CIRCULATING FIBROSIS MARKERS, EOSINOPHIL CATIONIC PROTEIN AND EOSINOPHIL PROTEIN X IN PATIENTS WITH Wuchereria bancrofti INFECTION: ASSOCIATION WITH CLINICAL STATUS


Summary:
We measured the concentrations of several circulating fibrosis markers (type I collagen, type III procollagen, hyaluronan) and eosinophil granule proteins (ECP and EPX) in lymphatic filariasis patients to investigate their relationship with clinical, parasitological and immunological data. This study was conducted in Polynesian patients with various stages of the disease (acute lymphangitis, chyluria, hydrocoele, elephantiasis), a closely related microbial lymphangitis and endemic controls. We observed modifications of the different markers in this pathology. Serum type I collagen and PIIINP were decreased. Serum hyaluronan, linked to peri-lymphatic granulomatous inflammation, was significantly increased in acute lymphangitis and elephantiasis patients. Serum ECP was also increased, at the limit of significance in our sample, in elephantiasis patients. These two last markers, already validated in another helminth disease, schistosomiasis, have potential interest in terms of follow-up of morbidity in these parastic diseases.

KEY WORDS: lymphatic filariasis, fibrosis markers, eosinophil proteins, French Polynesia.

INTRODUCTION

Lymphatic filariasis (LF) presents a broad clinical spectrum, including acute (adenolymphangitis, ADL) and chronic (mainly hydrocoele and disfiguring elephantiasis), triggered by adult worms of Wuchereria bancrofti in the lymphatics (Kumaraswami in Nutman, 2000). It is believed that, at least in part, the pathogenesis of LF (and other chronic helminth diseases) is related to a persistent immunological response of genetically susceptible person to relevant parasite antigens. Consequently, an extensive lymphoedema and deposition of fibrous material is seen in this chronic pathology, suggesting that connective tissue metabolism is modified during this long-term process. The search for an alternative to painful and sometimes hazardous, at least for the liver, biopsy has led to the development of serological and urinary markers of disease activity reflecting the turn-over of extracellular matrix (ECM). This non-invasive follow-up of fibrosis has been applied in parasitology, mainly in schistosomiasis-associated liver fibrosis (Secor et al., 1994; Ricard-Blum et al., 1998) chromoblastomycosis – (Ricard-Blum et al., 1998) and lymphatic – (Fleming-Hubert et al., 1997) associated elephantiasis.

We also measured the serum and urine levels of two eosinophil granule proteins supposed to reflect both the release of toxic cationic enzymes able to damage the larval forms and the eosinophil-rich inflammation against the tissue-dwelling helminth parasites. Eosinophil cationic protein (ECP) and the neurotoxin eosinophil peroxidase (EPX) are major constituent of secondary granules which usually correlate with peripheral eosinophil counts, even if the circulating pool is only a minor part of the global eosinophil population. Serum levels of ECP and EPX have been measured in patients with LF-associated elephantiasis and schistosomiasis mansoni (Tischendorf et al., 1996). In addition urinary levels of ECP are significantly correlated with intensity of infection and ultrasonographically detectable urinary tract pathology in schistosomiasis...
haematobium (Leutscher et al., 2000, Reimert et al., 2000).

In the present study, we measured the concentration of serum markers of fibrosis (type I collagen, N-terminal propeptide of type III collagen (PIIIP)) and glycosaminoglycan hyaluronan (HA), first to investigate their relationship with the clinical status of the patients and then to determine if they are useful to monitor the effect of specific treatment. Furthermore, a previous analysis of circulating adhesion (soluble ICAM-1, VCAM-1 and various selectins) and angiogenic (VEGF and endothelin) molecules (Esterre et al., 2005) provided us the possibility to investigate the relationship between these immunological markers and ECM metabolites or inflammatory markers (ECP, EPX). The main objective of these investigations is to delineate more precisely the immunopathological response underlying the pathogenesis of lymphatic filariasis, including the granuloma-associated fibrosis.

**PATIENT AND METHODS**

**Patients**

The 30 individuals included in this study were residents of the Society Archipelago, French Polynesia, a long-term identified area of LF transmission under incomplete control (Esterre et al., 2001), consulting a specialized clinic within the Malardé Institute, Papeete, French Polynesia. Prevalence (expressed as a percentage) and intensity (expressed as a geometric mean number of microfilaria (Mf) per milliliter of blood) of infection were performed by standardized methods, as the ELISA-based detection of filaria-specific IgG and circulating antigens. After confirmation of clinical LF and morbidity staging (WHO, 1992, Freedman et al., 1998), all patients (17 with acute filarial ADL, seven chronic elephantiasis, four hydrocele and two chyluria) were subsequently treated with diethyl carbamazine (DEC).

