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Characterization of immunosuppressive proteins of *Brugia malayi* microfilariae

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Summary

Inhibiton of Concanavalin A-induced lymphocyte proliferaton was used to monitor the partial purification and characterization of suppressor molecules from microfilariae of *Brugia malayi*. Suppressor activity was present in high molecular weight fractions of microfilarial extracts (Mr >50 kd on SDS-PAGE) and was protease-sensitive but resisted treatment with sodium periodate, indicating that it is associated with parasite proteins. Suppressor activity was released by microfilariae cultured in vitro and could be detected in peritoneal exudates of intraperitoneally-infected jirds and in lymph and sera from athymic C3H/HeN mice with patent *B. malayi* infections. These findings indicate that immune unresponsiveness during patent filarial infections may result from the in vivo release by microfilariae of high molecular weight proteins that suppress host immune responses.

Key words: *Brugia malayi;* suppressor factors; immune suppression; filariasis; microfilariae.

Introduction

Cellular and humoral immune responses to parasite antigens are more vigorous in amicrofilaremic than in microfilaremic animals and humans with lymphatic filariasis. This difference results in part from the activation of host cells that regulate immune responses, such as thymus-dependent (T) suppressor lymphocytes and adherent supressor cells by parasite molecules (Ottesen et al., 1977; Piessens et al., 1983b; Weller, 1978; Lammie and Katz, 1983a, b). The present study was undertaken to characterize such suppressor molecules pres-

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ent in microfilariae of *B. malayi* and to define their mode of action. When early results indicated that high molecular weight proteins with suppressor activity were released by in vitro cultured microfilariae, we also attempted to document the presence of these worm products in biological fluids from infected animals.

Materials and Methods

Preparation and fractionation of extracts of B. malayi microfilariae

Water-soluble extracts of *B. malayi* microfilariae prepared as described elsewhere (Wadee and Piessens, 1986) were concentrated by placing extract-containing dialysis bags (Spectrum Medical Industries Inc., Los Angeles, CA, molecular exclusion size 6000 daltons) in Aquacide (Calbiochem, San Diego, CA). Tenfold concentrated extracts were sterilized by filtration and stored at –20° C until used. Concentrated extracts were fractionated on Sepharose 4B or Sephadex G-200 columns as recommended by the supplier (Pharmacia Fine Chemicals, Uppsala, Sweden). The protein content of extracts and fractions derived therefrom was measured by the method of Lowry et al. (1951).

Lymphocyte proliferation assay

Mononuclear cells (MNC) were isolated from venous blood of uninfected human volunteers and of patients with clinical and/or parasitological manifestations of Brugian filariasis as previously described (Piessens et al., 1982b). In vitro proliferation of MNC induced by Concanavalin A (Con A, Calbiochem, San Diego, CA, 10 μg/ml) was measured by the uptake of ^{3}H-methylthymidine (Amersham, Chicago, IL, 24 Ci/mMole) in a standard microculture system (Piessens et al., 1982b). Proliferation is expressed as mean ± SD counts per minute (CPM) of radioactivity incorporated by triplicate sets of 2×10^5 cells cultured for 72 h. To monitor the purification of putative filarial suppressor molecules, cells were cultured in medium containing Con A and microfilarial extracts or fractions derived therefrom. The latter were standardized so that equal amounts of parasite protein present in the various fractions were added to the cultures. Percent suppression of the Con A response was calculated by the formula:

$$\% \text{ suppression} = 100 \times \left[1 - \frac{\text{CPM (Con A + parasite material)}}{\text{CPM (Con A alone)}}\right]$$

Assay of Interleukin 2 (IL-2)

To assess the effect of microfilarial extracts on IL-2 production, MNC from normal human donors were cultured with Con A in the presence or absence of 10 μg/ml parasite proteins. IL-2 activity in the cell-free supernatants of these cultures was assessed by measuring ^{3}H-thymidine incorporation by the IL-2 dependent CTLL-1 cell line as described by Gillis et al. (1978).

