiments.

ii. The microfilariae

The most common diagnostic procedure for onchocerciasis is the skin snip biopsy and the microscopic detection of the microfilariae in the biopsy tissue suspended in a liquid medium (commonly an isotonic salt solution or tissue culture medium, such as RPMI 1640). This test system was routinely employed in my work. The skin snip may be taken with a needle and a sharp razor blade, or even better with a corneo-scleral punch.

The optimum site for a relatively reliable skin biopsy is dependent upon the geographical strain of the parasite (WHO, 1976). Mengesha and Jembere (1975) in their work in the north-western part of Ethiopia - a savanna region - reported a greater yield of positive results in snips taken from the leg and thigh. We had previously shown (Taticheff et al. 1987) that in the south-western region of Kaffa, Ethiopia - a forest endemic zone - the best site for biopsy is the buttocks, and we used this site in the work reported here.

The skin snip procedure has been subjected to a number of studies in order to standardize the method and improve on its sensitivity (Scheiber et al. 1976, Braun-Munzinger and Scheiber 1977, Albiez et al. 1978, Taylor et al. 1987). The technique is easy and fairly reliable as an epidemiological and as a clinical tool.

An episode of allergic reactions following the administration of a single microfilaricidal dose of diethylcarbamazine (DEC) used as a provocative test was first described by Mazzotti (1948). The Mazzotti test has the draw-back of having a low specificity and sensitivity. The greatest shortcoming is, however, that the effects include the possibility of exacerbating or inducing ocular pathology (O'Day and Mackenzie 1985). For these reasons, the test as a diagnostic tool is recommended with reservations, and even this only in individuals in whom ocular involvements are absent and parasitologic tests have been repeatedly negative.

5. Clinical and pathological features

There is a wide spectrum of clinical responses associated with onchocerciasis, ranging from virtually no clinical change despite the

presence of numerous worms, to the occurrence of very severe tissue damage. Death of the microfilariae rather than the presence of the adult worms is associated with most of the tissue changes that contribute to the development of the various clinical manifestations (Mackenzie et al. 1985). In its classical form, onchocerciasis involves the skin, subcutaneous tissues, peripheral lymphatic vessels, lymph nodes and eyes. For this reason Ogilvie and Mackenzie (1981) have grouped the clinical appearances into four cardinal manifestations: dermatologic, lymphatic, general systemic and ocular.

At the dermal level, onchocercal dermatitis with itching and scratching, is the most important and early manifestation. Because of geographic and even individual variations of clinical manifestations (WHO 1987), the course of events following this early manifestation is variable. Macroscopic skin changes include altered pigmentation with or without associated papules. Eventually this progresses to atrophy where the surface of the skin has a shiny fragile appearance that has been compared to crushed tissue-paper. Collagen and elastin fibers are believed to be destroyed by the long-term presence of parasites in the skin with repeated occurrence of local pathology around dying parasites. Adult O. volvulus are relatively innocuous although they can produce discomfort due to nodules.

The second most common sign is lymphadenopathy. Affected glands are enlarged, firm, but not tender. Lymphoedematous changes may take place and are believed to be attributed to obstruction of the lymphatic drainage system.

There remains much to be discovered about the significance of O. volvulus microfilariae in the general systemic disease with the

findings of microfilariae in various body fluids (Buck et al. 1971; Fuglsang and Anderson 1974a) and deep organs (Rodhain and Gavrilov 1935; Connor et al. 1970). There is increasing evidence that severe onchocerciasis is not infrequently a systemic disease. Of special interest may be the relation of onchocerciasis with renal abnormalities (Couzineau et al. 1973). Proteinuria was encountered in some people with onchocerciasis included in the present study.

Ocular lesions are the most severe complications of onchocerciasis. Eye lesions may result in partial visual impairment or total blindness. Microfilariae in the eye can bring about changes in corneal, uveal and chorioretinal tissues as well as damage to the optic nerve. Total loss of vision results from sclerosing keratitis, iridocyclitis, chorioretinitis or optic atrophy (Anderson and Fuglsang 1977; O'Day and Mackenzie 1985). Because of the sequela pursuant to ophthalmic involvement the disease has been given the notorious name of "river blindness" by people residing near rivers in the savanna and forest zones of Africa. The actual cause of all the ocular lesions is, however, not known but it seems probable that they result in some way from the local death of microfilariae that have invaded the eye (Donnelly et al. 1985).

6. Treatment

Suramin and diethylcarbamazine (DEC) have for a long time been the mainstays in the treatment of human onchocerciasis. Suramin is the only adulticide but it has the major disadvantage of being intrinsically toxic and causes many adverse reactions, some of which may be fatal (Fuglsang and Anderson 1974b; Duke 1974).

DEC is a microfilaricide, but it also produces severe adverse effects associated with the destruction of microfilariae (Duke and Anderson 1972).

Progress in the search for and development of better drugs is impeded because of difficulties in maintaining O. volvulus experimentally. Many of the gains so far secured are based on studies using heterologous parasites. Models for primary and secondary screenings include a rodent/Dipetalonema and/or Brugia system and tertiary screening largely depends on the use of O. gibsoni and O. gutturosa, both of bovine origin (WHO 1987).

A milestone in the chemotherapy of onchocerciasis is the finding of ivermectin, originally a veterinary drug, to be a safe and efficient drug for human onchocerciasis. Ivermectin is a semisynthethic macrocyclic lactone derived from avermectins, which are fermentation products of the actinomycetes Streptomyces avermitilis (Campbell et al. 1983).

A single dose of ivermectin, given orally at 150 ug/kg body weight has been shown to be both effectively microfilaricidal and safe (Awadzi et al. 1985).

The mode of action of ivermectin against O. volvulus is still not clear (Bennett et al. 1988). Present belief is that the drug interferes with the neurotransmitter functions of the gamma amino butyric acid (GABA) system of the parasite. Ivermectin is presumed to enhance both the presynaptic release of GABA and the post-synaptic intensification of GABA-binding to a chloride channel-linked receptor. As a result of the chloride-ion channel opening, the microfilaria is considered to become paralyzed and eventually dies (Campbell 1985).

Surgical extirpation of onchocercal nodules is another plausible approach in the treatment of this filarial disease. The merit of nodulectomy has been appraised by Kale (1982) in Africa and Aoki et al. (1983) in Central America and conflicting results were obtained. Recent findings by Guderian et al. (1987) in Ecuador however, suggest that the removal of nodules is beneficial, both in terms of reducing skin microfilariae and minimizing the degree of the pathology.

It is clear that both suramin and DEC may result in disabling and unpleasant side-effects. In addition, the lengthy treatment, the need for a hospital based monitoring of untoward reactions and in the case of suramin the intra-venous route of drug administration, make the use of both drugs impractical and even dangerous for mass chemotherapy. Ivermectin offers an attractive alternative. Blackflies fed on ivermectin treated patients show a reduced uptake of microfilariae for up to six months (Cupp et al. 1986). This suggests that ivermectin has a potential to decrease disease transmission.

There has never been massive use of any drug against onchocerciasis in Ethiopia. The sporadic use of DEC has been the cause of some violent reactions including death (Oomen 1968), and the itching following its administration has often generated considerable resistance from patients undergoing treatment. The use of ivermectin in Ethiopia is being seriously considered and plans are now being finalized to introduce it early in 1989.

7. Control

In contrast to simuliid control aimed at alleviating nuisance (where a reduction in fly bites from thousands to hundreds may be considered satisfactory), when the goal is to interrupt onchocerciasis

transmission, a more stringent system is needed. Typically, simuliid biting density in virgin areas with productive larval breeding sites can be up to many thousands per man per year.

Success in the control of an arthropod-borne disease targeted against the intermediate host needs, as a pre-requisite, a thorough knowledge of the bionomics of the vector. Although much progress has been achieved, our understanding of the biology, ecology, taxonomy and vectorial capacity of the blackfly remains inadequate. Some highlights of relevance to control will be given. For a cohesive understanding of the black flies, the recent book edited by Kim and Merritt (1987) is recommended.

Adult Simulium range from 1.5-4 mm in length. They are relatively stout-bodied and when viewed from the side appear to have a somewhat humped thorax (Buffalo gnats). As their rather misleading name indicates, some are usually black in color. Nevertheless, flies with contrasting patterns of white, silvery or yellowish hairs on their bodies and legs and even some with predominently orange or bright yellow color do exist.

The mouth parts of the adults are adapted for piercing and sucking. Adult males feed on sap, but females exhibit duality in their feeding habit. Apart from being hematophagous, female flies also feed on plant juice. The former provides nutrient for egg maturation and the latter is used for flight energy and sustenance (Brenner and Cupp 1980). The digestion rate of blood in Simulium damnosum is about 72 hours at 23-25°C (Le Berre 1966) and in S. ochraceum 3-4 days (Porter and Collins 1985).

Of significance to the epidemiology and control of onchocerciasis

is the ability of the fly to travel a considerable distance from its origin. The distance travelled by the Simulium damnosum complex varies in different members, but may be over 500 km in some cytospecies (Garms et al. 1979).

Of the four stages; adult, egg, larvae and pupae, the black fly goes through to complete one biological cycle, the last three are entirely dependent on water. Therefore, especially important is the study of the physical and chemical character of water courses. Breeding rivers may be seasonal or perennial. Generally, maximum breeding occurs twice during the year, at the beginning and the end of the wet season (LeBerre 1966). Optimum conditions in water include a pH of 7-8, temperature of 20-30^{o}C, conductivity of 50-150 Ns/cm and a velocity of at least 0.8 m/sec. (Grunewald 1981). Generalization about physico-chemical conditions for all simuliids is dangerous because optimum requirements vary in different species/strains.

Patterns of oviposition by female simuliids under natural condition are variable. S. ochraceum oviposits by intermittently dipping its abdomen in water (Dalmat 1955), S. damnosum theobald by landing on a trailing vegetation (Marr 1971) and S. neavi roubaud by totally submerging itself under water (Wanson and Herard 1945).

Eggs of Afro-tropical species of blackflies, such as S. damnosum complex, hatch to larvae within four hours of deposition (Wanson and Herard 1945). At the end of the larval stage, pupation occurs in a cocoon from which the adult breaks out and escapes from the water into the air. Under favorable conditions, development of a new fly from egg to emergence from pupa takes about 12-15 days and longevity of adult flies is about one month (LeBerre et al. 1964). The gonotrophic cycle

is 3-6 days (Wenk 1981). Generally there are about 400-600 eggs laid per female (Bellec and Hebrard 1983).

The most aggressive step in the fight against onchocerciasis has been launched by the Onchocerciasis Control Programme (OCP) in the Volta River Basin. The objective of OCP is to reduce onchocerciasis to a level at which it no longer constitutes a public health problem. This was forseen to be attained by reducing the number of vectors through insecticide application. Results of the first ten years' activities indicate that transmission has been reduced to below the acceptable limit of tolerability in over 90% of the treated area (Philipon et al. 1984). An acceptable level of tolerability is defined as an Annual Biting Rate (ABR) of less than 1,000 and an Annual Transmission Potential (ATP) of less than 100 (Walsh et al. 1978). The success of this vector-focussed approach is also reflected in clinical findings with a 97% reduction in the occurrence of infection among children born since the inception of the OCP (Ba 1984).

The wide-spread use of insecticide is inevitably attended with the creation of resistant strains of vectors and the accumulation of pollutants in the environment. Although the impact of the latter was minimal, a major concern of OCP now is the development of resistance to conventional insecticides. A progressive shift to the use of a biocontrol agent, Teknar^{R} (Bacillus thuringiensis var. israelensis) is being made in areas where resistance to organophosphate insecticides is becoming a threat (WHO 1987).

Controlling onchocerciasis with larvicides has been going on for over ten years in many endemic areas. Monumental achievements have been made. Nevertheless, the problem is still there and it is a forlorn

hope to eradicate the disease only by vector control. Vector control integrated with chemotherapy, especially now that ivermectin is available, is the approach of the future.

There is no vertical control programme of onchocerciasis in Ethiopia at present.

B. Onchocerciasis in Ethiopia

Ethiopia is in the north-eastern corner of Africa (Figure 2). It has an area of about 1.25 million square meters, and according to the most recent census report by the Central Statistics Office (1984), the population is 42 million.

Although there are important variations from year to year and from one part of the country to the other, there are two rainy seasons; the "big rains" from July to September and the "small rains" from late February to early April. The remaining months are relatively dry.

Ethiopia's rivers, which are typical mountain streams over most of their length and form numerous waterfalls, rapids and chutes (conditions favorable for breeding of the simuliids) originate in the massive highlands with altitudes ranging from 1,500 to 4,600 meters. Many seasonal and perennial rivers contribute to the drainage system of the country. The most important are shown in Figure 2. Tropical rainforests are prominent in Kafa, Wollega and Illubabur, while in the low lands vegetation types include tropical savanna, tropical thorn and the riparian woodland.

Although the first accounts of the presence of simuliid vectors and onchocercal disease were made as early as 1939, (Giaquinto-Mira 1939; Bucco, 1965) onchocerciasis as a major public health problem was not recognized in Ethiopia until recently.

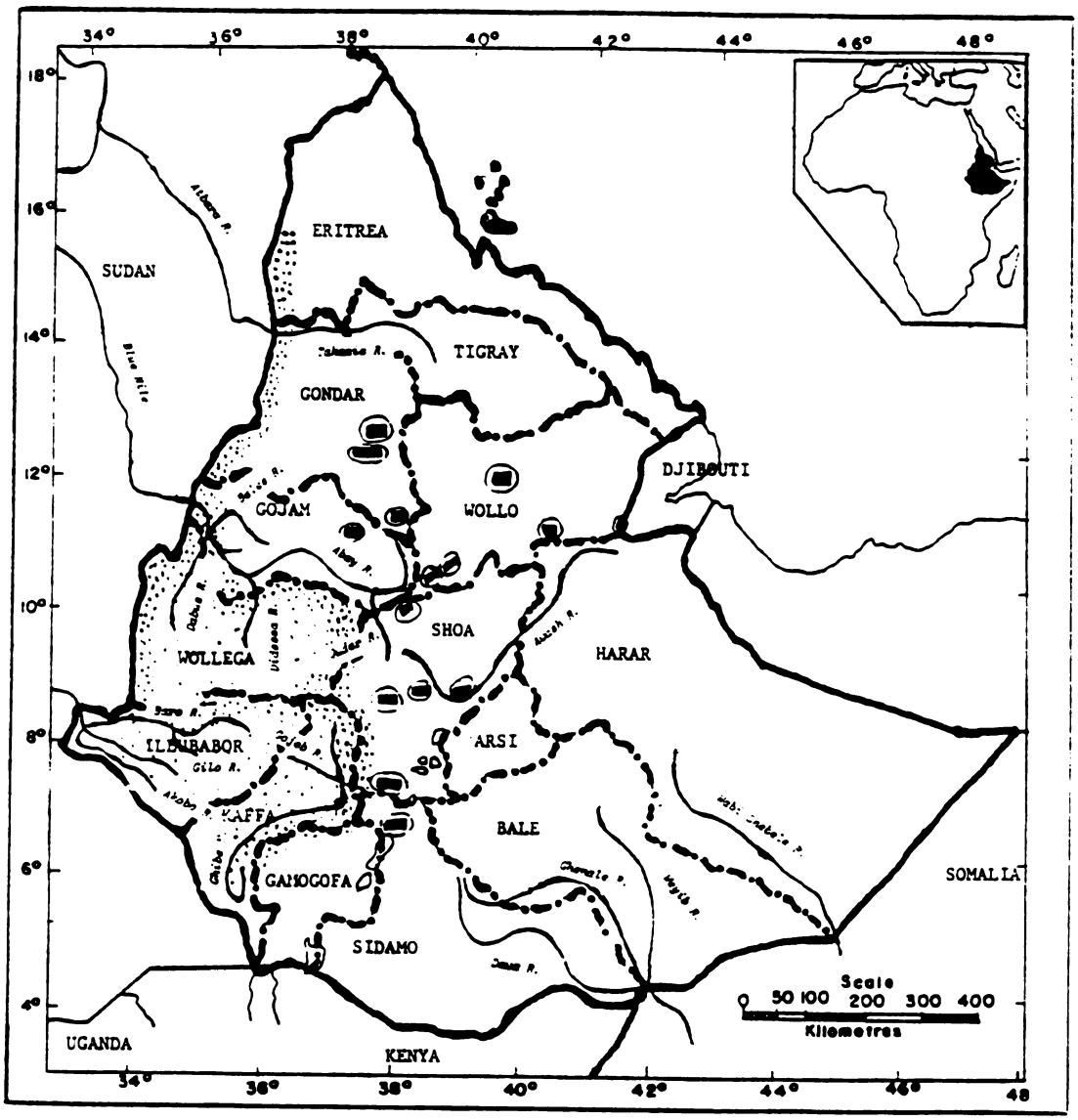

Figure 2. Ethiopia

Shown are approximate locations of:

= Major rivers
= Onchocerciasis endemic area
= Sites of potential Simulium vectors

Information on the entomology of onchocerciasis is still scarce. On circumstantial grounds however, flies of the Simulium damnosum complex probably occur along suitable waterways throughout the low lands of Southern Ethiopia and the S. naevi group is known to have a wide range distribution in the intermediate highlands of the south-western part of the country (Grenier and Ovazza 1956; Oomen 1969a; Ogata et al. 1970). According to Mebratu et al. (1979), potential vectors of O. volvulus breed in rivers flowing in over two thirds of the country. The approximate distribution of potential vectors is depicted in Figure 2. Attempts at determining the cytotype of the damnosum complex were made by R.W. Dunbar, and, as communicated to White (1977), they were unlike any of the named forms and species (Dunbar 1969; Vajime and Dunbar 1975).

