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Serum levels of eosinophil cationic protein, eosinophil-derived neurotoxin and myeloperoxidase in infections with filariae and schistosomes

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Abstract

The serum levels of three major granulocyte proteins were measured in patients with onchocerciasis, bancroftian filariasis and intestinal schistosomiasis and compared to controls from patients with malaria, Africans living in areas not endemic for these infections and healthy Germans. The investigation comprised the determination of the eosinophil granule proteins eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN/EPX), and the neutrophil/monocyte granule protein myeloperoxidase (MPO). ECP and EDN/EPX levels were found elevated only in the three helminth infections that are associated with eosinophilia, while MPO was found elevated in all tested disease groups. The levels of eosinophil granule proteins observed in the helminth diseases by far exceeded those described for bronchial asthma and atopic dermatitis. ECP, EDN/EPX and MPO serum levels reflect the ongoing disease and are related to functional activity of the respective leukopoetic system. ECP and EDN/EPX appear to be markers of the eosinophil effector system and MPO a marker of the neutrophil and/or monocyte/macrophage effector system. Significantly higher ECP levels in chronic hyperreactive onchodermatitis (sowda) versus generalized onchocerciasis seem to reflect an augmented degree of antigenic stimulation, eosinophil activation and eosinophil turnover rates, indicating a more active mechanism of parasite clearance in sowda patients.

Keywords: Cationic granule proteins; Eosinophil cationic protein; Eosinophil-derived neurotoxin; Myeloperoxidase; Helminth diseases

1. Introduction

The respective potent anthelmintic and antimicrobial activities of granulocytes and monocytes/macrophages are now well recognized. The toxic potential includes

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the release of stored granule cationic proteins, hydrolytic enzymes, proteins and peptides disrupting microbial functions, and the de novo generation of oxygen intermediates. Among the granular proteins are myeloperoxidase (MPO), lysozyme and elastase in neutrophils and monocytes/macrophages (Baggiolini et al., 1974; Ohlsson et al., 1977), and major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN/EPX) and eosinophil peroxidase in eosinophils (Ackerman et al., 1983). EDN is reported to be identical to EPX (Slifman et al., 1989). ECP and EDN/EPX constitute about 50% of the total granule protein and reside in the matrix of the eosinophil secondary granules (Peters et al., 1986). MPO is one of the major constituents of the neutrophil primary, azurophil granule. The proteins are immunologically distinct.

Eosinophils function as effectors to damage larval forms of helminth parasites (Butterworth et al., 1974; Medina-De la Garza et al., 1990; Brattig et al., 1991). Release of toxic enzymes leads to the killing and disintegration of helminth larvae by neutrophils and macrophages (Buys et al., 1984; Gutiérrez-Peña et al., 1996; Taylor et al., 1996). The remnant fragments of larvae and eggs are phagocytised by macrophages and giant cells. The levels of granule proteins in blood or local fluids may reflect the ongoing disease process. To determine the significance of granule proteins we investigated the levels of the cationic proteins ECP, EDN/EPX, and MPO in sera of patients with three tissue dwelling helminths using sensitive immunoassays. Sera from persons without infections with these three helminths were used as controls.

2. Patients and methods

2.1. Study population

The study comprised African patients with only one of the three most frequently observed chronic human infections with adult helminths living in the tissues or in the blood and not in the lumen of the intestine. In a hyperendemic area in southern Benin we examined 49 *Onchocerca volvulus* microfilaria carriers with common generalized onchocerciasis and 18 patients with rare chronic hyperreactive onchodermatitis (sowda) identified by the clinical features, antibodies against *O. volvulus* and sometimes microfilariae as previously described (Gallin et al., 1993). We investigated sera from 11 patients with chronic elephantiasis living in an area endemic for *Wuchereria bancrofti* in southern Benin. Sera from 20 persons excreting eggs of *Schistosoma mansoni* were collected in the hyperendemic fishing village Ruangara at the southern shore of Lake Albert in Uganda. The geometric mean of 718 eggs/g feces in a sample of 217 persons indicated a very high worm load in this focus.