Chemical modification of suppressor molecules

Samples were reduced and carboxyamidomethylated by a modification of the method of Bamburg et al. (1973). To 1 mg of lyophilized protein were added 400 μl of 8 M urea in PBS and 20 μl of 2-mercaptoethanol (2-ME). This mixture was incubated for 6 h at 37° C with occasional shaking. The sample was then cooled to 10° C. A freshly prepared mixture of urea (160 mg) and iodoacetamide (24 mg) in 3 mM Tris-HCl, pH 8.5 (1 ml) was added and the alkylation was allowed to proceed for another hour at 10° C. At the end of this period, 15 μl of 2–ME was added.

Degradation of carbohydrate moieties was performed according to Owhashi et al. (1983). Briefly, lyophilized filarial extracts were incubated in 0.05 M sodium metaperiodate in the dark with gentle mixing for 72 h at 4° C. Treatment with proteases was carried out by incubating Sepharose 4B eluates containing microfilarial proteins of high molecular weight with trypsin (300 μg/ml) or proteinase K (1 μg/ml, Boehringer-Mannheim, Indianapolis, IN) for one hour at room temperature. All treated samples were dialyzed for 48 h at 4° C against 4 changes of 2 liters of PBS.

Separation of proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed with 4% stacking and 10% separating gels as described by Laemmli (1970). One part of the gels was stained with Coomassie Brilliant Blue R250. Protein bands were eluted from the corresponding areas of unstained gels by placing gel slices into 10 ml of 5 mM $NaHCO_3$ containing 0.1% SDS on a slowly spinning wheel for 12 h at 37° C. SDS was then removed from the samples by making the protein solution 6 M with respect to urea by adding solid urea and passing it over a Dowex 1-X2 column equilibrated with 50 mM Tris-acetate buffer, pH 7.8, containing 10 mM 2-ME. Proteins obtained in this way were assayed for biological activity at 1 μg/ml culture medium.

Detection of suppressor activity in biological specimens

Twenty ml of PBS was injected into the peritoneal cavity of infected jirds. The exudate fluid was collected, freed of microfilariae and host cells as described elsewhere (Piessens and Dias Silva, 1982 a) and concentrated fivefold. Similarly processed exudates from uninfected jirds injected 5 days before harvest with saline or paraffin oil were used as controls.

Lymph samples of C3H/HeN nu/nu mice infected 6–12 months earlier with *B. malayi* were collected from dilated subcutaneous lymphatics, placed on ice for 30 min, and freed of cells and microfilariae by centrifugation. Sera from infected mice and from uninfected age-matched littermates were obtained by retroorbital venepuncture.

In vitro release of suppressor molecules by microfilariae

Microfilariae purified as described above were cultured in RPMI-1640 supplemented with 20 mM glucose for 24 h at 37° C in 5% CO_2. Microfilariae were then removed by centrifugation and the supernatants were tested for biological activity as described. Similarly prepared supernatants of cultures containing glutaraldehyde-fixed microfilariae were used as controls. Metabolically labeled excretory-secretory (ES) products were prepared by incubating 10^5 microfilariae in 1 ml Dulbecco's MEM buffered with sodium bicarbonate and made to contain 100 IU/ml of penicillin, 100 μg/ml streptomycin and 50 μCi 35S-methionine (Amersham, Chicago, IL). After 24 h of incubation, parasite-free supernatants were prepared, dialyzed against three changes of 2 liters of PBS at 4° C and concentrated as described above. The samples were then passaged on Sepharose 4B columns and tested for suppressor activity as described.

Results

Microfilarial extracts suppress Con A-induced proliferation of MNC from infected and uninfected donors

Mononuclear cells from amicrofilaremic or microfilaremic patients with Brugian filariasis or from uninfected human donors proliferate much less in medium containing Con A plus microfilarial extract (20 μg/ml) than in medium containing only the mitogen (Table 1). Experiments with cells from three microfilaremic and two control donors indicated that the kinetics of the response to Con A were not affected by the simultaneous addition of microfilarial extracts to the cultures (Results not shown).