There has never been a country-wide epidemiological investigation of onchocercal disease. Sasa (1976) reported that the endemic foci extend over 132,000 Km^2 in Southwest Ethiopia, including the Regional Administrations of Kafa, Illubabor and Wollega. There, of about 2.5 million people, half a million are estimated to be infected with the O. volvulus parasite. Recent additions to the endemic regions are Gamogofa and northern Gondar, bringing the overall estimated number of infected people to about 1.4 million (WHO 1987). Prevalences determined by skin microfilariae detection span from a high of 84% in a western endemic zone (Gundersen et al. 1988), through 56% in the south-west (Iwamoto et al. 1973), to a low of 19.5% in the northwest (Zein 1986). Therefore, based on the WHO (1966) standard level of endemicity, there are hyperendemic, mesoendemic and hypoendemic regions. Overall, the

prevalence of onchocerciasis is greater in the lowlands than in the highlands (Oomen 1969b; Gundersen et al. 1988).

Higher prevalence is reported in males than in females (Oomen 1969a Iwamoto et al. 1973, and Zein 1986) and those in the age group 15-45 are most affected (Ten Eyeck 1973; Taticheff et al. 1987).

The mean number of microfilariae per infected person varies considerably. Values of 16 microfilariae (mf) per 2-3 mm diameter of skin snip, 3.6 mf/mg of skin, and 15.6 mf/mg skin are reported from, respectively, hyperendemic (Gundersen et al. 1988), hypoendemic (Zein 1986) and mesoendemic (Taticheff et al. 1987) regions.

Onchocerciasis in Ethiopia, vis-a-vis other areas with comparable ecology, has its own unique features. Skin microfilarial loads are lower than in the Cameroon rain-forest and Nigerian Savanna (Woodruff et al. 1977).

Typically, clinical features include pruritus, pigment related skin changes, dermal atrophy and lymph nodes enlargement, but to a negligible level, eye involvement. Clinical signs and symptoms are not uniform throughout the range of endemic foci. In all of the areas investigated however, these clinical courses were apparently relatively benign (Oomen 1967a,b,; 1968, 1969a,b; Iwamoto et al. 1973, Mengesha and Jembere 1975, DeSole and Walton 1976, Menegesha and Tiruneh 1977, Zein 1986, Gundersen et al. 1988).

Onchocercal nodules with a prevalence of 11% (Oomen 1968) 6% (Iwamoto et al. 1973) and 36.7% (Woodruff et al. 1977) were found on the ribs, shoulders and pelvic girdle. The only known reports of onchocercomata on the head were those of Zein (1986) who found three cases in a study population of 1,366, and Gundersen et al. (1988) who

saw two cases in 182 skin microfilariae positive persons with 7% nodule carrier rate.

By all accounts, save identification of the strain of O. volvulus, conditions favoring onchocercal blindness exist in Ethiopia. Yet, blindness, even if defined in its broad sense (that is vision of less than 3/60 corresponding to inability to count fingers at a distance of 3 meters, WHO 1984) has never been attributed to this filarial parasite in Ethiopia (Gundersen et al. 1988). This is in contrast to the situation of neighboring Sudan where onchocerciasis is a major cause of ocular morbidity (Tizazu and Mburu 1983, El-Sheikh et al. 1985). Ocular anomalies with concurrent onchocercal infection have been reported in Ethiopia by Torrey (1966), Oomen (1968) and Woodruff et al. (1977). With the exception of a single case of the presence of live microfilariae in the anterior chamber of both eyes (Gundersen et al. 1988) there has never been conclusive evidence of ocular pathology where O. volvulus has been incriminated.

It is of interest to note that in the latest monograph by the WHO Expert Committee on Onchocerciasis (1987) 20,700 people in Ethiopia are considered blind as a result of onchocerciais. In view of the fact that there has never been a large scale assessment of the onchocercal ocular manifestations, and that the limited number of studies so far have provided little evidence of ocular disease in Ethiopia, the WHO document, although acknowledging its limited sources of data, appears to have grossly exaggerated the condition.

Does the Ethiopian strain of O. volvulus cause blindness? Is the problem of ocular disease in Ethiopia still to be identified? Is Ethiopian onchocerciasis any different from onchocerciasis elsewhere in

the world? It would be foolhardy to try to answer all these questions at this juncture. Carefully designed epidemiological, entomological and ophthalmological as well as immunological and clinical studies will be required to understand the ostensibly unique manifestations of onchocerciasis in Ethiopia.

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CHAPTER 1

HYPERSENSITIVITY TO SIMULIUM BITES

INTRODUCTION

Studies on hypersensitivity to black fly bites are few. However a review of reactions to bites by other arthropods, such as ticks and mosquitoes, provides a good background to this topic.

The classical study by Mellanby (1946) was probably the first to describe the sequential immunologic events following bites by arthropods. His results show that during a series of exposure to bites of mosquitoes over an extended period of time, five phases occur, namely: 1) a "no reaction" stage due to the lack of previous exposure to mosquito bite; 2) a delayed reaction stage which becomes evident after a latent period following initial sensitization; 3) a combined immediate and delayed reaction stage following continued exposure to similar bites; 4) a predominantly immediate reaction stage upon further bites, and; 5) a stage of complete desensitization in spite of persistent exposure to mosquito bites.

Two of the above stages may provide an apparent benefit to the host. On the one hand, people who are hyposensitive do not suffer from irritating allergic reactions to insect bites; and on the other, as demonstrated by Stebbings (1974), immediate hypersensitivity reactions may be important protective responses against blood-feeding arthropods. Allergic reactions presumably act by inducing avoidance behavior and/or by direct deleterious effects. The orderly sequence of immunological events described by Mellanby has been suggested to follow bites by most

medically important arthropods (Halliwell 1984).

From the point of view of "disease" causation, bites by the medically important arthropods should be considered from three angles: a) the induction of allergic conditions which are of variable intensities, with fatal reactions being possible; b) the introduction of pathogens through bites, and c) the consequence of the synergistic or counteractive effects of the hypersensitivity status on the bite-transmitted pathogens. Not much information pertaining to a and c is available in the literature on black flies, and their role as vectors has received almost all the attention in onchocerciasis. However, recent knowledge is being applied in the development of novel immunologic approaches for the control of allergies against other arthropods, as well as for the control of arthropod-borne diseases through immune reactions directed toward vector antigens.

For example, ticks of the Ixodoidea superfamily represent highly specialized blood-sucking arthropods that are important biological vectors of the agents of many human and animal diseases. Tick parasitism is known to induce resistance in various hosts. Acquired resistance has been shown to be immune mediated and accounts for many antagonistic effects on ixodid ticks including the reduction in the number of parasitizing ticks and diminished feeding, moulting, fertilizing capability and fecundity (Wikel et al. 1978; Wikel 1979). Host resistance to ticks, both acquired and induced, has also been shown to have the additional advantage of protecting hosts from tick-borne pathogens such as for instance, babesiosis in cattle (Francis and Little 1964). Vaccines against certain ixodid ticks are now being field tested.

Mosquitoes are notorious in causing allergic dermatitis and also in transmitting pathogens. The occurrence of both immediate and delayed type reactions in mosquito bites is attributed to antigens of salivary gland origin (McKiel 1959). Attempts to reduce the effects of mosquito bites were made by several workers using injections of saline extracts of the insect. Many years ago Benson (1936) reported a case where a single injection of mosquito extract conferred protection from a troublesome delayed reaction for four consecutive seasons. In another account, Alger et al. (1972) and Alger and Harrant (1976) reported successful protection of mice against Plasmodium berghei by immunization with salivary secretions from the vector.

Although the literature is scanty and studies of mechanisms very limited, black fly bites have been implicated in serious losses of live-stock in many parts of the world (Schmidt 1916; Hutcheon and Chivers-Wilson 1953; Towsend et al. 1977). The death of animals has been attributed by some to a "poison" from the salivary glands. In this connection, Georgevitch (1923) made an extract of the heads of black flies and upon injecting it under the skin of guinea pigs, rabbits and mice found it to be toxic. Laboratory animals which survived the initial dose developed a certain amount of immunity to subsequent injections. No comparable study has been done since then, but the inherent toxicity of the saliva was confirmed in studies by Remple and Arnason (1947) and Fallis (1964).

Black fly bites as a direct cause of cutaneous lesions in humans, especially depigmentation, have been reviewed by Stokes (1914), Loewenthal (1939), Brown (1960), Edungbola et al. (1983), and Fuglsang (1983). DeMeillon (1930) in an extensive description of the Ethiopian

Simuliidae, noted that black fly bites in humans are very potent irritants and cause edematous swellings which often end in sores. Goldman (1948) working in Mexico reported that many medically important arthropods, including Simulium species, cause "papular urticaria", a cutaneous clinical syndrome. Lastly, the haemorrhagic syndrome of Altamira (HSA), a disease characterized by localized and disseminated cutaneous hemorrhages, has been described as being produced by a hypersensitivity phenomenon or response to a toxin associated with intense black fly biting (Pinheiro et al. 1974). More recently, however, the notion that black fly bites are direct causes of cutaneous lesions has been challenged (Connor and Palmieri 1985).

Attempts to characterize the nature of the various bodily components of Simulium spp. have been made. In this respect, Hutcheon and Chivers-Wilson (1953), using extracts from whole black flies or the head and thorax, demonstrated the presence of histamine and anticoagulant activity. Yang and Davies (1973) have also shown the presence of agglutinating and anticoagulant factors in the salivary glands of adult females of S. decorum, S. venustum and S. vittatum.

Immunologic aspects of human-black fly relationships represent a fertile area of research. Some preliminary observations are available. Gudgel and Grauer (1954), in their study around Tokyo, noted that reactions to the bites of black flies consisted of immediate pruritic blood-crusted nodules, giant urticarial lesions associated with constitutional symptoms, eczematoid lesions and chronic changes. More recently, Ogilvie and Mackenzie (1981) have reported that a spectrum of reactions, ranging from immediate through a typical delayed type

hypersensitivity, exists and that individuals vary considerably in their responses to the bites of this arthropod.

The introduction of salivary secretions into a host is an inevitable event when an arthropod is blood feeding. The salivary secretions apparently function as the primary source of antigen(s) that stimulate the production of a hypersensitivity state. The questions of whether or not such an immune response to black fly bites has a role in aggravating or mimicking onchocercal lesions and whether or not such a hypersensitivity has a protective function against reactions to black fly bites and against onchocerciasis remain to be addressed. In this study, an attempt is made to answer some aspects of the first of these questions.

STUDY AREA

A. Ecology and Demography

The study site was Bebeka. It is located 590 Km south-west of Addis Ababa in the Kaffa Administrative region between $6^{o}51'$ and 7^{o} north latitude and, $35^{o}21'$ and $35^{o}3'$ east longitude (Figure 1). Its altitude ranges between 900 to 1100 meters.

Coffee, which incidentally received its name from Kaffa, the region where it was first found, growing wild, occupies a special place in the economy of Ethiopia. Bebeka is the site of one of the largest projects in the coffee plantation enterprise in Ethiopia. Started in 1979, the Bebeka Coffee Plantation and Development Project (BCPDP) has so far developed about 50% of its projected 10,000 hectare plantation land (BCPDP 1984-85). Topographically, Bebeka is level with gentle slopes in some areas. A longitudinal shallow depression bisects the area and in here the only perennial river, River Dobze, with many of its tributaries, flows south to north. Natural vegetation, except shade trees of various kinds including species of Acacia, Mellitia, Albizia, Sesbania and Leucaena, is cleared for the coffee plantation in the developed area. Pictorial presentations of Bebeka are given in Figures 2, 3, 4, and 5.

According to a 1987 report by the National Meteorological Services Agency, the average annual rainfall for the years 1980 to 1987 was 1,728 mm, with the months of June to September being the highest at a

monthly average of 212.3 mm. Lowest monthly rainfall, 47 mm, was from December to March. The remaining months averaged 172.7 mm.

Bebeka has a very heterogenous population totaling 12,319 people (BCPDP/Health Service 1984/1985). The indigenous people are members of the Shuro ethnic group and they, together with people from practically all of the 14 Administrative Regions of the country make up the working force of the plantation. Housing for workers and their families is mostly in apartment complexes or in small "tukuls", spread in the five farm camps of the project (Figures 2). All dwellings are numbered. Water for domestic use is provided from protected springs, wells and rivers and none is chemically treated. Health Services and elementary education are provided free of charge.

B. Prevalence of Tropical Diseases

Water-borne and poor sanitation related diseases account for over 1/3 of the health problems in Bebeka. (BCPDP/Health Service, 1984/85). Onchocerciasis has a point prevalence of 30.9% (Taticheff et al. 1987). Of the other vector-borne parasitic diseases, only malaria is rampant and neither leishmaniasis nor trypanosomiasis have ever been clinically documented. Sporadic cases of tuberculosis and leprosy are observed, and these do not constitute major health problems in Bebeka (BCPP/Health Service 1984/85).

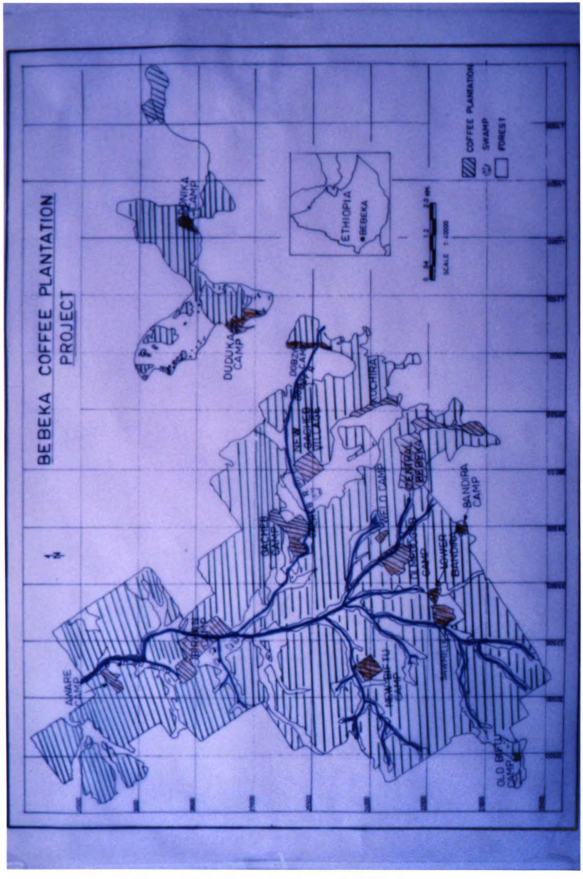

Figure 1. Map of Bebeka (Ethiopia)

Figure 2. Housing for coffee plantation workers in Bebeka, Ethiopia. A. Modern dwellings. B. Traditional tukuls.

Figure 3. Vegetation in Bebeka, Ethiopia.
A. Tall shade trees. B. Coffee trees.

Figure 4. Simulium breeding sites in Bebeka, Ethiopia. A. Natural (Riber Dobze). B. Man-made.

Figure 5. Man-*Simulium* contact sites, Bebeka, Ethiopia.

MATERIALS AND METHODS

An immunological study of onchocerciasis without adequate and recent information on concurrent tropical diseases, especially helminthiasis, would not be considered complete. For this reason, determinations of certain clinical laboratory test values, either directly or indirectly reflecting the prevalence of some tropical diseases, were made. Accordingly, in a sample of 425 people selected from one out of every 20 households, the following were carried out: stool examinations for intestinal parasites using the Ritchie (1948) concentration technique; thick and thin blood smears stained with Giemsa for malaria and other hemoparasites; total and differential leukocyte counts by manual methods; hemoglobin levels by the cyan-methemoglobinometry procedure; and urine sediments for Schistosoma haematobium and microfilaria of O. volvulus. In addition, serum analyses for HIV-1 by enzyme-linked immunosorbent assay ELISA (Wellcome^{R}) and Western Blot (Biorad^{R}) were done to determine the prevalence of AIDS.

The hypersensitivity study involved longitudinal observations made on three occasions. Since blackfly populations and, therefore blackfly bites, are seasonal the three rounds of observations were undertaken during the months of March, June and October, 1987, which correspond to pre-rainy, rainy and post-rainy seasons, respectively. The result of an

ancillary study showing patterns of S. damnosum bites and rainfall in Bebeka determined the selection of the study periods. This is shown in Figure 6.

Activities in the initial course of this study first consisted of a cross-sectional prevalence data collection on onchocerciasis from the 425 people randomly selected. Skin snip biopsies from right and left buttocks were collected from the sampled population using a sclero-corneal punch. The skin snips were first weighed, external surface down, on a torsion balance. They were then incubated at ambient temperature (22-26^{o}C) for not less than four hours in covered microtitration plates containing physiological saline solution. Microfilariae were counted with a tally counter under a 10x binocular microscope. Results are expressed as microfilariae per mg of skin (mf/mg).

Onchocercal dermal manifestations i.e., dermatitis, pigment related skin changes, texture related skin changes, lymphydenopathies and nodules were detected and appropriately documented. Such findings are denoted by numbers from 0 to 5, where 0 represents the absence and 1 to 5 represent increases in the number of positive manifestations. The format used to document these findings is given in appendix 1.

The second phase of the work in round one was the dermal hypersensitivity test. Because of scarcity of antigenic material and logistics problems, this aspect of the study was done only on a limited number of people, selected from the sample population. Criteria for selection included representation of different sexes, ages and onchocercal findings. Accordingly, 66 people were chosen, and on rounds two and

three follow-ups were done, with two repeats of skin snip biopsies, and repeat observation of onchocercal lesions.

Apart from the 66 people selected from the 425, approximately one out of every seven of the remaining population were further sub-sampled. There were 98 people in this category. These were followed for dermal manifestations and skin microfilariae only.

In summary, therefore, from the total population of 12,319 people in Bebeka, 425 were selected for the cross-sectional studies on the determination of certain clinical laboratory test values and the prevalence of onchocerciasis. Sixty six people out of the 425 and an additional 98 from the general population were, respectively, followed for skin reactivity and onchocercal morbidity.

Tests for skin reactivity were done by intradermal injection of 0.1 ml antigen on the volar surface of the forearm using disposable tuberculin syringes. The antigens, the preparation of which is described in appendix 2B were from O. volvulus third stage larvae (L3), adult blackfly homogenate (ABFH) and salivary gland of blackflies (SGA). Antigens for injections were diluted from the stock in sterile PBS pH 7.2, to give a final protein concentration of about 5 ug per 0.1 ml. Forty five people were injected three times with SGA and 15 people three times with L3 while 6 people were inoculated with ABFH. Results were recorded as either no reaction, delayed or immediate. Immediate reactions, detected by wheal formation were read during the first two hours at 30 minute intervals, and the delayed reactions identified by edematous type of swelling at the site of inoculation, were detected for up to 48 hours. The immediate reactions were further classified by

wheal size as small, medium, and large, corresponding to diameters in mm of, respectively, up to 5, >5 to 10, and >10.