Sera from persons without any of the three invasive helminth infections served as controls: 28 healthy Europeans (E.C.) and 7 Africans living outside the endemic areas in Cotonou in Benin (A.C.). Since the patients with helminth infections all lived in areas endemic for malaria, a third control group of 18 patients with malaria

were included (10 *Plasmodium falciparum*, 7. *P. vivax*, 1 *P. malariae*; 6 Africans and 12 Germans).

2.2. Serum collection

Venous blood samples were allowed to clot for 30 min at room temperature. After centrifugation at 2500 *g* for 10 min, serum was removed, kept in liquid nitrogen for transport to Hamburg and than stored at −20°C until testing.

2.3. Determination of granule proteins of eosinophilic and neutrophilic granulocytes

Serum levels of eosinophil cationic protein (ECP) were assessed by RIA (Pharmacia Diagnostics, Uppsala, Sweden) using a competitive double antibody assay (Peterson et al., 1990). ECP standards or ECP in the sample compete with a fixed amount of ^{125}I-labeled ECP for the binding sites of rabbit anti-human ECP IgG. The immune complexes were removed by Sepharose anti-rabbit IgG. The sensitivity of the assay is <2 μg/l; the normal range (95%) is 2.3–16 μg/l. The coefficient of variation was 5–11%. Radioimmunoassays of eosinophil-derived neurotoxin (EDN/EPX) and myeloperoxidase (MPO) were performed according to the same protocol as ECP. The sensitivity of the EDN/EPX and MPO assay was <3 μg/l and <8 μg/l, respectively. The normal ranges (95%) of EDN/EPX and MPO are 8.2–38.5 μg/l and 170–478 μg/l. The coefficients of variation for both assays were 5–11% and 6–12%.

2.4. Statistical analysis

The experiments were set up in duplicate and data are presented as medians and quartiles. For calculation of statistical significance of differences the Mann-Whitney U test was applied. For correlation the Spearman's rank-correlation test was used.

3. Results

Serologic determination of the granule proteins ECP, EDN/EPX and MPO were done for 49 patients with generalized onchocerciasis and 18 sowda cases, 11 patients with bancroftian filariasis, 20 with intestinal schistosomiasis, 18 with malaria, 7 Africans from non-endemic areas (A.C.) and 28 healthy Europeans (E.C.). In a representative sample peripheral blood eosinophils and neutrophils were counted. The findings and their statistical evaluation are presented in Table 1.

ECP and EDN/EPX levels were found elevated only in helminth infections with eosinophilia, while MPO was elevated in all tested disease groups. ECP was found significantly higher in helminth diseases than in malaria ($P<0.01$). The highest ECP levels were observed in schistosomiasis and sowda type onchocerciasis, ranging from 59 to 170 μg/l with a median of 130 μg/l and 120 μg/l, respectively (Table 1, Fig. 1C), which is significantly higher ($P<0.001$) than that of malaria and the other