The patients were compared to four bacterial ADL (typical erysipela closely mimicking filarial ADL, see Esterre et al., 2000) and 22 endemic controls, *i.e.* lifelong residents, of Maori or European origin, of the archipelago who had neither a positive parasitological nor immunological result. The description of these two groups is presented in Table I.

**Blood and urine sampling**

Informed consent was obtained prior to collection of blood or urine by the medical team. Blood sampling was organized by venepuncture and serum was obtained by centrifugation. Aliquots of serum and urine were immediately frozen at −20°C for subsequent laboratory analysis.

**Extracellular matrix metabolites**

The urinary concentration of the type I collagen was measured by an ELISA test (CrossLaps™, Nordic Bioscience, Herlev, Denmark) specific for a 8-amino acid sequence found in the C-telopeptide of the a chain of type I collagen (Bonde et al., 1994). The molecules measured in this assay are derived from degradation of collagen I and not from newly synthesized collagen type I. The reference range in healthy adults was 80-330 µg/mmol creatinin, urinary creatinine being measured by the Jaffé procedure (Sigma Diagnostics, St-Louis, MO). PIIIP is released in the circulation during the synthesis of type III collagen via lymphatic vessels. PIIIP was analyzed by radioimmunoassay (PIIIP RIAgnost4, Schering lab, Gif-sur-Yvette, France) and varied between 0.3 and 0.8 U/mL in healthy controls. Circulating HA, a marker of connective tissue destruction, was measured in the serum by high-affinity radiometric

<table>
<thead>
<tr>
<th>Endemic controls &amp; patients groups</th>
<th>No.</th>
<th>Age (mean ± SD) in years</th>
<th>Sex (no. of males/number of females)</th>
<th>Inclusion criteria</th>
<th>Immunologic criteria</th>
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<tbody>
<tr>
<td><strong>Patients</strong></td>
<td></td>
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<tr>
<td>Fill. group</td>
<td>17</td>
<td>48.7 ± 18.3</td>
<td>8/9</td>
<td>ADL, Mf*</td>
<td>IgG+CA*</td>
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<td>EleP group</td>
<td>7</td>
<td>38.2 ± 9.8</td>
<td>4/3</td>
<td>Typical elephantiasis</td>
<td>IgG+CA±</td>
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<tr>
<td>Hyd group</td>
<td>4</td>
<td>35.4 ± 10.9</td>
<td>2/2</td>
<td>Hydrocele</td>
<td>IgG+CA±</td>
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<tr>
<td>Chyl. group</td>
<td>2</td>
<td>ND</td>
<td>1/1</td>
<td>Chyluria</td>
<td>IgG+CA±</td>
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<tr>
<td>Microbial lymphangitis</td>
<td>4</td>
<td>ND</td>
<td>2/2</td>
<td>Typical erysipela, MF = 0</td>
<td>IgG-CA-</td>
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<td>(no. of females)</td>
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IgG: ELISA-based identification of anti-Brugia malayi antigen specific antibodies; CA: circulating antigens identified by immunochromatography (ICT test) and controlled by Og4c3 ELISA; MF: microfilaremia detected by membrane filtration; ADL: acute adenolymphangitis; ASLO: anti-streptolysin O antibodies; ASDB: anti-streptodornase B antibodies; ND: not done. Results are expressed as mean values ± standard deviations.

**Table I.** – Clinical patients and endemic controls included in the fibrosis study.
SERUM AND URINE ECP AND EXP

Eosinophil granule proteins were measured by a polyclonal sandwich type ELISA as previously described (Reimert et al., 1991a, 1991b), with serum reference ranges of 0.3-51.4 ng/mL and 8-38.5 ng/mL for ECP and EPX, respectively (Leutscher et al., 2000; Tischendorf et al., 1996). For urine samples, the reference ranges were 0.2-678 ng/mL and 70-1800 ng/mL for ECP and EPX, respectively (Leutscher et al., 2000; Reimert et al., 1993).