Venous blood samples were collected from all people during the three rounds. These were used for the immunoblot experiment described in Chapter 2.

Biopsies from the delayed and immediate reaction sites were taken for histological observations. Although it was desirable to biopsy as many people as possible, there were only very few volunteers. The histological study is described in Chapter 3.

A collateral study was also done in Sodere, a small resort area about 120 km south-east of Addis Ababa. This area is endemic for Simulium, but no onchocerciasis has ever been reported. Twenty four volunteers, with the only stipulation that they have resided in the area for the last three years were included. They were all subjected to the same kind of treatment except that the visit was done only once in the month of July, 1987. This period had a rain-fall and blackfly population pattern comparable with the second round study period in Bebeka. Eleven people were injected with L3 and 13 with SGA.

Contingency table analyses (x^2 and Fisher's exact test) were used as the statistical tool.

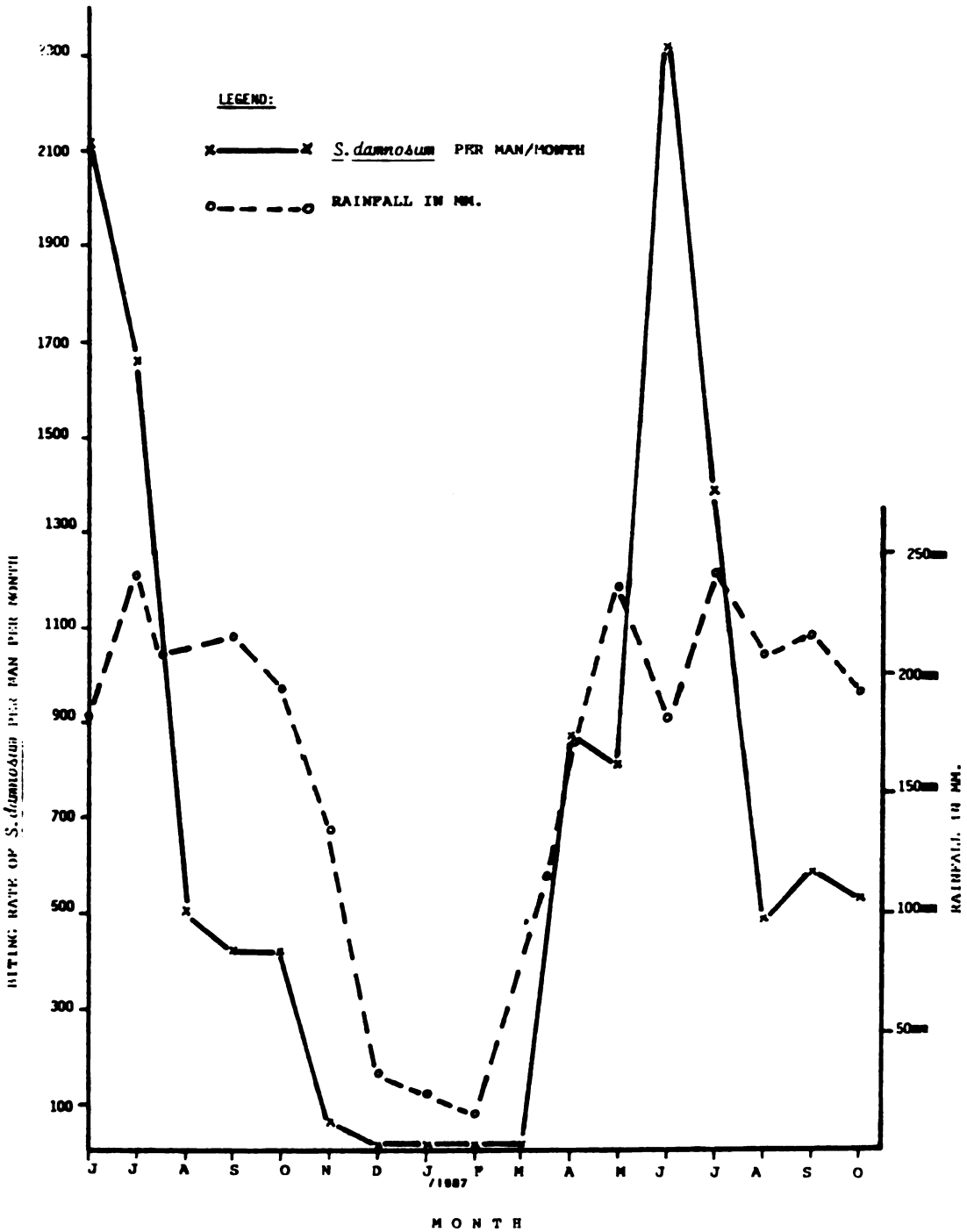

Figure 6. Monthly biting rates of S. damnosum per man in Bebeka, Ethiopia (June 1986 - October 1987)

RESULTS

A. Clinical Laboratory Tests

The results of the determinations of the clinical laboratory test values were as follow:

The total white blood cell count values range from 2,000 to 12,500 with a mean of 4,600 (SD=1300) cells per cu mm.

No parasites were detected in the blood smears and urine sediments.

Hemoglobin values ranged from 6 to 15.9 with a mean of 11.96 gm% and a S.D. of 1.8. Hemoglobin values below 10 gm% were found in 29 cases (6.8%)

Table 1. Differential white blood cell counts expressed in percent (relative values) in Bebeka (Ethiopia):

Cells	Range	Mean	S.D.
Neutrophils	22-88	59.92	10.84
Eosinophils	0-40	7.07	7.54
Lymphocytes	4-60	32.34	9.94
Monocytes	0-9	.52	1.27
Basophils	0-4	.13	.49

Eosinophil values greater than 10% were found in 102 cases (24%).

Table 2. Intestinal Parasite Survey in Bebeka (Ethiopia):

	Males (n=268)		Females (n=157)	
Intestinal Parasites*	No.	Pct.	No.	Pct.
Ascaris lumbricoides	169	63.1	103	65.6
Hook worm	121	45.1	67	42.7
Trichuris trichuria	66	24.6	40	25.5
Schistosoma mansoni	15	5.6	9	5.7
Strongyloides stercoralis	5	1.8	4	2.5
Hymenolepis sp.	8	2.9	5	3.1
Taenia sp.	2	0.7	1	0.6
Giardia lamblia	3	1.1	1	0.6
* Many people had multiple infections.				
Total negative/pct.	49	18.2	37	23.5
Total positive/pct.	219	81.8	120	76.5

Grand Total:

Negative = 86 (20.2%); Positive = 339 (79.8%)

Tests for HIV-1

Although 42 (9.9%) of the samples were positive on repeat ELISA, all were negative by Western blot and it was concluded that HIV-1 infection is not present in the study population.

B. Prevalence and Morbidity of Onchocerciasis

The result of the cross-sectional prevalence study of onchocerciasis in Bebeka show that onchocerciasis, determined by dermal lesions and skin snip biopsy, had a point prevalence of 38.4% (163/425) and 42.4% (180/425), respectively. Of the 163 people showing dermal lesions, 23 (14%), 97 (59%), 32 (20%), and 11 (7%) were in the age groups 1-14, 15-30, 31-45, and >45, respectively. Of the 180 people that were positive for microfilariae in the skin snip biopsy examinations, 18 (10%), 105 (58%), 44 (25%), and 13 (7%) were in the age groups: 1-14, 15-30, 31-45, and >45, respectively. In the total 425 people sampled for the cross-sectional survey, there were 268 males and 157 females. Onchocercal point prevalences determined by observation of the dermal manifestations and by skin snip examination were, respectively, 46.6% (125/268) and 53.7% (144/268) in males, and 24.2% (38/157) and 22.9% (36/157) in females. Therefore, the above observations indicate that males in the age group 15-30 followed by 31-45 are the ones showing the highest rates of onchocercal infection and disease in Bebeka. All the spectrum of onchocercal dermal lesions, including two cases of the possible Sowda variant, were observed. Inguinal lymph node enlargements were seen in 57/475 people (13%) while palpable nodules were detected in only 13/475 (3%). The mean number of mf/mg was 5.38 with a range of 0-79.

The morbidity longitudinal study in 98 people in Bebeka (Figures 1 and 2 in Appendix 3) showed an increase in both microfilarial positivity and dermatologic manifestation. Increases in dermatologic changes over the study period were highly significant ($P < 0.005$) compared with that in microfilarial positivity ($P < 0.1$). Positive dermatologic score rates were 49 (48/97), 53.1 (52/98), and 71.4% (70/98) in rounds 1, 2, and 3 respectively with dermatology scores of 1 contributing to most of the increase. In the same group of 98, microfilariae were present in 49 (50%), 61 (62%), and 65 (66%) of the people in round 1, 2, and 3, respectively, with the 0.1-10.9 mf/mg group contributing to most of the increase.

Over 98% of the cases with dermatology scores 1 and 2, both in the people followed for morbidity and those included in the hypersensitivity study, had papular eruptions (dermatitis) as one of their dermatologic manifestations. By combining the number of people with dermal scores of 1 and 2 (Figures 2A and 3A in Appendix 3), the sequential rates of dermatitis from round 1 through 2 to 3 were 37.8% (37/98), 43.9% (43/98), and 58.2% (57/98) in the longitudinal morbidity study group, and 35% (21/60), 45% (27/60), and 48.3% (29/60) in the group involved for the skin reactivity observation. Therefore, a progressive increase in dermatitis was observed in both groups.

Results of the cross-sectional and morbidity longitudinal studies along with pictures of various onchocercal manifestations found in this study are presented in Appendix 3.

C. Skin Reactivity

Blackflies as biting arthropods were specifically recognized by people in the study area at large. The sampled population reported seasonality in blackfly biting with increased intensity associated with heavy rains. Itching and scratching, as responses to bites were complained of by 5, 12 and 10% of the population studied in rounds 1, 2, and 3, respectively. None of the episodic complaints (reactions) were reported to last beyond 4 hours.

A summary of the observations made on skin reactivity to inoculations with salivary gland antigen (SGA), O. volvulus infective larvae (L3) and adult blackfly homogenate (ABFH) during round 1, 2, and 3 is presented in Table 3. These findings indicate that all three preparations induce an immediate type of reaction. Delayed response was seen only with SGA and L3 antigens. The most remarkable observation is that the number of people who gave immediate type reaction to both SGA and L3 showed an increase ($P < 0.005$ for SGA and $P < 0.1$ for L3) from round 1 through 2 to 3. Such periodic changes in reactivity to ABFH could not be assessed because all the people assigned to this group defaulted in the second and third round.

Pattern of wheal size variation per round in the immediate type responses to SGA, L3 and ABFH is depicted in Table 4. A progressive decrease in the number of people with small size wheals with a corresponding significant increase ($P < 0.001$ for SGA and $P < 0.05$ for L3) in those with medium and large size wheals was obtained both with the SGA and L3 antigens during the study period.

Table 5 shows the population studied for hypersensitivity reactions to SGA, L3 and ABFH antigens, classified in 4 groups, namely: 1)

dermatology and skin snip positive (D^+/S^+), 2) dermatology positive, but skin snip negative (D^+/S^-), 3) dermatology negative, but skin snip positive (D^-/S^+), and 4) both dermatology and skin snip negative (D^-/S^-). Immediate type responses both to SGA and L3 antigens appeared in more people with dermal lesions (D^+) i.e. D^+/S^+ and D^+/S^- groups than without (D^-) i.e. D^-/S^+ and D^-/S^- groups. Percentile values were 87 (D^+), 66 (D^-) and 89 (D^+), 78 (D^-) with SGA and L3, respectively. Differences of results in skin test responses (immediate and delayed) between people with positive dermatology (D^+) and negative dermatology (D^-) were significant ($P < 0.025$). Delayed type reactions with SGA developed in 11% and 7% in (D^+) and (D^-) people, respectively. However, all delayed type reactions to L3 injections were found in people without any onchocercal infection or disease (D^-/S^-).

Table 6 shows results of hypersensitivity reactions to all three antigens in people with graded dermatology scores of 1 to 5 and microfilaria load of 0.1 to 10.9, 11 to 50 and >50 mf/mg. Delayed type reactions were absent in people with microfilaria load >11 mf/mg tested with SGA and, in people with any dermatologic scores inoculated with the L3 antigen. Such an analysis for the immediate type responses to any of the antigens could not be made because of the small number of people present in the various subsets. Nevertheless, the higher rate of immediate type reactions (84% with SGA and 90% with L3) in people with dermatology scores of 1 suggests that immediate hypersensitivity to both SGA and L3 antigens was predominantly a selective response of people with low dermatology scores.

Results of hypersensitivity tests in Sodere (not shown) indicate that 6/13 (46%) and 4/11 (36%) people inoculated with, respectively,

SGA and L3, gave reactions of the immediate type. There were no delayed type responses to SGA and only one to L3. The immediate responses in Sodere were lower than the responses of comparable mf-/ asymptomatic people from the onchocerciasis endemic area where, 20/29 (69%) and 12/18 (67%) of the population gave an immediate type of reaction to SGA and L3, respectively (Table 5, D^-/S^- column). Nonetheless, the results in Sodere indicate that hypersensitivity reactions to Simulium bites are seen in the absence of a concurrent infection with O. volvulus parasite.

Photographs illustrating the types and degrees of responses observed following inoculation with SGA and L3 are presented in Figures 7 and 8.

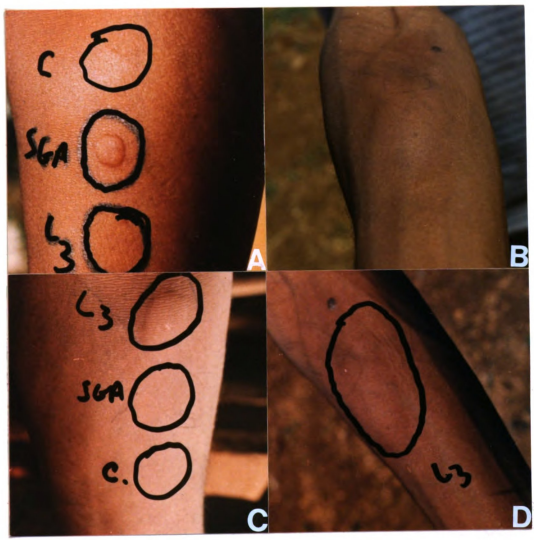

Figure 7. Hypersensitivity reactions in patients in Bebeka, Ethiopia.

A. 60 minutes, and B. 24 hours reactions with blackfly saliva.
C. 60 minutes, and D. 24 hours reactions with O. volvulus L3.

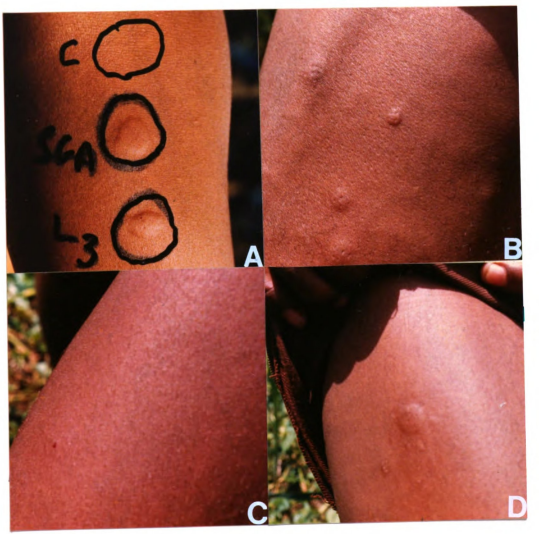

Figure 8. Hypersensitivity reactions in patients in Bebeka, Ethiopia

A. Simultaneous blackfly saliva and O. volvulus L3 antigens 60 minutes reactions.
B. Reactions to multiple Simulium bites.
C. 15 minutes, and D. 60 minutes Simulium bite reactions.

Table 3

Hypersensitivity to Salivary Gland Antigen (SGA), *O. volvulus* Third Stage Larvae (L3) and Adult Blackfly Homogenate (ABFH) in Rounds 1-3 in Bebeka (Ethiopia)

Round (n)	Antigen	Reactions		
		Immediate	Delayed	No Reaction
I (45)	SGA	29	2	14
II (45)	SGA	34	3	8
III (45)	SGA	36	8	1
I (15)	L3	10	1	4
II (15)	L3	13	2	0
III (15)	L3	14	1	0
I (6)	ABFH	6	0	0
II*	ABFH			
III*	ABFH			

*No data available for rounds II and III.

Table 4

Size Variation in the Immediate Type Reactions to Salivary Gland Antigen (SGA), O. volvulus Third Stage Larvae (L3), and Adult Blackfly Homogenate (ABFH) During Rounds 1-3 in Bebeka (Ethiopia)

Round (n)	Antigen	Size of Reactions		
		Small	Medium	Large
I (29)	SGA	20	5	4
II (34)	SGA	16	7	11
III (36)	SGA	6	16	14
I (10)	L3	6	3	1
II (13)	L3	3	8	2
III (14)	L3	2	8	4
I (6)	ABFH	2	2	2
II*	ABFH			
III*	ABFH			

*No data available for rounds II and III.