Table 1
Serum granule protein levels of study groups

Group	1	1a	1b	2	3	4	5	6	
Number	67	49	18	11	20	18	7	28	
Granule proteins	Onchocerciasis			Bancroftian filariasis	Schistoso-miasis	Malaria	Controls		Significance
	Total	Generalized	Sowda				Africans	Europeans	
ECP NR = 2.3–16 μg/l	70 (46; 130)	64 (41; 115)	120 (59; 150)	68 (59; 82)	130 (110; 170)	13 (7; 16)	5 (3; 12)	8 (5; 16)	**1, 1a, 1b, 2, 3 vs. 4, 5, 6 *1, 1a, 2 vs. 3 **1, 1a, 2 vs. 3 *1a, 2 vs. 1b
EDN/EPX NR = 8.2–38.5 μg/l	185 (98; 300)	165 (87; 275)	225 (120; 340)	100 (62; 143)	240 (220; 290)	34 (14; 42)	22 (7; 29)	21 (13; 26)	**1, 1a, 1b, 2, 3 vs. 4, 5, 6 *1, 1b, 3 vs. 2
MPO NR = 170–478 μg/l	800 (525; 1000)	725 (525; 1000)	850 (440; 950)	1350 (1044; 1700)	2100 (1450; 2700)	650 (390; 900)	235 (204; 284)	350 (285; 473)	**1, 1a, 1b, 2, 3, 4 vs. 5, 6 *2, 3 vs. 4 **1, 1a, 1b vs. 2 *1, 1a vs. 3

Data are given as medians (25%; 75% quartiles). $*P = <0.05$ and $**P < 0.01$ indicate statistically significant differences between groups as determined by use of Mann-Whitney U-test. NR = normal range (95%).

control groups. A significantly higher level also was observed for the patients with schistosomiasis versus patients with onchocerciasis (total group) and bancroftian filariasis ($P<0.001$), whereas patients with malaria had ECP levels essentially identical to those of E.C. controls. Sowda patients had about two-times higher serum concentrations of ECP as compared to generalized onchocerciasis ($P<0.02$) (Table 1; Fig. 1A).

Similar findings were observed for EDN/EPX (Table 1; Fig. 1B). The difference between sowda and generalized onchocerciasis, however, was not significant. All helminth diseases had significantly higher serum levels of EDN/EPX when compared to malaria and the other control subjects ($P<0.001$).

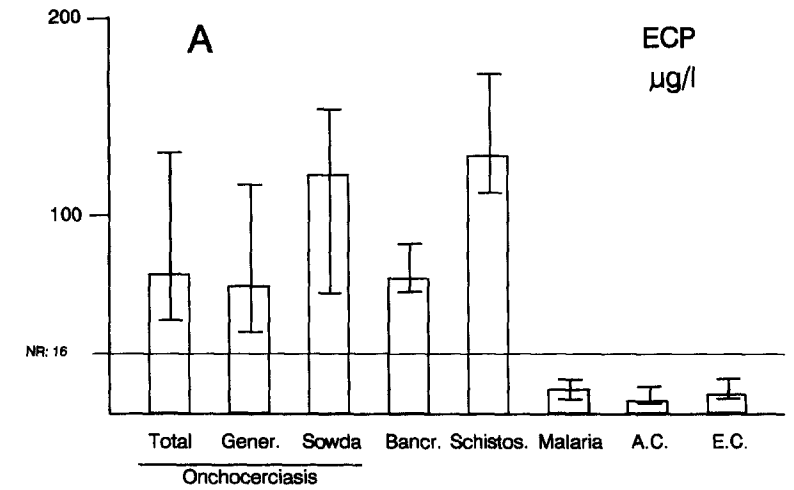
The sera were analyzed for their concentration of the neutrophil/monocyte granule protein MPO. Sera of patients from all disease groups as well as control groups exhibited significantly higher levels of MPO than ECP or EDN/EPX ($P<0.001$). The concentrations of MPO exceeded those of ECP and EDN/EPX by up to 20 and 13 times in helminth diseases and 47 and 17 times in control groups. In malaria patients MPO serum levels were 50 times higher than ECP and 19 times higher than EDN/EPX levels (Table 1; Fig. 1C).

