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USE OF AFFORDABLE TECHNOLOGY FOR THE SENSITIVE AND SPECIFIC DIAGNOSIS OF ONCHOCERCIASIS (river blindness)

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To Natalia

*"Don't forget that only dead fish
swim with the stream"*

Malcolm Muggeridge

SUMMARY

Onchocerciasis or river blindness is the most common infectious cause of blindness. Even in the absence of blindness, the skin effects caused are of social stigma. It is the goal of the World Health Organization to eradicate filariasis (including onchocerciasis). Eradication of onchocerciasis is to be undertaken by mass treatment with the microfilaricide ivermectin, combined with vector control. To date, the gold standard for diagnosis of onchocerciasis is through the parasitological identification of the organism, *Onchocerca volvulus*. As ivermectin treatment provokes a decrease in the microfilarial load of the individuals, the sensitivity of the assay for detection would need to be enhanced. Before and even after eradication is achieved, sensitive, specific, quick and affordable methods will be necessary for adequate diagnostics and epidemiological assessment. A number of recombinant proteins have been identified for use in diagnosis but the results have been disappointing. Using cocktails of recombinant proteins somewhat improved the sensitivity. However, production of recombinant material has proved to be a problem and costs have not even been discussed. Since onchocerciasis is a disease endemic in less well-off countries, the cost of reliable diagnostic methods is not a trivial matter. Therefore, there is definitely a need to re-address this problem of developing improved methods for monitoring the eradication programme in the context of sensitivity, specificity, availability and affordability.

Using manual ion exchange chromatography, we have isolated a fraction of low molecular weight proteins of *O. volvulus* (designated PakF), derived from adult female worms. This fraction, used in a Dot Blot Assay (DBA), proved to be highly sensitive and specific for the diagnosis of onchocerciasis in different geographic regions. We have tested the PakF-DBA in Guatemala (where no other filaria has been described), in Ghana (where other filariases are prevalent) and in an infectious Disease referral center in Sweden (a country not endemic for filaria). Additionally, we have tested for specificity using well-defined filarial sera from areas non-endemic for onchocerciasis. When used to analyze samples from different endemic scenarios, the PakF-DBA reached high levels of sensitivity (96%) and specificity (98%). Compared to methods that use recombinant material, the PakF-DBA offered similar or even better sensitivity and specificity values, but without the high costs involved in the production of recombinant proteins.

The PakF-DBA was worked out so it could provide specific and sensitive results and yet provide an affordable alternative to the use of more expensive or sophisticated diagnostic methods. In this regard, the method was simplified to use eluted finger pricked blood instead of serum as the source of antibodies to the PakF mixture. Using this technique, a positive correlation was found between the results obtained with the "gold-standard" skin snip test and the PakF-DBA, suggesting that the PakF-DBA could substitute, or at least provide a more sensitive tool when the parasitological examination is not possible, due to the reluctance of the individuals to undergo this procedure, or the lack of qualified personnel that performs the skin snip.

A closer look at the biochemical properties of this mixture of native proteins revealed that PakF is stable at room temperature, which in some endemic settings could reach 30-35° C. IgG_4 antibody response from infected individuals is infrequent and did not improve upon snesitivity, which adds an attractive feature in terms of costs, as anti-IgG_4 reagents tend to be expensive. Its stability at relatively high working temperatures makes PakF attractive under field conditions. In addition, heat inactivation of sera or eluted blood to reduce the risk of labile blood borne viruses does not affect the response to PakF. The relatively simple production of PakF, together with its stability, makes the PakF-DBA a serious contender in the quest for a reliable, yet affordable method for the diagnosis of onchocerciasis in areas where costs are an important issue and the disease threatens with recrudescence, even after years of control.

The results of this work speak for the potential of this protein mixture, PakF, as a contending diagnostic tool that, when put into the right format, could be a valuable tool for the monitoring of onchocerciasis control measures.

SUMMARY IN SPANISH

La oncocercosis (también conocida como "river blindness" ó Enfermedad de Robles) es la causa más frecuente de ceguera infecciosa. Aún cuando el resultado no es ceguera, las lesiones dérmicas son importantes debido a sus implicaciones sociales. Uno de los objectivos de la Organización Mundial de la Salud (OMS) es erradicar las filariasis (incluyendo a la oncocercosis). Dicha erradicación se basa en la distribución masiva de ivermectina, cuyo efecto es microfilaricida, combinada con el control de vectores. Ha la fecha, el método de diagnóstico de referencia para la oncocercosis (ó "gold standard") es el examen microscópico de biopsias de piel ("skin snip test") con el objeto de identificar la presencia del parásito *Onchocerca volvulus*, causante de la enfermedad. Como consecuencia del efecto microfilaricida de la ivermectina, la cantidad de microfilarias disminuye en los individuos infectados, resultando en pérdida de sensibilidad del método de diagnóstico basado en el conteo de las mismas. Por lo tanto, cualquier método de diagnóstico que deba ser considerado debe asimismo poseer una sensibilidad mayor que el examen de biopsias de piel. Antes, y aun después que la erradicación se haya logrado, será necesario contar con métodos de diagnóstico sensibles, específicos, rápidos y económicos, que permitan un diagnóstico adecuado, además de una mejor vigilancia epidemiológica. A pesar de que varias proteínas recombinantes de *O. volvulus* han sido identificadas y evaluadas como posibles herramientas en el diagnóstico de la oncocercosis, los resultados han sido poco satisfactorios. Aunque el uso de mezclas de esas proteínas (ó "recombinant cocktails") ha mejorado relativamente la sensibilidad, la producción de material recombinante ha resultado ser un problema por cuanto los costos involucrados no han sido motivo de discusión. Siendo la oncocercosis una enfermedad todavía endémica en países con pocos ingresos económicos, el costo de métodos de diagnóstico no puede considerarse trivial. Por lo tanto, existe la necesidad de reconsiderar el desarrollo de métodos de evaluación y monitoreo de las instancias de control y erradicación, en el contexto de una sensibilidad y especificidad mejoradas, asi como de disponibilidad y acceso econónico en las áreas afectadas.

Utilizando la cromatografía de intercambio iónico en un sistema manual, fue posible separar una fracción de proteínas de bajo peso molecular de *O. volvulus*, la cual fue designada "PakF", obtenida de vérmes adultas. Dicha fracción, utilizada en un inmunoensayo denominado "Dot Blot Assay" (ó PakF-DBA), resultó poseer alta sensibilidad y especificidad en el diagnóstico de oncocercosis utilizándo muestras de pacientes obtenidas en diferentes áreas geográficas. El PakF-DBA fue evaluado utilizando muestras de individuuos de Guatemala, uno de los países de las Américas donde la enfermedad es endémica y donde ningun otro tipo de filariasis humana ha sido reportada El ensayo fue evaluado utilizando también muestras de individuos de Ghana (país endémico para filariasis en Africa del Oeste), asi como muestras obtenidas de un centro de referencia en el diagnóstico de enfermedades infecciosas en Suecia, un pais donde la oncocercosis no es endémica. Adicionalmente, el ensayo fue evaluado utilizando muestras de individuos de áreas donde otras filariasis son endémicas pero no asi la oncocercosis. La evaluación del PakF-DBA con muestras de diferentes escenarios endémicos demostró que el ensayo posee un alto grado de sensibilidad (96%) y especificidad (98%). Comparado con otros métodos que utilizan material recombinante, el PakF-DBA ofreció niveles de sensibilidad y especificidad similares, y aún mejores en algunos casos, pero sin los costos elevados normalmente involucrados en la producción de proteínas recombinantes.

El ensayo PakF-DBA fue simplificado de manera tal que la sensibilidad y especificidad fueran alcanzadas sin los costos o la sofisticación inherentes a otros métodos de diagnóstico. A este respecto, en lugar de muestras de suero obtenidas de sangre venosa, el método se simplificó usando gotas de sangre obtenidas en papel de filtro, como fuente de anticuerpos que reconocieran las proteínas presentes en PakF. Utilizando esta técnica, se encontró una correlación positiva entre los resultados obtenidos con el "skin snip test" y los obtenidos con el PakF-DBA, sugiriendo que éste último podría substituir al "skin snip test", o al menos proveer una herramienta más sensible cuando el análisis parasitológico no es posible debido a la poca aceptación de éste cuando debe practicarse repetidamente, o la falta de personal capacitado para realizarlo.

Un examen más detallado de las propiedades bioquímicas de la fracción PakF, la cual contiene proteínas en forma nativa, reveló que dicha fracción es estable a temperatura ambiente, la cual en algunos lugares puede llegar a 30-35° C. La detección de PakF por anticuerpos IgG_4 de pacientes infectados con *O. volvulus* no fue frecuente y no mejoró la sensibilidad del ensayo, lo cual agrega una cualidad atractiva al PakF-DBA in términos de costo, debido a que los reactivos utilizados para detectar anticuerpos IgG_4 tienden a ser onerosos. Su estabilidad a temperaturas de trabajo

relativamente altas hace del PakF una opción atractiva en el contexto de pruebas de diagnóstico que puedan utilizarse en condiciones de campo. Adicionalmente, la inactivación por calor de muestras de suero o sangre eluida de papel de filtro no afecta resultados que se obtienen con PakF. La obtención relativamente simple de PakF, junto con su estabilidad, hacen del ensayo PakF-DBA un serio contendiente en la búsqueda de métodos de diagnóstico de la oncocercosis que sean confiables, pero a la vez accesibles en áreas donde los costos representan un problema, y done la amenaza de recrudecencia de la enfermedad permanence latente aun después de años de aparente control.

Los resultados de este trabajo describen el potencial de esta fracción de proteinas nativas de bajo peso molecular obtenidas de *O. volvulus*, y describen al PakF-DBA como un método de diagnóstico que, utilizado en un formato apropiado, podría convertirse en una herramienta valiosa en el monitoreo de las instancias de control de la oncocercosis.

LIST OF PUBLICATIONS

I   **Guzmán, G.E.**, Akuffo, H.O., Lavebratt, C., and Luján, R. 1997. Differential immune response to *Onchocerca volvulus*: IgG_4 antibody responses differ in onchocerciasis patients from Guatemala and Ghana. *Acta Tropica* 63(1): 15-31[+]

II   Lavebratt, C., Ljungström, I., **Guzmán, G.E.**, Thors, C., Eriksson, T., and Akuffo, H.O. 1997. Evaluation of serological assays for diagnosis of onchocerciasis. *Scandinavian Journal of Infectious Diseases* 29 (1): 65-70[++]

III   **Guzmán, G.E.**, Lavebratt, C., Luján, R., and Akuffo, H.O. 2002. Diagnosis of onchocerciasis using highly specific and sensitive native proteins. *Scandinavian Journal of Infectious Diseases* 34:583-590 [++]

IV   **Guzmán, G.E.**, Awadzi, K., Opoku, N., Narayanan, R. B., and Akuffo, H.O. 2002. Comparison between the skin snip and a simple dot blot assay as a potential rapid assessment tool for onchocerciasis in the post-control era in Ghana. *Clinical and Diagnostic Laboratory Immunology* 9(5):1014-1020. [+++]

V   **Guzmán, G.E.**, and H.O. Akuffo. Continued symptoms of onchocerciasis 20 years after leaving the endemic area. *Submitted.*

VI   **Guzmán, G.E.**, and H.O. Akuffo. Biochemical characterization of the immunodominant proteins of PakF, a highly specific and sensitive mixture of native proteins used in the diagnosis of onchocerciasis. *Manuscript.*

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CONTENTS

LIST OF ABBREVIATIONS

2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
AP	Alkaline phosphatase
APOC	African Programme for Onchocerciasis Control
cDNA	Complementary DNA
CP	Chronic Patient (*W. bancrofti*)
CTLA	Cytotoxic T-lymphocyte-associated
DBA	Dot blot assay
DEC	Diethylcarbamazine
DTT	Dithiotreitol
EDTA	Ethylenediaminetetraacetate
EE	Exposed European
ELISA	Enzyme-linked immunosorbent assay
EN	Endemic Normal (*W. bancrofti*)
Fc	Fragment crystallizable
GABA	gamma-amino butyric acid
GHmf+/-	Ghanaian microfilaria-positive/negative
Gly	Glycine
GST	Glutathione S transferase
GUmf+/-	Guatemalan microfilaria-positive/negative
HRPO	Horseradish peroxidase
IEF	Isoelectrofocusing
IFA	Immunofluorescence assay
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
kDa	Kilodalton
L3	Larval stage 3 (infective stage)
L4	Larval stage 4 (inmature worms)
LMW	Low molecular weight
LPS	Lipopolysaccharide
MALDI	Matrix-assisted laser desorption/ionization mass spectrometry
mf	Microfilaria(e)
MF	Microfildermic patient (*W. bancrofti*)
mfd	Microfilarial density
mM	Millimolar
Mw	Molecular weight
N-GU	Non-exposed Guatemalan
N-US	Non-exposed North American (United States)
OCP	Onchocerciasis Control Programme
OEPA	Onchocerciasis Elimination Programme for the Americas
OvAg	*Onchocerca volvulus* somatic antigen
OvGST	*Onchocerca volvulus* Glutathione S transferase
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction

P-GU	Guatemalan infected with other nematode parasites
PI	Putative Immune
Q-TOF	Quadrupole–orthogonal acceleration time-of-flight
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
ss-	Skin snip negative
ss+	Skin snip positive
TSF	Tris-buffer soluble fraction

1 INTRODUCTION

1.1 ONCHOCERCIASIS (River blindness)

Onchocerciasis is a parasitic disease caused by the nematode *Onchocerca volvulus* found in parts of tropical Africa, Latin America and the Arabian peninsula. The parasite is transmitted by blackflies of the genus *Simulium*, which breed in fast-flowing rivers, hence the alternative name "river blindness". Infection by the parasite produces three clinical manifestations: dermatitis, subcutaneous nodules and ocular lesions, with blindness being the most devastating outcome. The clinical pattern depends partly upon the intensity and duration of the exposure, but it also varies from individual to individual and from one geographic region to another. In some hyperendemic areas, where almost every person is infected, half the population become blind, dying within 10 years after getting this condition (1-3).

1.2 THE PARASITE *Onchocerca volvulus*

Onchocerca volvulus is a nematode worm found as a parasite in human beings and the blackfly vector *Simulium*. Within the genus *Simulium*, the main vectors *S. damnosum*, *S. neavei*, *S. ochraceum*, *S. metallicum*, and *S. exiguum*, are complexes of sibling species, which do not otherwise form a taxonomically close group of species. In Africa and the southern Arabian peninsula, onchocerciasis is associated with *S. damnosum*, and to a lesser extent, with the *S. neavei* group. In Central America, the primary vector is *S. ochraceum*. Interestingly, although onchocerciasis is said to be transmitted by "blackflies", *S. ochraceum* is a yellow (ochre) fly very distinct from the other *Simulium* species.

The adult Onchocerca worms (females 30-80 cm, males 3-5 cm) live in fibrous nodules, some of which are subcutaneous and palpable, while others lie deep in the connective and muscular tissues. With a life span of up to 16 years, the females produce abundant microfilariae, mf, (250-300 µm in length), which migrate from the nodules to invade the skin, eyes and other organs (Figure 1). The mf cause most of the disease manifestations and have a life span of 6-24 months (4, 5)
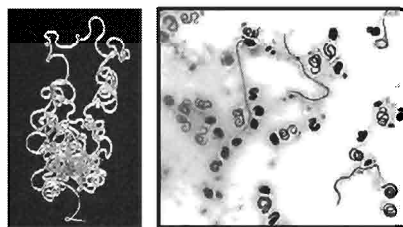

Figure 0. The parasite *Onchocerca volvulus*. Left: adult female worm isolated from a subcutaneous nodule (Photo courtesy Dr. R. Luján). Right: microfilariae obtained from skin snips (Photo courtesy Dr. Catharina Lavebratt).

1.2.1 The life cycle of *Onchocerca volvulus*

Humans are the only natural mammalian host of *O. volvulus*. Microfilariae ingested from the skin by blood feeding *Simulium* develop in the muscles of the vector over 6-12 days, without multiplication, to form infective larvae (L3), which can be inoculated into a new host when the fly subsequently feeds (6) (Figure 2). Flies are generally not infective until their third blood meal and usually die after their third reproductive cycle. Typically, two to six L3 enter the human host through a bite to the skin and migrate through subcutaneous tissues. Within several months, L3 molt twice, again without multiplying, and develop into female or male adult worms, which reside in subcutaneous nodules (onchocercomas), the characteristic subcutaneous granuloma resulting from the tissue reaction around adult worms. Adult worms can live in subcutaneous nodules for 10-15 years and produce millions of microfilariae (mf) (5, 7). The triggering mechanism for encapsulation is unknown; worms may release antigen, causing inflammation that leads to nodule formation, or the worm may be traumatized while migrating over bony prominences, provoking inflammation and encapsulation (4, 8). More recently, a role for parasite retinoid-binding proteins, particularly Ov-FAR-1 (previously known as Ov20), present in all states of *O. volvulus*, has been suggested in the development of onchocercomas, as well as ocular and skin manifestations of onchocerciasis (9). On average, a nodule contains approximately 2-4 living worms of various ages, however, .as many as 20-40 live adult parasites have been reported in hyperendemic areas (10). In rare cases, individuals have been identified having more than 100 worms.
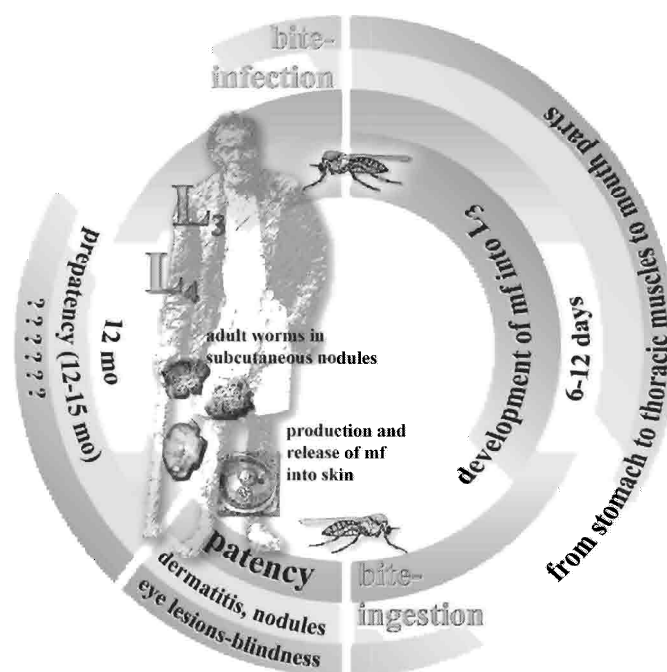

Figure 2. Life cycle of *Onchocerca volvulus*. Some components have been taken or modified with permission from the TDR picture library (WHO/APOC/TDR/Crump).

Although produced in large numbers, mf are not expelled by the female but leave the uterus by their own action. The first mf produced by adult females may appear in the skin some 10-15 months after infection (11, 12), known as the prepatent (asymptomatic) period. The production of mf is periodic with asynchronous cycles lasting 2-4 months (13) and different developing stages of mf may be present simultaneously in the uterus of the female worm. It has been estimated that 700-1500 mf per female, only part of the mf produced, succeed in escaping from the uterus every day (2, 14). Migration of mf from the nodules through the skin is facilitated by the secretion of enzymes, such as collagenase, which destroys the host's tissue (15). Free mf disseminate throughout the body, particularly the skin, eye and lymph nodes. To complete the cycle, a female adult blackfly must bite an infected person, taking up skin mf (16).

1.3 THE DISEASE

1.3.1 Distribution

Onchocerciasis is prevalent in 37 countries across Africa, the Americas and Yemen affecting >17.7 million people, of which an estimated 500,000 are visually impaired and another 270,000 are blind (Figure 3) (17, 18). The Onchocerciasis Control Programme (OCP), executed by WHO was initiated in 1974, and originally implemented in seven countries (Benin, Burkina Faso, Cote d'Ivoire, Ghana, Mali, Niger and Togo), and then in 1986, under its western extension, in four more nations (Guinea, Guinea Bissau, Senegal and Sierra Leone). The programme has been highly successful and the disease has declined dramatically in both prevalence and public health importance. The main public health problems remain the Sub-Saharan Africa, outside the OCP area, where the disease is both widely prevalent and severe. As a result, the African Programme for Onchocerciasis Control (APOC) was launched in 1995 and serves the 19 countries outside the OCP (Figure 3). The goal of APOC is to eliminate onchocerciasis as a disease of public health importance in the non-OCP countries (18).
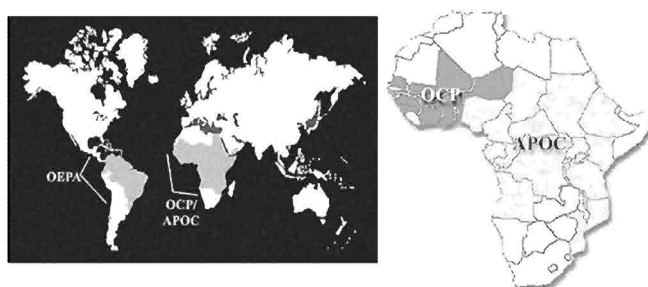

Figure 3. Geographical distribution of onchocerciasis. Left: countries covered by the Onchocerciasis Control Programme (OCP), the African Programme for Onchocerciasis Control (APOC), and the Onchocerciasis Elimination Program for the Americas (OEPA). Right: Detail of the original countries covered by OCP (dark shade) and APOC (light shade) in Africa.

3

The endemic foci for onchocerciasis in the six endemic countries in the Americas (Brazil, Colombia, Ecuador, Guatemala, Mexico and Venezuela) have different characteristics from those in Africa. Onchocerciasis in the Americas is probably the result of the introduction of *O. volvulus* into this hemisphere by the African slave trade in the last few centuries, thus leading to more recent host-parasite-vector relationships that are, perhaps, less well-adapted compared with those in Africa. Hence, onchocerciasis transmission is only well established in a few circumscribed foci and annual potential transmission for these areas are considerably lower than those observed in Africa (18). The special characteristics and the stability of the American foci promoted the creation of the Onchocerciasis Elimination Program for the Americas (OEPA) by the Pan American Health Organization (PAHO), with the aim of elimination of all morbidity from onchocerciasis, and, where possible, suppression of transmission in the Americas by 2007.