Table 5

Hypersensitivity Reaction to Salivary Gland Antigen (SGA), *O. volvulus* Third Stage Larvae (L3), and Adult Blackfly Homogenate (ABFH) in People Examined for Onchocerciasis in Bebeka (Ethiopia)

Antigen	Reaction	Onchocercal Diagnosis*			
		D^+/S^+ (n=45)	D^+/S^- (n=34)	D^-/S^+ (n=27)	D^-/S^- (n=29)
SGA	Immediate	38	24	17	20
SGA	Delayed	6	3	1	3
SGA	No Reaction	1	7	9	6
		(n=5)	(n=13)	(n=9)	(n=18)
L3	Immediate	4	12	9	12
L3	Delayed	0	0	0	4
L3	No Reaction	1	1	0	2
		(n=1)	(n=2)	(n=1)	(n=2)
ABFH**	Immediate	1	2	1	2

*D^+ = Dermatology Positive, D^- = Dermatology Negative, S^+ = Skin Snip Positive, S^- = Skin Snip Negative

**All immediate reactions

Table 6

Hypersensitivity Reactions to Salivary Gland Antigen (SGA), O. volvulus (L3), and Adult Blackfly Homogenate (ABFH) in Relation to Graded Dermatology and Microfilarial Load in Bebeka (Ethiopia)

Antigen	Reaction	Dermatology Score					Microfilarial Load (mf/mg)		
		1 (n=38)	2 (n=24)	3 (n=9)	4 (n=3)	5 (n=5)	0.1-10.9 (n=52)	11-50 (n=17)	>50 (n=3)
SGA	Immediate	32	16	9	2	3	39	13	3
SGA	Delayed	3	3	0	1	2	7	0	0
SGA	No Reaction	3	5	0	0	0	6	4	0
		(n=10)	(n=7)			(n=1)	(n=11)	(n=3)	
L3	Immediate	9	6	-	-	1	10	3	-
L3	Delayed	0	0	-	-	0	0	0	-
L3	No Reaction	1	1	-	-	0	1	0	-
		(n-1)	(n=2)				(n=2)		
ABFH	Immediate	1	2	-	-	-	2	-	-
ABFH	Delayed	0	0	-	-	-	0	-	-
ABFH	No Reaction	0	0	-	-	-	0	-	-

DISCUSSION

It is clear that the study area does not have typical features of a tropical rain-forest ecologic zone. However, because of the presence of many trees and other tropical vegetation in Bebeka and its vicinities and, because of the occurrence of rains, to some degree at least, in practically all the twelve months of the year (Figure 6), the study site may be considered more of a forest than a savannah zone in relation to onchocerciasis epidemiology. Distinctions between "rain forest" and "Sudan savanna" forms of onchocerciasis, based on differences in clinical manifestations and on differences in immunological studies, have been made by many authors (Anderson et al., 1974; Bryceson et al., 1976; Duke, 1981 and Lucius et al. 1987). Although there was a higher prevalence of people with nodules, the clinical manifestations and parasite burdens of onchocerciasis in Bebeka appear to be similar to those shown in "forest type" endemic zones in West Africa.

The very high prevalence of intestinal parasites, especially the preponderance of nematode infections such as Ascaris and hookworms, was surprising in view of the basic medical care provided by the plantation and the housing conditions for workers. It represents a complication to subsequent immunological studies because some cross-sensitization could be expected to occur in these subjects to antigens of O. volvulus. It may also be a determining factor in the hematological

profile of residents. Rather low hemoglobin values, particularly those less than 10gm% in 6.8% of the sample population, may be an indicator of morbidity due to these infections, and to S. mansoni and possibly malaria. Eosinophilia may also be the result of the high level of helminthic infection (Dessein and David, 1982).

Although malaria was a cause of much apprehension in Bebeka, no cases could be diagnosed by the parasitologic method. This unexpected finding may be attributable to suppression by the regularly administered bi-monthly chloroquine prophylactic treatment, coupled with the timing of the sample collection in March, a "non-malaria" season for this area.

The point prevalence of onchocerciasis in this village was determined in 1983 by Taticheff et al. (1987) and was then 30.9%. In the present study the prevalence of O. volvulus infection was 42%, an increase of over 11% in less than five years. The rates of occurrence of nodules (3%) and their distribution are compatible with the previous survey. The pattern of microfilarial intensity increases with age are typical of those seen in forest zones (Anderson et al. 1974). The morbidity associated with the disease is important in the health care of this community and the range and intensity of the clinical changes observed here suggest that the disease should have high priority in preventive medical programs at Bebeka. However, the absence of any ocular disease changes is both remarkable and very fortunate. The failure to detect any ocular onchocerciasis is consistent with all previous reports from Ethiopia. Whether this is a result of peculiarities of the local "strain" of pathogen remains to be determined. It is particularly noteworthy that to the west of this region in Sudan O.

volvulus infection is associated not only with dermatological problems, but with severe, often blinding ocular changes (Williams, et al. 1985). The recent emergence of molecular biological probes for the differentiation of strains of O. volvulus may eventually prove useful in addressing this enigma (Unnasch, 1987).

The clinical signs of onchocerciasis most frequently involved pruritus and, probably, some consequences of self-inflicted trauma. It is in this regard that the observations on hypersensitivity reactions to the various antigenic preparations tested here are most important. They permit several conclusions to be drawn. First, the enhanced and in some cases severe reactivity of individuals in Bebeka to the black fly derived antigens as compared to those in normal subjects is evidence that immunological sensitization takes place and is a factor in skin reactions. This is not to say that primary pharmacological effects of salivary components were not a contributing factor in the reactions as well; there is ample evidence in the literature of biological activity in Simulium saliva. However, the nature and timing of the lesions supports the conclusion that both immediate and delayed hypersensitivity to black fly antigens were expressed.

The increase in dermatologic problems encountered from the first round of data collected to the third was not directly related to increased O. volvulus microfilarial prevalence or intensity. Moreover, immediate type responses to salivary gland antigen were more frequent in patients with dermatological clinical signs than in those without. Wheal sizes increased with each successive round of observation. All this evidence supports the conclusion that these subjects' responses to Simulium were causal in the morbidity changes seen over

the course of the transmission season. That the pattern of temporal changes was not uniform is consistent with some individuals perhaps acquiring a degree of desensitization as a result of repeated bites. Pruritus and inflammatory changes with concommitant self-inflicted traumatic lesions due to hypersensitivity to Simulium could lead to pathological alterations that cannot be distinguished from those due to immunological sensitization to microfilarial antigens.

A key feature of immunological reactions is that they should be transferrable. Although the gross appearance and time course of the skin reactions seen here are in accord with categories of hypersensitivity described classically by Coombs and Gell (1976), no attempt was made here to determine transferability. However, Piessens and Mackenzie (1982) and Mackenzie (Personal communication) have commented on experiments in which cutaneous hypersensitivity to S. damnosum was successfully transferred to the skin of sub-human primates by means of the Prausnitz-Kustner (PK) reaction. This suggested that reaginic homocytotropic antibodies were present in the sera of patients showing immediate hypersensitivity to Simulium bites. Possibly antibodies of types other than IgE eventually modulate these reactions in the desensitization process (Ottesen, et al. 1981).

The immediate type hypersensitivity manifested toward O. volvulus L3 may be a result of inherently allergenic components of the worms, but could also be brought about by adherent Simulium allergens contained in the O. volvulus 3L antigenic preparation. This phenomenon has been postulated previously by Wikel (1982), and he suggested that reactions to these adherent antigens might even have a role in protection against the vector borne pathogen. Further work on the

immunochemistry of surface components of O. volvulus L3 would be necessary in order to pursue these questions. What was clear and very striking, however, was the restriction of delayed type hypersensitivity reactions to antigens of L3 to those individuals who had no demonstrable parasitological or clinical evidence of onchocerciasis. There is evidence of depressed cell mediated immunity both to parasite derived and unrelated antigens in O. volvulus infection and that this defect is reversed following a chemotherapy-induced elimination of microfilariae (Greene, et al. 1985). This could explain the absence of delayed hypersensitivity reactions in infected patients. On the other hand, it is conceivable that there is a causal relationship involved and that delayed hyperensitivity reactions provide some protective mechanisms which limit the success of 3L in their development process in human tissues in vivo. Parasite-negative people would then be delayed hypersensitivity positive as an expression of their resistance. This is most important in terms of current efforts to develop immunoprophylactic approaches to control of onchocerciasis (Parkhouse, et al. 1987). We cannot conclude that the allergens involved in this delayed hypersensitivity response are necessarily all exclusive to O. volvulus, because adherent Simulium constituents could also be involved.

Hypersensitivity reactions to Simulium could also be important in enhancing the success of O. volvulus larvae under some circumstances. For example, hyperemia and changes in vascular permeability might improve hematogenous movement of larvae from injection sites, or might make available certain esential host nutrients through extravasation around the parasite. Although apparently mediated by primary pharmacological activities of Lutzomyia longipalpis saliva, the recent work of

Titus and Ribeiro (1988) has shown that such events as local hyperemia can be important in enhancing the success of sandfly transmitted pathogens (Leishmania major). It is apparent that local events in the skin at the site of O. volvulus infective larval deposition may indeed be affected by immunological and non-immunological phenomena related to Simulium saliva.

The data collected here do not resolve definitively the continuing controversy over the effect of Simulium bites, but they do support the existence of heterogenous hypersensitivity reactions to blackfly bites in exposed subjects, and their aggravation or modulation temporally as a consequence of repeated exposure. Further specific immunological probes would be needed to define more critically the role of salivary constituents in onchocercal disease, but this would appear to be well worth pursuing. An important practical consequence of Simulium induced pathologic change is that O. volvulus control efforts based on the microfilaricidal effects of ivermectin may not reduce the pruritus and other manifestations normally attributed to O. volvulus as much as might be expected; some continued dermatological problems might persist due to repeated attacks by Simulium in the absence of vector control.

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CHAPTER 2

HUMORAL IMMUNE RESPONSES IN HUMAN ONCHOCERCIASIS

INTRODUCTION

Serological responses to antigens of Onchocerca volvulus have been detected by a wide variety of techniques aimed at enhanced diagnosis, epidemiological surveillance, and the identification of correlates between laboratory tests and clinical status and prognosis (Buck et al. 1973; Weiss et al. 1981; 1982a; Kawabata et al. 1983; For reviews see Mackenzie et al. 1986; Greene et al. 1983). However the antigenic composition of O. volvulus is remarkably complex (Williams et al. 1985) and specificity of all test procedures so far, including those based on monoclonal antibodies, has not been satisfactory (Lucius et al. 1986; 1987). Neither has there been any convincing demonstration of reliable clinical correlations between antibody specificities or antibody isotypes and disease characteristics, although a recent report claims to have incriminated IgG3 antibodies in the Sowda form of onchocerciasis (Parkhouse et al. 1987).

Almost all studies done to date have used a cross-sectional approach, whereby a sample of the population under study was examined parasitologically and clinically, and blood was then drawn for laboratory examination. Only a few observations have been made on samples drawn serially (usually annually) for diagnostic evaluation. There is therefore no information available about dynamic changes in antibody response to the parasite over the course of a transmission season, and there is nothing in the literature regarding detection of

related antibody responses to antigens of saliva of the Simulium vector over a similar time span. No data at all are available on immune responses to S. damnosum antigen in Ethiopian patients.

The immunoblot, or a Western blot technique, has become a very popular analytical tool for identifying the types of antigens recognized by antibodies in patients' sera, and for determining the class or sub-class of patient antibody involved in the reaction. The technique is extraordinarily sensitive so it can be applied in systems where antigen is in very short supply. By combining the opportunity to sample and examine patients serially in this study in Ethiopia, with the availability of the sensitive Western blot technique, several unique objectives were approached. First, the question of seasonal influences on antibody profiles to O. volvulus was examined by using sera collected serially from well characterized patients pre, during, and post transmission in an endemic area. Second, the possibility that circulating antibodies might develop against antigens of whole S. damnosum and of S. damnosum saliva was examined, and similarly pursued longitudinally over three successive visits to the field. Finally, an attempt was made to establish if detectable changes in antibody profile were relatable to altered clinical or parasitological status.

MATERIALS AND METHODS

Sampling procedure and sample handling

The study site and population characteristics are described in Chapter 1. Venous blood samples were collected with Venoject^{R} serum tubes. Blood samples were left to clot at room temperature for about two hours and then centrifuged at 2,000 RPM for five minutes. Clear serum was pipetted off and aliquotted in duplicate in polystyrene tubes and transported in either LN_2 or dry ice. The tubes were stored at -70^{o}C until used.

Although blood samples were collected three times from all 66 patients referred to in Chapter 1, because of constraints due to time and the limited quantity of antigenic material, only selected samples could be tested serially.

Western blotting procedure

Methods employed for this test are described in detail in Appendix 4. Data in the following table represent the number of immunoblots done per antigen preparation and per immunoglobulin isotype. Methods of preparing antigens are described in Appendix 2.

Table 1. Number of Immunoblots conducted with various antigenic preparations and immunoglobulin probes.

	Antihuman immunoglobulin isotypes				
Antigens	IgG	IgG1	IgG2	IgG3	IgG4
Adult O. volvulus homogenate	60	30	30	30	42
Adult Blackfly homogenate	81	42	42	42	51
Salivary Gland Antigen	78	30	24	24	36

Additionally, 24 sera collected at Sodere (Chapter 1), from 13 and 11 people tested once only for hypersensitivity to SGA and L3, respectively, were used for the detection of IgG against all three antigens.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) of antigens was performed according to Laemmli (1970) in 7.5% to 15% (W/V) gradient slab gels. Some gels were stained with Coomassie blue and some were used for immunoblots. Immunoblotting was carried out by adapting methods described by Towbin et al. (1979) using horse radish peroxidase conjugated anti-human IgG (AHIgG), or anti-human IgG1, 2, 3, and 4 monoclonal antibodies sandwiched with peroxidase conjugated anti-mouse IgG. All anti-sera were procured from ICN Immunobiologicals^{R}. Immunoglobulin IgG1-4 concentrations in certain sera from adults in Bebeka (18) and Sodere (7) were measured by radial immunodiffusion using the Accra Assay^{TM} kit (ICN Immunobiologicals, Lisle, IL, USA). Normal adult Ethiopian sera (4) were used as controls for standards.

Immunoblot results were recorded for certain antigens in terms of the relative molecular weight (Mr) of the bands, determined by

comparison with bands of molecular weight standards (MWS). Where appropriate, intensity of band coloration was estimated visually and noted. The objective was to try to establish some quantitative aspects of the exceedingly complex pattern of antibody responses.

RESULTS

SDS/PAGE Protein Composition of Antigens

A. Adult O. volvulus Homogenate (AOVH)

Separation of AOVH proteins by SDS/PAGE yielded a complex pattern of up to 34 bands stained with Comassie blue, with molecular weights ranging from 14 to 200 KD. (Figure 1B). Particularly prominent bands appeared at 21, 29, 33, 42, and 50 KD.

B. Adult Blackfly Homogenate (ABFH)

In ABFH, up to 25 bands could be seen over a narrower range, 13 to 95 KD, with most prominent ones at 25, 30, 34, 37, 47, and 49 KD (Figure 1C).

C. Blackfly Salivary Gland Antigen (SGA)

Electrophoresis of SGA resulted in the separation of fewer bands (17), and these covered approximately the same range as the ABFH. A limited number of bands, 36, 49, and 50 KD, showed prominence (Figure 1D).

Antibody Response Against Separated Antigens

A. AOVH

The IgG antibody response to AOVH was quite variable between patients in the study group. A range of antigenic polypeptides was

recognized, from approximately 14 to 200 KD (Figure 2), with a prominence of bands in the 14-40 KD range. There was no clear relationship between the parasite burden and the number or intensity of bands, nor with the clinical scores assigned for severity of lesions. This is exemplified in Figure 2 where "A" was a sample from a patient with dermal lesions, but with a negative skin snip; "B" was serum from a patient with dermatologic scores of 0, 1, 2, and microfilarial load of 0.6, 0.3, and 1 mf/mg in, respectively, rounds 1, 2, and 3; and "C" was sera from a patient with a microfilarial count of 6, 10, and 8 mf/mg, in rounds 1, 2, and 3, respectively, but a dermal score of 0 during the three rounds.

Of the antibody immunoglobulin isotypes responsible for the immunoblot reactivity, IgG4 was by far the most important contributor (Figure 3). More bands were in general recognized in the blots probed with anti-IgG4 isotype and they were more intense. The changes in reactivity were not relatable to alterations in parasite burden or clinical severity. This is demonstrated in Figure 3 where, "A" was from a patient with a 24.5 mf/mg and zero dermatology score, "B" was from a person with no microfilaria and no dermatologic manifestations, and "C" was from a patient with a dermatology scores of 0, 1, 2, and a microfilarial load of 6, 0, 22 mf/mg in, respectively, rounds 1, 2, and 3.

Changes in immunoblot reactivity patterns were apparent when serum samples collected from individuals in round 1, 2, and 3 were compared. In some instances band intensity and number increased (Figures 2A and 3C); in certain patients reactivity was not clearly apparent in the early stage of the season, but by the end several antigenic bands were

detected (Figure 2C). A pattern of fluctuating reactivity was evident in some individuals who had marked antibody recognition patterns in round 1, but these were variable in rounds 2 and 3 (Figure 2A).

B. ABFH

The IgG antibody response to ABFH as well as the intensities of recognized bands was variable between patients in the study group. The range of antigenic polypeptide identified was narrower than that of the AOVH and included bands with approximate molecular weight of 14 to 92 KD. This is depicted in Figure 4. Serial increases in intensity and number of bands were evident. Some showed no response in round 1 followed by a progressive increase in antigen recognition during rounds 2 and 3 (Figure 4C), while others showed sequential changes mostly related to prominence in bands (Figure 4B). No substantial difference in antigen recognition was observed between people with different dermatologic scores and microfilarial loads. In Figure 4, lanes 1 and 4 were from patients positive only for, respectively, dermatologic manifestation and microfilariae while lanes 2, 3, 5, and 6 and lanes 7, 8, and 9 were from patients, respectively, with positive and negative dermatology and skin snip examinations.

Reactions with IgG1, 2, 3, and 4 appear to follow a similar pattern of antigen recognition. With all isotypes, recognized antigens most commonly ranged in molecular weight from 38 to 50 KD and these appeared to be influenced by season, both in terms of increase in number and intensity of bands (Figure 5). Responses to the antigens were not relatable to any onchocercal findings.

C. SGA

lhe IgG antibody response to SGA was very heterogeneous in sera from patients in Bebeka. Antigenic recognition ranged from proteins with approximate molecular weight of 14 to 80 KD with a prominence of bands of 21, 50, 60, 70, and 80 KD (Figure 6). Some patients showed intense bands in round 1 which faded in round 2 and the color re-intensified in round 3 (Figure 6A), and others showed a gradual increase in number and intensity of bands over the course of the study period (Figure 6C). Sequential and progressive increase in the number of intense bands was evident (Figure 6B, C). In Figure 6 (where each triplet of strips represents a serial run for an individual) strips 1 and 2, and strips 3 and 9 were done with samples from, respectively, patients with only microfilariae and only dermal lesions while strips 7 and 8, and strips 4, 5, and 6 were done with samples from, respectively, people with both dermatology and microfilariae positive and negative findings. These depict the overall response to IgG and show that the immunoblot results did not relate to any specific dermal or parasitic findings.