STATISTICAL ANALYSIS

Data are presented as mean ± SD. Statistical significance of between groups difference was assessed by the non-parametric Mann-Whitney U-test. The relationship between two parameters was assessed by Spearman rank correlation coefficient. As usual, the significance level was defined as P < 0.05 and the statistical analysis performed with dedicated softwares (Statmed, Medical computer lab., Faculty of Medicine, Nancy, France and StartViewII, Abacus Concepts, Berkeley, CA). In addition, the potential relationship between circulating ECM metabolites or eosinophil markers and adhesion molecules (Esterre et al., 2005) was investigated in parallel.

RESULTS

CIRCULATING FIBROSIS MARKERS IN ENDEMIC CONTROLS

Mean circulating concentrations of the different fibrosis markers were in the normal range for the 22 endemic controls: 250.1 ± 148.8 µg/L, 0.95 ± 0.21 U/mL and 20.1 ± 12.1 µg/mL for collagen I, PIIIP and HA, respectively.

CIRCULATING FIBROSIS MARKERS IN FILARIAISIS PATIENTS

We did not observe any correlation between fibrosis markers levels and the infection status (low vs high parasitemia).

Collagen I urine levels in filarial (146.8 ± 74.1 ng/mL) and microbial lymphangitis (118.9 ± 69.2 ng/mL) patients were significantly decreased (t = 4.05, P = 0.0076 and t = 2.43, P = 0.022, respectively) by comparison with controls. The levels observed with chyluria (165.8 ± 24.2 ng/mL), hydrocoele (181.8 ± 132.9 ng/mL) and elephantiasis (155.8 ± 20.8 ng/mL) were not significantly different from the control values (Fig. 1). Surprisingly, the PIIIP values were significantly lower (t = 12.32, P < 0.001) for the hydrocoele group (0.60 ± 0.5 U/mL) than in the controls (0.95 ± 0.21 U/mL), the microbial (1.10 ± 0.3 U/mL) and the filarial (0.97 ± 0.22 U/mL) lymphangitis patients (Fig. 2). Unfortunately, elephantiasis samples were not available for this analysis. More interestingly, the HA values (Fig. 3) were significantly increased with the microbial (104.5 ± 92.1 µg/mL) and filarial (65.1 ± 36.0 µg/mL) lymphangitis groups by comparison with the controls (t = 3.94, P = 0.006 and t = 5.37, P < 0.001, respectively). The same significant increase (t = 6.77, P < 0.001) is observed with the elephantiasis group (139.1 ± 52.8 µg/mL), although the levels observed in the hydrocoele were not different from the control values (Fig. 3). As expected, it is interesting to note that the ‘elephantiasis’ levels were significantly increased by comparison with the ‘filarial lymphangitis’ and ‘hydrocoele’ levels (t = 3.42, P = 0.005 and t = 3.58, P = 0.009, respectively).

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As observed on urine taken during a follow-up of seven patients, the levels of collagen I slightly decrease (from 146.2 ± 91.4 µg/L to 133.9 ± 54.0 µg/L) six months after treatment with DEC (data not shown). Unfortunately the sampling was incomplete (n < 5) for PIIIP and HA.

ECP AND EXP IN CONTROLS

Mean serum concentrations of the two eosinophil molecules were in the normal range for the 22 endemic controls (32.2 ± 29.1 ng/ml and 30.5 ± 5.5 ng/mL, for ECP and EPX respectively). The urine levels were not measured in the control group. A positive (r = 0.958) and highly significant correlation (n = 22, P < 0.001), was observed between serum, but not urine, levels of ECP and eosinophilia (Y = – 0.06 + 0.084X) calculated in the haematology laboratory.

ECP AND EPX IN FILARIASIS PATIENTS

We did not observe any correlation between eosinophil molecules levels and the infection status (low vs high parasitemia). The serum levels for ECP (Fig. 4) and EPX for the acute filarial lymphangitis (42.9 ± 29.1 ng/mL and 33.3 ± 11.4 ng/mL, respectively), chyluria (6.6 ± 0.7 ng/mL and 40.1 ± 14.5 ng/mL, respectively), hydrocoele (23.6 ± 24.4 ng/mL and 28.3 ± 20.0 ng/mL, respectively) patients were not significantly different from the controls and microbial lymphangitis patients. The only interesting trends, although of borderline statistical significance, were observed with elephantiasis patients who had increased serum ECP levels (96.8 ± 88.2 ng/mL) by comparison with hydrocoele patients (see Fig. 4, t = 1.97, P = 0.070) and controls (t = 1.94, P = 0.071).