To exclude the possibility that components of these extracts adsorbed Con A and thereby decreased the amount of mitogen available to stimulate the cells, the following experiments were performed. Cells from uninfected donors were incubated with 10 μg/ml of either microfilarial extract or bovine serum albumin (BSA) for 3 days, after which the lymphocytes were recovered, washed exten-

Table 1. Microfilarial extracts (MFX) suppress Concanavalin A-induced lymphocyte proliferation

Status of cell donor	No. tested	% suppression of Con A response
Uninfected	8	45 ± 12
Infected: microfilaremic	14	47 ± 19
Infected: amicrofilaremic	15	51 ± 18

MNC were cultured in medium containing Con A alone or Con A plus 20 μg/ml microfilarial extract. Addition of 10 μg/ml bovine serum albumin to culture medium reduced the Con A response by <10% in all donor groups.

Table 2. Microfilarial extracts (MFX) activate suppressor cells

Addition to indicator culture	CPM ^{3}H-TdR incorporated by indicator cells
None	1291 ± 982
Con A	22761 ± 1887
Con A plus BSA-pretreated cells	27778 ± 3252
Con A plus MFX-pretreated cells	*15468 ± 4224* (44)

Data shown are mean ± SD of 5 experiments. Underlined values are significantly different from corresponding controls ($p < 0.05$ by "t" test of paired samples). Number in parenthesis is mean % suppression of Con A response.

sively, irradiated at 3000 rads and mixed (1 : 1 ratio) with freshly isolated MNC from the same donor. This cell mixture (2.5×10^5 cells) was then cultured in medium containing or devoid of Con A for 72 h, after which cell proliferation was assessed. Microfilarial extract-pretreated lymphocytes markedly suppressed the response of autologous indicator cells to mitogenic stimulation (Table 2). Microfilarial extracts also suppressed the proliferation of MNC prestimulated for 1 h with Con A in the absence of parasite extract (mean ± SD suppression in 3 experiments: 55 ± 16%). Finally, such extracts did not inhibit Con A-mediated agglutination of human red cells, indicating that they do not inhibit the activity of Con A as a lectin (Results not shown).

Parasite proteins eluted from Sepharose 4B columns in three broad peaks, but eluate fractions containing proteins with molecular weight between 190 and 340 kd were the only ones that suppressed Con A-induced proliferation of human lymphocytes. Peak biological activity was observed with the 240 kd eluate (Fig. 1). As little as 0.1 μg protein of this fraction was suppressive, but maximal inhibition was observed with 2 μg eluate protein/ml culture medium (Results not shown).

The 240 kd eluate fraction was further subjected to SDS-PAGE under reducing conditions. This yielded 6 major protein bands with estimated Mr of 30, 50, 70, 90 and 190 kd; the sixth band did not migrate sufficiently into these