Responses with anti IgG1, 2, 3, and 4 were also variable. Increases in reactivity, mostly in intensity of bands over the study period was observed in most sera. This is depicted in Figure 7 where each triplet of strips represents serial samples arranged sequentially. No association could be made between isotype responses and clinical or parasitological results.

Immunoblots in Sodere

Immunoblot results with samples from Sodere showed reactivity with the AOVH antigen probed with IgG, but the patterns were different. Some people from Sodere recognized up to 8 bands. The molecular weights of most proteins recognized ranged from 28-92 KD (Figure 8A).

In the ABFH probed with IgG, all patients showed reactions. Approximate molecular weights of the antigens recognized ranged from 30 to 72 KD with bands 31, 40, 45, and 50 KD being more prominent than others (Figure 8B). In the SGA probed with IgG, similar findings to those with ABFH were obtained except that certain bands were more intense (Figure 8C).

In Table 2, values of the IgG1-4 concentrations are given.

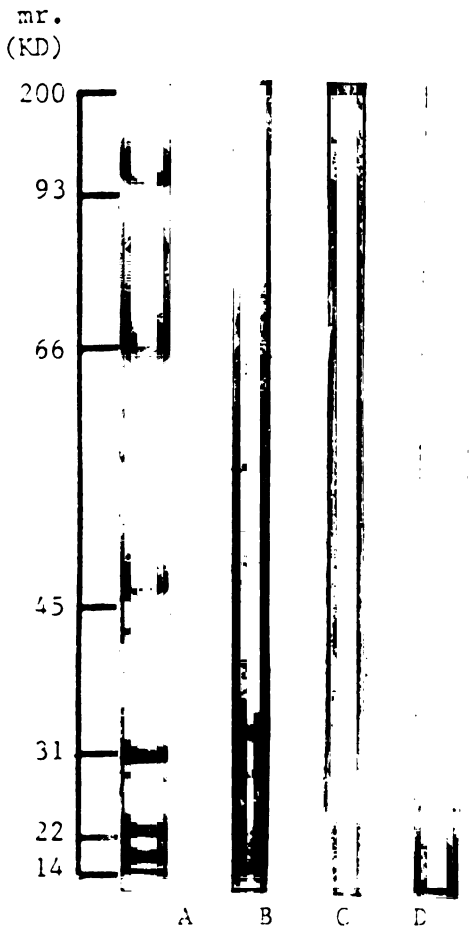

Figure 1. Acrylamide gels stained with Coomassie Blue Stain.
A. M.W. markers
B. Adult O. volvulus homogenate (AOVH)
C. Adult blackfly homogenate (ABFH)
D. Blackfly salivary gland antigen (SGA)

Figure 2. Adult O. volvulus homogenate (AOVH) IgG immunoblot analyses with serial (rounds 1-3) samples from onchocerciasis patients in Bebeka, Ethiopia.

A. Sera from a patient with dermatologic scores of 2 and 3 collected in, respectively, rounds 1, 2, and 3, and with negative skin snip results in all 3 rounds.

B. Sera from a patient with dermatologic scores of 0, 1, 2, and microfilarial loads of 0.6, 0.3, and 2 mf/mg during, respectively, rounds 1, 2, and 3.

C. Sera from a patient with no dermal lesions in all three rounds and microfilarial loads of 6, 10, and 8 mf/mg in , respectively, rounds 1, 2, and 3.

D. Control; "normal" human serum substituted for patient serum.

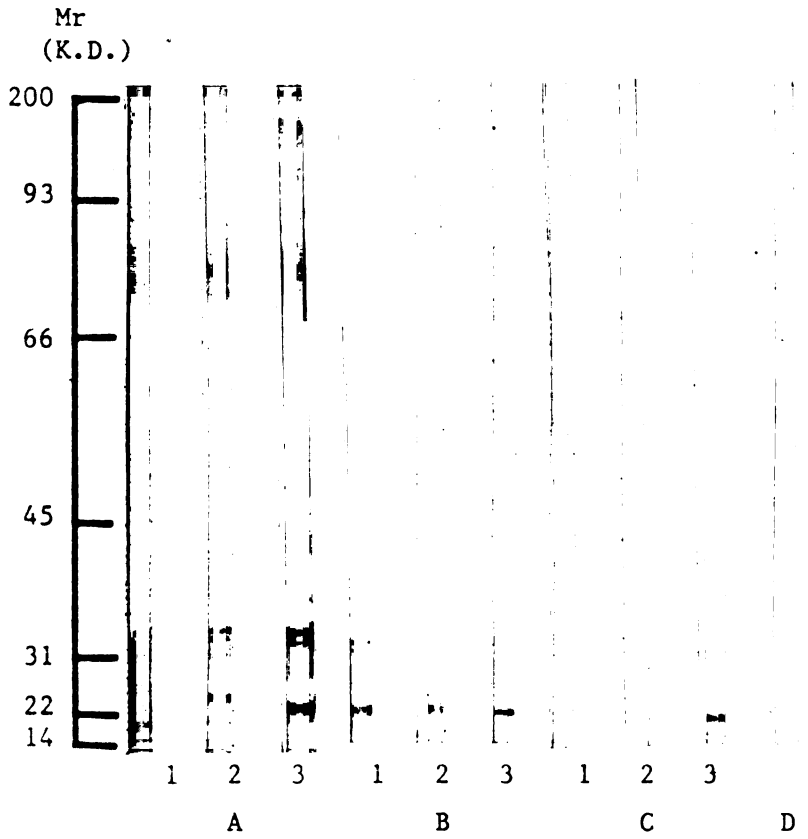

Figure 2.

Figure 3. Adult O. volvulus homogenate (AOVH) IgG1-4 immunoblot analyses with sera collected from onchocerciasis patients in Bebeka, Ethiopia.

A. Serum from a patient with a dermatologic score of 0 and a microfilarial load of 24.5 mf/mg analyzed for IgG1-4, arranged consecutively from left to right.

B. Serum from a patient with no dermal lesions and no skin microfilariae analyzed for IgG1-4 arranged consecutively from left to right.

C. Sera collected serially from a patient with dermatologic scores of 0, 1, 2, and microfilarial loads of 6, 0, and 22 in, respectively, rounds 1, 2, and 3 analyzed for IgG4.

D. Controls; "normal" human serum substituted for patients sera and analysed for IgG1-4 arranged consecutively from left to right.

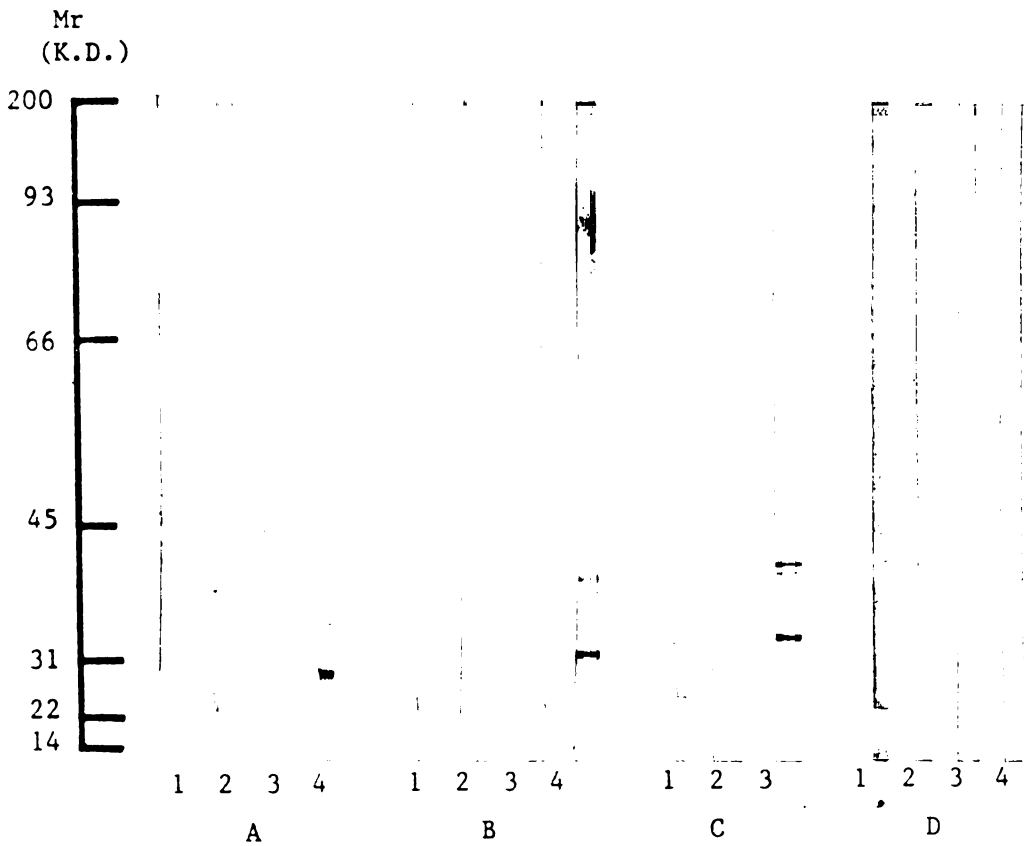

Figure 3.

Figure 4. Adult blackflie homogenate (ABFH) IgG immunoblot analyses with serial (rounds 1-3) samples collected from onchocerciasis patients in Bebeka, Ethiopia.

A. Sera from a patient with dermatologic scores of 1, 1, and 3 in, respectively, rounds 1, 2, and 3, and with negative skin snip test results during all three rounds.

B. Sera from a patient with dermatologic scores of 0, 1, and 2 and microfilarial loads of 2, 0, and 0 mf/mg in, respectively rounds 1, 2, and 3.

C. Sera from a patient with no dermal lesions, but microfilarial loads of 13, 4, and 20 in rounds 1, 2, and 3, respectively.

D. Control; "normal" human serum substituted for patient serum.

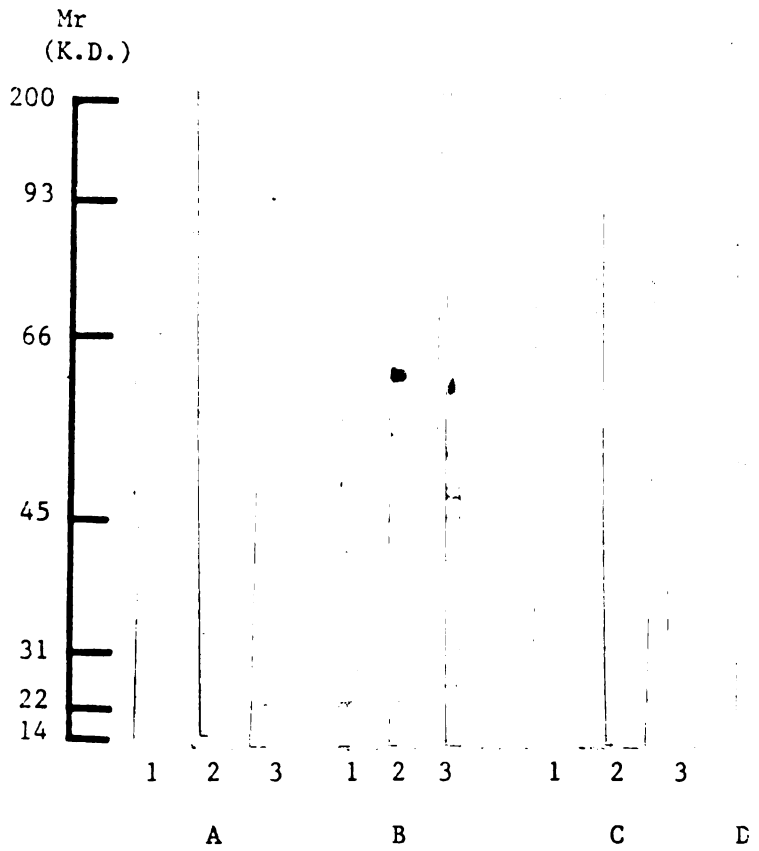

Figure 4.

Figure 5. Adult blackflie homogenate (ABFH) IgG1-4 immunoblot analyses with serial (rounds 1-3) samples collected from onchocerciasis patients in Bebeka, Ethiopia.

A. Sera from a patient with dermatologic scores of 1, 0, 1, and microfilarial loads of 0, 10, and 17 mf/mg in, respectively, rounds 1, 2, and 3 analyzed for IgG1.

B. Sera from a patient with dermatologic scores of 0, 1, 1, and microfilarial loads of 6, 4.5, and 0 mf/mg in, respectively rounds 1, 2, and 3 analyzed for IgG2.

C. and D. Sera from a patient with dermatologic scores of 0, 1, 2, and microfilarial loads of 0, 0, and 5 mf/mg in, respectively, rounds 1, 2, and 3 analyzed for IgG3 (C) and IgG4 (D).

E. Controls; "normal" human serum substituted for patients sera and analyzed for IgG1-4, consecutively from left to right.

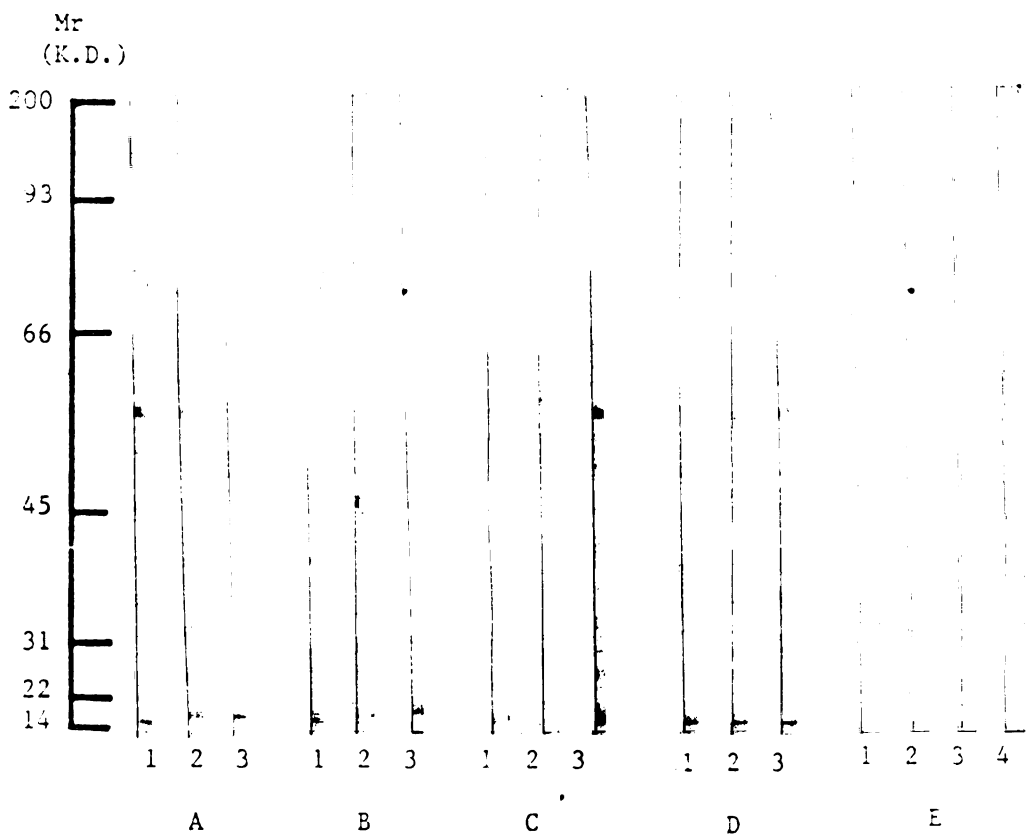

Figure 5.

Figure 6. Black fly salivary gland antigen (SGA) IgG immunoblot analyses with sera serially collected from onchocerciasis patients in Bebeka, Ethiopia.

A. Sera from a patient with dermatologic scores of 0, 0, 1, and microfilarial loads of 10, 11, and 0 mf/mg in, respectively, rounds 1, 2, and 3.

B. Sera from a patient with no dermal lesions and no microfilariae in all three rounds.

C. Sera from a patient with dermal scores of 2, 2, 3, and microfilarial loads of 4, 6, and 0 mf/mg in, respectively, rounds 1, 2, and 3.

D. Control; "normal" human serum substituted for patient serum.

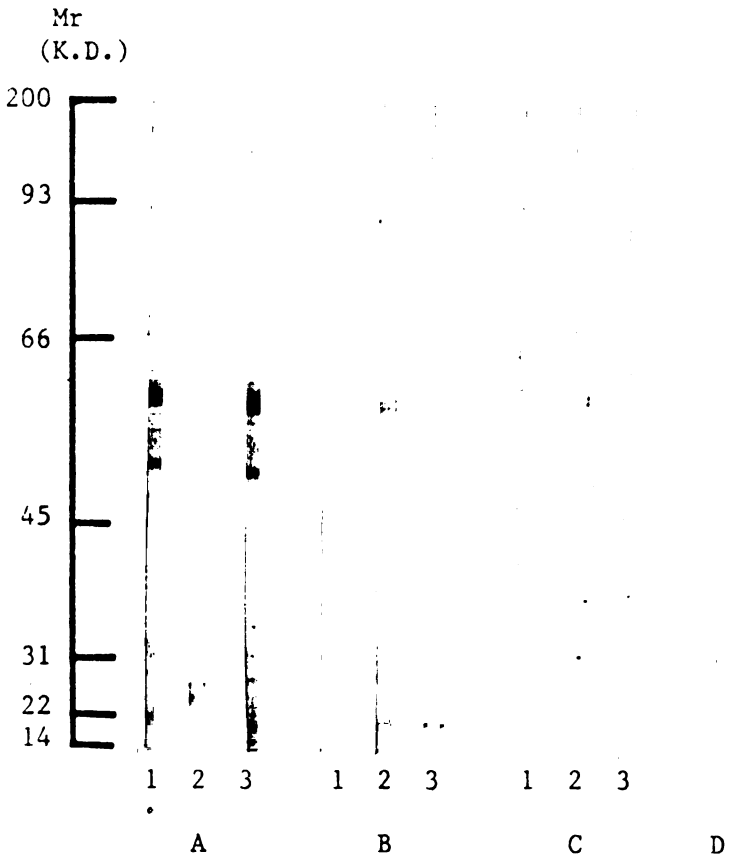

Figure 6.