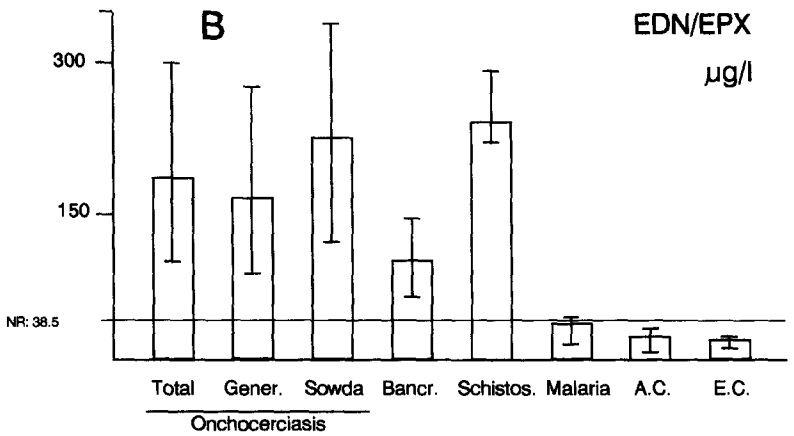
A positive correlation was observed between ECP and EDN/EPX levels in the sera of the patients with helminth diseases ($r=0.66$, $P<0.001$) (Fig. 2A). EDN/EPX levels in these diseases were significantly correlated to the number of peripheral blood eosinophils ($r=0.43$, $P<0.05$) (Fig. 2C), whereas no such coefficient of correlation was demonstrated for ECP and the eosinophil counts (Fig. 2B). Helminth diseases and in particular sowda showed higher peripheral eosinophil but lower neutrophil counts than European (E.C.) and malaria controls. In malaria, a positive correlation for MPO serum levels and the number of peripheral blood neutrophils was observed ($r=0.46$, $P<0.05$).

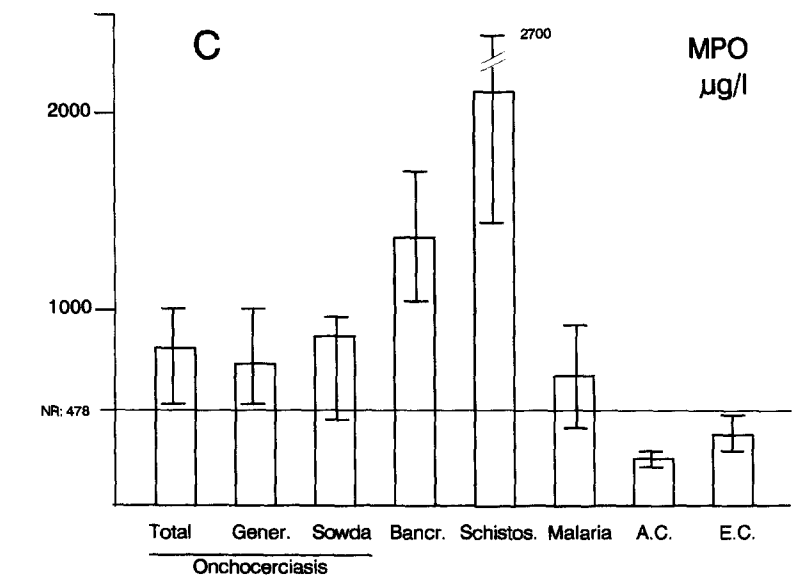
4. Discussion

In the present investigation we have measured the serum levels of the three granule proteins ECP, EDN/EPX and MPO in three helminth infections (onchocerciasis, bancroftian filariasis, intestinal schistosomiasis) and malaria. It was shown that ECP and EDN/EPX levels were elevated only in diseases that are associated with eosinophilia, i.e. helminth infections, while MPO was also slightly elevated in malaria patients.

MPO is a component with broad spectrum antimicrobial activity within neutrophils and monocytes/macrophages. It catalyses reactions between hydrogen peroxide and chloride ions that lead to formation of hypochlorous acid and chloramines (Lehrer and Ganz, 1990). The enzyme also kills newborn larvae of *Trichinella spiralis* in vitro (Buys et al., 1984), and it is secreted on the surface of. *O. volvulus* microfilariae in the skin (Gutiérrez-Peña et al., 1996). ECP is cytotoxic to helminth larvae. Thus, ECP achieves killing of schistosomula of *S. mansoni* (McLaren et al., 1981; Ackerman et al., 1985), newborn larvae of *T. spiralis* (Hamann et al., 1987), and microfilariae of *Brugia pahangi* and *Brugia malayi* in vitro (Hamann et al., 1990).