The available information and the quality of the data for the endemic countries on the number of people infected vary considerably from country to country. In some surveys, for example, visual impairment has not been assessed. Current figures of blind or severe visually disabled, as well as infected or at risk individuals certainly underestimate and do not fully reflect the importance of the disease and its implications.

1.3.2 Clinical Manifestations

The manifestations of onchocerciasis are predominantly dermal, lymphatic and ocular in character. Several other features of uncertain association, etiology or pathogenesis have also been described, including low body weight, general debility, diffuse musculoskeletal pain and, in Africa, epilepsy and hyposexual dwarfism.

The mf stage is the cause of substantial morbidity. Blindness, the most devastating disease manifestation, is caused by inflammation in the eye because of mf dying in the cornea. With visual impairment in 500,000 and blindness in 270,000 humans, onchocerciasis has remained the second most frequent cause of preventable blindness in Africa. Second in its impact on life, but a much more frequent disease manifestation, is dermatitis, ranging from acute episodes of papular inflammation to chronic atrophy, lichenification and leopard skin (19). The dermatitis so caused can have extreme social and psychological effects on the individual.

The extent and distribution of skin and lymphatic lesions allows classification of the disease into generalized and local forms. Generalized onchocerciasis is the usual presentation characterized by symmetrical cutaneous lesions which may be more marked in the lower, or less commonly, the upper part of the body. The localized form, also called hyperreactive onchocerciasis, is asymmetric and may be confined to a circumscribed part of the body, such as one limb (Figure 4). The chronic form of hyperreactive onchocerciasis, also known as "*sowda*" is characterized by frequent acute

4

exacerbations and tends to occur in new residents and in people from outside the endemic areas.
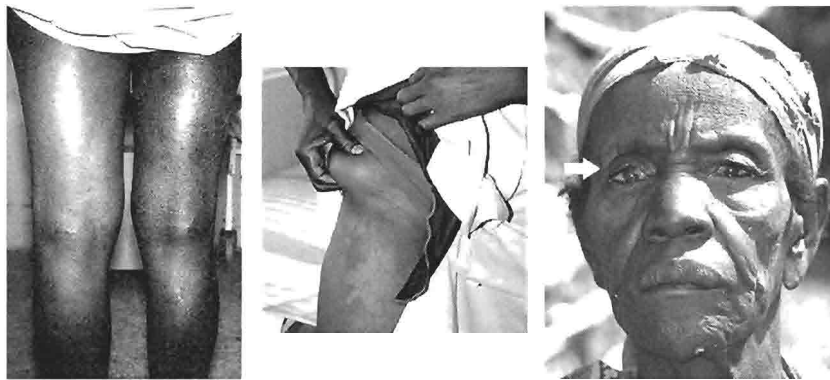

Figure 4. Left: Hyperpigmentation of one limb (sowda) due to localized onchocerciasis. Note the darker pigmentation of the right leg. Center: large onchocercal nodule in a patient with onchocerciasis (Photos courtesy Dr. H. Akuffo). Right, Ugandan elderly woman with her sight badly impaired as a result of ocular damage that occurs due to long-term infection (Photo: WHO/APOC/TDR/Crump, reproduced with permission).

1.3.2.1 Microfilariae in skin

In the skin, mf are most frequently seen in the upper dermis, characteristically with no evidence of surrounding tissue reaction. Aside from mf in the dermis, early changes include localization of inflammatory cells around vessels and dermal appendages and an increase in dermal fibroblast and mast cells. Subsequent changes include hyperkeratosis, focal parakeratosis, and acanthosis with melanophores and increased mucin in the upper dermis, accompanied by dilated lymphatics and tortuous upper dermis vessels. End-stage disease is characterized by advanced atrophy of the epidermis with loss of elasticity and only very thin layers of remaining epidermis and keratin overlaying the dermis (20).

1.3.2.2 Nodules

Onchocercal nodules (onchocercomas) are fibrous encapsulations of adult worms in host tissue; they are hard, unless recently formed, and are not tender or painful. Some may be fixed to skin, bones or joints (10). The localization of nodules on a person differs between geographical regions and between age groups. It has been suggested that the differences may partly be related to where the blackfly bites, which may be an intrinsic feature of the vector or may be related to clothing habits. In West Africa, the predominant sites of nodules are the bony prominences such as the pelvic girdle (including iliac crest, the trochanter and the sacro-coccygeal region) and the knee

5

(Figure 4). In Guatemala, Central America, the majority of the nodules are found on the head of the person (10, 21).

1.3.2.3 Ocular symptoms

The ocular signs of onchocerciasis are related to the presence of living or dead mf; these can be seen with a slit lamp and have been demonstrated in all tissues of the eye. As in other aspects of this disease, regional influences play an important role in determining the clinical picture of onchocerciasis. Evidence of geographical variation of the parasite have been reported, showing differences between the *O. volvulus* that can be found in savanna regions, compared to that found in forest areas (22, 23). Phylogenetic studies at the genomic level not only confirmed these findings but suggested that the parasite strains found in the Americas (specifically in Guatemala and Brazil) seem to be more related genetically to the savanna-type of *O. volvulus*, which has been related to a higher prevalence of blindness and more severe ocular lesions (24). This is consistent with the reports from surveys in the United Cameroon Republic showing the prevalence of sclerosing keratitis and blindness to be much higher in savanna than in forest areas (25). However, surveys conducted in Sierra Leone revealed the prevalence of blindness and sclerosing keratitis to be higher in the forest than the savanna areas of that country (26, 27). Reasons for the higher prevalence of blinding lesions in forest than savanna regions of Sierra Leone may include the setting of villages away from *S. damnosum* breeding sites and less dense and more scattered populations in the northern savanna. Blindness in the hyperendemic savanna areas was previously attributed mainly to sclerosing keratitis, but a subsequent study showed that optic nerve disease plays a significant role in some localities (28). In the forest areas, blindness is said to be due mainly to posterior segment disease. In contrast, in Guatemala and Mexico, where foci are found in forest-type areas, blindness is relatively rare, and when present, it is often a result of anterior uveitis (29).

Live mf in the eye, as in the skin, cause little inflammatory reaction and may be found in conjunctiva, cornea, sclera, and anterior and posterior chambers, vitreous, uveal tract, retina, optic nerve, and sheath. At any one time, few mf are dying and so the inflammatory response is limited. Over time, however, the cumulative effect causes scarring and inflammation, which then can lead to significant visual impairment (30). Recent studies using a murine model for river blindness where soluble extracts of *O. volvulus* were injected into the corneal stroma, demonstrated the involvement of species of symbiotic *Wolbachia* bacteria in the predominant inflammatory response in the cornea (31).

1.3.3 Diagnosis of onchocerciasis

1.3.3.1 Clinical

Clinical diagnosis is not difficult in endemic areas, especially when patients present clinical manifestations such as subcutaneous nodules, hanging groin, "leopard skin"

and skin atrophy. Pruritus in the absence of skin lesions may be onchocercal in origin. Conditions such as streptocerciasis, scabies, insect bites, miliaria rubra (prickly heat), contact dermatitis, hypersensitivity reactions, post-traumatic or inflammatory depigmentation, tuberculoid leprosy, dermatomycoses and treponematoses, need to be excluded. Nodules need to be differentiated from lymph nodes, lipomas, fibromas, ganglions, other parasitic cysts, and juxta-articular nodes in jaws. Examination for nodules is mainly performed by palpation as the alternative is by ultrasound, which requires expensive equipment (32). Limitations in the use of nodule detection for diagnosis are that patients carrying infections without mature female worms have no nodules and sowda patients may appear without palpable nodules (33). In non-resident visitors to an endemic area, asymmetrical pruritic lesions with or without limb swelling or lymphadenitis are suggestive of onchocerciasis. The diagnosis may be missed because of the long interval of 1-3 years that may elapse between exposure and the onset of symptoms. The diagnosis of ocular onchocerciasis requires ophthalmologic evaluation to determine visual function, the presence of intraocular mf and pathological changes attributable to the infection. Although ocular examinations are time-consuming and require specialized techniques and an ophthalmologist, demonstration of mf in the eye leads to a definite diagnosis of onchocerciasis (2).

1.3.3.2 The Mazzotti test

The Mazzotti test is the deliberate provocation of the Mazzotti reaction using a limited dose of diethylcarbamazine (DEC). The Mazzotti reaction is an acute inflammatory response, which occurs when there is synchronous death of mf (34). This reaction is characterized by pruritus and maculopapular eruption that coalesce into red plaques of induration. Exfoliative dermatitis and generalized edema with tender lymphadenopathy can develop and patients often have generalized symptoms of headache, myalgia, arthalgia, and they can be febrile with tachycardia and tachypnea. The ocular changes include punctate keratitis, uveitis and optic neuritis, with a marked increase in the number of live and dead mf seen in the cornea. A permanent loss of vision can result. In severe cases hypotension, collapse, and loss of consciousness can occur, and occasional deaths have been attributed to DEC treatment (3). This test should be used only when onchocerciasis is suspected but the parasite cannot be demonstrated in the skin or eye. A non-invasive test based on the Mazzotti reaction, the DEC patch skin test, has been developed with the objective of replacing the skin snip test, the current parasitological diagnostic method being used for onchocerciasis (35).

1.3.3.3 Parasitological diagnosis

The demonstration of mf in skin snips is the classic and gold standard method of determining the prevalence and intensity of infection by *O. volvulus*. Superficial biopsies of $\geq$ 1 mg are taken from the uppermost layer of the skin using a puncher, a pair of scissors or another sharp tool (Figure 5). The skin snips are incubated in physiological saline at room temperature and subsequently examined for mf emerging into the saline. Although the sensitivity can be improved by prolonging the incubation from 15 min to 24 h, it has been shown by collagenase digestion of the skin snips that, even after 24 h, 20-30% of the mf are still in the skin snip and would not be detected.

(36). Besides the low sensitivity, this method is associated with patient discomfort and risks from inadequate sterilization of the snipping tools. However, when positive, this test has a very high specificity.

Figure 5. Parasitological diagnosis of onchocerciasis. Left: skin snip taken from the calf area or a volunteer youngster from the district of Ho, Ghana (Photo: G. Guzmán). Center: skin snip taken from the scapular area (Photo: Courtesy Dr. Ricardo Luján). Right: counting of microfilariae that had emerged form the skin snips after 24 hr incubation in saline (Photo: G. Guzmán).

1.3.3.4 Immunodiagnosis

The main drawback of serodiagnostic methods for onchocerciasis is their poor specificity or sensitivity, with abundant cross-reactions against related lymphatic filarial nematodes, such as *Wuchereria bancrofti* (37). However, due to the potential of serological techniques, great efforts have been put into developing serodiagnostic tests that overcome these limitations. Specificity was first increased by using semi-purified low molecular weight fractions of somatic *O. volvulus* extracts (38), and then by detecting IgG_4 instead of IgG antibodies because of the special prominence of the former in onchocercal infections (39-41). The prominence of this isotype and its lack of response against cross-reactive nematode carbohydrate-containing antigens, such as phosphocholine-containing polysaccharides (42), coupled with the finding that that IgG_4 does not recognize carbohydrates, became the basis for the use of this isotype to enhance the specificity of serodiagnostic test for onchocerciasis. However, it has been demonstrated that IgG_4 antibodies may also recognize carbohydrate structures, showing that such responses are not immuno-restricted to peptide epitopes (43).

Further research with cloned antigens or synthetic peptides corresponding to specific epitopes that represented determinants recognized by a large majority of individuals in target populations offered ideal possibilities for diagnosis. However, work with recombinant antigens from filarial nematode parasites showed that filarial peptide epitopes are commonly cross-reactive and that for most clones, the human humoral response is extremely heterogeneous (44). The heterogeneity in recognition may also reflect polymorphism or divergence in the respective native proteins. A differential screening of a cDNA expression library constructed from *O. volvulus* obtained in Mali,

8

followed directly by screening with individual patient sera (also from Mali), provided a rapid method for isolating clones that expressed proteins with immunologically specific epitopes. Nonetheless, the varying degree of of recognition of cloned peptides was noticed again, thus indicating the requirement for a combination of specific peptides in a cocktail to detect antibodies in all patients (45).

In 1993, as a first step in the development of immunodiagnostic tests for onchocerciasis, seven collaborating centers evaluated 37 recombinant antigens against coded serum samples from the WHO Filariasis Serum Bank (46). Of these, 10 were selected for further study because they exhibited high specificity. These antigens were evaluated in a second round testing both against serially collected sera from children shown to have acquired onchocercal infection during a longitudinal study in a hyperendemic area of Mali, and against sera of experimentally infected chimpanzees. For this second screening, the antigens were selected for high sensitivity in detecting early infections during either the prepatent or the early patent period. As a result of this selection process, three recombinant antigens, namely Ov-7 (45, 47), Ov-11 (45), and Ov-16 (48), were chosen as components of an "antigen-cocktail"-based antibody-detection assay used in a study of recrudescence in Pendié, Burkina Faso, and in a study on transmigrants exposed to onchocerciasis in the Vina Valley, Cameroon. Both areas were under vector control at that time. Preliminary results showed that seropositivity rates were directly correlated with the rate of parasitologically confirmed new infections, but in all the villages studied these rates were higher than the prevalence of positive skin snips. This discrepancy resulted from the fact that 30-50% of the exposed individuals who subsequently converted to having a positive skin snip within the following 12 months were found to be serologically positive, in addition to 60-80% of those with early patent infection. The important results of this multicenter study were (i) that no single recombinant antigen was recognized by all sera from patients with onchocerciasis, and (ii) samples from 20-40% of children (5-15 years of age) with detectable mf in skin responded negatively to the cocktail of recombinant antigens. Another 50-75% of adult cases with prepatent infections turning mf positive within the following 12 months (2, 49) were also negative in their response to the cocktail of recombinant antigens. This selection of candidate antigens to be included in serodiagnostic tests, nonetheless, was made based on results obtained in well-equipped western laboratories with a limited number of carefully selected sera. Thus, the true diagnostic potential of these antigens in various endemic settings often remained to be determined under more realistic field conditions (46).

After the multicenter study in 1993, different recombinant antigens have been used in different settings to evaluate their true potential as diagnostic tools. In a later study, a different cocktail of recombinant antigens, used in an ELISA test, was evaluated for its potential to predict the endemicity of onchocerciasis infection in hyper, meso- and hypo-endemic areas in Cameroon (50). The results of this study showed that if antibody detection assays are sensitive to the endemicity/intensity of infection in a community, it might be possible to calibrate the assay and provide a serologic index that would measure and predict the endemicity in different areas. More recently, Ov-16 was used in a rapid-format immunochromatographic antibody card test (ICT) to evaluate its sensitivity and specificity with well-characterized human sera (51). The development of an ICT test for onchocerciasis built upon the rapid success of a similar

9

test used for the detection of *W. bancrofti* antigen in human blood (52-54). Rapid antigen-detection tests similar to what is available for lymphatic filariasis would be preferable because they would indicate active infection as opposed to exposure to infection. Unfortunately, antigen tests for *O. volvulus* are apparently not on the horizon (18).

Table 1 shows some of the recombinant antigens that have been used in diagnostic tests for onchocerciasis. Despite the progress made in identifying potential candidate antigens to be used in a commercial diagnostic test for onchocerciasis, the actual manufacture and deployment of such materials has not yet been achieved. Problems with high expenses in the production of recombinant antigens have been reported (49), and this fact together with inconclusive results from other well-characterized molecules motivates the search for other, alternative assays. Therefore, at present, there is still place for the development of assays based on original parasite material, especially for use in endemic countries since this material is not limiting in such areas. Recently, a simple immunoassay based on the recognition of a mixture of native, low molecular weight proteins, obtained by partial purification from a somatic *O. volvulus* Tris-buffer extract, was shown to be as good as or superior to presently available diagnostic tests (55). The assay also fulfilled the criteria for a good screening method for onchocerciasis (56, 57). This assay has several advantages over the existing ELISA-based techniques in terms of simplicity, cost, easy production and low consumption of the antigen, as it utilizes 10-20 ng of protein mixture (in a 0.5 µl volume) per duplicate test, compared to 150 ng (in a 50-100 µl volume) used in a routine ELISA for filarial diagnosis. It also offers advantages over the ordinary Western blot, which requires even more parasite material.

Table 1. Some recombinant *Onchocerca volvulus* antigens evaluated for diagnostic potential. See ref. 46 for a complete list of antigens tested.

Antigen	Assay	Specificity	Sensitivity
Ov10	DBA	100	61
Ov29	DBA	100	43
Ov20	DBA	100	32
Ov21	DBA	100	11
Ov16	ICT	97	90.5
Ov27	DBA	96	68
Ov103	rELISA	96	68
Ov11	DBA	96	54
Ov7	rELISA	79	82
Ov5	DBA	96	50
Ov3	DBA	96	43
Oc3.6/Ov33	WB	75	86
Oc9.3	WB	83	68
Ov30	DBA	88	68
Ov34	DBA	88	50
Ov32	DBA	75	75
Ov37	DBA	75	61
OvTOT (OvAg)	ELISA	21	100

1.3.3.5 DNA-based diagnosis

Techniques based on the Polymerase Chain Reaction (PCR) using species-specific probes for the "Oncho-150" repeat have been shown to be effective for diagnosing *O. volvulus* infection by detecting parasite DNA in routine skin snips (58). The technique, although more sensitive in detecting low-level infections than standard skin methods, still requires the taking of skin snips and does not give any indication of the number of skin mf or their viability. In research laboratories, PCR-based assays for diagnosis of onchocerciasis have great potential as the costly equipment and reagents are often available, and contamination of the samples can be prevented. However, under conditions where most of routine diagnosis of onchocerciasis is performed, the high cost, the poor availability of the reagents and the difficulties in controlling contamination limit the applicability of PCR-based techniques (59, 60). In addition, minor changes in a highly specific PCR protocol may result in amplification of fragments, which can easily be misinterpreted as *O. volvulus* products or gene fragments resulting in false diagnosis (61). A recent adaptation of a paper chromatography hybridization assay (PCHA) to detect amplified O-150 DNA was compared to two established methods, namely agarose gel electrophoresis (AGE) and hybridization enzyme-linked immunosorbent assay (ELISA) (62). Although this method detected PCR products in 30 minutes without electricity or special equipment, and it claims that such technology brings DNA detection a step closer to widespread use in field settings, the dependence of the initial PCR protocol to obtain amplified DNA is still evident.

1.3.4 Treatment

For years, surgical removal of nodules (nodulectomy) has been undertaken in the endemic areas as a way to reduce the burden of adult worms in infected individuals, and for aesthetic reasons. However, as a treatment, nodulectomy is not efficient because it is difficult to locate all nodules. The invasive nature and the risk of secondary infections require experienced personnel, equipment and careful monitoring of the patients. Nonetheless, large nodulectomy campaigns in districts of Guatemala and Mexico between 1935 and 1979 have, in combination with vector control and urbanization programs, contributed to the decrease in nodule prevalence from 24 to 9% among the people in these areas (29).

At present, there is no effective drug that kills the adult worm without the risk of severe side effects. Suramin, which kills the adult worm, has been administered through repeated intravenous injections. This practice, however, is limited because it requires careful monitoring and there is risk of fatal side effects (63, 64).

Diethylcarbamazine (DEC), a microfilaricide, was the drug of choice for the treatment of onchocerciasis until the mid 1970s. Although it has limited effect on mf *in vitro* (65), the enhancement of the immune responses associated with the pathological changes in onchocerciasis is believed to be the basis of the microfilaricidal effect of

DEC (66). However, this same property is the negative aspect of the use of this drug, which in patients with high parasite load can lead to devastating Mazzotti reactions.

The current drug of choice for the treatment of onchocerciasis is ivermectin, a mixture of macrocyclic lactones produced by *Streptomyces avermitilis*. Before being used to treat onchocerciasis, ivermectin has been used in veterinary medicine since 1981. The drug is effective against a number of nematodes, including *Ascaris lumbricoides*, *Stronglyloides stercoralis*, hookworms and the filarias *W. bancrofti*, *Brugia malayi*, *Loa loa* and *Mansonella ozzardi*, all of them human parasites. After its clinical trials (67) ivermectin was registered in 1987 for use against onchocerciasis and suitability of this drug for large-scale treatment opened up prospects for chemotherapeutic control of the disease (18).

Initial studies on the mode of action of ivermectin suggested that the biological activity was mediated via an interaction with a ligand-gated chloride channel, as one mechanism of toxicity of the drug appears to be the action on ion channels in cell membranes. This causes influx of negatively charged ions in cells resulting in muscle paralysis. It was speculated that ivermectin interacts with gamma-amino-butyrate (GABA, 4-aminobutyrate) –gated chloride channels (68), but later work showed that ivermectin affects a non-GABA-gated chloride channel (69). GABA is an amino acid formed by decarboxylation of glutamate. In certain neurons, amino acids including glycine, aspartate, glutamate and GABA, serve as transmitter substances that are stored in the synapses and released upon stimulation. The released transmitters regulate the electrical behavior of neighboring neurons, either by inducing or by inhibiting the generation of an action potential. Most neurotransmitters and neuromodulators stimulate the opening of ion channels, whereas only a few stimulate closure. The type of ion channel involved determines the direction of the change in the membrane potential of the postsynaptic cell. A decrease in the membrane potential due for example, to opening of sodium channels stimulates postsynaptic action potentials whereas an increase due to opening of chloride channels inhibits their formation (70).

Through a combination of biochemical, molecular and electrophysiological techniques, the interaction of ivermectin with its gated chloride channel was elucidated. Those results suggested that low concentrations of ivermectin could potentiate the interaction of glutamate with its receptor-binding site. Therefore it appears that ivermectin at high concentrations (>10 nM) directly opens the channel whereas ivermectin at low concentrations (<10 nM) potentiates channel opening by glutamate (69, 71). The avermectins are highly selective for invertebrate chloride ion channels and only interact with vertebrate receptors at high concentrations. They appear to act on a GABA-gated chloride channel in vertebrate brain but through a low affinity receptor. Since avermectins do not readily cross the blood-brain barrier, only very low concentrations ever reach vertebrate receptors. The combination of lower affinity and compartmentalization of host receptors may account for the low incidence of host toxicity with this class of compounds (72).

Microfilariae in skin snips and eyes from treated individuals have shown a reduced motility compared to those from untreated individuals (66). Such alternative effects on the mf might result in their clearance through the lymphatic drainage (73, 74).