Figure 7. Black fly salivary gland antigen (SGA) IgG1-4 immunoblot analyses with sera serially collected from onchocerciasis patients in Bebeka, Ethiopia.

A. Sera from a patient with dermatologic scores of 0, 1, 2, and microfilarial loads of 0, 0, and 7 mf/mg in, respectively, rounds 1, 2, and 3 analyzed for IgG1.

B. Same sample as in A, but for IgG2.

C. Same sample as in A, but for IgG3.

D. Same sample as in A, but for IgG4.

E. Controls; "normal" human serum substituted for patients sera and analyzed for IgG1-4 arranged sequentially.

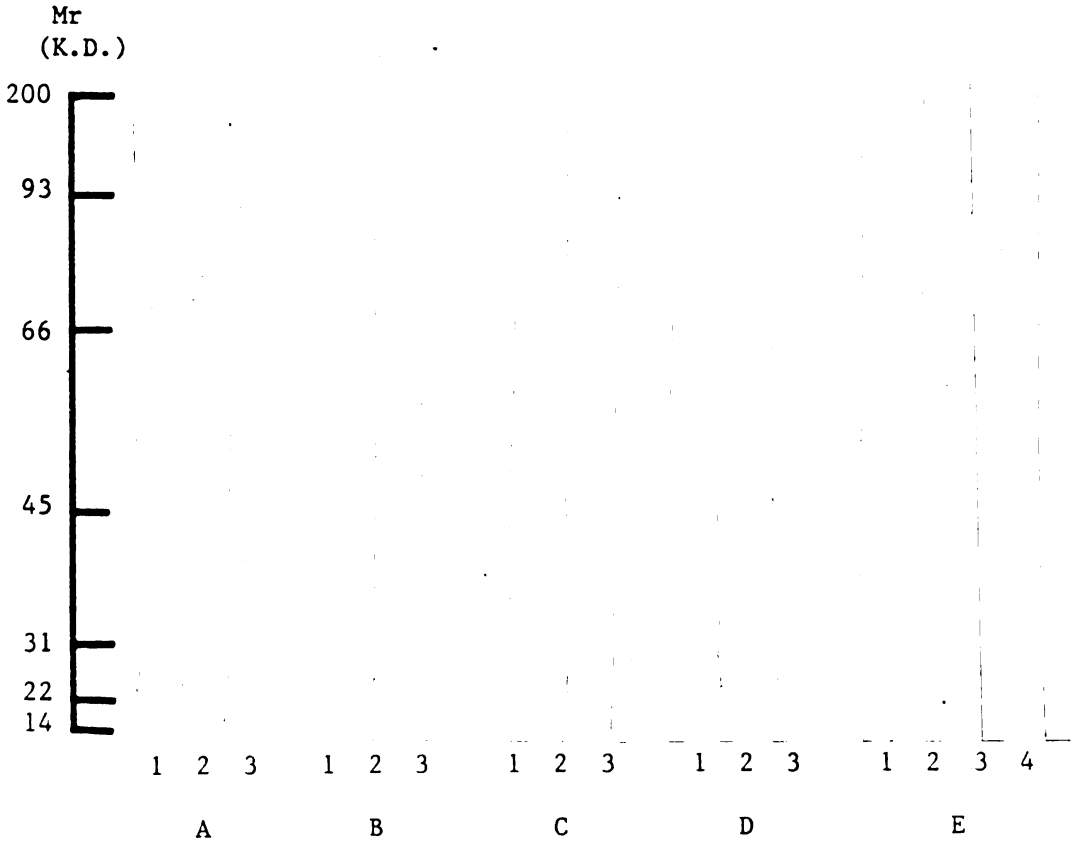

Figure 7.

Figure 8. Immunoblots with sera collected from people in Sodere, Ethiopia.

A. Adult O. volvulus homogenate (AOVH) IgG immunoblot analyses in four samples.

B. Adult black fly homogenate (ABFH) IgG immunoblot analyses with the same samples as in A.

C. Black fly salivary gland antigen (SGA) IgG immunoblot analyses with the same samples as in A.

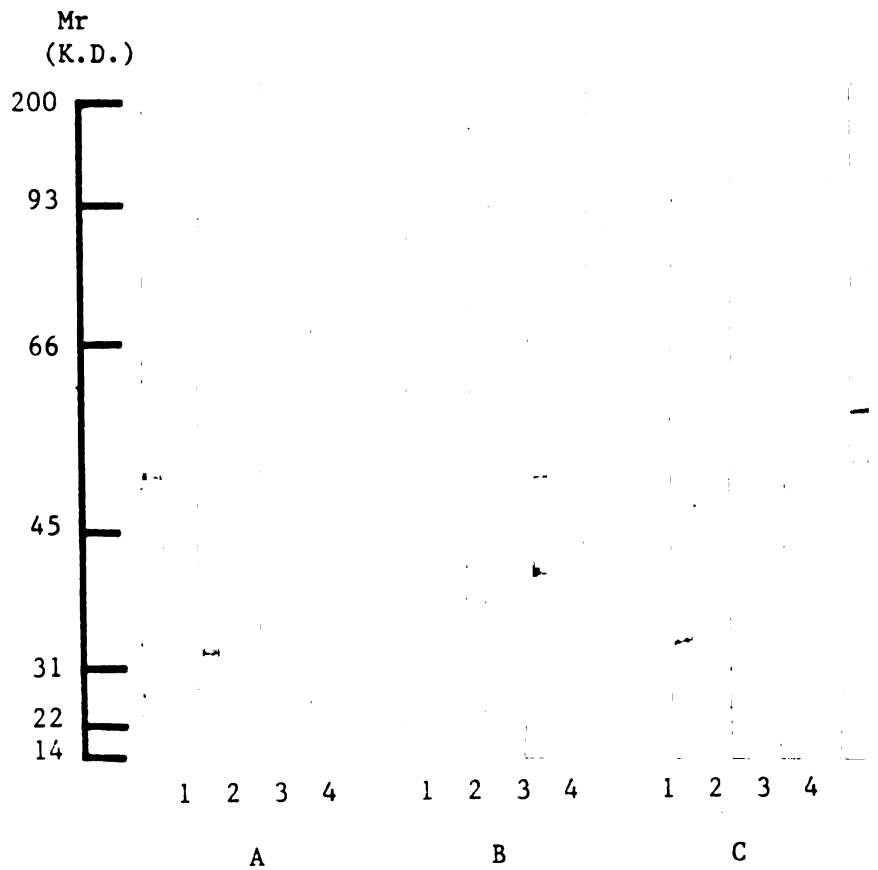

Figure 8.

Table 2. Serum IgG Subclasses Concentration by Radial Immunodiffusion (Mean values and range in mg/dl.)

IgG Subclasses	Bebeka (n=18)	Sodere (n=7)	"Normal" Ethiopians (n=4)	"Normal" Westerns*
IgG1	539 (124-1680)	600 (200-1300)	690 (240-1100)	540 (280-1020)
IgG2	215 (166-600)	472 (270-716)	330 (105-850)	210 (60-790)
IgG3	170 (99-420)	150 (85-360)	70 (30-150)	58 (14-240)
IgG4	750 (140-1350)	90 (30-940)	70 (30-360)	60 (11-330)

*ICN^R values

DISCUSSION

These results permit several important conclusions to be drawn about the differential characteristics of immunological responses to O. volvulus and to the vector which transmits it. They also emphasize the rapidity with which antibody profiles may change in an individual; this raises serious questions about cross-sectional approaches routinely used to try to relate antibody activity to parasitological and/or clinical status in this disease.

Overall, the antibody responses seen against O. volvulus crude antigens in these patients was heterogeneous, but not anywhere near as complex as responses seen in sera from patients in savannah regions (Lucius et al. 1987, Weiss et al. 1982b,). Antibody activity in patients with Ethiopian onchocerciasis in general seems to be directed to a number of relatively low M.W. polypeptides, and there is a dominant involvement of IgG4 isotype immunoglobulins. This preponderance of IgG4 is beginning to appear to be a common feature of antibody responses in human helminthiasis (Oxelius 1984; Ottesen et al. 1985; Hussain and Ottesen 1986; Hussain et al. 1986). Other isotypes contributed to a much lesser degree in the serological reactivity, and quantitatively it is clear that total IgG4 concentrations were very much higher in sera from these patients than in normal western population sera, or in normal Ethiopians. It is not at all evident, however, why this preferential stimulation of IgG4 comes about or what

benefit or otherwise it confers upon the responding host. Immunoglobulins of this type have been associated with "blocking" types of functions in immediate hypersensitivity (Ottesen et al. 1985), but they have also been incriminated in a causal role in certain food allergies (Oxelius 1984). They normally participate to a very limited degree in serological reactions to most pathogens and are by far the least concentrated immunoglobulins of the IgG type in human sera. Recently, IgG4 antibodies have been shown to dominate the antibody response to Schistosoma mansoni, and have been detected in high levels in sera from onchocerciasis patients by Parkhouse et al. 1987, and ElKhalifa (personal communication). They also dominate in the sera of patients with lymphatic filariasis (Ottesen et al. 1985). Although IgE concentrations were not measured in these Ethiopian sera they are likely to be much higher than normal based on previous studies on human filariasis, and the IgG4 antibodies may serve in some modulating or blocking way.

The changes in blot profiles over the relatively short intervals covered in this study were often remarkable. Generally increasing complexity became apparent as the season progressed, presumably as a result of continuing exposure to infective larvae. More O. volvulus bands were seen and with greater intensity in many cases after the transmission season was underway or had been completed. These changes were not correlated with clinical score or increases in microfilarial intensity, so it is not possible to incriminate any particular antigen-antibody system in the pathological process. However, as with previous studies (Lucius et al. 1986; Lucius et al. 1987), prominent bands appeared in many sera at 21 KD, as well as at 33 KD and these

appear to be immunodominant constituents. Lucius et al. (1988) have recently targeted the 21KD component for diagnostic immunoassay development.

Specificity of immunoblot profiles of onchocerciasis patients was obviously undermined by the cross-reactivity resulting from widespread helminthiasis due to other intercurrent parasite infections. The bias toward recognition of other antigens in sera from Sodere, for example, suggests nevertheless that certain antigenic components of O. volvulus could eventually be valuable as specific epidemiologic indicators of exposure in populations. Since the reactivities are so heterogenous within the population, can quickly change over a transmission season, and do not bear, at least in this study, an obvious relationship to clinical or parasitological status, it seems unlikely that antibody tests can serve as the basis for diagnostic testing in individual patients.

There was very little antibody activity expressed in these sera by IgG3 immunoglobulins. Antibodies of this type are associated with the Sowda form of the disease, according to Parkhouse et al. (1987). In Bebeka the onchocercal disease tends towards the mild and generalized form, although some cases showing the more intense and asymmetric localization of lesions of "Sowda" were seen. The strict discrimination between generalized and localized onchocerciasis that has been propagated in the literature is not as clear in the field as might be expected (Mackenzie and Williams 1985), and this is obviously true in Ethiopia. The absence of IgG3 reactivity may not therefore be as important a finding as one might believe at first sight. The evidence for its incrimination in "Sowda" cases remains scanty.

Antibodies against Simulium, and especially against the saliva, were readily demonstrated in both O. volvulus infected and non-infected individuals in this study. Gel profiles of polypeptides from saliva confirmed the complexity of this fluid, and the blot profiles confirm the existence of multiple antigenic components in the preparation used for antibody detection. Remarkably, there was evidence of antibody formation in all IgG isotypes, with no dominance of any particular type. Nor was there any relationship between onchocercal lesions and western blot profiles. Nevertheless, band recognition increased in prevalence and some bands, especially that of 60KD, were seen more intensely as the season progressed. This may represent the dynamics of exposure to repeated antigenic experiences through blackfly bites over the season. The fact that patients in Sodere also commonly reacted to salivary antigens suggests that antibody reactions are commonly provoked by Simulium bites, although it was not possible to sample serially at that site.

Although these data illustrate for the first time the complexity and heterogeneity of antibody responses to Simulium, the results shed little light on the mechanism of the severe reactions in the skin of Simulium-bitten patients, or of reactions to the intradermal administration of antigen. These reactions are most likely to involve reagenic homocytotopic antibodies of the IgE type. The latter were not detected in the immunoblots prepared here; preliminary experiments aimed at demonstrating IgE reactions with anti IgE reagents gave very unsatisfactory results, and unacceptable non-specific binding. However, absolute IgE concentrations are typically very much lower than those of all other immunoglobulin classes, and an assay involving purified

radiolabelled salivary antigen may be required to identify and quantitate specific anti-*Simulium* IgE in future work. Once again, some of the antibody activity in other classes and subclasses might be responsible for modulating and eventually desensitizing patients exposed repeatedly. These antibodies could also be important in neutralizing the biological activities of saliva that have primary proinflammatory or anticoagulant effects in their own right. *In vitro* studies on lymphocyte reactivity to *Simulium* saliva might also provide valuable insights into the dynamics of hypersensitivity events in people in endemic areas.

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CHAPTER 3

HISTOLOGICAL OBSERVATIONS OF THE SKIN RESPONSES TO ANTIGENS OF BLACK FLY SALIVA (SGA) AND *O. VOLVULUS* INFECTIVE LARVAE (L3)

INTRODUCTION

Over 70 years ago, Stokes (1914) wrote an extensive account on the blackfly histology made on himself. The most prominent features he reported were vascular dilatation, perivascular edema, and cellular infiltrates, particularly eosinophils. No other similar work has ever been done since then and no information on the histology of skin responses to O. volvulus infective larvae (L3) is available in the literature. Histologic observations on reactions to other insects provided some background to this study.

Those most extensively studied are reactions to ticks where, at the site of the ectoparasite attachment, eosinophils and degranulated mast cells have been observed (Pavlovskii and Alfreva, 1941; Moorhouse and Tatchell, 1969; Theis and Budwiser, 1974; Alen et al., 1977; Rubaire-Akiki and Mutinga, 1980; Brossard and Fivaz, 1982). Similar histologic events were documented in louse-infested mice (Nelson et al. 1977). Furthermore, Dvorak et al. (1986) have described cellular infiltrates of lymphocytes, eosinophils, basophils and neutrophils in a chigger-mouse system. Cutaneous responses to chigger feeding were considered to have an immunological basis (Wright et al. 1988). Biopsies following bites by mosquitoes have also shown edema and perivascular infiltration of neutrophils, eosinophils, lymphocytes and plasma cells (Rockwell and Johnson 1952). Willadsen (1980) and Wikel (1982) have reported intense

eosinophilia and increase in tissue basophils as host responses to hematophagous arthropods in general.

The significance of these cellular infiltrates is not clear. However, degranulated mast cells and basophils suggest immediate hypersensitivity responses while eosinophils are known to play a regulatory role in these reactions (Weller and Goetzl, 1979).

In this study, attempts were made to show that SGA and L3 are capable of eliciting dermal reactions and to demonstrate that the sequella of these reactions might, at the histological level, mimic onchocercal lesions.

MATERIALS AND METHODS

Materials were obtained from 15 volunteers in the hypersensitivity group described Chapter 1. There were five and four biopsies made from people injected with SGA with, respectively, immediate and delayed type reactions. The corresponding numbers for those inoculated with L3 were 3 and 1. Only two biopsies were collected from people tested for ABFH response and giving immediate reactions. The biopsies were made with 3 mm biopsy punches from the center of the reaction sites (immediate and delayed). The periphery of the reaction sites was injected with a local anesthetic. The materials thus collected were placed in labelled polystyrene vials and kept in LN2 for transportation. Individual biopsies were then mounted in OCT and processed by Dr. C.D. Mackenzie of the London School of Hygiene and Tropical Medicine.

RESULTS

Although it was initially planned to take 1 biopsy per person/round, from as many people as possible, this was not achieved because of lack of volunteers. Therefore, these results do not represent sequential observations.

The histology of the reactions in people inoculated with salivary gland antigen (SGA) showed an impressive degree of edema in those with immediate type responses. Those that gave delayed type reactions to the skin tests showed cellular infiltrates with both eosinophils and mononuclear cells, and mast cell degranulation was a dominant feature. One sample from the immediate type responders showed vascular dilatation. SGA also produced some eosinophil degranulation in the vascular walls.

Observations in samples collected from people inoculated with the L3 antigen indicate a similar pattern to that of the SGA.

On the whole, immediate type responses with both antigens did not give pronounced cellular change at 60 minutes, but edema and vascular dilatation were present. By 24 hours, samples of patients given both SGA and L3 gave strong eosinophil reactions. The histologic observations are depicted in Table 1, and Figures 1 and 2.

Table 1

Histological Observations of Skin Responses to Blackfly Salivary Gland Antigen (SGA), O. volvulus Third Stage Larvae (L3) and Adult Blackfly Homogenate (ABFH) in Bebeka (Ethiopia)

		Histological Findings				
Antigen Injected	Time of Biopsy	Edema	Eosino-philia	Degran-ulated Mast Cells	Mono-nuclear Cells	Vascular Dilatation
SGA	60 min.	++	0	0	0	++
	60 min.	++	0	0	0	0
	60 min.	++	0	+	±	0
	60 min.	+	0	++	±	0
	60 min.	0	++	0	0	0
	24 hours	0	±	0	++	0
	24 hours	+	0	++	+	0
	24 hours	±	++	+	++	0
	24 hours	0	++	++	+	0
L3	60 min.	0	+	±	0	0
	60 min.	++	0	0	0	++
	60 min.	++	0	++	±	0
	24 hours	0	++	0	++	0
ABFH	60 min.	+	0	+	0	0
	60 min.	+	0	+	+	0

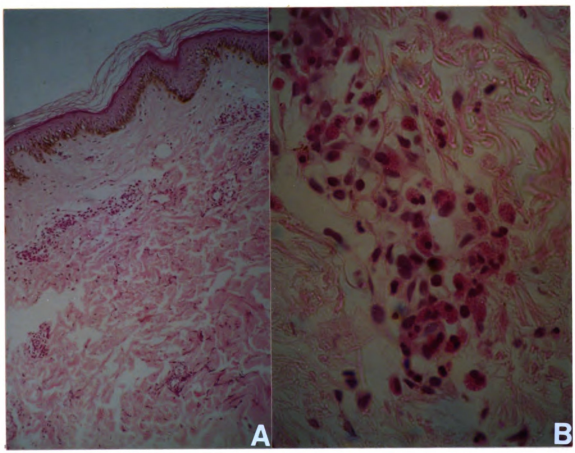

Figure 1. Histological appearance of biopsy taken 60 minutes (A) and 24 hours (B) after injection with salivary antigen.