A
ECP
µg/l
200
100
NR: 16
Total
Gener.
Sowda
Bancr.
Schistos.
Malaria
A.C.
E.C.
Onchocerciasis


B
EDN/EPX
µg/l
300
150
NR: 38.5
Total
Gener.
Sowda
Bancr.
Schistos.
Malaria
A.C.
E.C.
Onchocerciasis


C
2700
MPO
µg/l
2000
1000
NR: 478
Total
Gener.
Sowda
Bancr.
Schistos.
Malaria
A.C.
E.C.
Onchocerciasis

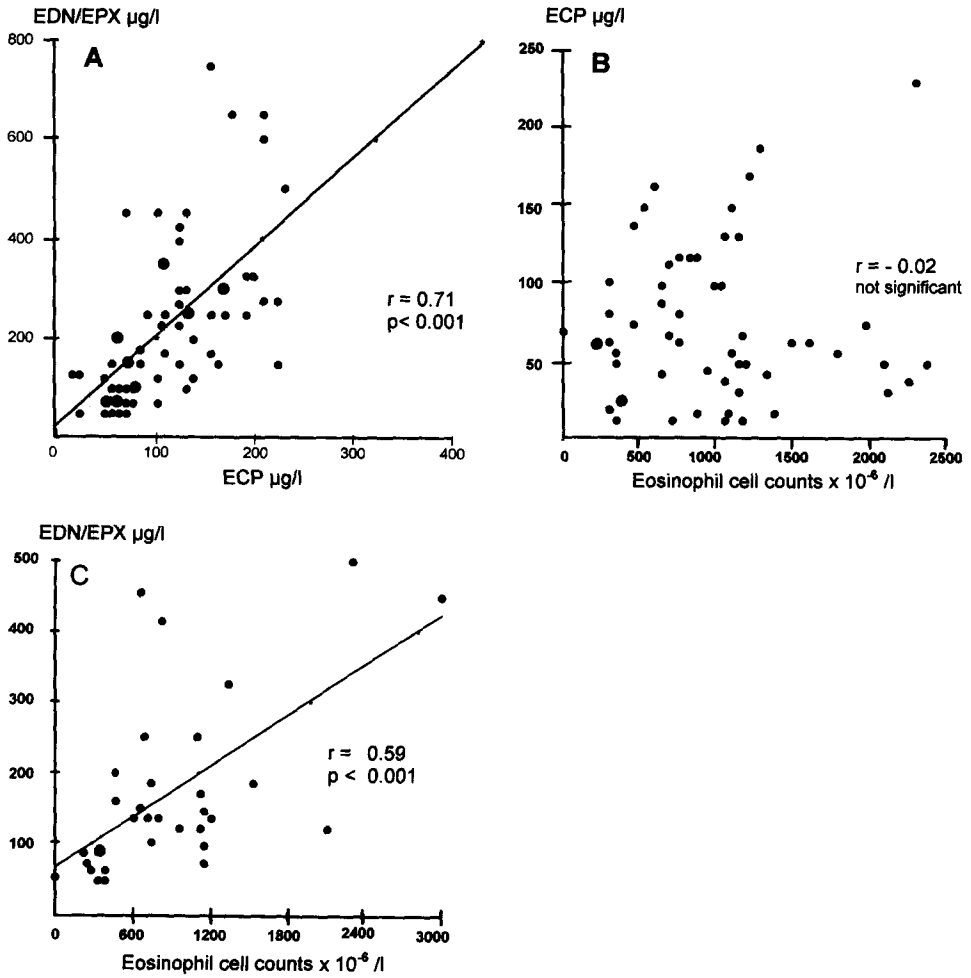


Fig. 2. Relationship between the serum levels of ECP and EDN/EPX (A), and between the levels of the respective granule proteins and the blood eosinophil count (B,C) in 68 individuals with filarial diseases. Multiple values are given as bigger points.