12

Ivermectin treatment results in a rapid reduction of the number of mf in the uppermost skin layer during the first days. A minority of these mf are believed to migrate to deeper skin layers, whereas the majority has been shown to totally disappear from the skin (73). A very low mf level persists for several months with a subsequent gradual increase to a pre-treatment level after about one year. The long-term persistence of low levels of mf may be explained by an effect on their release from the adult female worm. Intrauterine microfilariae were reported to be inhibited from leaving the uterus for about one month after treatment (13).

When ivermectin was registered as the drug of choice for onchocerciasis, model predictions indicated that larviciding activities need only be maintained for a period of 14 years (instead of 20 years) or 12 years when combined with ivermectin distribution (75). Since the OCP is a control, not an eradication effort, it is expected that low levels of prevalence in the program areas will remain after OCP closes in 2002. These parasite populations alone are thought to be insufficient to maintain transmission despite the increase in blackfly abundance that will occur following cessation of vector control activities. However, there is concern about possible recrudescence (or re-occurrence) of onchocerciasis transmission through re-invasion of blackflies or migration of infected persons into the former OCP areas (18). The ONCHOSIM model, a computer program for modeling the transmission and control of onchocerciasis (76), suggests that, if recrudescence is detected before exceeding 1.0-1.5% annual infection incidence, then annual ivermectin distribution for >15 years should lead to suppression of transmission and prevent a nascent parasite population from re-establishing itself (77).

It is believed that ivermectin could prevent blindness. However, in a review of randomized controlled trials that included 3,810 participants, no statistically significant difference was observed in any trial (reporting visual acuity outcome) between ivermectin and placebo groups for visual acuity loss. The effects of ivermectin on lesions affecting the eye are uncertain and data on whether the drug prevents visual loss are unclear (78).

1.4 IMMUNE RESPONSES IN ONCHOCERCIASIS

The clinical presentations of onchocerciasis can be thought of as a spectrum of manifestations from a generalized form to a localized one (Figure 6). On one extreme of the spectrum, adult worms in the subcutaneous nodules and millions of dermal mf characterize those individuals with generalized infection, mostly from hyperendemic areas. Despite this high antigen load, individuals with generalized onchocerciasis often present weak skin inflammation and exhibit relatively mild alterations such as atrophy and pigmentary changes. By contrast to those with generalized onchocerciasis, there are few individuals in hyperendemic areas who remain free from disease despite equivalent heavy exposure to parasites and assumed infection with L3. These people are the ones termed putatively immune, PI, and would be in the middle of the spectrum. On the other extreme of the spectrum, there are those with the localized form of the disease,

13

sowda, which includes the minority of individuals with chronic hyperreactive onchodermatitis presented unilaterally. These patients suffer from papular dermatitis, hyperpigmented lichenified lesions, pruritus and lymphadenitis. They have low mf loads and low numbers of adult worms resident in large nodules. This condition is assumed to be the result of a hyperreactive inflammatory response leading to the killing of mf at the expense of skin integrity.

The key to the understanding of resistance and susceptibility in human onchocerciasis is to recognize that there are different types of immune reactions that must be considered when studying the immune responses in onchocerciasis. Studies on the immune responses of patients with onchocerciasis have yielded three major conclusions. First, and most importantly, a cellular immune hyporesponsiveness to onchocercal antigen exists in patients with microfiladermia. Second, in contrast to this cellular hyporesponsiveness, specific antibody responses are vigorous in all patients; the highest antibody levels being found in those with localized (sowda-form) onchocercal dermatitis. Third, seemingly uninfected "endemic normal" individuals (who are considered to be immune) generally have higher cellular responses (proliferation and cytokines) to onchocercal antigen than those with generalized microfiladermia, but tend to have lower specific antibody responses (79). Most of the pathology is the direct consequence of localized host inflammatory responses, although some may derive from pathogenic molecules elaborated by the parasite itself. Pathology in the host can be viewed as the overt inadequacy (or at least insufficiency) of anti-inflammatory mechanisms. In human onchocerciasis, this pathology is found primarily in the skin and eye, although lymphatic, renal, nervous and other systems can be affected.
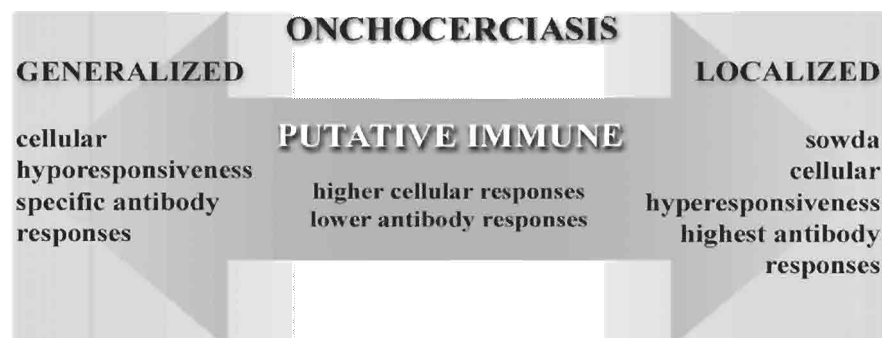

ONCHOCERCIASIS

GENERALIZED   **LOCALIZED**

cellular   **PUTATIVE IMMUNE**   sowda
hyporesponsiveness   cellular
specific antibody   higher cellular responses   hyperresponsiveness
responses   lower antibody responses   highest antibody
                                                             responses

Figure 6. Spectrum of immunological characteristics of onchocercal infection. Generalized onchocerciasis, mostly in hyperendemic areas, is characterized by the presence of adult worms in the subcutaneous nodules, and millions of mf. Cellular hyporesponsiveness is common to all infected individuals, as well as the production of specific antibodies. However, the highest antibody responses have been observed in individuals with localized onchocerciasis (sowda), presented unilaterally. These individuals show low levels of mf loads and low numbers of adult worms in large nodules. In the middle of the spectrum, putatively immune individuals, who also live in the endemic areas, present higher cellular and lower antibody responses, and remain free of infection, despite heavy exposure to parasites. (Illustration: G. Guzmán).

1.4.1 Cellular hyporesponsiveness

Human studies have shown that there is a suppression of cellular immune responses in onchocerciasis patients with mf in the skin (74, 80, 81), but different results have been reported as to the specificity of this suppression. Impairment in the general cellular immune responses (as defined by the response to the T cell mitogen PHA and to the Mycobacterial antigen PPD) in people infected with *O. volvulus* was demonstrated both by skin testing and *in vitro* lymphocyte activation assays (80, 82). However, others could not reproduce the same generalized immunosupression (74). The phenomenon of suppression appears to be more prominent in people with mf distributed throughout the skin and little cutaneous reaction (generalized) as compared to those with highly reactive skin disease and few mf demonstrable (sowda). Furthermore, lymphocyte reactivity in infected young people (12-16 yr) was greater than that in older infected individuals, suggesting that there is acquired down-regulation of the cellular immune response accumulated over time (80). The precise mechanisms by which dampening of the cell-mediated immune response occurs is as yet unknown, although more recently, an alternative mechanism has been proposed that is associated with specific cell hypoproliferation (19).

1.4.2 Antibody responses

Infection in humans leads to the formation of antibodies against multiple antigens of the parasite. Because the onset of infection is usually unknown, and there are few studies in children, data are not available that define the evolution of the antibody response. However, with chronic infection, antibodies against a multiplicity of parasite antigens develop. In addition, antibodies of various classes, especially IgG, IgM and IgE, with no apparent specificity for *O. volvulus* are elevated and it is likely that the infection leads to polyclonal B cell activation (83). Early data from a chimpanzee model of onchocerciasis has yielded insights into the dynamics of the immune response. Within one month following infection, antigens of developing larvae elicited both an antibody and a cell-mediated immune response against crude parasite antigen. This was manifested by IgG recognition of multiple bands on Western blot, and by a quick and active blastogenic response to soluble adult worm extract (20). In almost all reported studies, patients with generalized onchocerciasis had significantly higher specific IgG and IgG subclass responses to onchocercal antigen than the endemic normals living in the same region (84-88), and non-endemic normal individuals have minimal or no antibody reactivity of any isotype to the parasite antigen. Attempts to correlate specific antibody levels with skin mf counts or skin pathology produced interesting findings. Skin mf were found to correlate directly with both specific IgG_4 antibodies and antibodies detected by immunofluorescence on mf, while inverse correlations were noted between skin mf levels and complement-fixing antibodies (IgG_1, IgG_2 and IgG_3 subclasses), and specific IgM (88). The degree of skin pathology has also been reported to be proportional to the level of the specific IgG (and each IgG subclass) response to onchocercal antigen; indeed, those with the localized sowda skin manifestations have the highest specific IgG and IgG subclass antibody responses (89). IgG_4 was shown to increase in response to chronic antigen stimulation, such as during onchocerciasis (90), although not all individuals apparently have the capacity to

produce an enhanced IgG$_4$ antifilarial responsiveness (91). In generalized onchocerciasis, the IgG directed against *O. volvulus* shows a differential distribution among the subclasses and both IgG$_4$ and IgG$_1$ have the highest concentrations (92).

The protective role of antibodies in the human immune response against onchocercal infection has been difficult to demonstrate, although evidence of IgG$_3$ involvement in acquired immunity was reported (87). *In vitro* experiments have shown that sera from infected persons promote granulocyte adherence to mf and infective larvae (93, 94), and destruction of mf was demonstrated. Acquisition of protective immunity after prolonged exposure to *O. volvulus* was circumstantially supported by epidemiological data, which showed that after middle age, the intensity of infection decreases with increasing age (95).

1.4.3 Immunity to onchocerciasis

The existence of immunity to infection with *O. volvulus* has not been demonstrated conclusively in humans but is suggested by epidemiological and clinical data. It is only recently that protective immunity has been shown to be induced in animals (96). Acquisition of protective immunity in humans has been suggested by the plateau seen in the skin mf levels of subjects aged 20-40 yr (concomitant immunity) (97), and the identification of persons in hyperendemic areas who have had lifelong exposure to high transmission rates but appear to remain infection-free, variously termed putatively immune (PI) or endemic normals (29, 86). The term putative describes the prospective nature of analysis because PI should be re-examined for a correct diagnosis to exclude prepatency at the time point of immune characterization (98). The identification of truly infection-free persons is made difficult by the existence of low-level microfiladermia and subclinical infections, neither of which is detectable using parasitological and clinical techniques.

1.4.4 Cytokines

Published information on cytokine responses in patients with onchocerciasis is not clear-cut. Endemic normal individuals have been described as having both higher (86) and equivalent (99) interleukin (IL)-2 responses to onchocercal antigen compared with patients with generalized onchocerciasis, whose responses do not differ from those of non-endemic normals (86, 100). There was also discrepancy between studies on gamma-interferon (IFN-γ) production by endemic, non-endemic normal, and individuals with generalized onchocerciasis. After stimulation with onchocercal antigen, peripheral blood mononuclear cells (PBMC) from patients with generalized onchocerciasis also appear to produce more IL-4 than do PBMC from non-endemic normals (100), and more IL-5 but equivalent amounts of IL-10 compared with cells from endemic normal persons (99).

1.4.5 Clinical presentations and their immune effector pathways

Recent advances in the understanding of the immune effector pathways associated with the different clinical presentations, and particularly from further analysis of the suppressor immune reactions in onchocerciasis, suggest that the apparent discrepancies in the results obtained from studies with infected individuals are indeed cases of a counterbalance between suppressor and effector immune reactions, trespassing the strict dogma of the Th1 and Th2 dichotomy.

The immune effector mechanisms against mf and L3 are complex. In untreated generalized onchocerciasis, eosinophilic granulocytes and macrophages are found in the tissue around degenerating or dead mf (101). Increased accumulation of cells in the nodules is dependent on the presence of mf (102). Histological studies with skin biopsies taken from patients after microfilaricidal treatment has shown that integral, non-degenerated mf are attacked by eosinophils, but this was not usually seen in untreated generalized onchocerciasis (103).

The killing of mf without previous antifilarial treatment is regularly observed in infected sowda patients. These patients have a restricted number of parasites and few, large nodules, characterized by massive inflammatory infiltrates of lymphocytes (including several plasma cells), mast cells, eosinophils, neutrophils and macrophages (104). Eosinophils, neutrophils and macrophages all possess the capacity to kill mf (101, 104). The difference in immune attack against mf between sowda and generalized onchocerciasis suggests that mf are killed *in vivo* by an interplay between inflammatory cells, which are strongly activated in sowda but are partially suppressed in generalized onchocerciasis. T cells and antigen-presenting cells apparently play important regulatory roles in directing these responses (19).

Evidence from *in vitro* studies confirmed the capacity of neutrophils and eosinophils to kill mf and to immobilize L3 in infected patients (94). Whereas L3 were immobilized by eosinophils after complement activation by normal serum, opsonization by specific antibodies is needed for immobilizing mf (105). The mechanisms for immune destruction of L3, on the other hand, are difficult to analyze using human material *ex vivo* (histology). From studies in animal models, it has been shown that antibody-dependent eosinophils mediate the destruction of L3 shortly after entry into the host and during the molt stages to L4, but not of the later developmental stages of the parasite, in accordance with *in vitro* observations (105). The presence of antibodies and effector cells seems essential, and the dependence on IL-4 and IL-5 qualify this reaction as mediated by T helper (Th) 2 cells (19).

The paradigm of the two cross-regulating subgroups of Th cells (Th1 and Th2), demonstrated first in mice in the 1980s, was later applied to humans, and results from individuals infected with helminths, including *O. volvulus*, served as examples for studying human Th2 responses (106). This prompted the exploration of the possibility of a Th1 and Th2 dichotomy being associated with different clinical presentations of onchocerciasis. However, the reports showed mixed results. In one report, a Th1-type response was observed in a subgroup of putative immune (PI) individuals (99).

17

However, in most other reports, PBMCs from PI or endemic normals displayed mixed Th1 and Th2 responses (107, 108), which were elevated when compared with those from generalized onchocerciasis, and IFNγ and IL-5 were the major cytokines detected in PI. In other studies, a Th1-type response was characteristic after antifilarial treatment (74) or subclinical infections (92). The idea of a Th1-like response came from studies with PI, thus suggesting immunity to invading L3, the most probably targetted stage in PI. The mechanism, nonetheless, remains unclear. PI individuals provide an immunological picture completely different from those with generalized onchocerciasis. The levels of Fc receptor-and-complement binding IgG$_3$-type antibody levels are higher in PI (87) compared to those in generalized onchocerciasis individuals, who characteristically produce elevated IgG$_4$ antibodies against a water-soluble extract from $O.$ $volvulus$ worms (5). These antibodies were shown to be of a blocking isotype (109). Even though the impact of IL-4 and IL-12 on IgG$_4$ is well documented, the relationship between IgG$_3$ and Th1 has not been reported.

Potentially different co-infections were thought to be the explanation for increased IFNγ in PI. However, infections with gastrointestinal nematodes have often been found in PI and individuals with generalized onchocerciasis in equivalent frequencies (107, 110), and the suggestions that other co-infections potentially reduce a Th1 response against onchocerciasis were largely excluded (110).

Sowda individuals, on the other hand, show a characteristic Th2-type response (111). This is in contrast to the overall mixed Th1 and Th2 response in PI. Sowda patients also produced elevated levels of IgG$_1$, IgG$_3$ and IgE against $O.$ $volvulus$ (89, 112). In addition, the levels of IgG$_3$ antibodies against somatic $O.$ $volvulus$ antigen correlate negatively with mf loads (88). The destruction of mf is thus assumed to be a Th2-mediated, antibody-dependent and granulocyte-dependent response, which also leads to skin inflammation in sowda.

Evidence has been put forward that attempts to explain the hyporesponsiveness in generalized onchocerciasis, without ascribing it to a necessary Th2-type response. Two facts are considered for this; one is the mixed type of effector response seen in PI and sowda, and the other is the recovery in cellular proliferation after antimicrofilarial treatment by ivermectin, which increases both Th1 and Th2 responses, rather than inducing an overall shift from Th2 to Th1 (108, 113). Instead, an alternative regulatory mechanism has been suggested, based on the evidence of a third arm of a Th cell reaction, which is associated with OvAg-specific hypoproliferation and is able to suppress both Th1 and Th2 responses. T cells of this type have been termed Th3 or T regulatory 1 (Tr1) (Figure 7). It has been suggested that the downregulatory response in onchocerciasis be defined (110) as Th3/Tr1 in order to emphasize that downregulation in chronic helminth infection requires a novel, non-Th1 and non-Th2 type of response. T-cell effector pathways comprising mixed Th1 and Th2 cells have been reported in lymphatic filariasis and schistosomiasis, thus raising the possibility of finding Th3/Tr1 cells in such infections.

Two cytokines have been associated with cellular hypoproliferation to specific antigen: IL-10 and another downregulatory cytokine, transforming growth factor β (TGF-β). Given that IL-10 seems to have a role in downregulating Th2 driven allergic responses,

18

it is worthwhile to consider this type of response as a negative regulator of Th2-driven immune pathways. Of importance, a downregulatory molecule, cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) was a key marker for this new type of T cell, is upregulated spontaneously in mf-infected individuals, and is induced by antigen from mf but not from other parasite stages (19).

Ultimately, the effector pathway that is suppressed along with cellular hyperactivity in peripheral blood seems to be T-cell suppression of Th1 and Th2 responses, which probably diminishes the presence of both effector cells and antibodies and alters the antibody subclass, thus preventing the attack against mf in generalized onchocerciasis.

Even though the balance between effector and suppressor mechanisms in onchocerciasis can be explained with the presence of Th3/Tr1 cells, the question of how the immune response is induced still remains.
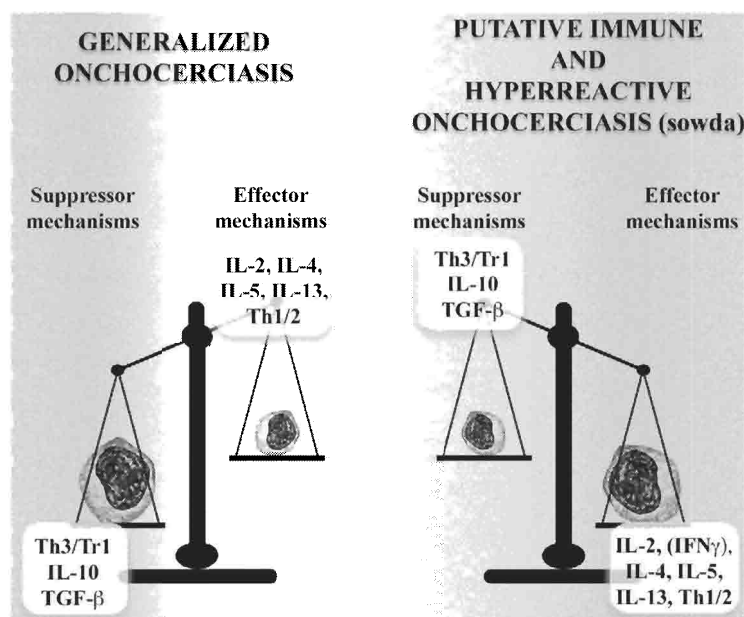

Figure 7. Model for the balance between effector and suppressor mechanisms in onchocerciasis. In generalized onchocerciasis, the Th1- and Th2-dependent effector reactions are suppressed by IL-10 and TGF-β, and antigen-specific T-regulatory cells (Th3/Th1) can be found. In putatively immune individuals and patients with the hyperreactive form of onchocerciasis (sowda), the T helper (Th)1 and Th2 effector mechanisms prevail, leading to immune attack against L3 (putative immunity) or mf (sowda). Abbreviations: INFγ, interferon γ; IL, interleukin; Th3, T helper 3 cells; TGF-β, transforming growth factor β; Tr1, T regulatory cell 1. Illustration: G. Guzmán.

It is known that complex organisms like *O. volvulus* present a plethora of antigens to their hosts, including immunomodulatory proteins (114) or proteins homologous to mammalian downregulatory cytokines (115). However, non-protein molecules, such as bacterial DNA, lipopolysaccharide (LPS) or oligosaccharides also drive T-cell

responses into the different effector pathways. Of particular importance is the fact that those molecules are also found in filarial species. Molecules derived from the mutualistic bacteria *Wolbachia* are found in most human and animal filarial species, including *O. volvulus* (116). Bacterial products, both LPS-related and LPS unrelated, stimulate the release of pro-inflammatory and anti-inflammatory cytokines, and in the case of *O. volvulus*, they are the main inducers of the neutrophil response around adult worms (117), which is Th1-dependent in mice. Sugar moieties and phosphorylcholine, present on filarial L3 and mf surfaces, promote Th2 responses (118). Therefore, the parasite can provide the host with both, Th1 and Th2-inducing molecules, and supposedly Th3/tr1-inducing molecules as well (115).

2 THE SCIENTIFIC SCOPE OF THIS THESIS

2.1 OBJECTIVES

- To identify and compare the immunodominant components of Guatemalan and Ghanaian *Onchocerca volvulus* worms in terms of the IgG_4 response elicited in infected individuals (**Paper I**).

- To compare the efficacy of a semi-purified antigenic fraction of proteins (designated PakF) found in both Guatemalan and Ghanaian *O. volvulus* isolates, used in a simple dot blot assay to other diagnostic methods for filariasis routinely used in a non-endemic country like Sweden (**Papers II & VI**).

- To evaluate the IgG and IgG_4 antibody response from infected individuals to a semi-purified mixture of immunodominant antigens (designated PakF) during patent infection (**Paper III, V**).

- To compare and evaluate the performance of a simple dot blot assay (PakF-DBA) to the "gold standard" skin snip test, for the rapid assessment of onchocerciasis in the post-control era (**Paper IV**).

- To characterize the nature of the components comprising the native mixture of low molecular weight proteins in PakF (**Paper VI**).

2.2 METHODOLOGY

2.2.1 Samples and sample preparation

2.2.1.1 Sera and onchocercal nodules

Peripheral blood samples were obtained from mf+ patients and healthy controls. In addition, samples were obtained from donnors infected with filariasis other than onchocerciasis and other nematodes. Serum samples were prepared from clotted venous blood and kept frozen at $\leq 20°$ C until used. In some cases blood was collected on filter paper and serum obtained by elution. Non-calcified nodules were surgically excised from onchocerciasis patients under local anesthesia and transported to the laboratory in liquid nitrogen and stored at -80° C until use.