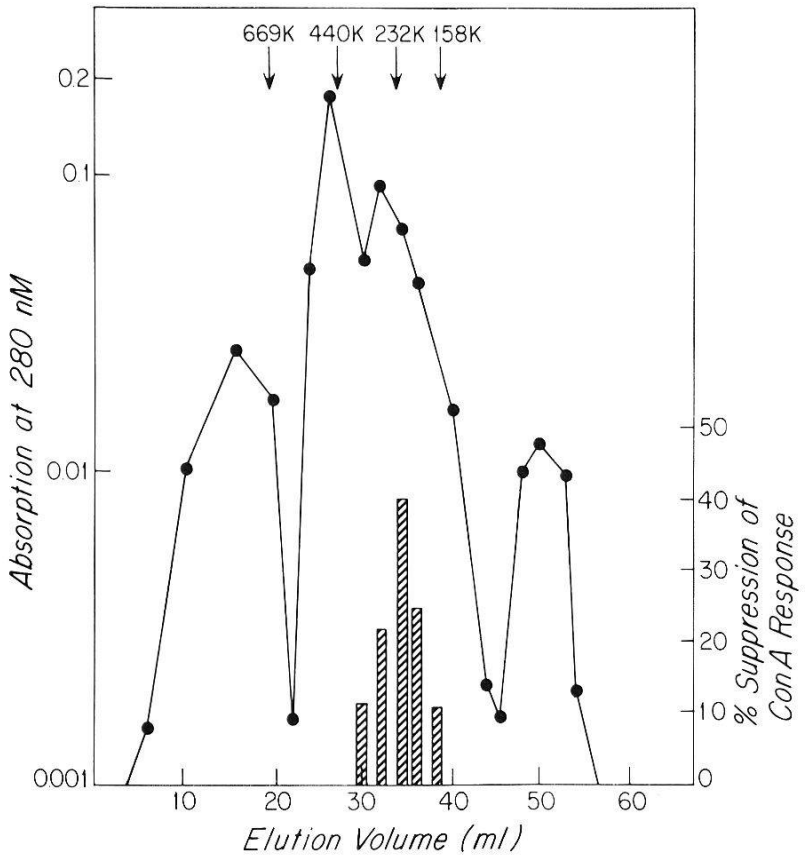

Fig. 1. Elution profile of microfilarial extracts from Sepharose 4B columns. Solid line represents absorption at 280 nM. Mean percent suppression of Con A response in 3 experiments is indicated by vertical bars. Biological activity was tested by adding 1 μg eluate protein to cultures. Elution of molecular weight markers is indicated at the top of the figure.

gels to allow accurate estimation of its molecular weight. These bands were eluted from the gels, concentrated and tested for biological activity. Of the 6 major protein bands, only those with Mr of 190, 90, 70 and 50 kd suppressed Con A-induced lymphocyte proliferation (Table 3).

Suppression of lymphocyte proliferation is due to a microfilarial protein

To further characterize suppressor factors of microfilariae, the 240 kd eluate from Sepharose 4B colums was assessed for biological activity after it had been subjected to treatments that would degrade either carbohydrate or polypeptide portions of glycoproteins. Treatment with sodium metaperiodate had no effect, but treatment with proteases almost completely eliminated the suppressor activity of this eluate fraction (Table 4). This supports the view that suppression of Con A responses is mediated by a microfilarial protein.

Microfilarial suppressor factors inhibit IL-2 production

Supernatants from cultures of human MNC stimulated with Con A in the presence of the 240 kd eluate fraction from sepharose 4B columns contained significantly less IL-2 than supernatants of cells stimulated with the mitogen only. In contrast, addition of the same parasite fractions to supernatants con-

Table 3. SDS-PAGE fractionation of reduced and alkylated suppressor activity eluted from Sepharose 4B columns

Culture conditions	CPM ^{3}H-TdR incorporated by indicator cells
Lymphocytes alone	1762± 510
Lymphocytes plus Con A	2142±2991
+ HMW eluate	9914±3634 (54)
+ 190 kd fraction	11421±2966 (47)
+ 90 kd fraction	10191±1964 (52)
+ 70 kd fraction	11123±2190 (48)
+ 50 kd fraction	11566±2111 (46)
+ 30 kd fraction	22461±1994 (0)
+ 14 kd fraction	21612±2107 (0)

Data shown are mean±SD of three experiments. Numbers in parentheses are mean % suppression of Con A response.

Table 4. Polypeptide nature of filarial suppressor activity

Culture addition	CPM ^{3}H-TdR incorporated	% suppression of Con A response
Concanavalin A	21421±2991	
+ untreated eluate	9914±3636	54
+ NaIO4-treated eluate	10491±4211	51
Concanavalin A	25657±1246	
+ untreated eluate	11740±3942	54
+ trypsin-treated eluate	26538±3211	0
+ protease K-treated eluate	28724±3661	0

Data shown are mean±SD of three experiments. See text for definition of eluate fractions.

taining preformed IL-2 did not reduce the biological activity of the lymphocyte mediator (Fig. 2).