The cytotoxic and neurotoxic potency of EDN/EPX is far less than that of ECP, and it does not seem to be as efficient as ECP in killing of various helminths (McLaren et al., 1984). EDN/EPX and ECP have, in addition, ribonuclease activity (Gleich and Adolphson, 1986).

Fig. 1. Serum levels of ECP (A), EDN/EPX (B), and MPO (C) in patients with onchocerciasis (total group, $n=67$; generalized, $n=41$; sowda type, $n=18$), Bancroftian filariasis ($n=11$); intestinal schistosomiasis ($n=20$) and malaria ($n=18$) as compared to African (A.C., $n=7$) and European (E.C., $n=28$) normal control subjects. The upper limit of the normal range (NR, 95%) of the respective granule protein is marked. The results of statistical evaluation are listed in Table 1.

The serum concentrations of these proteins appear therefore to mirror the functional activity of the respective leukopoetic effector system in the particular host. Levels of ECP and EDN/EPX were found to be elevated in the serum of patients with bronchial asthma (Dahl et al., 1978; Durham et al., 1989; Carlson et al., 1990, Griffin et al., 1991) and atopic dermatitis (Sugai et al., 1992). In both diseases a significant correlation was observed between serum levels of ECP and blood eosinophil counts (Sugai et al., 1992). Only a few reports deal with serum levels of eosinophil granular proteins in parasitic diseases, one with elevated MBP levels in bancroftian filariasis after treatment with diethylcarbamazine (DEC) (Ackerman et al., 1981) and one short report with ECP levels in untreated onchocerciasis (Tischendorf and Brattig, 1992). The values for ECP and EDN/EPX in bronchial asthma and atopic dermatitis did not exceed geometric means of 50 μg/l, which is significantly lower than those observed in the helminth diseases of the present study.

The plasma concentration of MPO was reported to significantly correlate with the total number of neutrophils in the blood (total blood granulocyte pool) as well as with the neutrophil turnover rate, using a radioimmunoassay (Hansen et al., 1975). These authors tested 31 patients with non-parasitic diseases covering a wide range of blood neutrophil counts and turnover rates, including leukemias and pancytopenic states. The correlation is highly suggestive that neutrophilic granulocytes are the main source of MPO in the serum. In circulation of normal individuals about 95% of the cellular MPO is contained in the neutrophils whereas the remaining MPO is contributed by the less numerous monocytes (Bahner and Johnston, 1972; Bos et al., 1978). In skin, nodules and lymph nodes of onchocerciasis patients MPO is observed in the neutrophils around the filariae (Gutiérrez-Peña et al., 1996 and unpublished data).

From our data it may be concluded that eosinophils, neutrophils and monocytes/macrophages are involved in parasite clearance in patients with worm diseases as indicated by the simultaneous serum elevation of the eosinophil marker proteins ECP and EDN/EPX, and the neutrophil/monocyte marker protein MPO.

Previous research demonstrated a relationship between the development of peripheral blood eosinophilia and changes in serum levels of MBP after DEC treatment (Ackerman et al., 1981).

The results of the present study show a significant correlation of the levels of EDN/EPX but not of ECP with peripheral blood eosinophil counts in untreated filarial diseases (Fig. 2). The lack of a positive correlation of ECP with the peripheral eosinophil counts indicates, that serum granule proteins may also be derived from eosinophils in tissues like lymph nodes or bone marrow. The circulating pool of eosinophils represents only a minor part of the total eosinophil mass. Most of the clearance of helminths apparently occurs in liver, spleen, lungs, gut, skin and lymph nodes in association with a mixed inflammatory cell reaction with large numbers of macrophages and eosinophils surrounding the dying microfilariae (Woodruff, 1951; Wildenburg et al., 1994) or schistosome eggs (McCully et al., 1976).