2.2.1.2 Antigen preparation

2.2.1.2.1 Tris-buffer Soluble Fraction, TSF

Intact, non-calcified nodules were digested by the collagenase method of Schultz-Key *et. al.* (119) using RPMI 1640 medium. Female worms were washed first with RPMI followed by washing with PBS, and then frozen at -70° C in RPMI containing gentamycin (0.2 mg/ml). Tris-soluble fractions (TSF) were prepared by carefully washing the worms twice in 10 mM Tris-HCL pH 8.0. The worms (pools of 3-5) were placed in Eppendorf vials and mechanically homogenized in homogenizing buffer (20 mM Tris-HCL, pH 8.0, 0.1 M NaCl, 0.1% Tween-20, 1 mM EDTA) at 4° C, using 100 µl per vial of worms. The homogenate was centrifuged at 14,000 rpm for 5 min and the supernatant collected. The pellet was homogenized and centrifuged five more times as before and the supernatants combined to obtained a final volume of approximately 500 µl. After the extraction, supernatants from all pools were evaluated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) for differences between worm pools, and stored at -20° C until used. The total protein concentration was estimated by the Bradford assay (120).

2.2.1.2.2 PakF fractions

PakF fractions were prepared separately from Guatemalan and Ghanaian TSF (TSF-GU, and TSF-GH, respectively), by manual ion exchange chromatography or with the assistance of a peristaltic pump. A 1-ml HiTrap-Q FF™ anionic column (Amersham-Pharmacia, Uppsala) was equilibrated with five volumes of equilibrium buffer (50 mM Tris-HCL, pH 9.0 containing 0.05% Tween-20) at a flow rate of 1 ml/min, then washed with five volumes of washing buffer (same composition as equilibrium buffer but containing 0.5 M NaCl), and re-equilibrated with five volumes of equilibrium buffer. After equilibrium, 100 µl of TSF, mixed with an equal volume of equilibrium buffer were passed through the anion exchanger. When done manually, a 1-ml syringe was used as a buffer reservoir. The sample was eluted with equilibrium buffer at 0.5 ml/min and the eluate collected in eight-350 µl aliquots before changing to washing buffer. Two final 350-µl aliquots were further collected using the buffer containing

22

NaCl. Each fraction was tested for antigenicity and purity using a dot blot assay and SDS-PAGE (see below).

2.2.2 Biochemical techniques

2.2.2.1.1 One-dimension SDS-PAGE

O. volvulus TSF antigens were separated in 12, 15, or 17.5%T, 3.3% C polyacrylamide gels using the discontinuous method of Laemmli (121). A volume corresponding to 30 μg of total TSF diluted 1:2 in sample buffer [4.9% 2-mercaptoethanol, 2% (w/v) SDS, 12.3% (v/v) 0.5 M Tris, pH 6.8, 10% (v/v) glycerol, and 0.02% (v/v) of 1% bromophenol blue prepared in 1% ethanol], kept in boiling water for 5 min, and then loaded onto the gels at a concentration of approximately 0.4 μg/mm stacking gel. Electrophoresis was performed in a vertical Mini-Protean II system (BioRad, Richmond, CA), containing electrode buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3). Samples were separated at 20-50 mA (constant current), and 10-15° C until the dye front reached the bottom end of the gel (approximately 1.5 h). After electrophoresis, the resolved proteins were either transferred onto nitrocellulose for immunodetection, or stained with silver or Coomassie blue. Alternatively, the samples were separated in 18% Tris-HCl Criterion gels in a Criterion Electrophoresis unit (BioRad, Stockholm) following the manufacturer's instructions.

2.2.2.1.2 Two-Dimension get electrophoresis (2D-PAGE)

PakF proteins were separated by isoelectric focusing (first dimension) using 7-cm Immobiline DryStrip gel strips (Amersham-Pharmacia) with a pH gradient 6-11, following the manufacturer's instructions. Before isoelectric focusing, the gel strips were rehydrated overnight with 125 μl of rehydration buffer (8 M Urea, 2% CHAPS, 0.5% IPG buffer pH 6-11, DTT 2.8 mg/ml, and a few grains of bromophenol blue) containing 40 μl of PakF, or 20 μl of 2D-Molecular weight standards (BioRad). After rehydration, the strips were placed on a Multiphor II horizontal electrophoretic cell (Pharmacia) and the proteins focused at 3500 V, 2 mA, and 5 W for 4.5 hours. After the first dimension, the strips were equilibrated for 15 min in 50 mM Tris-HCl, pH 8.8 containing 6 M Urea, 30% glycerol, 1% DTT and a few grains of bromophenol blue. The strips were placed on an 18% Tris-HCl Criterion gel (BioRad) and the second SDS-PAGE dimension carried out at 40-60 mA until the moving boundary reached the bottom end of the gel. Resolved proteins were either transferred to nitrocellulose for immunodetection, stained with silver or Coomassie blue for posterior treatment and N-terminal sequencing.

2.2.2.1.3 Proteinase-K treatment

Twenty-five microliters (approximately 0.5 μg) of PakF fraction were incubated with Proteinase K (18.8-150 μg/ml) at 37° C for 30 min and analyzed by SDS-PAGE and immunoblotting.

2.2.2.1.4 Heat treatment

Fifteen microliters (approximately 0.3 μg) of PakF samples were heated at 95° C for 5-25 min using PCR-type vials in a common thermocycler, and immediately dotted on nitrocellulose for dot blot analysis. Heated and non-heated samples were dotted on nitrocellulose for dot blot analysis.

2.2.2.1.5 Glycoprotein detection

In-Gel staining was performed using GlycoGel Staining kit (Pierce Chemical Co., Springfield, IL), based on periodic oxidation and fuschin acid staining, following the manufacturer's instructions. Alternatively, a similar method was used to oxidize glycoproteins for subsequent labeling with biotin hydrazide and further chemiluminescent detection with horseradish peroxidase-labeled streptavidin.

2.2.2.1.6 Protein Staining

Polyacrylamide gels were stained with Coommassie brilliant blue G-250 as described by Sasse and Gallagher (122). Briefly, after electrophoresis, the gels were fixed for 15 min in 25% isopropanol, 10% acetic acid and stained with 0.006% Coomassie brilliant G-250 dissolved in 10% acetic acid. The gels were left overnight in Coomassie solution and destained with 19% acetic acid for 30 min to 1 hr.

Silver staining of protein was made following essentially the method described by Heukeshoven and Dernick (123), with the modifications described by Kierdorf et at (124). Immediately after electrophoresis, the gels were immersed in fixing solution (40% ethanol, 10% acetic acid) for 30 min with gentle agitation. After fixing, the gels were incubated for 40 min in incubation solution (0.2% sodium thiosulphate, 4.1% sodium acetate anhydrous, 0.52% of glutaraldehyde, 30% ethanol). After incubation, the gels were washed three times, 5 min each, with distilled water and then left in impregnating solution (0.1% Silver nitrate, 0.02% formaldehyde) for 20 min. The impregnating solution was replaced with developing solution (2.5% sodium carbonate, 0.01% formaldehyde, 20% ethanol) and the bands were developed with several changes of this solution. Once the bands had reached appropriate intensity, the reaction was stopped by leaving the gel in stop solution (1.46% EDTA disodium salt) for 10 min to up to 2 hr. The stained gels were washed for 10 min with several changes of distilled water before photography or drying. Alternatively, the gels were sequentially stained with Coomassie after silver staining.

2.2.3 Immunological techniques

2.2.3.1.1 Immunodetection of proteins electrotransferred onto nitrocellulose

After 1D or 2D-PAGE, O. volvulus proteins were electrotransferred onto nitrocellulose membrane (Western Blot) essentially as described by Towbin et al (125). The gel was equilibrated in cold (approximately 10 °C) transfer buffer (25 mM Tris, 192 mM Glycine, pH 8.3, 001% SDS, 10% Methanol) for 15 min to allow temperature and size stabilization. The Mini-Protean II, or Criterion Transfer units (BioRad) was assembled

24

and proteins were transferred for 2 hr at 100 mA, constant current in cold transfer buffer. After transfer, the unit was disassembled and the membrane let to air dry until used. In some instances, the membrane was used to produce 3-mm wide strips for immunodetection of proteins with individual sera or anti-sera, and for total protein detection. For Dot Blot, droplets of 0.5 µl PakF were applied in duplicate onto nitrocellulose strips (4 mm wide, 30 mm long) and allowed to air dry.

Nitrocellulose strips with transferred or dot-blotted proteins were placed on plastic mini-incubation trays (BioRad) and were blocked with 1% skimmed milk powder in Phosphate buffer saline pH 7.4 containing 0.05% Tween-20 (PBST) for 1 hr on a rocking shaker. After blocking, electrotransferred proteins were probed overnight with first antibody (sera, polyclonal, or monoclonal antibodies) in a dilution properly determined. Typically, sera were diluted 1:2000, polyclonal and monoclonal antibodies were diluted 1:500 in PBST. Strips were washed three times, 5 min each, with PBST and then incubated with second antibody diluted in PBST for 1 hour. After washing three times with PBST and twice with PBS alone, the immunoreactive bands were developed with BCIP/NBT for 10 minutes. The color reaction was stopped with several washes with distilled water and the strips were left to air dry in the dark.

2.2.3.1.2 Antibody elution

Human antibodies were eluted according to the method described by Rosfjord (126) with some modifications. Briefly, nitrocellulose discs (Ø = 6mm, two sides, 56.5 mm^2) or strips (4 x 50 mm, two sides, 400 mm^2) were immersed in PakF or solution containing individually electroeluted proteins for 10 minutes and let air-dry. The membrane was blocked with 1% milk powder in PBST for 1 hr and then immersed, overnight and with gentle agitation, in serum pools from infected or non-exposed individuals diluted 1:40 in PBST. The membranes were soaked twice, 5 min each in PBST, and then immersed in 3 ml of elution buffer (0.05 M Gly-HCl for 5 min with gentle agitation. The membrane was removed and the elution solution neutralized for 5 minutes with 1 M Tris-HCl pH 7.2, 3.4% NaCl. Blocking solution was added up to 5% of total volume (i.e., 200 µl to 4 ml of antibody solution). When smaller volumes were used, the proportions of the reagents were adjusted accordingly. The antibody solution was concentrated once when necessary using concentrator tubes (Pall Life Sciences, Lund, Sweden).

2.2.3.1.3 Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates were coated with a crude phosphate-buffered saline (PBS)-soluble fraction of homogenized female adult *Acanthocheilonema viteae* (1.5 µg total protein/ml) overnight. Two-fold dilutions of human serum (starting with 1:400) were incubated in the plates for 2 hr. Plates were washed and the antibody response to the antigen was detected with horseradish peroxidase-conjugated sheep anti-human Ig (SBL, Stockholm, Sweden). Hydrogen peroxide and 5-aminosalicylic acid were added after washing and the optical density (OD) read at 450 nm. Cut-off values were determined by analysis of sera from healthy Swedish controls.

2.2.3.1.4 Immunohistochemistry

Eluted antibodies were used to detect *O. volvulus* proteins in 5-µm cryosections obtained from adult female worms. Sections were placed on four-well microscope slides and kept at -70 °C until use. Sections were fixed with cold acetone for 15 minutes, and then blocked for 15 minutes with 1% bovine serum albumin (BSA)

containing 0.05% Tween-20. The sections were then washed twice for 5 min with PBST, and then incubated with eluted antibodies without dilution. After washing twice for 5 min with PBST, the sections where incubated for 15 minutes with goat anti-human IgG labeled with alkaline phosphatase, diluted 1:2000 in PBS. After a final set of two washes (5 min each) with PBS, the sections were developed with BCIP/NBT substrate for 30 min until the reaction was stopped with 3 washes with distilled water (1 min per wash). The sections were counterstained with Bismarck Brown, which stains yellow the areas of the section where no phosphatase activity is detected, thus no immunostaining resulting in blue areas against yellow tissue.

2.2.4 Proteomics

2.2.4.1.1 In-gel digestion with trypsin

Spots of interest were cut out from Coomassie-stained 2D gels and digested with trypsin essentially as described by Opperman *et al* (127). All steps were performed at 37 °C. Briefly, gel plugs were put into 500 µl PCR tubes (Costar, Corning, NY, USA) and washed for 1 h in 200 µl 0.2 M ammonium bicarbonate/acetonitrile (1:1 vol). After a second wash in 200 µl 0.05 M ammonium bicarbonate/acetonitrile (1:1 vol) for 1 h, gel plugs were dried under vacuum for 15 min and re-soaked for 10 min in 5 µl 0.1 µg/µl porcine modified trypsin (Promega, Madison, WI, USA) in 0.2 M ammonium bicarbonate. The plugs were covered with 20 µl 0.2 M ammonium bicarbonate and incubated over night. Digestions were stopped by adding 0.5 µl concentrated trifluoroacetic acid (TFA) and the supernatant collected. Peptides were extracted stepwise from the gel plugs for 2 x1 h in 100 µl 0.1 % TFA in 60% acetonitrile (v/v). A final extraction was performed using 100 µl 0.1% TFA in 40% acetonitrile (v/v) before all 4 supernatants were pooled and concentrated under vacuum to a final volume of 10 µl. The peptide mixes were desalted and further concentrated using reversed-phase C18 micro-columns (ZipTip, Millipore, Bedford, MA, USA).

2.2.4.1.2 Identification of proteins by mass spectrometry

MALDI mass spectra were recorded using a Voyager DE-Pro (PE Biosystems, Framingham, MA, USA) instrument and a Bruker Reflex 3 (Bruker, Leipzig, Germany) using α-cyano-4-hydroxycinnamic acid as the matrix. The peptide mass maps were searched against simulated tryptic digests of proteins in Swissprot using the EMBL software at the address above or in Genbank using MS-FIT (http://prospector.ucsf.edu/).

Nano-ES spectra of selected peptides were recorded on a hybrid quadrupole-orthogonal acceleration time-of-flight (TOF) tandem mass spectrometer (Q-TOF, Micromass, Manchester, England) fitted with an orthogonal-sampling nano-ES interface (Z-Spray, Micromass, Manchester, England). Mass spectra were recorded at a resolution of 6000 (full width at half maximum height, FWHM, definition), which corresponds to approximately 3000 on the 10 % valley scale. The spectra were analyzed and multiply charged peptides, not associated with fragments of digested trypsin or known medium contaminants, were subjected to tandem mass spectrometry (MS/MS) analysis. MS/MS spectra were recorded by selecting the precursor isotopic cluster of interest with the quadrupole mass filter, focusing the ions into a hexapole collision cell containing argon gas and accelerating undissociated precursor and fragment ions into an orthogonally

26

arranged TOF analyzer. The collision voltage employed was in the range of 20 – 35 V. Amino acid sequence tags were generated manually and compared with the translated sequences from filarial proteins in Swissprot using the PepSea, Peptide Mass and Prosite utilies for proteomic analysis at ExPASy (http://www.expasy.ch).

2.2.5 Densitometry

Dried gels or nitrocellulose membranes were laser-scanned with Personal Densitometer SI and analyzed using ImageQuaNT software (Molecular Dynamics, Sunnyvale CA) or green-light-scanned with a Sharp XJ-610 color scanner and analyzed with the BioImage's Whole Band Analyzer and 2D-Analyzer software (BioImage, Ann Arbor, MI).

2.2.6 Statistical Analyses

Significance of differences in sensitivity and specificity between serological assays, as well as the relationship between mf density and recognition of immunodominant antigens was analyzed by the Chi square-test. Correlation between eosinophil count and the result from the antibody-based assays were determined using the correlation test from StatView™ SE + Graphics (Abacus Concepts, Inc., Berkley, CA). Correlations between prevalences obtained with skin snip test and IgG-PakF-DBA were determined by calculating the Pearson's correlation coefficients (r), and their significance tested using the Fisher's z transformation.

2.3 RESULTS AND DISCUSSION

2.3.1.1 Guatemalan and Ghanaian *O. volvulus*: the same parasite? (***Paper I***)

The comparison of Guatemalan and Ghanaian derived *O. volvulus* worms in terms of both the polypeptide composition and the antigenic recognition of these polypeptides showed differences that may be evidence of an apparent geographic variation of the parasites. The polypeptide composition analyzed by one and two-dimensional gel electrophoresis showed that TSF fractions from Guatemalan and Ghanaian worms, prepared in the same way, differed mostly in the region of 30-45 kDa. Using highly sensitive silver staining, which allows polychromatic staining of the polypeptides, it was shown that the staining pattern of the polypeptides in the Guatemalan and Ghanaian homogenate differed in both the color and sharpness of the resolved bands (Figure 8). These differences were reproducibly detected when worms from different nodules were analyzed.

The chromatism exhibited by basic proteins has been described and explained in terms of differences in protein-silver ion complex formation, related to the amino acid composition (128). Under silver staining, the relative abundance of yellow-orange proteins in TSF-GU, observed in both one and two-dimensional SDS-PAGE, may indicate the presence of basic proteins, difficult to stain with the silver staining method of choice in this work. Possibly, TSF-GU contains more basic and/or acidic as well as more glycosylated proteins than TSF-GH.
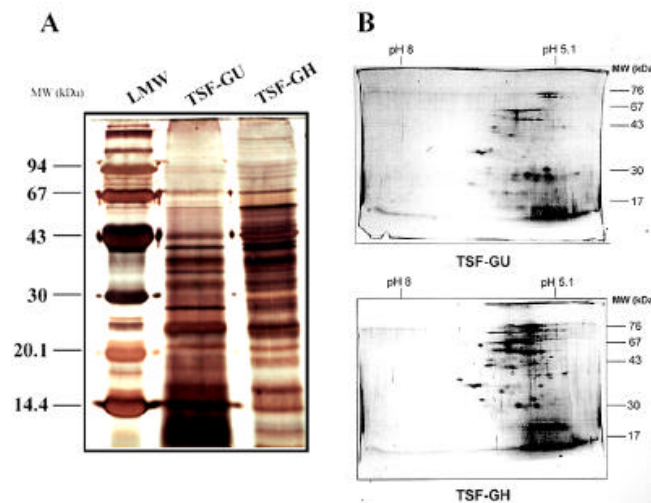

Figure 8. **A**: Silver stained polyacrylamide gel showing the chromatism exhibited by proteins due to differences in protein/silver ion complex formation, related to amino acid composition. Careful control of the developing step allows proteins to stain differently, resulting in a range of colors from yellow to dark brown. Glycosylated proteins, for example, stain yellow. Such chromatism was used to detect differences between TSF-GU and TSF-GH. **B**: Two-dimensional electrophoresis later confirmed that TSF-GU and TSF-GH differ in polypeptide composition, and relative amount of proteins that could be focused in a pH range 5-8.

28

Only minor differences between isolates of *O. volvulus* from savanna and rain forest have been described (23, 129), although the pathological pictures induced by these two strains are distinct. Careful and quantitative analysis of silver stained polypeptides from Guatemalan and Ghanaian isolates allowed the identification of the most variable regions of the electrophoregram. Figure 9 shows the electrophoretic patterns of Guatemalan and Ghanaian TSF preparations with equal amounts of protein (14 µg each) loaded on a gradient gel. The apparent low abundance of high molecular weight proteins in the Guatemalan preparation suggested very small amounts of those proteins in the sample. Thus, electrophoresis was carried out loading double the amount of TSF-GU. Doubling the amount of TSF-GU resulted in an increase in the intensity observed in the other parts of the polypeptide profile (not shown). Using analytical densitometry, the differences between the patterns were quantified and expressed as a percentage of similarity. Figure 9A shows that the patterns were more similar in the high (>50 kDa) and low (<30 kDa) molecular weight regions, and differed most in the region of medium molecular size (30-50 kDa). Glycoprotein detection showed that TSF-GU contained more glycosylated proteins than TSF-GH, most of them within the medium range (30-50 kDa) and high molecular weights (>85 kDa) (Figure 9B).
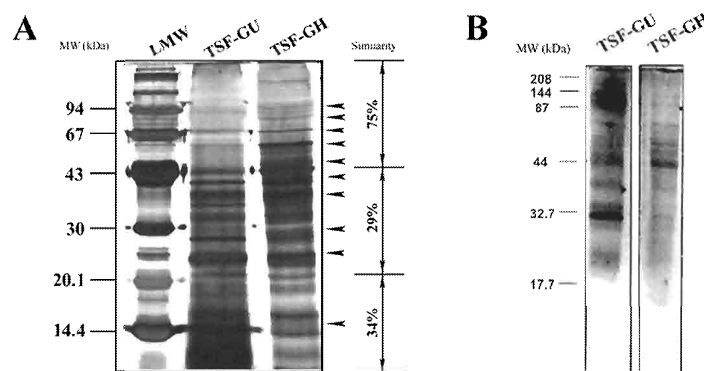

Figure 9. A Silver stained polyacrylamide gradient gel showing the electrophoretic polypeptide patterns of equal amounts of TSF-GU and TSF-GH proteins (14 µg each). LMW indicate the position of low molecular weight standards. Arrows indicate the main visual differences between the two patterns. The percentage of similarity between the indicated sectors along the polypeptide profiles is given on the right as calculated by GelComnpar3.1®. **B**: Chemiluminescent detection of glycoproteins in TSF-GU and TSF-GH, showing more glycosylated proteins in TSF-GU than TSF-GH within the medium size (30-50 kDa) and high molecular weights (>80 kDa).

The parasite populations used in this doctoral work were obtained from topographically similar regions of Ghana and Guatemala. The clinical picture of onchocerciasis in Guatemala and that in the area of Ghana where samples were taken do, however, tend to differ, with severe skin manifestation being more common in Ghana than in Guatemala. The analysis of the polypeptide proteins indicate that Guatemalan and Ghanaian *O. volvulus* differ, even though they are found in similar topographical regions. Studies by others, addressing the evolutionary history of *O. volvulus* using DNA sequencing demonstrated significant differences between the forest and savanna

strains of Africa (24), which appear to cause disease of different severity. Interestingly, using those methods, parasite populations from the New World (Guatemala and Brazil) were indistinguishable from savanna strains obtained in Africa. Although the results presented in this thesis may suggest a geographical variation between worms found in Guatemala and those found in Ghana, the direct relationship of such differences with distinct clinical manifestations seen in the two countries remains unclear.