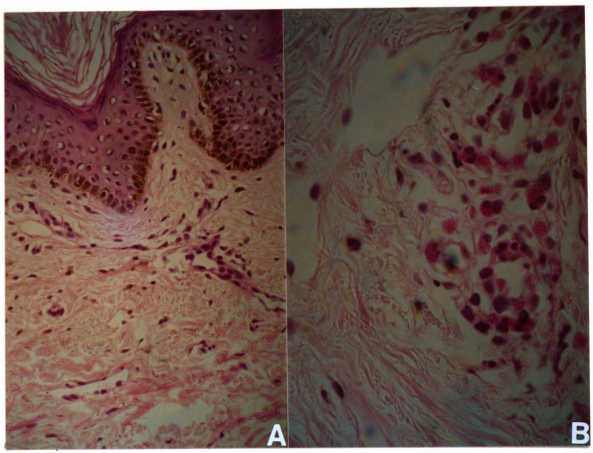

Figure 2. Histological appearance of biopsy taken 60 minutes (A) and 24 hours after injection with L3 antigen.

DISCUSSION

On the basis of these histology results, we can only speculate on what might have happened at the antigen-host interface. Earlier works have shown that bites by arthropods stimulate immune responses (Wikel 1982) and edema as well as histopathologic changes involving cellular infiltrates associated with hypersensitivity reactions have been observed (Benjamini and Feingold, 1970; Goldman et al. 1952; and Theis and Budwiser, 1974). In this study, we may reasonably presume that an antigen-antibody reaction has occurred at the site from which the biopsy was made. Indications of the immune responses were edema, vasodilatation, cellular infiltrates and mast cell degranulations. These responses are known to be mediated by histamine and other inflammatory mediators (Vagt, 1974; Beaven, 1976).

Tissues from people with the immediate and the delayed reactions showed a good deal of similarities. Nevertheless, edema was the essential characteristic in samples from people with the immediate reactions while increase in cellular infiltrates was dominant in those with the delayed response. Because of the small number of biopsy materials, a comparison between findings in those inoculated with similar and different antigens as well as difference in histologic results between people of varying dermatologic scores and microfilariae load could not be justified. In the absence of this information there is much yet to be done. However, the histology observations, i.e.

edema (with all antigens), the preponderance of eosinophils and mononuclear cells and the presence of degranulated mast cells indicate that there were common histological bases for the skin reactions seen in the hypersensitive people described in Chapter 1.

The death of the O. volvulus microfilariae as a possible cause of immunologically mediated pathology has been proposed (Bryceson, 1976). In view of the occurrence of comparable inflammatory responses, we speculate that an immunological mechanism is also involved in bringing about cutaneous lesions following bites by blackflies. It is also apparent that additive or even synergistic effects might occur in the skin as a result of hypersensitivity to infective larvae saliva and microfilariae and these might all contribute to the disease process.

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APPENDIX 1

RECORD SHEET FOR INDIVIDUALS IN THE STUDY

APPENDIX 1

RECORD SHEET FOR INDIVIDUALS IN THE STUDY

A. General Information:

Identification Number_____ Name_____

Age_____Sex_____Farm unit_____Occupation_____

Nativity_____Years in Bebeka_____

Do you have scratching complaint_____. If yes, is it seasonal ____yes/____no/____don't know. What month(s) of the year does it get worse_____. Do you have vision problems_____.

B. Dermatologic Observation

	Present (site)	Absent
Dermatitis		
Pigment related skin changes		
Texture related skin changes		
Lymphadenopathies		
Nodules (number)		

C. Laboratory Investigations

i. Stool for parasites_____

_____

ii. Hemoparasite_____

_____

iii. Hgb_____, WBC_____, D.C._____

iv. Urine microscopy_____

v. Skin snip (Mf/mg skin)_____

vi. Simulium bite reaction_____

vii. SDS/PAGE_____

_____

D. Pictures Taken

Film(s) identification No._____

Anatomical region(s) photographed_____

_____

E. Others:_____

_____

_____

_____

APPENDIX 2

ANTIGEN PREPARATION

A. Preparation of Adult O. volvulus Homogenate (AOVH)

B. Preparation of Adult Black fly Homogenate (ABFH), Salivary Gland Antigen (SGA) and O. volvulus Third Stage Larvae (L3)

APPENDIX 2

ANTIGEN PREPARATION

A. Preparation of Adult O. volvulus Homogenate (AOVH)

Nodules from patients in Bebeka were surgically removed, placed in 50 ml Corning^{R} polypropylene tubes and directly frozen in LN_2 to be transported to the central laboratory where they were kept at -50°C until used.

Individual nodules were thawed and excess tissues removed, leaving the thin layer of the capsule. The nodules were then submerged in RPMI 1640 medium (Gibco) containing 2-3 mg/ml collagenase (Sigma) and 100 U/ml of a streptomycin/penicillin suspension and were left over night at 37°C in a rocking incubator. Digestion was considered complete when the tissue surrounding the worms was cleared. Individual worms were then picked out with a pair of forceps, washed several times with PBS buffer, pH 7.2 and checked for exogenous tissues under a dissecting microscope. No attempt was made to separate male from female worms. Clean worms were placed in a chilled manual glass homogenizer and ground for a minimum of 30 minutes while adding small amounts of cold PBS buffer. The milky-opaque homogenate was then decanted in a sterile centrifuge tube, centrifuged at 5000 RPM for 30 minutes and the supernatant pooled in a small vial. The pellet from the centrifuge was dislodged by tapping, rinsed into the homogenizer for further grinding

and subsequent centrifugation. This process was repeated until the pellet was too small for more homogenization. The pool of homogenate was mixed with a vortex^{R} for more than 3 minutes and alliquoted in 200 ul volume. Total protein determined by the Lowry (1951) method was 0.66 gm%.

B. Preparation of adult black fly homogenate (ABFH), Salivary gland (SGA) and O. volvulus third stage Larvae (L_3) antigens

Black flies were collected, in Bebeka, by landing capture from 6:00 am to 11:00 am and from 4:00 pm to 6:00 pm during the height of the fly season. Daily collections were kept in capped containers and stored in $^{LN}_2$ until used.

Flies mounted on a glass slide with a drop of sterile PBS, pH 7.2, were dissected under a dissecting microscope. Any larvae from head and/or thorax that were considered morphologically indistinct from O. volvulus L_3 were picked out with a needle and transferred to an iced vial containing PBS and labelled L_3. From dissected flies showing no L_3, the salivary glands were carefully removed intact and collected in a separate vial labelled SGA. Other flies, again with no L_3, had their wings removed and the bodies were collected separately and identified as ABFH. Over 3000 flies were processed as above and each preparation was homogenized, cold, using a sterile manual glass homogenizer. Homogenized antigens were then each filtered using Millex GS 0.22 nm filter unit (Millipore Products Division), double-checked for sterility with inoculation on blood agar media and aliquoted in 200 ul volumes. Protein determinations were made using Chemo-mat^{R} (Coultronics) automated method based on the Lowry (1951) principles. Protein values were 0.9 gm%, 0.2 gm%, and 0.1 gm% for ABFH, SGA and L_3, respectively.

All antigens were serially diluted with sterile PBS, pH 7.2, solution and tested for potency by intradermal injection on people from onchocerciasis endemic and non-endemic areas (Figure 1).

Dilutions that gave positive reactions in onchocerciasis endemic people, but no reaction in "normal" people were used for the hypersensitivity study. "No reaction" was defined by comparison with a PBS solution injected side-by-side with the antigen.

References

Lowry, O.H., Rosebrough, N.R., Farr, A.L. and Randeull, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Figure 1. Salivary gland (A) and O. volvulus 3rd stage larvae (B) antigens titration on persons from onchocerciasis non-endemic area. Left upper most circles represent lowest antigen dilutions.

Skin test results with lowest salivary gland (C) and L3 (D) antigens dilution on persons from onchocerciasis endemic area.

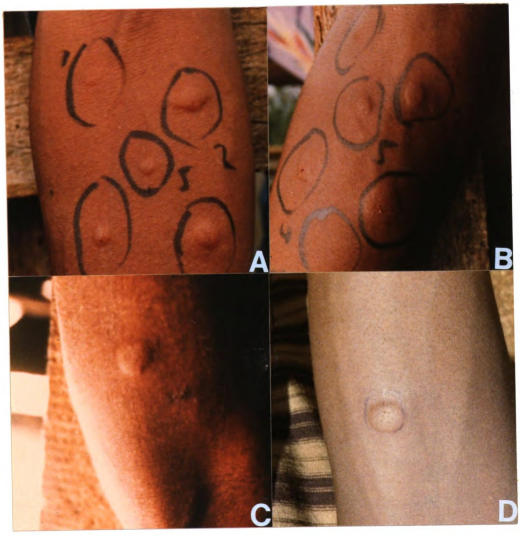


Figure 1.

APPENDIX 3

ONCHOCERCIASIS IN BEBEKA, ETHIOPIA

Table 1. Onchocerciasis cross-sectional study in Bebeka (Ethiopia). Graded dermatological lesions microfilarial load by age group.

Dermatology Scores	Age Groups				mf/mg	Age Groups			
	1-14	15-30	31-45	>45		1-14	15-30	31-45	>45
0	67	136	56	3	0	72	128	44	1
1	15	67	16	4	0.1-10.9	16	66	21	8
2	6	19	14	2	11-50	2	36	21	5
3	2	5	1	2	>50	0	3	2	0
4	0	5	1	1					
5	0	1	0	2					
TOTAL	90	233	88	14		90	233	88	14

Appendix 3

ONCHOCERCIASIS IN BEBEKA, ETHIOPIA

A. Results of onchocerciasis cross-sectional study - graded dermatologic lesions and microfilarial load by age group.

B. Results of onchocerciasis cross-sectional study - graded dermatologic lesions and microfilarial load by sex.

C. Results of onchocerciasis longitudinal studies in Bebeka - Pattern of dermatologic lesions and skin snip examination changes in 98 people from round 1, through 2 to 3.

D. Results of onchocerciasis longitudinal studies in Bebeka - Pattern of changes in graded dermatologic lesions and microfilarial load in 98 people from round 1, through 2 to 3.

E. Results of onchocerciasis longitudinal study - Pattern of graded dermatologic lesions and microfilarial load changes in 60 people included for hypersensitivity test from round 1, through 2 to 3.

F. Pictorial presentation of some clinical forms of onchocerciasis in Bebeka.

Table 2. Onchocerciasis cross-sectional study in Bebeka (Ethiopia). Graded dermatological lesions and microfilarial load by sex.

Dermatology Scores	Sex		mf/mg	Sex	
	Male	Female		Male	Female
0	143	119	0	124	121
1	72	30	0.1-10.9	87	24
2	34	7	11-50	52	12
3	9	1	>50	5	0
4	7	0			
5	3	0			
TOTAL	268	157			

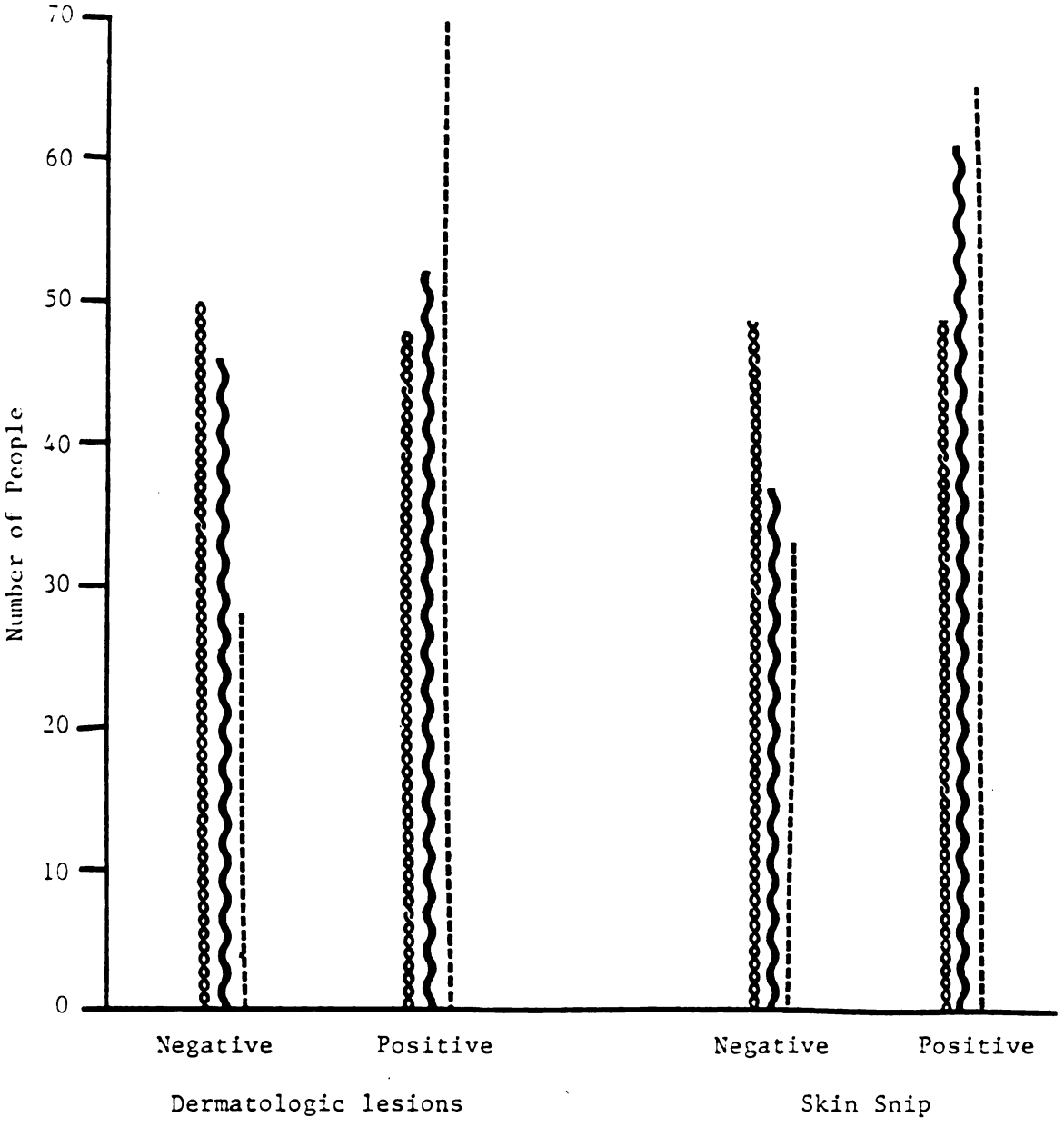
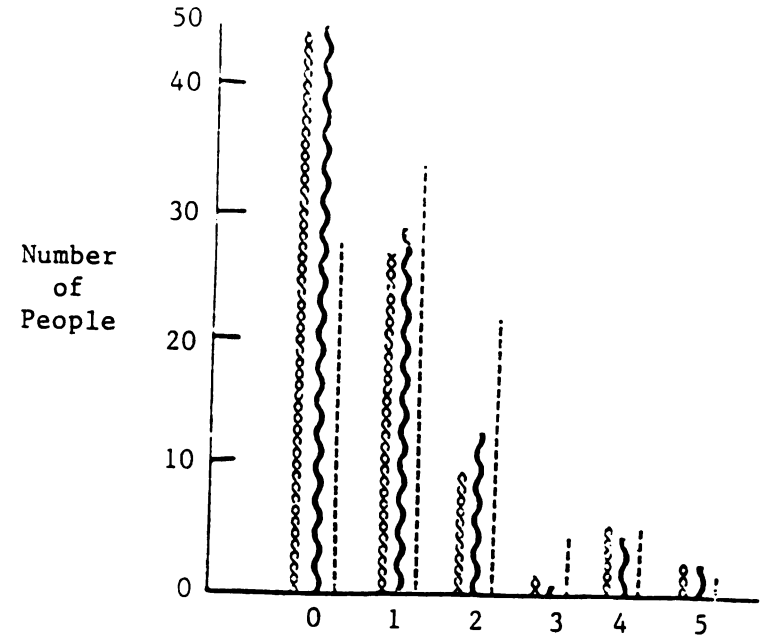
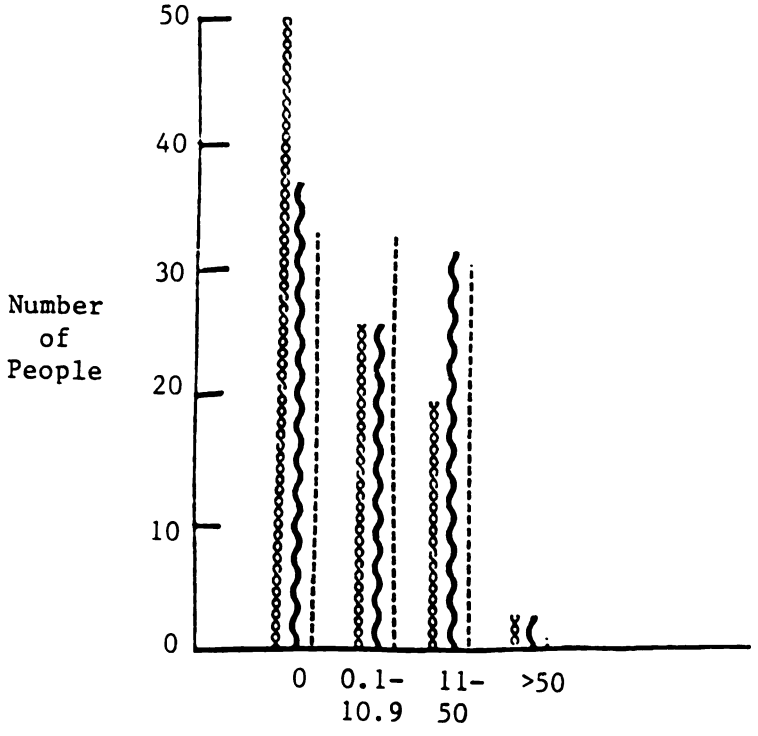


Figure 1. Onchocerciasis longitudinal study in Bebeka, Ethiopia. Pattern of dermatologic lesions and skin snip examination changes in 98 people from round 1 ∞∞∞, through 2 ∿∿∿ to 3 ▬▬▬ .