Suppressive proteins are secreted by microfilariae

Supernatants from in vitro cultures of viable microfilariae suppressed Con A-induced lymphocyte proliferation, whereas similar supernatants from cultures of glutaraldehyde-fixed microfilariae failed to do so (Table 5). Suppressor activity coeluted with metabolically labeled ES products of high molecular weight from Sepharose 4B columns (Fig. 3), indicating that the biological activity in these supernatants was of parasite origin.

Because these experiments indicated that microfilariae released suppressor molecules in vitro, we examined whether these could be detected in biological fluids from infected animals. Peritoneal exudates from *B. malayi*-infected jirds and sera and lymph fluids from microfilaremic nude mice suppressed Con

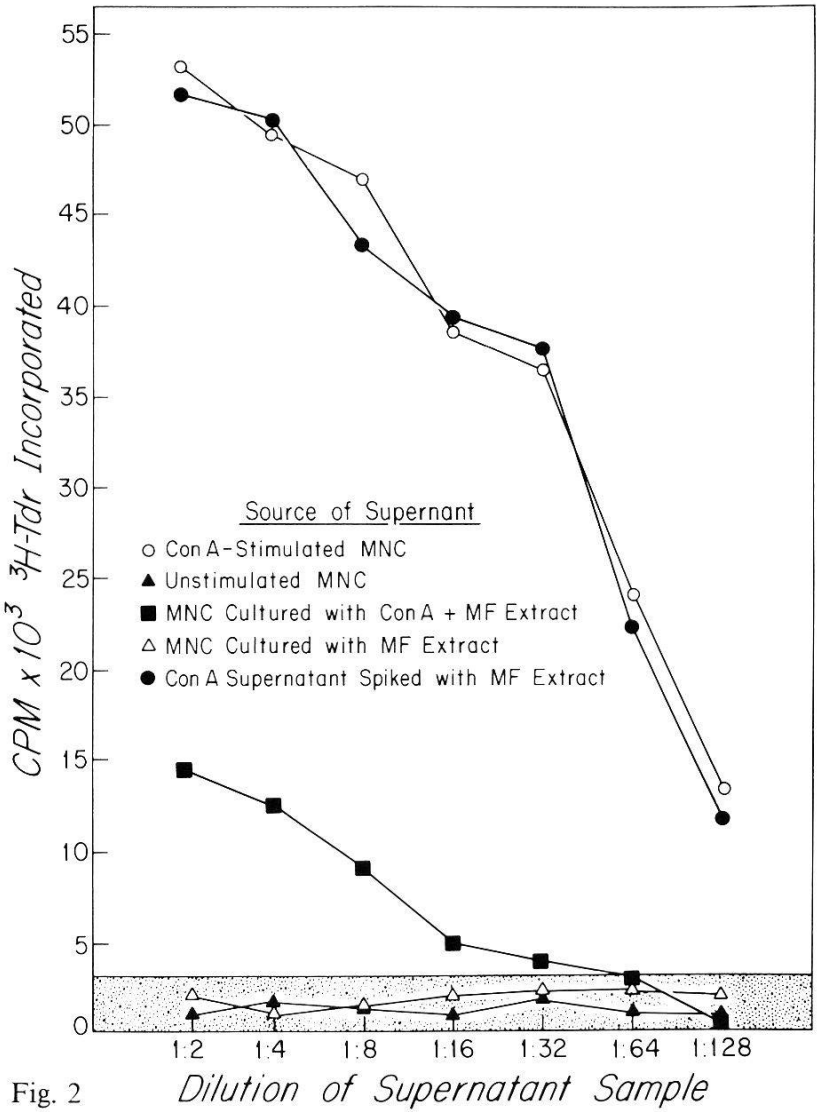

Fig. 2

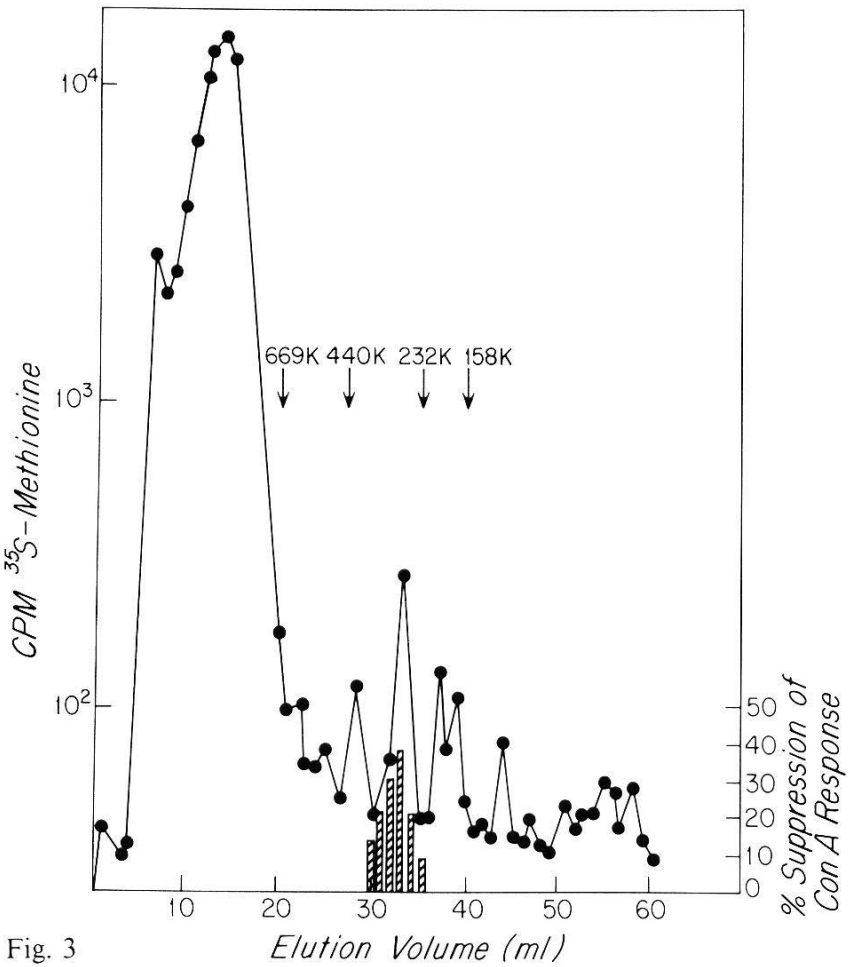

Fig. 3

Fig. 2. Microfilarial extracts inhibit mitogen-induced production of Interleukin-2. Results shown are mean of triplicate determinations of IL-2 content of supernatants of human MNC stimulated as indicated. Hatched area represents 3H-TdR incorporation in plain culture medium.

Fig 3. Sepharose 4B elution profile of 35S-methionine-labeled ES products of *B. malayi* microfilariae. Solid line represents CPM radioactivity. Mean percent suppression of Con A response in 3 experiments is indicated by the vertical bars. Position of molecular weight markers is shown at the top of the figure.

Table 5. The effects of peritoneal exudates and supernatants of 24-h microfilarial cultures on Con A-induced lymphocyte proliferation

Culture conditions	CPM ^{3}H-TdR incorporated	
	Experiment 1	Experiment 2
Lymphocytes alone	2784	2521
Lymphocytes + Con A	31527	27728
Lymphocytes + Con A + peritoneal exudates from normal jirds	33717 (0)	27038 (2)
Lymphocytes + Con A + peritoneal exudates from *B. malayi*-infected jirds	19365 (39)	9141 (67)
Lymphocytes + Con A + supernatants from glutaraldehyde microfilariae	28436 (8)	31246 (0)
Lymphocytes + Con A + supernatants from viable microfilariae	10303 (67)	11261 (59)

Data shown are mean of triplicate determinations. Numbers in parentheses are mean % suppression of Con A response.