In an era where great effort is being put into finding relevant antigens that can be used to diagnose onchocercal infection with more sensitivity than the skin snip test without jeopardizing specificity, or as vaccine candidates, the analysis of polypeptide patterns has to be complemented with the analysis of the immune response to such polypeptides.
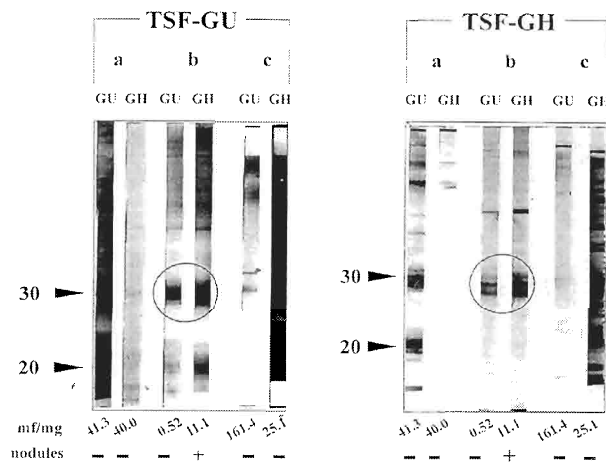
Figure 10. A: Representative Western blots of age and sex matched Guatemalan (GU) and Ghanaian (GH) mf+ sera showing different intensities in the recognition of TSF-GU antigens. Pair A, higher intensity of response in GUmf+ than in the corresponding GHmf+ matched sera; this kind of pattern was observed in the majority. Pair B, comparable intensity of GU and GHmf+. The position of the 30 and 20 kDa antigens, the microfilarial density (MFD, in mf/mg skin) and the presence of nodules is indicated. **B:** Detailed recognition of the 30 kDa antigens. Guatemalan and Ghanaian sera recognized the antigens in TSF-GU as a broad, diffuse band, whereas in TSF-GH, the 30 kDa antigens were recognized as sharp distinct bands.
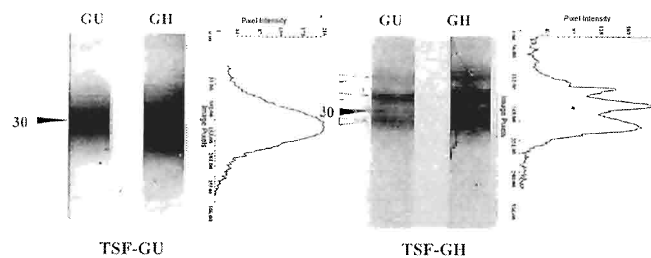
It has been shown that IgG_4 improves the specificity of immunological techniques such as immunoblotting, particularly when whole parasite soluble extracts are used. The general IgG_4 antibody pattern of antigen recognition of TSF-GU by age- and sex-matched Guatemalan and Ghanaian onchocerciasis sera was essentially similar although some obvious differences were also noted (Fig. 10). Sera fom mf+ Guatemalan (GUmf+) and Ghanaian (GHmf+) individuals consistently recognized two

groups of antigens of 20 and 30 kDa (Fig 10). Sixty-one percent (19/31) of GUmf+ sera showed a more intense response against TSF-GU than GHmf+ sera (Fig. 10A, pair a), while the opposite was true in 29% of the sera compared (Fig. 10A, pair c). Comparable intensities were seen in 10% of the matched sera (Fig 10A, pair b). The group of antigens at 30 kDa was recognized as a broad band made up of, at least, three distinct bands, the most prominent of which was approximately 33 kDa, while the group of antigens at 20 kDa was recognized as a more diffuse pattern, consisting of at least two polypeptides (Fig. 10B). Some individual Guatemalan and Ghanaian sera detected other minor antigens in the high and low molecular weight regions. Control sera from individuals outside the endemic area showed no response against TSF antigens. Thus, the antigens at 20 and 30 kDa were taken as markers of onchocerciasis.

As shown in Table 2, the majority of mf+ sera from both groups (97% Guatemalan and 74% Ghanaian, respectively) reacted against the major group of antigens at 30 kDa. Significantly more GUmf+ sera recognized the 30 kDa antigens in TSF-GU than the corresponding matched Ghanaian sera. These antigens were also detected by 41% of Guatemalan and 45% of Ghanaian mf- (GUmf- and GHmf-, respectively). Significantly more GUmf+ sera (84%) reacted to 20 kDa antigens than the corresponding matched GHmf+ sera (48%). A few mf- sera from both groups (27% and 18%, respectively) showed positive reactivity against these antigens. The majority of GUmf+ (81%) recognized both the 30 and 20 kDa antigens whereas only 45% of the GHmf+ showed a combined reactivity to both groups of antigens, but this difference was not statistically significant. In general, the same sera that recognized the 30 kDa antigens also showed positive reactivity to the group at 20 kDa as well. The opposite situation was not necessarily evident. There was a strong relationship between recognition of the 20 and 30 kDa antigens present in TSF-GU, separately or in combination, and the presence of mf in skin of both GUmf+ and GHmf+ individuals.

Table 2. Most prominent Onchocerca volvulus antigens recognized by IgG$_4$ antibodies from individuals in endemic areas from Guatemalan and Ghana

| MW (kDa) | Guatemalan sera | | Ghanaian sera | | Control sera | | | |
	mf$^+$	mf$^-$	mf$^+$	mf$^-$	NC-GU	NC-GH	P-GU	NEC
Guatemalan TSF								
30	30/31 (97)	9/22 (41)	22/31 (74)	10/22 (45)	0	0	0	0
20	26/31 (84)	6/22 (27)	15/31 (48)	4/22 (18)	0	0	0	0
20 and 30	25/31 (81)	6/22 (27)	15/31 (48)	4/22 (18)	0	0	0	0
Ghanaian TSF								
30	25/31 (81)	11/22 (50)	16/31 (52)	15/22 (68)	0	0	0	0
20	14/31 (45)	7/22 (32)	12/31 (39)	4/22 (18)	0	0	0	0
20 and 30	14/31 (45)	7/22 (32)	12/31 (39)	4/22 (18)	0	0	0	0

Numbers in parenthesis represent the percentage of sera in each group responding to the antigens NC-GU, normal controls from Guatemala; NC-GH, normal controls from Ghana; P-GU, other parasite controls (only from Guatemala); NEC, normal European controls (from Sweden).

Despite the observed variation in the polypeptide distribution in TSF-GU and TSF-GH, the main immunostained bands in both preparations were similar. Crossed analysis of Guatemalan and Ghanaian sera with Guatemalan and Ghanaian parasite extracts revealed that the immune response to TSF-GH antigens could also be divided into the same three distinct groups as with TSF-GU (Fig 10). The three patterns of intensity in the IgG_4 response were observed in essentially the same Guatemalan and Ghanaian sera that reacted against TSF-GU. However, in contrast to the recognition pattern observed in TSF-GU, the multiple antigens at 30 kDa in TSF-GH were recognized as three separate bands by mf+ sera; some reacted against all the three whereas others only reacted against separate bands. None of the control sera showed reactivity against any of these antigens present in TSF-GH. Significantly more GUmf+ sera recognized the 30 kDa antigens in TSF-GH than the corresponding matched GHmf+ sera (Table 2). In this case, although GUmf+ also showed a strong relationship between recognition of the 30 kDa antigens, and the presence of mf in skin, such relationship was not observed with the 20 kDa antigens in TSF-GH. Ghanaian mf+ sera did not show a relationship between the presence of mf in skin and the recognition of the antigens at 30 or 20 kDa.

Even when mf+ sera from different geographic regions recognized antigens of similar molecular weight in apparently geographically different parasites, the detailed pattern of recognition of these antigens by homologous and heterologous sera differed depending on the parasite material used. This suggests a different polypeptide composition of the most immunogenic antigens, even when they are located in the same molecular weight regions. Broad immunostaining patterns often indicate the presence of glycoproteins, which is consistent with both silver staining and glycoprotein detection patterns. Taken together, these results could suggest that the immunodominant antigens at 30 kDa in TSF-GU are glycosylated polypeptides. In contrast, the immunostaining and electrophoretic patterns of the immunodominant antigens in TSF-GH suggest that these polypeptides are less glycosylated (if at all) than the antigens of Guatemalan parasites. Such differences between the protein glycosylation in this molecular weight region, as well as the variation in immunostaining patterns could be due to changes at the transcriptional, translational or post-translational level, as discussed by Lobos and Weiss (23).

Since the three patterns of intensity in the IgG_4 response were observed to both TSF-GU and TSF-GH by the respective sera, this suggests that the varying patterns were not due to differences in TSF preparation. The most frequently observed pattern of recognition was characterized by higher intensity of response within the GUmf+, than the matched GHmf+ sera. Differences in antibody responses against *O. volvulus* by different ethnic groups have been reported in Ecuadorian Indians (Amerindians) and Blacks (130). In that study, Amerindians demonstrated a more intense IgG response and more frequent recognition of low molecular weight antigens than Blacks of African origin from the same area. In addition, significantly higher levels of immunoglobulins against *O. volvulus* were present in the Amerindians, compared to the Black population.

Selecting the antigens at 30 kDa as a marker for onchocerciasis allows for an estimation of sensitivity and specificity of the IgG4-based Western blot technique for the diagnosis of onchocerciasis. Sensitivity using TSF-GU and GUmf+ sera was 97% (30/31) and 74% (22/31) when GHmf+ sera were used. Sensitivity using TSF-GH and GUmf+ was 81% (25/31), whereas using GHmf+ sera produced a sensitivity of 52% (16/31). Specificity based on Guatemalan control sera (N-GU and P-GU) and using either TSF-GU or TSF-GH was 100% since none of them gave a positive reaction against the 30 kDa antigens. These results suggest that using IgG_4 responses, which have been shown

to enhance the specificity of onchocerciasis diagnosis (39, 40) may not be as suitable for use in one geographical area as in another since only 50% of GHmf+ had a detectable IgG4 response to homologous TSF and those responses tended to be weaker than that of the GUmf+ sera.

Although a correlation of antigenic patterns of *O. volvulus* with differential clinical manifestations of onchocerciasis was not explored, differences in the polypeptide composition were demonstrated between Guatemalan and Ghanaian parasites, as well as differences in the detailed immune recognition of their common antigens. The implications of these observations, together with a variable IgG_4 response to *O. volvulus* antigens when sera from different geographic regions are studied, suggest that the nature of the antigens used for diagnosis and the antibody class detected need to be evaluated in different geographic settings before their extensive use.

2.3.1.2 Can diagnosis of onchocerciasis be made in an affordable, simpler way? (*Papers II & III*)

Evidence of possible geographic variation between *O. volvulus* obtained in different geographic areas prompted the study of onchocercal antigens that do not seem to vary, regardless of the origin of the parasites. An example of such antigen, designated PakF, present in Ghanaian TSF preparations and later found to be present also in Guatemalan parasites (132), was described by Lavebratt *et al* (131). A simple dot blot technique using PakF-GH was described, in which a minute amount of the antigen prepared by ionic exchange chromatography of TSF was used to detect mf+ individuals. Those early studies also reported low sensitivity values when IgG_4, but not IgG responses were evaluated in populations from West Africa.

Table 3. Results from analyses of sera with different filariasis assays

| Designation | Detected microfilariae/nodules | Sample origin | Proportion positive | | | | | |
			DBA	IgG4 TSF-GH[a]	IgG4 TSF-GU[a]	$ELISA_{0.2}$	$ELISA_{0.3}$	IFA
Oncho patients	yes/yes	Ghana I	31/32	17/32***	24/32*	32/32	31/32	n.d.
Oncho patients	yes/yes	Ghana II	15/15	n.d.	n.d.	15/15	15/15	14/15
Endemic controls	no/no	Ghana I	9/24	15/24	10/24	23/24***	18/24**	n.d.
Normal controls	no/no	Ghana I	1/10	0/10	0/10	9/10***[b]	4/10**[b]	n.d.
Normal controls	n.d.	Ghana II	2/22	n.d.	n.d.	17/22***	8/22*·**[b]	5/22
Ivermectin treated	no/yes	Ghana I	10/10	n.d.	n.d.	10/10	10/10	n.d.

[a] In the IgG_4 assays response to either of the 20 kDa or the 30 kDa antigen groups was noted as positive

[b] The p-values given was obtained combining Ghana I and Ghana II.

*** Significantly different ($p < 0.001$, ** $p < 0.01$, * $p < 0.05$) compared with the DBA by χ^2-test.

Further studies compared an IgG-based dot blot assay using PakF-GH (IgG-PakF-DBA) to three serological diagnostic methods (ELISA, and IgG_4-based immunoblot assay, and Immunofluorescence Assay-IFA) to evaluate the efficacy of the IgG-PakF-DBA (**Paper II**). In the first part of these studies, the IgG-PakF-DBA was compared using sera from an area of Ghana known to be endemic for onchocerciasis, as well as

Ghanaian endemic and normal controls plus a large pool of sera collected in Sweden, from individuals of different nationalities suspected of filarial infection. The results are shown in Table 3. Of the 47 sera from the confirmed mf+ individuals, 98% (46/47, combined results from Ghana I and Ghana II) were positive by the DBA assay. The IgG_4 immunoblot assay for the detection of the antigen groups of 20 and 30 kDa from TSF-GH was able to identify only 41% and 53% of the parasitologically confirmed individuals, respectively. The use of TSF-GU in the assay resulted in a corresponding detection by 50 and 75% of the mf+ individuals. None of the sera recognized the 20 kDa antigen group alone. Thus if the recognition of at least one of the two groups of antigens was defining a positive sample, the number of mf+ identified was 53% and 75% for the TSF-GH and TSF-GU, respectively. Among the endemic controls, nine recognized antigens from both TSF preparations. The endemic controls positive in the IgG-PakF-DBA included seven of these as well as two controls that recognized only TSF-GH. The IgG_4-based assays had significantly lower sensitivity than the IgG-PakF-DBA. This and other reports (131, 133) suggest that the detection of IgG_4 may not be appropriate for the diagnosis of onchocerciasis in all populations from different geographical areas. This is further supported by the finding that the induction of IgG_4 response has been shown to be genetically controlled (130).

Table 4. Results with the different filariasis assays of sera obtained in Swedish hospitals from patients with suspected filarial infection. The patients were grouped according to their main clinical symptom

Clinical symptom	Proportion positive		
	DBA	$ELISA_{0.2}$	IFA
Dermatological (incl. itching)	8/36	17/36	17/36
Ocular	0/5	2/5	1/5
Swelling (incl. lymphangitis)	1/14	9/14	9/14
Other	5/57	31/57	27/57
Totals	14/112	59/112	54/112

The ELISA response was evaluated using two different cut-off points. In $ELISA_{0.2}$ (cut-off at OD $\geq$ 0.2) all mf+ samples were positive. However, 96% of the endemic controls and 81% of the normal controls were also positive in $ELISA_{0.2}$. Thus, although highly sensitive, the $ELISA_{0.2}$ was associated with poor specificity. Increasing the cut-off value to O.D. 0.3 for negative samples resulted in an enhancement of the specificity. However, as expected, this result was coupled with a decrease in the sensitivity of the assay. The IFA was significantly more specific than the $ELISA_{0.2}$, but still less sensitive than the IgG-PakF-DBA. Taken together, the IgG-PakF-DBA, ELISA and IFA were significantly more sensitive than the IgG_4-Western blot assay, which uses a whole worm extract of O. volvulus. On the other hand, the specificity of the IgG-PakF-DBA and IFA were significantly higher than that of ELISA.

When the group of sera from individuals of various nationalities was analyzed, 12% were DBA+ (Table 4). Eight of those individuals had history of travel to areas of the world endemic for onchocerciasis. Among those DBA+, 8 had dermatological complaints, one had swelling of limbs, which in most cases was diagnosed as lymphangitis and 5 had other symptoms. Patients with symptoms possibly arising from non-*O. volvulus* filariasis, or other parasite infections, responded positively in the ELISA and IFA to a higher extent than in the IgG-PakF-DBA.

Taken together, the comparison of the IgG-PakF-DBA to other serological methods showed that a method as simple as the dot blot, using PakF as antigen, is useful for enhancing the specificity in the diagnosis of onchocerciasis in a non-endemic setting. The ELISA and IFA routinely used in the diagnosis of filarial infections, including onchocerciasis, apparently did not distinguish onchocerciasis from other filariasis, even though they where highly sensitive for filarial infections in general. High sensitivity tends to have priority over high specificity in patient care. The advantage of an assay like the IgG-PakF-DBA, resides in the possibility that a specific (and sensitive) diagnosis of onchocerciasis is important for optimal treatment.

The present challenge in the quest for reliable and affordable diagnostic methods for onchocerciasis is to overcome the frequent cross-reactivity between *O. volvulus* and other filarias and nematode parasites. Elevated IgG levels, specifically those of the IgG$_4$ isotype, are reported to characterize onchocerciasis and other worm infections (39-41).

Production of recombinant proteins held promise to eliminate the problems of sensitivity and enhance the specificity of diagnostic assays for onchocerciasis. However, high specificity was often achieved at the cost of sensitivity and improvement in specificity sometimes still required the use of IgG$_4$ antibodies for detection (40, 134). Having established that the IgG-PakF-DBA was more sensitive and specific than other routinely used serodiagnostic methods (**Paper II**), and that the detection of IgG$_4$ antibodies did not improve upon specificity of the dot blot or immunoblot assays (**Papers I & II**), a series of studies to assess the potential use of the IgG-PakF-DBA were undertaken. The assay was further simplified, its reproducibility evaluated, and its sensitivity and specificity assessed using well-defined sera from individuals with filariasis other than onchocerciasis, as well as sera from a region where onchocerciasis is the only filariasis reported (**Paper III**). The rationale for this study was that the assessment of specificity of the IgG-PakF-DBA could not be achieved using sera from inhabitants of areas where onchocerciasis and other filarias co-exist or where continuous migration between areas with ongoing transmission and areas without onchocerciasis could not be ruled out. Guatemala is a country where the only human filarial worm known to exist is *O. volvulus*.

In Guatemala, the most common nematode parasite that is co-endemic with *O. volvulus* is *Ascaris lumbricoides*. Sera from a panel of individuals with no history of visiting onchocerciasis endemic areas but infected with *A. lumbricoides* (group P-GU), was selected as control for cross-reactivity due to the presence of carbohydrate-containing antigens. Such cross reacting antigens tend to plague serological diagnostic methods based on IgG detection (39-41). In addition, onchocerciasis foci are stable and circumscribed, thus migration to those areas can be established in order to select normal controls without history of residence in the endemic areas. The analysis of well-characterized sera from individuals with other filarial infections, allows the possibility

to assess the potential of the IgG-PakF-DBA in areas where other infections overlap with onchocerciasis.

Analytical densitometry was used to validate the visual examination of the DBA results. The densitometer could measure the intensity of the spots, whereas the visual evaluation could only classify them as "positive" or "negative". While the intensity of the spots varied among individuals, a positive result always showed a spot of reactivity and a negative result did not, since there was a clear-cut difference between infected and non-infected individuals (Fig 11A). Densitometry also allowed to represent the DBA results in a more graphic manner (Fig 11B). The significance of these results is that a densitometer, or any other sophisticated readout device, which is expensive and impractical is not necessary for accessing the DBA results. This is important if the assay has a potential to be used under field conditions. The visual evaluation of the IgG-PakF-DBA is an attractive feature of the assay. As shown graphically in Fig 11B, the intensities of non-infected Guatemalan as well as visually DBA-negative sera, fell into the intensity range 0-5 (arbitrary units of integrated intensity). Those sera that were visually positive but weak, which were scored as weak positives or borderline, fell into the intensity range of 5-10.

As reported in the previous studies (**Papers I & II**), the IgG$_4$ response against PakF was less intense than the IgG response. Only 16% of the mf+ sera recognized PakF-GU, one of which was visually scored as visually weak or borderline. Eighty-four percent were negative. Testing PakF-GH in parallel showed that 25% of the mf+ sera were positive (2 of them weak or borderline) and 75% were negative. None of the Guatemalan normals or the Ascaris controls reacted against either PakF (Fig 11B). Interestingly, 75% of the group of mf- sera from the endemic areas in Guatemala was positive in the IgG-PakF-DBA (data not shown). Although mf- sera were included in the analysis, the significance of the results of this group is limited since mf- implies that no mf were observed in these individuals' skin snips. The reasons why the skin snip may be negative are several. If mf are dead inside the skin snip, for instance, they do not come out into the saline, thus rendering the diagnosis negative. If, on the other hand, the microfilarial density is too low, and since their distribution in the body is not uniform, the possibility of missing mf at the time of sampling exists. This is what makes the skin snip insensitive to detect prepatent disease. The fact that the majority of mf- sera recognized PakF may indicate that the assay is detecting prepatent disease in those individuals. However, this possibility can only be explored by studying a cohort of mf- individuals.

A panel of 25 sera from the WHO Serum bank (6 from Congo, infected with *Loa loa*, 6 from Kalimantan infected with *Brugia malayi*, and 13 from Sri Lanka infected with *Wuchereria bancrofti*) was tested to assess the specificity of the IgG-PakF-DBA in the presence of antibodies against other filarias. When tested initially at the normal working dilution of 1:2000, used in previous assays, there was a weak reactivity in 4 *L. loa*, 13 *W. bancrofti*, and 3 *B. malayi* sera. When the sera were diluted 1:4000, only one sample with *L. loa* was weakly positive in the IgG-PakF-DBA. A group of 14 mf+, including sera from individuals with high (29-203 mf/mg skin) and low (0.11-12 mf/mg skin), were also diluted 1:4000 and re-tested for their reactivity to PakF. The results clearly showed that the mf+ sera remained positive even when tested at the higher dilution (Fig 12). These results suggest that the weak reactivity observed in the WHO sera tested at dilution 1:2000 is due to non-specific reactivity and probably unrelated to filarial infection. It was encouraging to observe that of the 6 *L. loa* sera tested, only one was weakly positive in the IgG-PakF-DBA assay. These *L. loa* serua

36

were from Congo, an area where it is impossible to rule out co-infection with *O. volvulus*. Onchocerciasis-associated uveitis as well as a high prevalence of onchocercal nodules has been reported in Kinshasa and in the Sankuru River Valley, respectively (135, 136).
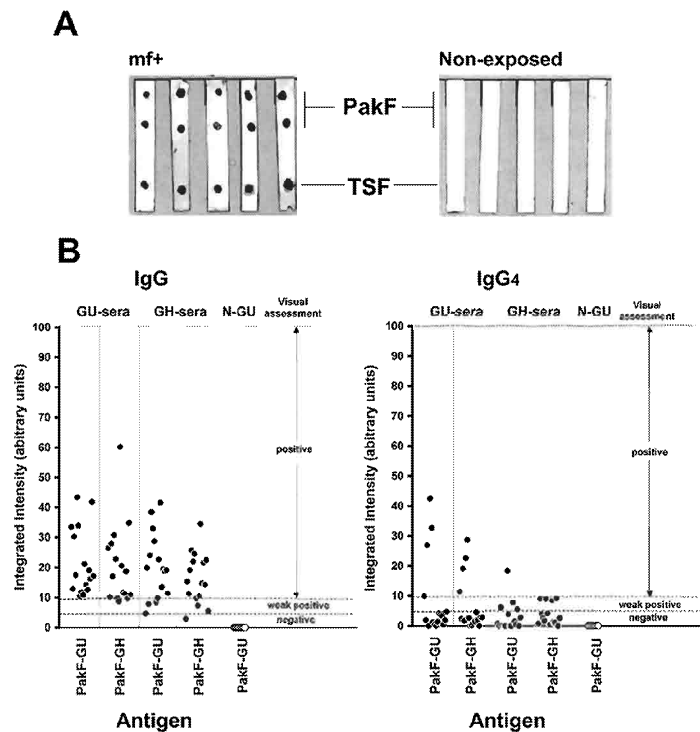

Figure 11. **A**: Digitized images of a representative DBA, showing the intensity of recognition of PakF antigens, by mf+ sera, using the IgG DBA. A set of 8 N-GU sera (non-exposed) from the control group is shown for comparison. TSF was used as an extra assay control. **B**. Graphical representations of the IgG DBA (left) and IgG$_4$ DBA (right) responses to PakF produced from Guatemalan or Ghanaian worms. Cross analysis was made using GU and GH onchocerciasis mf+ sera (filled circles) and N-GU (open circles).