Figure 2. Onchocerciasis longitudinal study in Bebeka, Ethiopia. Pattern of graded dermatologic lesions (A) and microfilarial load (B) changes in 98 people from round 1 , through 2 to 3 .



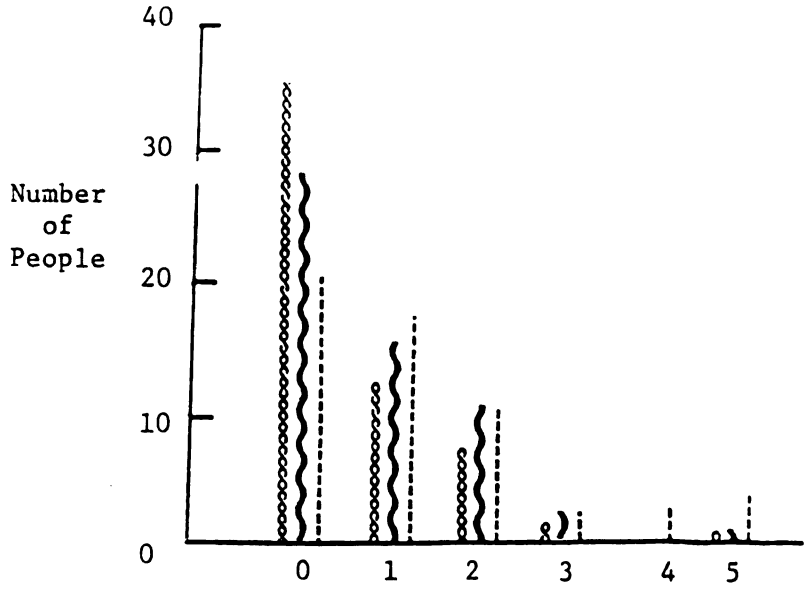
A. Dermatologic scores



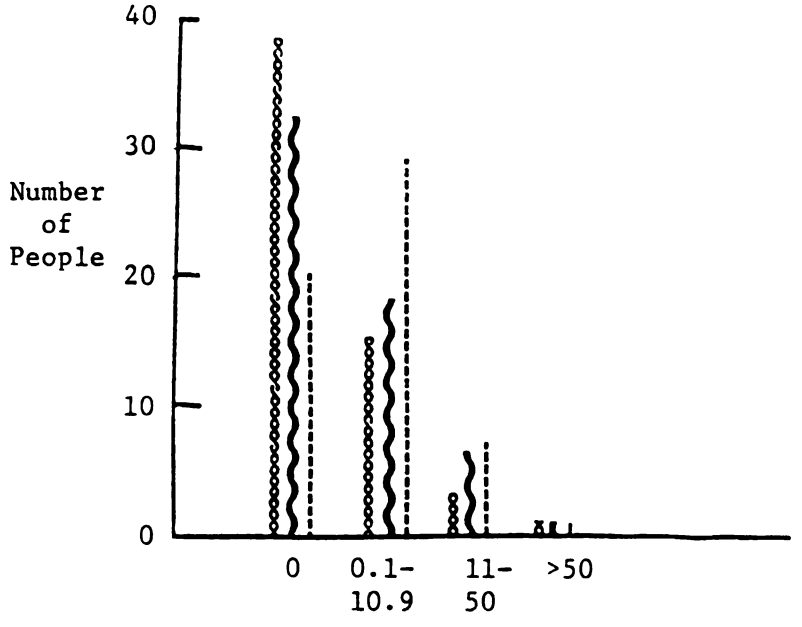
B. Microfilariae per mg.

FIGURE 2.

Figure 3. Onchocerciasis longitudinal study in Bebeka, Ethiopia. Pattern of graded dermatologic lesions (A) and microfilarial load (B) changes in 60 people included for the hypersensitivity test from round 1 , through 2 to 3 .



A. Dermatologic scores



B. Microfilariae per mg.

FIGURE 3.

Figure 4. Patients with onchocerciasis in Bebeka, Ethiopia

A. "Clean skin" in a boy age 14 with 30 mf/mg

B. Discrete papular rashes

C. Pruritic papules

D. Typical nodule on iliac crest

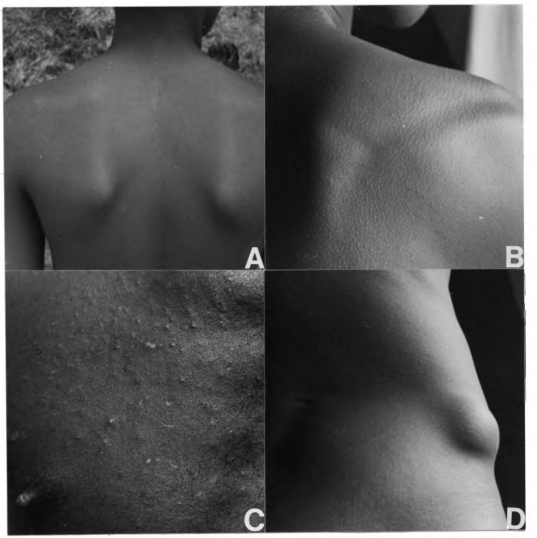


Figure 4.

Figure 5. Patients with onchocerciasis in Bebeka, Ethiopia

A. Hyperpigmentation on the back with papular eruptions

B. Premature aging of the skin (crushed tissue paper)

C. Advanced pruritic lesions

D. Inguinal bilateral lymphadenopapthy (hanging groin)

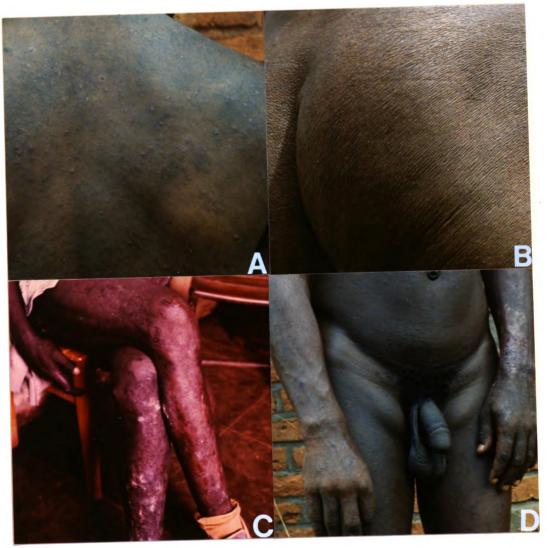


Figure 5.

Figure 6. Patients with onchocerciasis in Bebeka, Ethiopia

A. Nodule, lymph gland enlargement and elongated scrotum

B. Hypopigmentation on the shins (leopard skin)

C. and D. Suspected "Sowda" cases with unilateral lesion development.

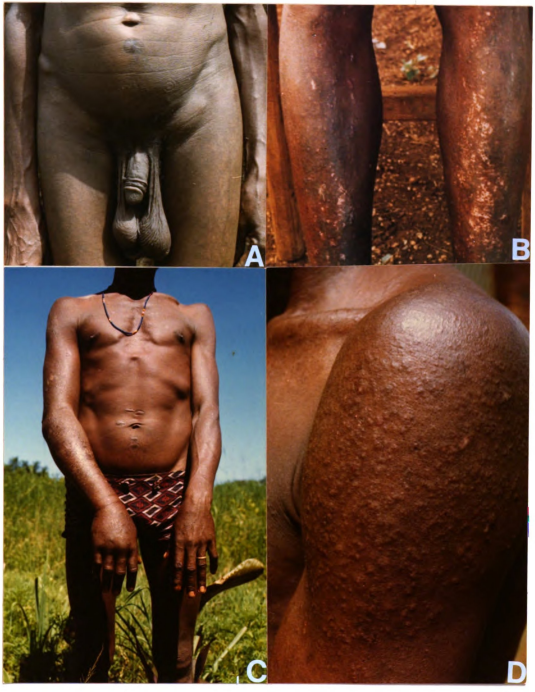


Figure 6.

APPENDIX 4

CHARACTERIZATION OF ANTIGENS BY WESTERN BLOTTING

APPENDIX 4

CHARACTERIZATION OF ANTIGENS BY WESTERN BLOTTING

The purpose of this technique was to demonstrate recognition by human immunoglobulins (antibodies) of the individual components of O. volvulus adult homogenate (OVAH), adult blackfly homogenate (ABFH) and Simulium salivary glands (SGA) antigens. Three steps were required for this procedure.

a. Protein separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE).

b. Western Blotting (WB), or transfer, of the electrophoretically separated proteins from the gel to nitrocellulose paper (NCP).

c. Visualization of antigen/antibody reaction with an enzyme-linked immunosorbent probe on NCP.

A detailed description of the principles, materials and methods of SDS/PAGE and Western blotting is given in the manual of instructions by Hoefer Scientific Instruments. The following is therefore only a brief outline of what was done in the present study.

A. SDS/PAGE

i. Equipment and accessories: All equipment and materials used

in this experiment are as provided by Hoefer Scientific Instruments.

ii. Reagents:

1. 30% acrylamide stock solution: acrylamide 29.2 gm., Bis (N'-N' bis methylene acrylamide) 0.8 gm. dissolve in about 70 ml deionized water, filter, bring volume to 100 ml. and store in a dark container at 4°C. Note: Acrylamide is neurotoxic; one must use gloves and masks when handling.
2. Separating gel buffer: 1.5 M Tris-HCL, pH 8.8.
3. Stacking gel buffer: 0.5 M Tris-HCL, pH 6.8.
4. 10% Sodium dodecyl sulfate (SDS)
5. 10% Ammonium persulfate (prepared fresh)
6. 75% glycerol in deionized water.
7. N,N,N'-N'-tetramethylethylenediamine (TEMED)
8. Low molecular weight (LMW) protein standards (from Bio-Rad Laboratories, Richmond, CA) with MW operating range of 10,000-100,000 Daltons.
9. Electrophoresis buffer (EB):

Tris base	3.0 gm	Deionized water	1.0 L
Glycine	14.4 gm	Adjust pH to 8.3	
SDS	1.0 gm		

10. Sample dilution buffer:

Deionized water	4.0 ml	10% SDS	1.6 ml
0.5 M Tris-HLC pH 6.8	1.0 ml	Mercaptoethanol	0.4 ml
Glycerol, pure	0.8 ml	0.05% bromphenol blue	0.2 ml

11. Coomassie stain:

Coomassie blue R-250	1.25 gm	Methyl alcohol	227.0 ml
Glacial acetic acid	46.0 ml	Distilled water	500.0 ml

12. Coomassie destaining solution:

Glacial acetic acid	10 ml
Methyl alcohol	30 ml
Distilled water	60 ml

ii. Procedure:

-Assemble glass plates as directed by Hoefer Scientific Instruments.

-Prepare 7.5 and 15% gel for gradient casting as follows:

	Volume for two gels of	
Stock solutions	7.5%	15%
30% acrylamide	3.75 ml	1.625 ml
1.5 M Tris-HCL pH 8.8	3.75 ml	0.95 ml
75% glycerol	--	0.9 ml
Deionized water	7.5 ml	--
10% SDS	150 ul	33.5 ul
10% Ammonium persulfate	37.5 ul	17.5 ul
TEMED	3.4 ul	2.5 ul

-Cast gel using a gradient apparatus and a peristaltic pump.

-Overlay gel with deionized water, cover and let stand at room temperature over night.

-Discard overlaid water and rinse with EB

-Insert teflon combs in the gel sandwiches.

-Prepare stacking gel as follows (for two gels)

30% acrylamide	1.0 ml
Deionized water	6.3 ml
0.5 M Tris-HCL, pH 6.8	2.5 ml
10% SDS	100 ul
10% Amonium persulfate	100 ul
TEMED	3.4 ul

-Add stacking gel until all spaces are filled and let stand for about one hour for polymerization to complete.

-Remove combs, rinse twice and fill with EB

-Make a 1:20, 1:10 and 1:2 dilution of AOVH, and ABFH or SGA, respectively and 1:20 dilution of MWM, with the sample dilution buffer. Boil for five minutes. The dilutions of the primary antigens correspond to a protein concentration of about 20 ug/ml.

-Underlay 30 ul of diluted samples in each well using a 100 ul Hamilton syringe. Of the 20 wells, the first, the middle and the last are filled with the diluted MWM.

-Displace any trapped air bubbles by further addition of EB.

-Mount the upper buffer chamber on the gel plates, fill it with EB and place the entire set in the lower chamber containing EB.

-Electrophorese at constant current first at 15 mA/gel till the dye front reaches the interface and then at 30 mA/gel until electrophoresis is complete.

On Completion the gel is either stained and mounted for permanent record or blotted on NCP.

For staining, gel is placed in a pan- containing Coomassie blue stain and left for 3-4 hours at room temperature. It is then destained and dried on either filter paper or covered with cellophane preserving sheet. In either case drying is effected with a gel dryer.

B. WESTERN BLOT (WB)

i. Equipment and accessories:

All equipment and accessories used are as provided by Hoefer Scientific Instruments.

ii. Reagents:

1. WB buffer

Glycine	4.4 gm
Tris-base	3.0 gm
Deionized water	1.0 L

Adjust pH to 8.3 and add 200 ml. methanol.

2. Amido black stain

Amido black	200 mgm	Glacial acetic acid	20 ml
Methyl alcohol	90 ml	Deionized water	90 ml

3. Amido black destain

Methyl alcohol	45 ml
Glacial acetic acid	10 ml
Deionized water	45 ml

iii. Procedure:

-Pour about 1.5 liters of the WB buffer into a tray big enough to hold the cassette. Place the gel holder in it and wet 4 sheets (15 x 15 cm) filter paper and NCP.

-Place one scotch brite (foam sponge) on the inner side of the open gel holder then place two sheets of filter papers and the NCP on top.

-Carefully, place the gel on the NCP, cover with a pair of prewetted filter papers, then the second scotch brite and finally close the gel holder. Apply gentle pressure to effect good contact and to remove air bubbles.

-Put the mounted gel holder in place in the blotting chamber filled with the WB buffer. Care should be taken in the orientation of the electrical flow. In this set-up the NCP was anodic to the gel.

-Place the cooling system in its appropriate slot.

-Cover chamber with the lid and connect the electrodes to power source.

-Effect transfer at 30 volts per gel for four hours.

-Using gloves, remove NCP from assembly.

-Cut strips corresponding to the size of the wells made with the comb.

-Stain the MWM strips and two sample strips with amido black stain for about 45 minutes.

-Destain until background is clear, dry strips in air and save.

C. ENZYME-LINKED IMMUNOSORBENT PROBE ON NCP

i. Equipment and accessories:

1. Tube mixer/rotator
2. Small forceps
3. Falcon^{R} polystyrene conical 15 ml tubes
4. Corning^{R} 50 ml polypropylene tubes

ii. Reagents:

1. PBS - tween buffer

Sodium phosphate, (Na_2HPO_4)	14.196 gm
Potassium phosphate (KH_2PO_4)	13.6 gm
Sodium chloride	1.5 gm
Deionized water	2.0 L

Adjust pH to 7.2 and add 6.0 ml tween-20.

2. Blocking (saturating) buffer (BB)

0.01 M Tris-HCL pH 7.2	100 ul
Triton X-100	50 ul
Bovine serum albumin	2 gm
Gelatin	0.5 gm

3. Horse radish peroxidase conjugate antihuman globulin (from ICN^R).

4. Substrate: Diaminobenzidine (DAB) 0.5 mg/ml in PBS-tween with 5ul per ml 30% H_2O_2 added just before use. Note: DAB is a potential carcinogen; must be handled with gloves and mask.

5. Serum from study population.

iii. Procedure:

-Place all the NCP strips in the 50 ml disposable tube filled with BB and incubate overnight with shaking at room temperature.

-Make a 1:200 serum dilution with BB in the 15 ml centrifuge tubes each labelled with the serum identification number.

-Using the forceps, transfer as many strips as required to the tubes containing diluted serum. The number of NCP strips depends on whether one or more than one immunoglubulin isotype is to be detected in a single serum sample.

-Incubate for two hours while shaking at room temperature.

-Wash three times, 15 minutes each with PBS-tween.

-Prepare a 1:2000 dilution of conjugate with PBS-tween and dispense in the 15 ml centrifuge tubes labelled with the isotype and serum identification.

-With the forceps, place one strip in each tube containing the immunoglobulin probe and incubate for two hours with shaking at room temperature.

-Wash three times 15 minutes each, with PBS-tween.

-Prepare required amount of DAB (about 10 ml. per tube).

-Dispense DAB to all tubes and incubate for 15-45 minutes with shaking at room temperature

-Remove each strip from tube with a forcep, dip it in cold distilled water to stop reaction and drain to dry in air.

NOTE: 1. Appropriate modifications of the above procedure were made when using non-labelled monoclonal antibodies.

2. Time, materials and reagents were economized by:

   a. Incubating all strips in one tube for a serum to be tested for different immunoglobulins.

   b. Identifying strips with different colors at the tip. More than ones strip could be placed in one tube while incubating with conjugate and DAB.

3. Several trial and error tests were done to establish the optimum conditions (dilutions of serum, primary antigens, immunoglobulin probes, conjugate, and incubation time) for best resolution.
4. Controls, including incubation of blotted strips without serum and with "normal" serum, were done parallel with all daily runs.