Table 6. Sera and lymph from *B. malayi*-infected nude mice contain suppressor activity

Culture conditions	CPM ^{3}H-TdR incorporated
Lymphocytes alone	1907± 541
Lymphocytes plus Con A	29098±2369
+ uninfected nude mouse serum	31541±3559 (0)
+ microfilaremic nude mouse serum	19896±1643 (32)
+ amicrofilaremic nude mouse lymph	29869±3214 (0)
+ microfilaremic nude mouse lymph	11326±2616 (61)

Data shown are mean±SD of 3 experiments, Sera and lymph fluids were used at a final concentration of 10% (v/v). Numbers in parentheses are mean % suppression of Con A response.

A-induced lymphocyte proliferation, whereas control exudates or sera from uninfected mice did not do so (Tables 5 and 6). This biological effect was mediated by moieties of similar high molecular weight as those present in extracts or ES products of *B. malayi* microfilariae, as evidenced by the similarity of the elution profiles of suppressive molecules present in these various preparations (Results not shown).

Discussion

We report here that microfilariae of *B. malayi* contain and release proteins of high molecular weight that suppress mitogen-induced in vitro proliferation of lymphocytes from infected and uninfected human donors. Suppressor

activity coelutes from sizing columns with metabolically labeled parasite proteins, is inactivated by proteases and resists treatment with periodate, indicating that it is associated with polypeptides synthesized by *B. malayi* microfilariae. These suppressor molecules decrease the IL-2 content of supernatants from cultures of mitogen-activated lymphocytes, but whether this is due to decreased production or increased consumption of IL-2, an essential component of T cell responses to antigenic and mitogenic stimuli (Farrar et al., 1982), cannot be determined from our studies.

Our finding that suppressive microfilarial proteins are present in vivo is consistent with the thesis that these molecules contribute to the state of immune unresponsiveness that characterizes patent lymphatic filariasis in humans and in animals. In animal models, filarial antigen-specific and nonspecific forms of immune suppression appear to be mediated by distinct cell types that are differentially distributed among various organs and tissues of the host (Lammie and Katz, 1983b). Whether or not the microfilarial products identified in the present study activate both types of suppressor cells remains to be determined, but this possibility is suggested by our previous finding that crude microfilarial extracts can induce "specific" and "nonspecific" suppressor cells in vitro, depending on the concentration of parasite material used (Piessens et al., 1982b). The observation that filarial antigen-specific T cell clones augment the in vitro production of antiparasite antibody and of nonspecific immunoglobulins further supports the idea that specific and nonspecific effects can be mediated by single cells stimulated with parasite antigens (Nutman et al., 1984).

Treatment with diethylcarbamazine restores the ability to react to parasite antigens of patients with Brugian filariasis in whom microfilaremia is terminated by the drug (Piessens et al., 1981). This suggests that microfilariae are a major source of molecules with immunosuppressive potential. The present study confirms that microfilariae indeed contain and release such moieties. It is unlikely, however, that microfilariae are the only source of these materials because immune suppression in animal models of filariasis occurs long before the development of patency (Schrater and Piessens, 1982). Whether other developmental stages of filarial helminths contain immunosuppressive molecules similar to the ones decribed here remains to be determined.

The microfilarial proteins with direct suppressor activity described herein are distinct from parasite mitogens that might indirectly contribute to immune suppression via polyclonal activation of the host's immune system. The exact in vivo role of high molecular weight suppressor proteins from microfilariae in the resistance to and the pathogenesis of lymphatic filariasis remains to be determined. However, both types of parasite molecules are present in differing amounts in microfilarial extracts (Wadee and Piessens, 1986, and this report). This could explain the apparent paradox of polyclonal hypergammaglobulinemia in patients with patent filariasis who lack specific antibodies to parasite antigens.

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