A total of 125 mf+ (71 GUmf+ and 54 GHmf+), and 85 non-exposed (including 29 N-GU, 31 P-GU, and 25 WHO sera) were tested with the IgG-PakF-DBA. When examined visually, 123/125 mf+ sera were positive (98.4%), whereas only 1/85 non-exposed reacted to PakF. The analysis of these now extended pools of mf+ and non-exposed sera allowed for assessment of sensitivity and specificity, and further comparison with published data from other proteins (recombinant or from a crude extract). Table 5 shows the results of this comparison.

As seen from table 5, the sensitivity and specificity obtained with the IgG-PakF-DBA using sera from endemic areas in Ghana and Guatemala, as well as sera from areas with filariasis other than onchocerciasis, are comparable to those obtained with tests like the ICT card test, recently reported, that uses a recombinant antigen (51). The summary in Table 5 also shows that the IgG-PakF-DBA, whilst being a mixture of native proteins, offers similar values of sensitivity and specificity when compared with other highly purified molecules. This comparison is useful when considering issues like availability of recombinants, and the costs involved, not only in their production but also in the transfer of the technology to produce them in large amounts in the areas where the disease is still endemic. Although the challenge of new diagnostic methods is being internationally addressed, with at least three diagnostic tests for onchocerciasis under development, (118, 137), future studies are needed to confirm the utility of these antigens in onchocerciasis control programs (138). Thus, the possibility to use native *O. volvulus* antigen preparations, when available, should not be generally dismissed in favor of presently expensive, unavailable recombinant proteins.
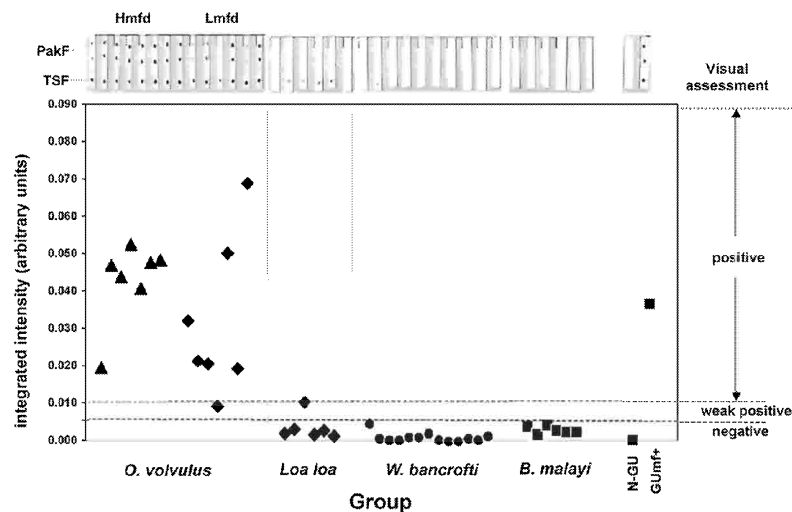
Figure 12. Sensitivity of the IgG-PakF-DBA was assessed using sera from individuals with other filarial infection tested at 1:4000 serum dilution. Fourteen sera from Guatemalan onchocerciasis-infected individuals with high or low microfilarial densities (Hmfd and Lmfd, respectively) were used for comparison. The graph shows that the integrated intensity of all but one of the non-onchocerciasis sera is not visually detectable. Intensities were measured with the Whole Band Analyzer software (BioImage). For comparison, most of the DBA strips have been aligned at the top of the graph to appreciate the difference between visually positive and negative samples.

The fact that onchocercal nodules have to be removed from humans to obtain the parasite material may rise ethical issues. However, it is worth noting that nodulectomy is still an ongoing practice in most endemic areas. In both Guatemala and Ghana, worms are surgically removed for therapeutic and cosmetic reasons, making the

availability of worm material not a limitation in these countries as long as the infection prevails.

Table 5. Specificity and sensitivity of the PakF dot blot compared to other published recombinant and crude preparations

Antigen[a]	Assay	Specificity (No. of samples tested)	Sensitivity (No. of samples tested)
PakF	DBA	98.8 (85)	98.4 (125)
Ov LMW	ELISA	88 (24)	89 (28)
Ov7	ELISA	79 (24)	82 (28)
Ov11	DBA	96 (24)	54 (28)
Ov16	ICT card	97 (105)	90.5 (116)
Ov26	ELISA (?)	96 (24)	56 (28)
Ov29	DBA	100 (24)	43 (28)
Ov3.6/Ov33	WB	75 (24)	86 (28)
Ov103	ELISA	75 (24)	68 (28)

[a] Except for PakF, and Ov16 in the ICT card assay (See Weil et al, 2000), all the antigens and their values have been reported by C.P. Ramachandran (See Ramachandran, 1993). The antigens are published recombinant proteins except PakF and the crude low molecular weight extract, Ov LMW.

Further simplification of the assay, such as the use of finger-pricked blood instead of serum, did not affect the sensitivity of the assay, while reducing the time of analysis and improving the compliance of the individuals (**Paper IV**).

2.3.1.3 Can the IgG-PakF-DBA be used in different endemic scenarios? (*Papers IV & VI*)

The encouraging results of sensitivity and specificity obtained with sera from infected individuals and well-characterized sera representing other filarial infections prompted the immediate question as to the potential use of this technique as a diagnostic tool in different endemic scenarios. Preliminary studies with samples from Ghanaian individuals treated with ivermectin showed an increase in the antibody titers (Paper II) and the intensity of their response in the IgG-PakF-DBA, 28 days after a single dose of ivermectin (Fig. 13). Antibodies do wane in the absence of re-infection as shown by the DBA responses of European individuals (Fig. 14), who had been infected with *O. volvulus*, moved out of the onchocerciasis endemic areas and subsequently treated and cured, and who were negative when samples taken 20 yr later were analyzed (**Paper V**). This suggested the possibility of using the IgG-PakF-DBA to monitor the effect of long-term treatment with ivermectin, by detecting the change in intensity of response with time, thus providing a way to evaluate the impact of control measures. To do this, a group of Guatemalan sera was selected from communities who had received at least 3 rounds of ivermectin treatment, and their DBA responses evaluated.
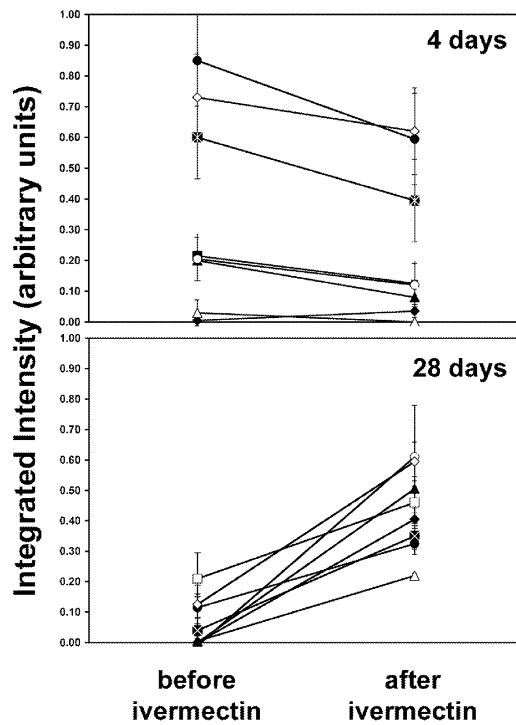
Figure 13. Short-term evaluation of a single dose of ivermectin using IgG-PakF-DBA showing an apparent change in the intensity of response 4 and 28 days after a single dose of the anti-microfilaricidal.
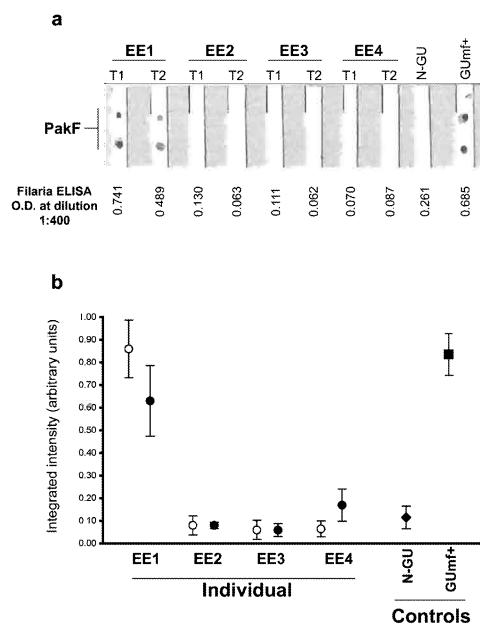

Figure 14. Immune response against *O. volvulus* antigens. **a)** Visual assessment of the PakF-DBA results from Exposed Europeans (EE). For EE1 the first sample, T1 (O), was 20 yr after diagnosis. For EE2, EE3 and EE4, T1 was 19 yr. T2 (●) for all subjects was 27 yr after diagnosis and subsequently living outside onchocerciasis endemic areas. The optical densities of the filaria ELISA are shown. **b)** Graphic representation of the PakF-DBA results after scanning each spot of reactivity on the DBA strips, showing each spot of EE1 comparable to that of the positive controls from mf+ individuals. The error bars are the standard deviation of the mean integrated intensity from duplicate DBA spots

40

As can be expected, due to the effect of ivermectin, mf density decreased (Fig. 15). However, the intensity of the DBA remained virtually unchanged. This may reflect both the continuous release of antigens from dying mf and exposure of individuals to re-infection in the area. It is worth noting that the area where the Guatemalan samples were taken had not been under vector control. Alternatively the half life of the relevant antibodies may be long. Other studies, using different recombinant *O. volvulus* antigens have shown, however, that the antibody levels decrease after both vector control and ivermectin treatment (50). Sera of exposed European individuals, which were not only treated with ivermectin but also had left the endemic areas, showed that after disappearance of the adult worms, antibody levels probably revert to those compared to healthy controls (Fig. 14). These results indicate that in this context, the IgG-PakF-DBA may be useful to monitor clearance of onchocerciasis by long-term intervention. After moving out of the endemic areas, these individuals stopped being exposed to re-infection, probably resembling the situation in areas clear of onchocerciasis, where ivermectin and vector control are combined.
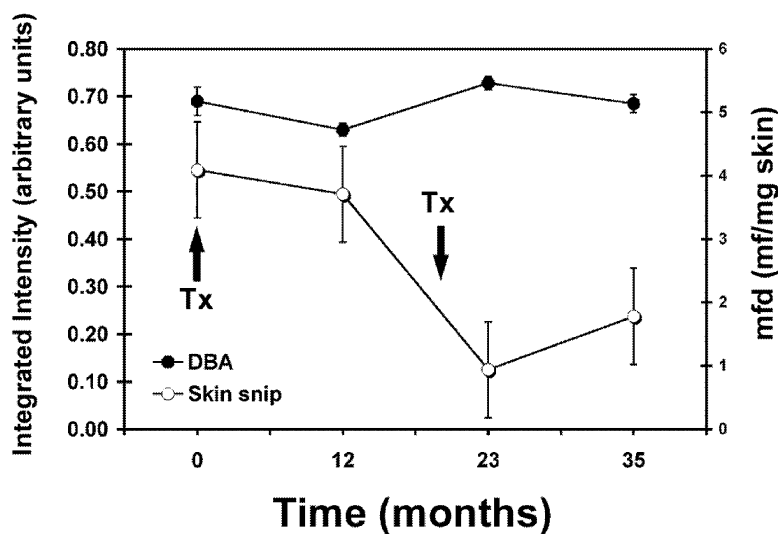
Figure 15: Evaluation of repeated treatment with ivermectin using IgG-PakF-DBA shows no difference in the intensity of response, even when the microfilarial density is decreased as a result of the treatment (Tx).

Interestingly, one of such non-exposed Europeans still showed a strong positive response in the IgG-PakF-DBA, as well as in ELISA, years after moving from the endemic area, suggesting that the estimation of the life span of the adult worm, currently accepted to be of about 16 yr, might be inaccurate since immunity to this infection is neither sterilizing nor concomitant.

Thus far, the IgG-PakF-DBA seemed able to differentiate between infected and non-infected individuals in endemic areas (Fig. 11), and exhibited high sensitivity and specificity (Table 5). However, when individuals are treated with ivermectin, the assay

is limited in its ability to detect changes in the antibody response based on the visual assessment of the DBA results (Figs. 13 and 15). Possibly, the advantage of the clear-cut results between positive and negative results obtained with the IgG-PakF-DBA makes it useful to be used in areas where transmission has been interrupted long enough (at least as long as the life span of the adult worm) after repeated treatment with ivermectin. However, the assay must first be tested in comparison with the "gold standard" in order to assess its reliability. Selecting areas with mixed endemicities offers the opportunity to evaluate the potential of the IgG-PakF-DBA when low mf densities are expected. Therefore, a good correlation must be achieved between the prevalence obtained by the DBA (using only visual results), and the skin snip test. Once this correlation is established, a further attempt to bring the assay one step forward, that is using it in areas where ivermectin has been given long enough so the transmission has been interrupted, can be envisaged.

Areas of mixed endemicities comprised those where ivermectin has been given, and those still with ongoing transmission. In those areas where ivermectin has not been given it is expected to find mainly positive individuals, whereas in those areas where ivermectin has been given, the results would be mixed. Since ivermectin is not effective against the adult worm, those individuals that have been exposed long enough so that they carry adult worms would be expected to test positive by the IgG-PakF-DBA, whereas those individuals born after transmission has been interrupted would be expected to test negative, since they would not have adult worms. This thus bears the consideration of different age ranges into the study design.

Table 6. Comparison of the PakF-DBA and skin snip test in the diagnosis of onchocerciasis in different age ranges.

Age (yr)	Skin snip[a]		PakF-DBA[b]		PakF-DBA sensitivity (%)[c]
	No. (%) POS	No. (%) NEG	No. (%) POS	No. (%) NEG	
5–10	21 (14.0)	35 (23.3)	35 (23.3)	21 (14.0)	166
11–20	15 (10.3)	5 (3.3)	19 (12.7)	1 (0.7)	126
21–30	11 (7.3)	1 (0.7)	12 (8.0)	0 (0.0)	109
31–40	15 (10.3)	4 (2.7)	18 (12.0)	1 (0.7)	120
41–50	17 (11.3)	5 (3.3)	20 (13.3)	2 (1.3)	118
51–60	12 (8.0)	1 (0.7)	13 (8.7)	0 (0.0)	108
61–70	6 (4.0)	0 (0.0)	6 (4.0)	0 (0.0)	100
71–80	2 (1.3)	0 (0.0)	2 (1.3)	0 (0.0)	100
Total	99 (66.0)	51 (34.0)	125 (83.3)	25 (16.7)	

[a] POS, mfd > 0 mf/mg of skin; NEG, MFD = 0 mf/mg of skin

[b] POS, visually positive; NEG, visually negative.

[c] Sensitivity of DBA in individual age groups is defined in comparison with the skin snip results

The IgG-PakF-DBA was used to evaluate samples from inhabitants of localities with diverse endemicities in the Volta Region of Ghana, and the results compared with those obtained by the skin snip test. The study region of Ghana has pockets of high and low transmission of onchocerciasis and mass treatment has been practiced in some of these. Screening for onchocerciasis in these areas has always been done using the skin snip test, however, repeated ivermectin treatment results in decreased mf load, making the skin snip test less sensitive and reliable. A simplification was introduced in the dot blot assay through the use of finger-pricked blood instead of serum. In addition, the potential cross-reactivity of the assay with other filarial infections was evaluated using a panel of sera from patients with circulating *W. bancrofti* mf (from India).

Thirty individuals from each of five localities (LOC1 to LOC5) from the District of Ho, in the Volta Region, who volunteered to participate were selected (15 youngsters under 17 yr, and 15 adults from each locality) to make a total of 150 individuals. Parallel to finger pricking, four-site skin snips were taken from all volunteers. The IgG-PakF-DBA was performed blind and thus the results from the skin snip were not made available to the person performing the assay until after all the analyses had been done.

Of the 150 individuals tested, 99 were skin snip positive (ss+) and 51 were ss-. A cluster of 40 negative individuals was found in the age range 5-20 and the remaining 11 were distributed between 21 and 60 yr of age (Table 6). Stratified by age, the results clearly show that the IgG-PakF-DBA followed closely the skin snip results in all age groups. However, the DBA consistently showed more positive individuals in all groups except the older age groups, with the biggest discrepancy between the DBA and skin snip results seen in the lower age groups.

Table 7a. Performance of PakF-DBA compared to skin snip test for diagnosis of onchocerciasis in the whole study population (5 to 78 years old)

| Locality | n | Skin snip[a] | | PakF-DBA[b] | |
		No. (%) POS	No. (%) NEG	No. (%) POS	No. (%) NEG
LOC1	30	19 (63.3)	11 (36.7)	24 (80.0)	6 (20.0)
LOC2	30	13 (43.3)	17 (56.7)	21 (70.0)	9 (30.0)
LOC3	30	23 (76.7)	7 (23.3)	27 (90.0)	3 (10.0)
LOC4	30	21 (70.0)	9 (30.0)	24 (80.0)	6 (20.0)
LOC5	30	23 (76.7)	7 (23.3)	29 (96.7)	1 (3.3)
Total	150	99 (66.0)[c]	51 (34.0)[c]	125 (83.3)[c]	25 (16.7)[c]

[a] POS, mfd > 0 mf/mg of skin; NEG, mfd = 0 mf/mg of skin

[b] POS, visually positive; NEG, visually negative.

[c] Mean value of all localities

Table 6 shows that the parasitological prevalence tends to peak in the age ranges 31-40 and 41-50 but tends to decrease above the age of 50. This dynamics is not reflected in the dot blot results since a correlation between the intensity of the DBA spots and the mf density could not be found. A comparison of the prevalences obtained by skin snip and dot blot (using visual examination) is shown in Tables 7a and 7b. The prevalence of mf+ by skin snip varied from 43.3% to 76.7% in the five localities, whereas the prevalences obtained with the DBA varied from 70-96.7%, although following the same trend with LC2 being the lowest (Table 7a). A closer look at the age group 5-17 years in the individual localities (Table 7b), showed a similar trend between the DBA and skin snip, with LOC2 having the lowest DBA positivity but LOC5 showing DBA prevalence akin to LOC3.

Table 7b. Performance of PakF-DBA compared to skin snip test for diagnosis of onchocerciasis in the population of 5-17 yr old

Locality	n	Skin snip[a]		PakF-DBA[b]	
		No. (%) POS	No. (%) NEG	No. (%) POS	No. (%) NEG
LOC1	15	5 (33.3)	10 (66.7)	10 (66.7)	5 (33.3)
LOC2	15	1 (6.7)	14 (93.3)	6 (40.0)	9 (60.0)
LOC3	15	13 (86.7)	2 (13.3)	13 (86.7)	2 (13.3)
LOC4	15	8 (53.3)	7 (46.7)	10 (66.7)	5 (33.3)
LOC5	15	6 (40.0)	9 (60.0)	14 (93.3)	1 (6.7)
Total	75	33 (44.0)[c]	42 (56.0)[c]	53 (70.7)[c]	22 (29.3)[c]

[a] POS, mfd > 0 mf/mg of skin; NEG, mfd = 0 mf/mg of skin

[b] POS, visually positive; NEG, visually negative.

[c] Mean value of all localities

The IgG-PakF-DBA detected more positive individuals compared to the skin snip test. Moreover, 98/99 ss+ individuals were detected by DBA for a sensitivity of 99% and a predictive value of 78%. A positive correlation (Pearson's correlation coefficient Pcc, r= 0.899, p<0.05) between the prevalence estimated with skin snip and the IgG-PakF-DBA was found when the whole study population was analyzed, which did not vary appreciably when the population 5-17 yr was analyzed separately (r = 0.893, p<0.05). There was no significant correlation between the prevalence estimated by the two methods among those older than 18 yr (r = 0.102, p>0.05). The strong correlation found in the population 5-17 yr may influence the results for the entire study population. The fact that there is no correlation in the population older than 17 yr could be the result of the particular dynamics of parasite loads in the areas under study. Others have reported that the intensity of infection with age can vary from region to region (50). For example, in some areas, the mean mf density increased continuously with age, whereas in others a plateau or a decrease above the age of 30 followed the progressive increase in prevalence up to the age of 21-30 yr. A positive correlation between the prevalence estimated by DBA and that by skin snip means that when the

parameter increases using the skin snip then it does also when using the dot blot. However, in the age groups above 17 yr, the differences in mf density detected by skin snip are not followed by differences in the number of positive individuals detected by the IgG-PakF-DBA, which seems to have higher sensitivity,

The fact that the DBA results were comparable to those obtained by skin snips indicates the possibility that the overall estimates of prevalence generated by DBA from a sentinel populations could replace those generated by skin snips. The major difference in the number of positive individuals detected by DBA and skin snip was observed in the groups aged 5-17 yr (Table 7b). When analyzed separately, a positive and significant correlation between the prevalence estimated with the IgG-PakF-DBA and the skin snip was found for the population aged 5-17 yr. However, no such correlation was observed in the group of adults studied (>17 yr). Due to the long life span of the adult worms, the continuous exposure in areas with ongoing transmission of onchocerciasis, and the unknown duration of antibodies against the proteins contained in PakF, it is not likely that an antibody-based assay like the IgG-PakF-DBA would show a better performance than the skin snip when an age group > 20 yr is selected as a sentinel population to assess the efficacy of control measures. The results presented here are in accordance with other studies that have shown that the most useful indicator group for detecting recrudescence, as well as for determining the intensity of infection in a community is the population 5-15 years of age (35, 50). The reason why IgG-DBA-PakF may be positive in some specimens which are negative in the skin snip may be explained in a number of ways, including higher sensitivity, lower specificity, or the capacity to detect even early infection (prepatent). The accumulated results, however, suggest that the IgG-PakF-DBA has a higher sensitivity coupled with high specificity.