In the onchocerciasis patients of the present study the significantly higher ECP levels in the hyperreactive sowda form compared to the immune-suppressed generalized form seem to reflect an augmented degree of eosinophil activation and turnover

rate and consequently killing of microfilariae. Pronounced cellular and humoral immune responses are documented in sowda (Büttner et al., 1982; Brattig et al., 1987; Brattig et al., 1994; Rubio de Krömer et al., 1995). The most striking feature of sowda is the low microfilarial density in skin and lymph nodes.

In the generalized form, ivermectin treatment leads to a migration of microfilariae from the skin to the lymph nodes and to reinforced killing of microfilariae by cytotoxic cells (Darge et al., 1991; Duke et al., 1991; Wildenburg et al., 1994). The lymph nodes from treated patients with generalized onchocerciasis had over ten times more eosinophils as compared to those from untreated patients with a peak of eosinophil density at 40–48 h following treatment. In sowda patients eosinophil cationic proteins were lacking almost completely before and after treatment in the enlarged regional lymph nodes, indicating that in sowda the killing and disintegration of microfilariae by eosinophils happens in the nodules and in the skin and does not depend on therapy alone (Wildenburg et al., 1994). This would explain the observation of higher serum ECP levels in our untreated sowda patients.

The observations of Venge et al. (1978) and Hällgren et al. (1979) implicate similar mechanisms. They found high levels of serum ECP in acute infection and the inflammatory reaction of the type seen in acute myocardial infarction in spite of the absence of circulating eosinophils, indicating that eosinophils are actively involved in tissue- dwelling inflammatory reactions.

After DEC therapy eosinophils have been described as the predominant infiltrating cells in the first 24 h after treatment in onchocerciasis (Gibson et al., 1976; Ackerman et al., 1990). Although the exact mode of action of DEC remains to be defined, in vitro studies on purified eosinophils and neutrophils of patients with onchocerciasis revealed an enhancement of the effector function of the cells after exposure to the drug, thus suggesting an in vivo action of DEC on host's effector cells as well as on the parasite itself (Medina-De la Garza et al., 1990). Other anthelmintic drugs like ivermectin, amocarzine, (CGP 6140) and CGP 20376 revealed a dose-dependent modulatory in vitro effect on the respiratory burst of eosinophilic effector cells indicating that these compounds too may modulate the host defence in vivo (Tischendorf et al., 1993). Degranulation of cytotoxic granular proteins would be a consequence of eosinophil-mediated killing of the parasite resulting in elevated blood levels of these proteins.

Besides eosinophilic also neutrophilic granulocytes adhere to *O. volvulus* microfilariae, but their immobilizing capacity is less pronounced as compared to that of eosinophils (Medina-De la Garza et al., 1990; Gallin et al., 1995; Gutiérrez-Peña et al., 1996). Neutrophils are the predominant cells adjacent to adult *O. volvulus* (Gallin et al., 1995 and unpublished data), and they are found in the infiltrates around damaged adult *W. bancrofti* (Galindo, 1971). Neutrophil infiltrates were also observed in filarial lymphoedematous skin (Olszewski et al., 1993). The superior role of eosinophils as microfilaricidal effector cells is reflected by the observation that inflammatory infiltrates surrounding microfilariae in situ predominantly comprise eosinophils and macrophages but seldom neutrophils (Gibson et al., 1980). Possibly, serum MPO is contributed also by patient's macrophages. The source of MPO in serum of parasitic diseases is currently under investigation.

Blood leukocytosis due to neutrophils and/or monocytes is the substrate of malaria (Boros and Boros, 1960). The elevated MPO and normal ECP serum levels observed in our cases are in agreement with these haematological data.

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