Urine ECP levels were slightly increased compared to normal values (as urine concentrations should be below 5 ng; see Reimert et al., 2000) for filarial lymphangitis (3.2 ± 5.6 ng/mL), chyluria (0.7 ± 8.3 ng/mL), hydrocoele (3.3 ± 11.1 ng/mL) and elephantiasis (1.75 ± 1.5 ng/mL). Its interesting to note that the microbial lymphangitis patients had also a normal level of serum ECP (69.9 ± 28.1 ng/mL). Urine ECP levels were within the normal range for chyluria (731.3 ± 238.9 ng/mL) and hydrocoele (861.4 ± 337.1 ng/mL), and slightly increased in filarial lymphangitis (2169 ± 383.3 ng/mL) and elephantiasis (2075 ± 1181.5 ng/mL) patients.

RELATIONSHIP BETWEEN LEVELS OF SOLUBLE MOLECULES

It seems appropriate to use regression analyses in order to compare the levels of the different fibrosis and eosinophil-associated proteins as previously done for adhesion proteins (Esterre et al., 1998, 2005). No relationship was found between serum PIIIP and urine collagen I levels. A positive relationship was observed between serum PIIIP and HA levels (r = 0.46, P = 0.0027, Y = – 82.5 + 125.0X) on 23 patients. More interestingly, there was a negative correlation (P < 0.001) between urine collagen I and serum HA levels (r = – 0.499, Y = 213.2 – 0.9X) observed on 41 couples. No relationship was found between serum and urine levels of ECP and EPX.

ASSOCIATION BETWEEN FIBROSIS- AND EOSINOPHIL-ASSOCIATED PROTEINS AND THE OTHER POTENTIAL MORBIDITY MARKERS

Correlations between levels of fibrosis- or eosinophil-associated proteins and other biochemical markers (adhesion and angiogenic factors) have been actively sought but did not revealed any significant result. A trend, although not statistically significant in our sample (r = – 0.22, n = 42, P = 0.165), was noticed between
ECP and VEGF levels (Esterre et al., 2005). The same was true \((r = 0.23, n = 36, P = 0.169)\) between ECP and HA levels.

**DISCUSSION**

This is, to the best of our knowledge, one of the few study comparing eosinophil-related (Tischendorf et al., 1996) and ECM markers (Fleming-Hubertz et al., 1997) with parasitological and clinical status in LF. Serum HA was considered as a potential marker of schistosomiasis-associated morbidity, as precisely quantified by ultrasonography (Ricard-Blum et al., 1999), and was well-correlated with the best immunological marker (sICAM-1: Esterre et al., 1998). Interestingly, the circulating levels were significantly increased in elephantiasis and ADL patients but also, at a lower level of significance, in microbial lymphangitis patients. Up to 90 % of HA is degraded through the lymphatics (Fraser et al., 1998), increased serum levels likely reflect tissue damage around the lymphatics harbouring the adult worms. It might constitute a potential marker of morbidity in this pathology, as previously indicated in a pioneering study in Tanzania (Fleming-Hubertz et al., 1997). An intriguing finding was that significant correlations were found between serum HA level and urine level of collagen I (negative correlation) or serum level of PIIP (positive correlation). Serum PIIP levels were significantly decreased in hydroceles but, unfortunately, not tested on elephantiasis patients. Urine collagen I levels were significantly decreased in ADL and microbial lymphangitis patients. The increase of serum, but not urine, ECP levels was at the limit of significance probably due to a limited sample.

Another potential morbidity marker is serum ECP. Indeed we observed an increase, unfortunately at the limit of significance probably due to a limited sample, of serum but not urine ECP levels in elephantiasis. This trend, also observed in African elephantiasis patients (Tischendorf et al., 1996), indicated a different pathogenetic mechanism for this chronic form of the disease (Freeman, 1998; Kumaraswami, 2000). However the precise pathogenesis of lymphatic disease in filariasis-endemic areas is not fully elucidated, particularly in long-standing mass chemoprophylaxis areas such Polynesia, the single exception being likely streptococcal-associated acute lymphangitis (Esterre et al., 2000). Correlations between levels of fibrosis or eosinophil-associated proteins and other biochemical markers (adhesion and angiogenic factors), studied in the companion paper (Esterre et al., 2005), did not revealed any significant result confirming that the granulomatous and fibrosis processes are independantly regulated.

**REFERENCES**


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