An emphasis is made in the WHO strategy on the need of surveillance methods to be highly specific even at the cost of low sensitivity. The problem of defining specificity is that of the quality of the "gold standard". If the gold standard has low sensitivity then assays with higher sensitivities can be viewed as having low specificities. Besides the patients' discomfort, this is the main drawback with the highly specific, but less sensitive skin snip test. However, we have previously established the specificity of the IgG-PakF-DBA to be 100% using a panel of sera of non-infected individuals from areas where no other filaria is present. In addition, we ruled out cross-reactivity due to the carbohydrate-containing antigens widely shared among nematodes. Analysis of well-characterized sera from the WHO serum bank provided further evidence of the assay's specificity (**Paper III**). An additional panel of 40 sera from Indian individuals with bancroftian infection, and with apparently no exposure to *O. volvulus* was used to further expand the pool of non-onchocerciasis sera in order to re-assess the specificity of the IgG-PakF-DBA. This additional panel of non-onchocerciasis controls included 9 microfilaremic (Bancrofti mf+, MF), 16 patients with chronic infection but no circulating mf in the blood (CP), and 15 individuals living in the same area with no evidence of filariasis (endemic controls, EN). As shown in Fig 16, none of the MF was positive by IgG-PakF-DBA. However, 2 CP and 2 EN sera reacted positively to PakF. Pooled GUmf+ and non-exposed sera, used as positive and negative controls, were positive and negative, respectively, when tested at the same dilution. In total, 4/40 Indian sera were positive by IgG-PakF-DBA, resulting in 90% specificity of the assay. Of importance, however, is the fact that those positive sera in the IgG-PakF-DBA were not the individuals with circulating Bancroftian mf, where 9/9 sera were negative by the DBA. Combined analysis using these results and the data from panels of sera from other areas endemic for other filarias but not onchocerciasis (**Paper III**), resulted in 96% specificity. Together with its ability to detect onchocercal infection, these results

also suggest that the IgG-PakF-DBA could be useful in screening children and adolescents born after control measures have been established, since they may form a desirable sentinel population to detect recrudescence of infection in controlled areas.
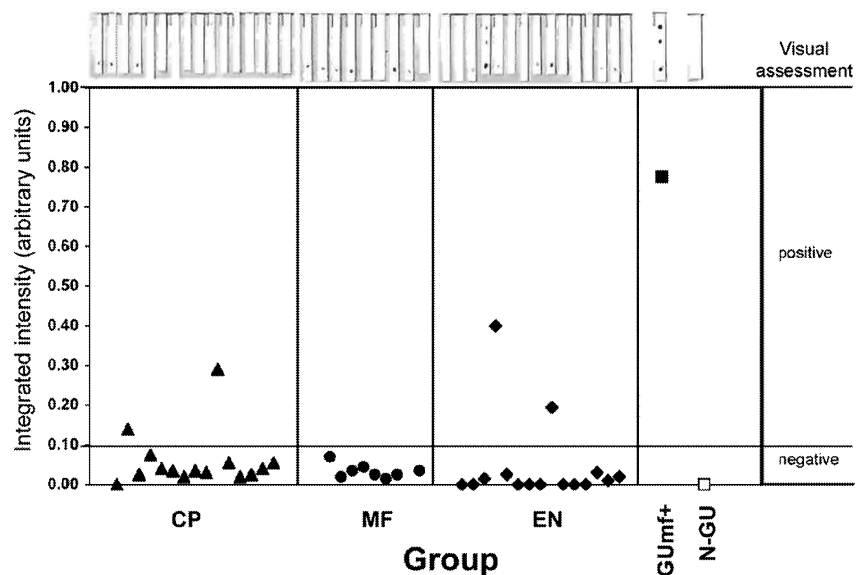
In the endemic areas, the need for assays that provide rapid results is advocated. The time needed to obtain qualitative results from the PakF-DBA was comparable to that of the rapid version of the skin snip test, i.e. 3 hours. Thus, although a quantification of the level of infection is beyond the capabilities of the dot blot assay, in terms of a rapid assessment for surveillance purposes, it represents an affordable alternative that offers more sensitivity than the skin snip test. Moreover, since the test can be performed using only a few drops of finger prick blood, this represents an advantage in terms of a higher compliance from the individuals in areas under constant screening to evaluate the impact of control measures.

Figure 16. Specificity of the IgG-PakF-DBA. The graphic representation of densitometric data shows that the PakF-DBA is highly specific when sera from individuals with filarial infections, other than onchocerciasis are analyzed. Two *W. bancrofti* chronic patients (CP), and two endemic normals (EN) were positive by DBA, whereas none of the microfilaremic (MF) individuals reacted to PakF. Pools of Guatemalan infected (GUmf+) and non-exposed (N-GU) were used as positive and negative controls, respectively.

Several promising diagnostic methods, based on detection of specific antibodies, parasite DNA, or parasite antigens in clinical specimens have been developed for onchocerciasis in recent years (48, 58, 139, 140). However, none of these have been implemented under field conditions in the endemic areas. Antibody tests using recombinant antigens have been based on a standard, indirect ELISA, which can be performed in a simple laboratory. The antigen- and DNA-detection methods are

performed over 2-3 days, and they require more in terms of laboratory infrastructure, equipment and expensive reagents than ELISA (134). In either case, the costs involved in both, the acquisition of instrumentation and the transfer of the technology are not trivial, thus making the development of less expensive, stable and reliable methods, a necessity in low-income countries where onchocerciasis is being controlled or still exists. Simple, yet reliable approaches like the use of a less expensive diagnostic technique that could be implemented under field conditions, is of particular importance especially when the successor to OCP, the African Programme of Onchocerciasis Control (APOC), is facing funding difficulties which could threaten its future success (141).

A possible disadvantage, compared to antigen- or DNA-detection methods is that the IgG-PakF-DBA does not distinguish between past or current infections, but this is a problem of all antibody-based assays. Nevertheless, the PakF-DBA may still be a suitable tool for following changes in transmission when used in younger age groups. Furthermore, antibody testing still holds great promise as a means of monitoring changes in the transmission of *O. volvulus* after mass treatment of populations (134).

Criticisms against the use of native human-derived material in the PakF-DBA assay have previously been made in terms of reproducibility of material and safety. PakF has been prepared repeatedly and the reproducibility in performance in the DBA has been high. A reliable, sensitive and specific recombinant protein would be ideal, but in its absence, native proteins still have a role.

Concern about the availability of parasite material to prepare PakF is also justified. However, in areas where no control measures have been established, the relative abundance of nodules from which the worms are freed makes it possible to obtain large amounts of PakF to be used in rapid screening of sentinel populations. In my hands, the amount of TSF obtained from a batch of 1-3 worms is enough to run a HiTrap-Q column up to 10 times. Each round of purification produces 350 µl of PakF that can be applied onto nitrocellulose strips without further treatment. The yield after 10 purification rounds (3.5 ml), theoretically allows for the analysis of at least 3,000 samples using the format of duplicate PakF spots per strip. Even if the format is changed to use 1 µl per spot, the amount of samples that could be tested is large enough considering that the material comes from only 1-3 worms. In the endemic areas where nodulectomy is a common practice not only for control but also for aesthetic purposes, the availability of worms for PakF production would not be an insurmountable difficulty.

As reported before, there seem to be variations in the protein composition of *O. volvulus* from different geographic areas (**Paper I**). However, PakF has been obtained from worms isolated both in Ghana and Guatemala, used it in crossed analysis using sera from the two countries, and obtained similar results regardless of the origin of the parasite material (**Paper III**). Therefore, geographical variations would not be of concern in screening a particular area with parasite material obtained from worms from another region.

The evidence thus indicates that it is still possible to implement simple techniques for the monitoring of onchocerciasis which offer high sensitivity and specificity, while being affordable in financially challenged areas. Where costs are an issue, and in the absence of more elaborated diagnostic tests available in the endemic areas, simple tests

47

like the PakF-DBA could still deliver reliable information that can be used to monitor changes in the transmission of onchocerciasis.

2.3.1.4 What exactly is PakF?(*Paper V*)

PakF is a mixture of native proteins ranging from 10 to 40 kDa, from which a 22 kDa polypeptide can be distinguished as the most prominent under silver staining and the most immunoreactive under immunoblot experiments. (Fig 17).
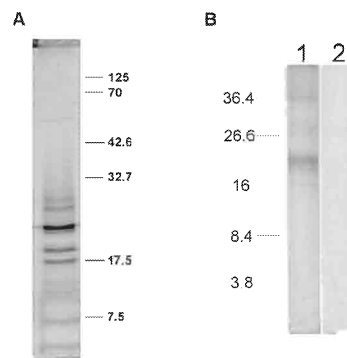
Figure 17. **A.** Components of PakF detected by silver staining. **B.** Western blot of PakF showing the antigenic proteins recognized by sera. Lane 1: pool of GUmf+ sera; lane 2: pool of N.GU sera. The positions of the low molecular weight markers are indicated.

An important characteristic of PakF is its ability to withstand heat. Heat treatment experiments, subjecting PakF to temperatures as high as 95° C for 25 min did not affect its recognition by sera from infected individuals. The apparent stability to high temperatures displayed by the PakF components could be due of a number of reasons. First, the proteins may be in an unfolded state, thus insensitive to heat denaturation. When a protein unfolds, the buried non-polar side chains are brought into contact with water. In order to accommodate these side chains, cages of water molecules surround them, so that the extent of hydrogen bonding is increased. The heat capacity (a property related to the amount of heat necessary to produce changes in conformation) of the unfolded protein is greater than that of the folded protein (142). Regardless of the state of the polypeptide, the epitopes recognized by antibodies from the infected individuals were preserved during the process of PakF preparation. The fact that these polypeptides may be in an unfolded state reduce the risk of further denaturation or instability, another attractive feature for potential diagnostic products. Second, stability due carbohydrate moieties contained in the immunogenic part of PakF. However, the data did not support this. Whereas treatment with 2-mercaptoethanol resulted in partial loss of antigenicity (not shown), treatment of PakF with Proteinase K resulted in a total loss of antigenicity as assessed by DBA and Western blot (Fig 18B). One and 2D-PAGE did not show the presence of broad bands characteristic of carbohydrate-containing glycoproteins (143), but a more definitive proof was obtained by in-gel

glycoprotein staining which could not detect any glycoprotein in PakF, although some were present in the whole TSF (Fig 18A).

The lack or relatively low amount of glycoproteins in the molecular weight range where the PakF components migrate in one-dimensional electrophoresis was also confirmed by the more sensitive chemiluminescent detection of glycoproteins (Fig. 9B).
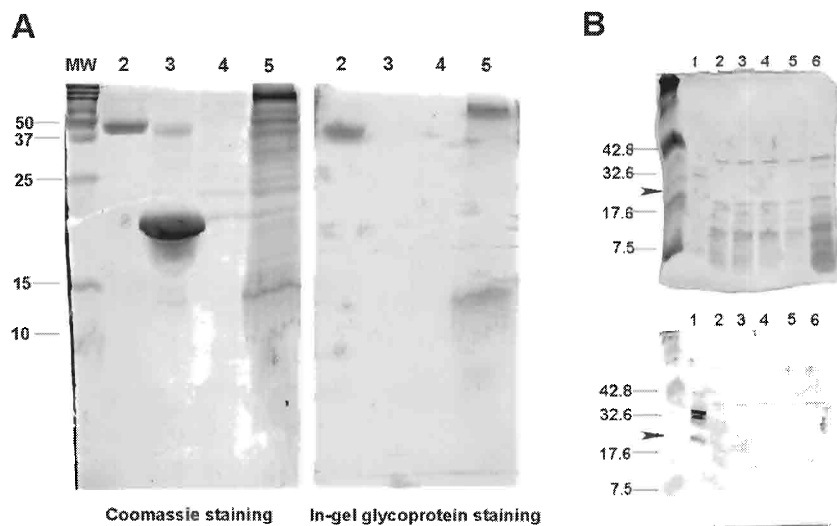
Figure 18. A. In-gel glycoprotein staining for assessing the presence of glycoproteins in PakF. Lane 1: Molecular weight markers; lane 2: glycoprotein positive control (Horseradish Peroxidase); lane 3: glycoprotein negative control (Soybean trypsin inhibitor); lane 4: PakF; lane 5: TSF. B: Treatment of PakF with increasing concentrations of Proteinase K. Lane 1: PakF without Proteinase K; lane 2: 150 µg Proteinase K; lane 3: 75 µg Proteinase K; lane 4: 37.5 µg Proteinase K; lane 5: 18.5 µg Proteinase K; lane 6: 300 µg Proteinase K. Top: Coomassie blue stained gel; bottom: Immunodetection with a pool of sera from mf+ individuals. The arrow indicates the PakF proteins without Proteinase K. The immunogenic band at 22 kDa was lost even at low concentration of Proteinase K.

The molecular weight range in which the components of PakF migrate make them likely to include some of the previously described molecules (45, 47, 48), including the 21 kDa breakdown product of Ov33, (144). However, monoclonal antibodies anti-Ov33 (4A7) (145) did not recognize any of the components of PakF (Fig 19). Furthermore, Ov33 has previously been described as specific and sensitive when used in an IgG_4-based serodiagnostic test (40) and the IgG_4 response to PakF was weak or nonexistent in those infected with *O. volvulus*. In addition, Ov33 is highly heat-sensitive and degrades at room temperature to produce a degradation product of 21 kDa.

Polyclonal antibodies to another onchocercal protein, OvGST1 (146), reacted against PakF (molecular weight range 32-36 kDa) indicating the presence of glutathione S-transferase in the fraction (Fig 20). OvGST1, however, was not the main immunoreactive protein of PakF. In this regard, it is important to mention that endoglycosidase studies in *O. volvulus* homogenate followed by detection of OvGST1 with specific antibodies indicated that the enzyme possesses at least two N-linked oligosaccharide chains, although the enzymatically active recombinant OvGST1 produced in *E. coil* is a non-glycosylated dimer (146). Another *O. volvulus* GST sequence, designated Ov24 (a GST of the π-class) ans published earlier (147), predicted no N-glycosylation sites. Similarly to OvGST1, Ov24 also shows bands at 32-36 kDa when resolved on an SDS-PAGE. The fact that glycosylated components could not be detected in PakF may indicate their relatively low abundance or the presence of a non-glycosylated GST, similar to Ov24. The polyclonal antiserum (Rabbit anti-Ov-GST1) could be detecting common epitopes of GSTs, rather than the glycosylated part of the molecule.
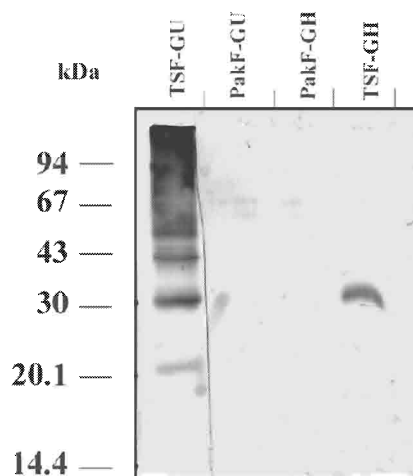
Figure 19. Western blot analysis of Guatemalan (GU) and Ghanaian (GH) *O. volvulus* antigens (TSF and PakF) using the monoclonal 4A7 anti-*O. volvulus* Ov33 (33 kDa). In contrast to the lack of response to PakF, this monoclonal identified a 33 kDa product in both TSF preparations but also a major proteolytic sub-product of Ov33 at 21 kDa, as well as a broad pattern at high molecular weights in the TSF-GU. The positions of molecular weight markers are shown on the left.

This could explain the Western blot pattern observed in Fig 18B and the apparent lack of carbohydrate moieties in PakF. GSTs are ubiquitous enzymes in aerobic organisms performing reactions essential for protection of cell constituents from oxidative attack (148). The presence of either OvGST, even if in low concentration, is of interest since cross-reacting GST antibodies arising from other nematode infection may interfere with the specificity of serological assays using a mixture where GST may be present. A GST has been isolated from *Ascaris suum* (149) and in Guatemala; infections with *A. lumbricoides* are highly prevalent in the areas where onchocerciasis is present. However, there was no cross-reactivity when a panel of 22 sera from individuals with *A. lumbricoides*, with no history of exposure to *O. volvulus*, was used to probe the PakF proteins (**Paper III**). Although Brattig and co-workers (107) reported IgG and very low IgG₄ antibody responses to OvGST2 (150), polyclonal antibodies to this protein did not recognize either of the PakF components.

When probed in a one-dimensional Western blot, a polyclonal antibody from *Dirofilaria immitis* (Di22U) (151) recognized a 22 kDa protein in the PakF fraction (Fig. 20), although mf+ serum antibodies and the anti-Di22U antibodies recognized different proteins in a two-dimensional Western blot (Fig 21).
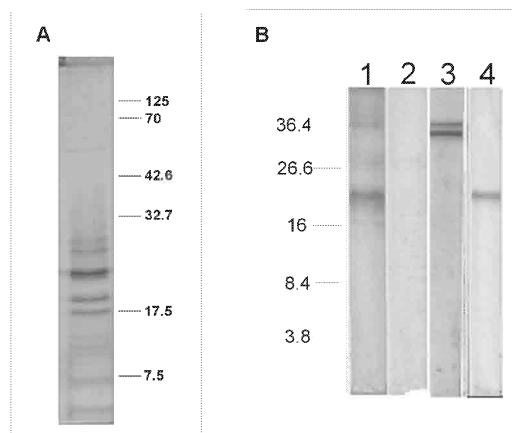
Figure 20. A. Components of PakF as detected by silver staining. **B.** Western blot of PakF showing the antigenic proteins recognized by different sera. Lane 1: pool of infected Guatemalan (GUmf+) sera; lane 2: pool of normal, uninfected Guatemalan (N-GU) sera; lane 3: rabbit anti-OvGST1 serum; lane 4: rabbit anti-Di22U serum. The position of the low molecular weight markers is shown on the left.

Since the molecular weight range corresponding to the PakF components has been extensively studied over the years, it would not be surprising to find that PakF contains various amounts of some of the previously described molecules that have been produced by recombinant methods,

Preliminary sequencing experiments of proteins resolved in 2D gels resulted in the identification of a stretch of five amino acids (MLVDS), also found in the Ov16 antigen precursor (48). However, it is important to emphasize that even if all the components of PakF were identified and produced by recombinant methods, it is apparently the mixture that renders PakF highly specific and sensitive for the diagnosis of onchocerciasis. The biochemical characteristics of the mixture designated PakF, such as its apparent stability to high temperature, posses an interesting alternative to the use of well defined molecules, which tend to be labile. Pursuing the identification and complete characterization of the components of PakF that have not yet been described, may provide more tools in the future to explore the biology of the parasite and its interaction with its host. Moreover, it may provide means to explore possible vaccine candidates. However, in terms of a practical diagnostic tool, which can be made accessible and affordable in the areas where the disease is still endemic and those areas where the impact of control measures need to be evaluated, the use of PakF may prove more valuable, even when the methodology involved in its production does not involve sophisticated technology or infrastructure that requires an investment still out of reach

of many countries that currently succumb to the morbidity and social stigma of onchocercal infections.
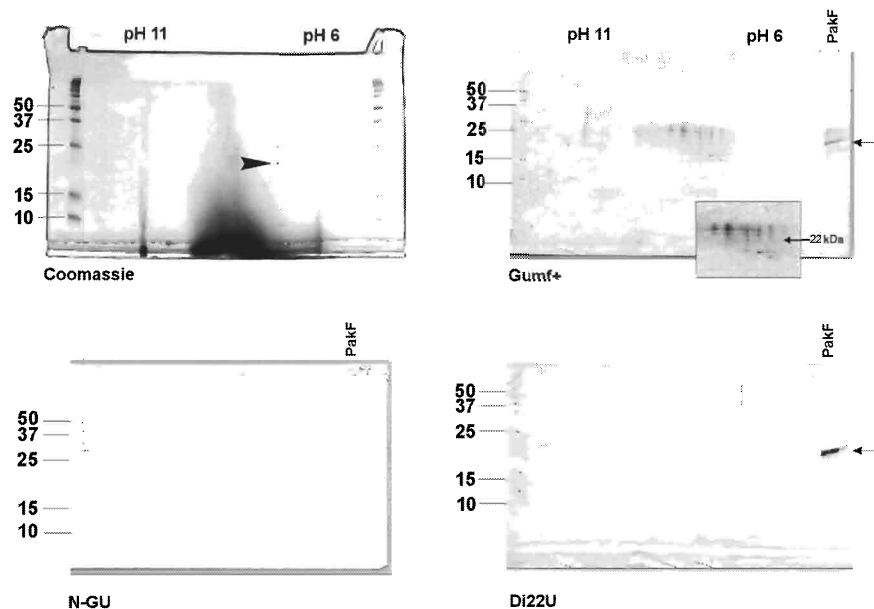
Figure 21. Two-dimensional electrophoresis and Western blot analysis of the proteins in PakF. Top panel: Commassie blue stained gel and immunodetection of transferred proteins by a pool of Guatemalan infected (GUmf+) individuals. Insert: detail of the recognition pattern of the immunogenic proteins (15-25 kDa) with the pH range 6-11. Bottom panel: two-dimensional Western blot using a pool of sera from non-infected Guatemalans (N-GU) and rabbit serum anti-Di22U. The solid arrow on the Coomassie stained gel points at the most immunogenic protein recognized in one-dimensional Western blot (thin arrows on the right).

The identification of the 22 kDa immunodominant component (temporarily designated OvP22) has proved elusive, although attempts to sequence it utilizing protein spots resolved in two-dimensional electrophoresis have produced two five-amino acid sequences (FQSGD, FLGLE) that are consistent with a conserved sequence of the cyclosporin-binding region of cyclophilins (peptidylprolyl *cis-trans* isomerase, PPI, EC 5.2.1.8). Cyclophilins (CYPs) are ubiquitous proteins involved in protein folding which have been identified in various species including the nematodes *C. elegans* and *B. malayi* (152). Although the significance of these results needs to be further explored, it is interesting that the cyclophilin identified in *B. malayi* belongs to a divergent form of CYPs that have an altered cyclosporin binding domain (CBD), and extended C and N-terminal domains. CYPs of that kind possess a more specific function, and the altered CBD has been hypothesized to correlate with substrate specificity (153).

Preliminary studies using antibodies eluted from PakF-adsorbed nitrocellulose indicate that the primary site of antibody binding in sections of female adult worm is in the

hypodermis but no appreciable binding was detected in intrauterine or other organelles (fig. 22)

Of interest, glutathione-binding antigens have been shown in the wall of the seminal receptacle and spermatozoa, but not oocytes (147). Since the eluted antibodies react against the mixture of proteins in PakF, it is possible that the staining seen in Fig. 22 represents the reactivity of antibodies against the main immunogenic component, OvP22, and also a GST-like protein similar to the π-class, non-glycosylated Ov24, for example. However, the very weak reactivity of Gumf+ sera in the region where OvGST migrates in the electrophoregrams suggests that the eluted antibodies are binding mainly to the 22 kDa antigens in the hypodermis. Indirect evidence that OvP22 may not be Di22U comes from the comparison of sections probed with anti-Di22U with those shown in Fig. 22. Figure 23 shows that, in contrast to the eluted ant-PakF antibodies, anti-Di22U also binds to intrauterine structures such as oocytes, and early stages of mf and eggshells. The definitive answer should come from studying the binding of antibodies raised against isolated proteins.
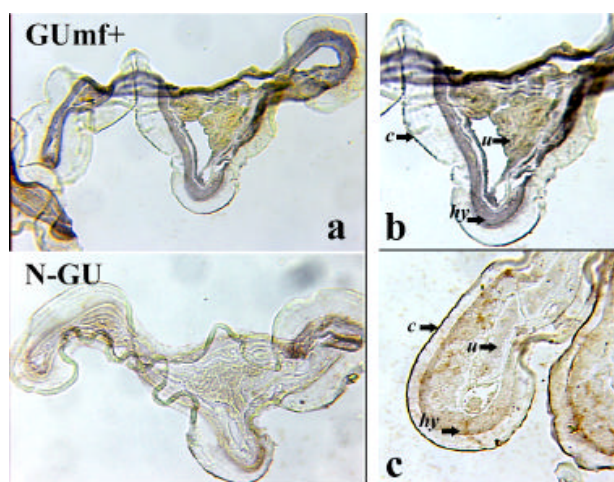
Figure 22. a: Immunolocalization of PakF antigens by eluted antibodies from pools of sera from infected (Gumf+) and non-exposed (N-GU) individuals, on cryosections of isolated female *O. volvulus*. **b**: Eluted anti-PakF antibodies bound to the hypodermis, *hy*, and not to the intrauterine structures, *u*. **c**: Detail of a section probed with antibodies eluted form a pool of non-exposed individuals showing no specific binding to the hypodermis or uterine structures. The cuticle, *c*, is shown.

Studying different developmental stages of the parasite would also give information about the differential regulation of the expression of such proteins. Thus far, these results suggest that the main immunodominant protein comprised in PakF is not present in early intrauterine states of the parasite. If PakF proteins are mainly localized in the hypodermis of adult worms (and possibly late mf stages before they leave the uterus), this could explain in part why individuals that are treated with ivermectin (a drug

53

effective only against mf), continue to react against PakF, even after several rounds of treatment.
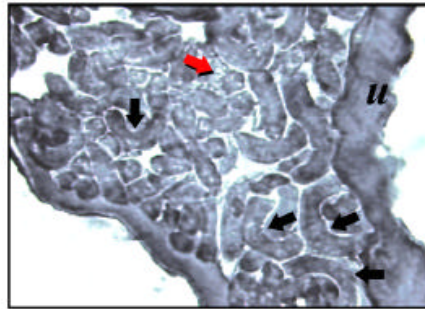
Figure 23. Immunolocalization of *O. volvulus* antigens with crossreactive antibodies. Anti-*D. immitis* Di22U antibodies bind to advanced embryos (tadpole stage, black arrows), and the uterine wall, *u*. Some degenerated embryos are shown (red arrows)

2.4 CONCLUDING REMARKS

When considering onchocerciasis as a public health problem, the evidence for the success of the control measures (mainly through ivermectin treatment) are very clear. However, when considering the elimination of transmission of onchocerciasis, many uncertainties still remain. The risk of recrudescence makes necessary the development of more effective tools for both, control and monitoring of onchocerciasis.

Control has been achieved mainly by ivermectin treatment, and in the future, the availability of macrofilaricidal compounds will help eliminate the parasite reservoir. However, monitoring when microfilarial loads and number of adult parasites decrease, calls for the development of highly sensitive and specific, yet affordable assays that can be used to evaluate the impact of control and elimination efforts. It is in the latter scenario that this work has revolved: the use of affordable technology for the sensitive and specific diagnosis of onchocerciasis.

Through the study and use of low molecular weight, native proteins of *O. volvulus*, a simple assay, which offered high sensitivity and specificity in the diagnosis of onchocerciasis, has been developed, further simplified and evaluated in different endemic scenarios. The particular characteristics of this protein mixture, designated PakF, have made it an attractive product to be used in endemic areas, as well as in areas now under control, providing not only a tool for rapid assessment, but also for monitoring recrudescence of onchocerciasis when appropriate populations are selected, particularly sentinel populations in the age range 5-17 years.

Although the technology involved in the production and utilization of PakF could have been one of high sophistication, this work shows that, when costs are not a trivial issue, more affordable technologies can achieve similar, and even better results than costly methods that are not likely to be available where the disease still exists.

The evidence showed that, in contrast with currently accepted dogmas, IgG_4-based immunodiagnostic methods may not always improve upon specificity in the diagnosis of onchocerciasis, and IgG-based methods can still deliver high sensitivity and specificity, when the antigenic material to be detected does not seem to elicit an IgG_4 response. This warrants further study of the PakF components and their significance in terms of the immune response (or lack thereof) from individuals infected, or individuals equally exposed but apparently immune to onchocercal infection.

Due to the simplicity of the PakF-DBA, its stability in temperatures where other materials require special care, and the positive correlation found between its results and those from skin snip tests, an evaluation on a larger scale is warranted. The cost of transferring this type of technology would not be insurmountable, since the basic infrastructure and human resources are already in place in the endemic areas. The implementation and use of this technique in those areas, in parallel to the routine practice of the skin snip, could allow for a better evaluation under field conditions with the possibility of allowing a future transition to a less invasive way of monitoring onchocerciasis in the post-ivermectin era. This same model of adaptation of relatively simple technologies could preferably be attempted for other parasitic diseases. Such efforts could result in the development of "in house" methods that could compete well with other approaches, which bare the costs of high technology and marketing strategies.

3 THE PHILOSOPHICAL SCOPE OF THIS THESIS

3.1.1.1 The 10/90 Gap

Parasitic diseases are victims of what is called "The 10/90 Gap", which is best explained in the words of Els Torrele, co-chair for an initiative announced by Medecins Sans Frontieres: "*Less than 10% of the world-wide expenditure on health research and development is devoted to the major health problems of 90% of the population*"[1]. Various fora have addressed this fundamental mismatch, expressed as millions of lives lost each year between human needs and scientific innovation. Although the 10/90 Gap is more evident in diseases with high mortality rate, it is also important to address the same problem in diseases that do not cause death, but have an impressively high morbidity rate. Onchocerciasis (or river blindness), besides being commonly regarded as a blinding disease, is also the cause of social stigma due to the skin manifestations caused by the exacerbated immune response of the host.

The control measures undertaken by the Onchocerciasis Control Programme (OCP), the African Programme for Onchocerciasis Control (APOC) and the Onchocerciasis Elimination Programme for the Americas (OEPA), have resulted in one of the most successful endeavors that have combined science and technology to achieve the goals set more than two decades ago: to eliminate onchocerciasis as a public health problem. When considering the control of onchocerciasis, the evidence for the success of ivermectin treatment is clear: the community microfilarial load values (CMFL, a measure of intensity of infection and an index of the public health importance of the disease) everywhere to be reduced to zero or close to zero, and a generally quite low prevalence of infection. However, when interruption of transmission is considered, although in some areas it appears that transmission might have been interrupted, in other areas even after 10-12 years of ivermectin treatment, transmission is still ongoing. Therefore, many uncertainties remain and perhaps one of the most important to address is the risk of recrudescence.

This doctoral work has revolved around the use of affordable technologies that can help in the sensitive and specific diagnosis of the disease and it proposes that such diagnosis can be accomplished without necessarily relying on sophisticated methods. The sole idea of keeping the level of sophistication to a minimum, without necessarily neglecting the science behind the development of such techniques, has brought opposition. At the dawn of the 21st century, it is inconceivable for some that there are still regions of the world where technological advances have not yet made their presence, or are simply out of the majority's reach. All too often, such opposition hinders the discussion of the scientific merit of new ideas based not on how sophisticated our research can be, but on how our ideas can be applied and effectively translated into something useful by those living in less well-off countries, where the burden of parasitic diseases, such as onchocerciasis, have their most devastating consequences.

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[1] **Lewis, R.** 2002. Fighting the 10/90 Gap. *The Scientist* **16** (10):22-24.

As it happens in many scientific fields, the arrival of new ideas that challenge the current dogmas, thoughts and axioms produces an almost automatic rejection. Claude Bernard once wrote: "*If an idea presents itself to us, we must not reject it simply because it does not agree with the logical deductions of a reigning theory*", which exemplifies one of the most important driving forces of scientific advancement: no accepted idea or established principle should be regarded as beyond being questioned if there is an observation challenging it.

Although this work does not attempt to offer a solution to the 10/90 Gap, it does attempt a different perspective of scientific research, based more on the application than the generation of knowledge as the main objective. In a sense, it is not so much in exploring the frontiers of the knowledge in onchocerciasis as it is in developing the findings of such knowledge.

3.1.1.2 Today's science for today's society?

It is been said that Alfred North Whitehead once asked: "*Has the modern biochemist, in analyzing the organism into parts, so departured from reality that his studies no longer have biological meaning?*" to which Sir Frederic Gowland Hopkings responded: "*So long as his analysis involves the isolation of events, and not merely of substances, he is not in danger of such departure...*"

During our scientific training, we face one question several times, and the answer to that question may help us find the direction of our scientific research: "What do I want from my doctoral work?" In a sense, that question is not too different from Whitehead's. The same question will present itself as our knowledge expands, our skills develop, and our taste for science finds its favorite condiment, largely divided between applied or fundamental science. I have come to the conclusion that one cannot be totally independent from the other.

At least in the biological sciences, every fundamental concept bares an application, even if not an immediate one. Reason helps us to extend the significance of fundamental concepts and imagination helps us to find their application into the right context. However, science is a natural social activity of man. It is an integral part of man's life, and an activity, which at best, is a blend of logic, intuition, art and belief.

My answer to the question of "what do I want from my work" was shaped and re-defined along the road, and this work is the result of such re-definition of my scientific goals, as a researcher yes, but also as a person whose research has been oriented towards the ultimate beneficiary of science: *society*. Here, further refinement of my goals have helped me identify the group to which I attempt to offer the outcome of my work, based on the premise that in science even a very moderate capacity can contribute to a supreme achievement. Such groups are those living where onchocerciasis is still endemic or where it has been controlled but who still need an affordable way to establish their own surveillance activities and/or rapid epidemiological assessment. Not surprisingly, those groups also live in countries where the investment on health is not a priority.

The OCP is facing its ending and the control efforts are now to be transferred to the country's own health systems to carry out surveillance and control activities. That

eliminating transmission of onchocerciasis would be more difficult than eliminating the disease as a public health problem was predicted by simulation models. The need for the development of more effective tools for onchocerciasis (macrofilaricides, less invasive but sensitive and specific diagnostic tools), as well as to better understand the long-term impact of ivermectin treatment in different epidemiological situations in order to optimize treatment strategies, comprise the current research strategy for onchocerciasis. It is there where I see the application of the findings described in this doctoral work.

3.1.1.3 Why is this important?

In the case of onchocerciasis diagnosis, even though public research is producing a wealth of new knowledge on the causing agent, and some ideas have been developed into potential tools, their deployment has not yet been materialized. In most cases, such deployment requires strategic decision and commitment within a commercial company. Such commitment implies marketing or at least an expectation of profit.

Figures 25 and 26 show a different way of looking at the world in terms of health and money. Countries where OCP, APOC and OEPA operate have been highlighted from the rest of the world. For comparison, two developed countries (Sweden and the United States of America) have been highlighted as well. The chart in Fig. 24 shows the discrepancy of total expenditure on health as a percentage of the countries' gross domestic product (GDP). In countries like Guatemala, with a low GDP, total expenditure on health roughly reaches 2.5%. Even in countries with higher GDP, such as Colombia, Brazil or Gabon, the total expenditure on health does not reach 10% of their GDP. It is important to recognize that in these countries, onchocerciasis may not be the priority in terms of health expenditure. This means that the resources are likely to be spent in infrastructure or in treating diseases with more priority, such as those causing high infant mortality rates. Figure 25 shows that the health expenditure per capita in most of the countries where onchocerciasis is still endemic is minimal and correlates well with the GDP per capita. However, in some other countries, mainly in the Americas, health expenditure per capita tends to be higher (around 300 USD). In any case the total health expenditure per capita reaches 4% of the GDP per capita, compared to almost 15% spent in countries like the United States. The charts in Figs. 25 and 26 suggest that if the deployment of diagnostic tools for onchocerciasis in those countries that need them depends on an expected market for such products, the likelihood of their availability is poor, since there seems to be no potential market. The discrepancy illustrated in Figs. 24 and 25, in terms of health expenditure, makes it unlikely that the development of commercial diagnostic products for onchocerciasis can spark the interest in the private sector to invest in those areas. The significance of this is that even when such products can be developed at low cost, the possibility of still being too expensive in some areas where health expenditure is minimal exists. When someone's income is very low, even a relatively low cost test can be unaffordable. The fundamental mismatch between human needs and scientific innovation, the discrepancy in health and life expectancy between the richest and the poorest countries can be interpreted as one of the greatest disappointments in biological sciences in the 20th century. Our effort should continue to understand in detail our relationship with parasites, but also to make an impact; perhaps it is the reliance on giving that will make the difference. This doctoral work attempted to address a minute part of a big problem with a slightly different approach to research, which is best put in the words of

Brailsford Robertson: "*It is not the talents we possess so much as the use we make of them that counts in the progress of the world.*"
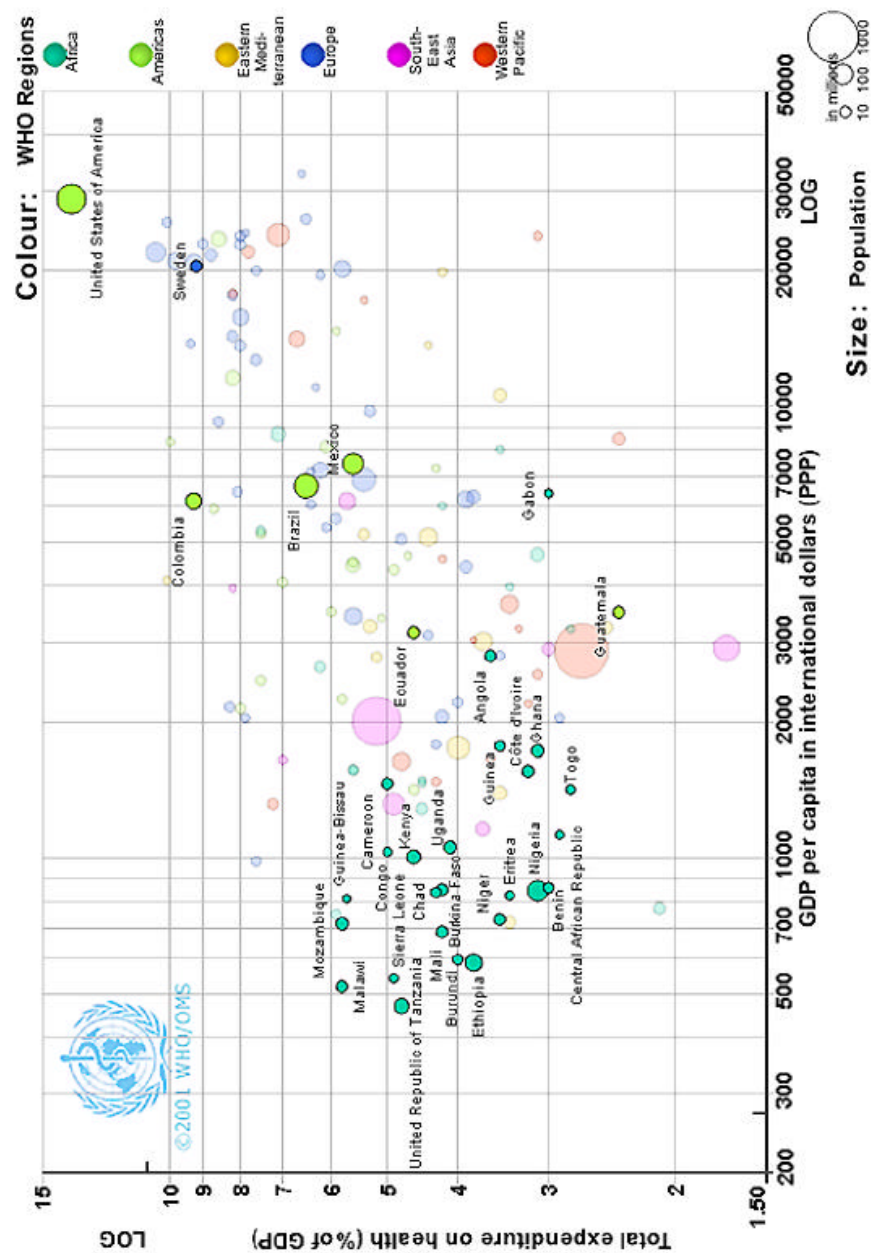
Figure 24. World Health Chart of total expenditure on health (as percentage of Gross Domestic Product, DGP) in the countries where onchocerciasis is still endemic or is being controlled. An international dollar has the same purchase power over GDP as the USD in the United States of America (USA). Two developed countries from Europe and the Americas (Sweden and USA) are shown for comparison. The chart was made using World Health Chart Public Beta 0.1. Data display corresponds to WHO data of 1999.

60
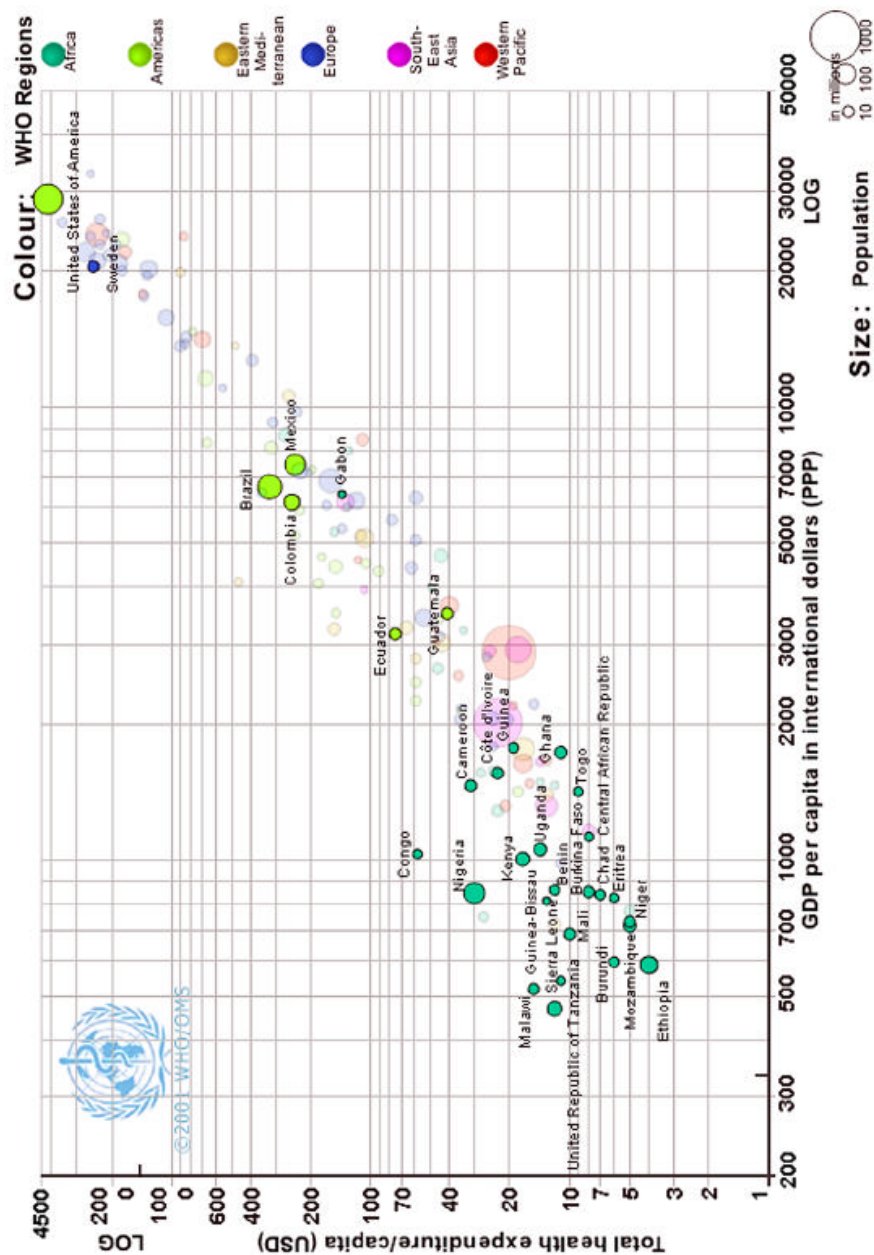
Figure 25. Total health expenditure per capita in the countries where onchocerciasis is still endemic or is being controlled. Two developed countries; Sweden and USA have been highlighted for comparison. The chart was made using World Health Chart Public Beta 0.1. Data display corresponds to WHO data of 1999.

61

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