CIRCULATING FIBROSIS MARKERS, EOSINOPHIL CATIONIC PROTEIN AND EOSINOPHIL PROTEIN X IN PATIENTS WITH *WUCHERERIA BANCROFTI* INFECTION: ASSOCIATION WITH CLINICAL STATUS

ESTERRE P.*, PLICHART C.*, HUIN-BLONDEY M.O.**, NGUYEN L.N.**, HARTMANN D.***, GUERRET S.***, REIMERT C.M.**** & RICARD-BLUM S.*****

Summary:

We measured the concentrations of several circulating fibrosis markers (type I collagen I, 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 perilymphatic 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 parasitic diseases.

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

Résumé : MARQUEURS DE FIBROSE ET PROTÉINES DE L'ÉOSINOPHILE DANS LA FILARIOSE LYMPHATIQUE

Les dosages d'un certain nombre de marqueurs circulants de fibrose (collagène I, procollagène III, hyaluronane), déjà validés sur plusieurs fibroses d'origine parasitaire, et de protéines des granules de l'éosinophile (protéines ECP et EPX), impliquées dans l'immunité anti-larves, ont été réalisés chez des patients polynésiens présentant différents stades de filariose lymphatique (adénolymphangite aigue, chylurie, hydrocèle, éléphantiasis). Ces résultats ont été comparés avec ceux observés dans une lymphangite microbienne cliniquement très proche (érysipèle) et des contrôles endémiques. Deux marqueurs potentiels de morbidité, déjà validés en matière de bilharziose, ont été identifiés : le hyaluronane sérique et la protéine ECP ; le premier de manière significative aussi bien dans les lymphangites aiguës que dans les éléphantiasis chroniques.

MOTS CLÉS : filariose lymphatique, marqueurs de fibrose, protéines de l'éosinophile, Polynésie Française.

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 lymphodoema 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

* Immunology & ** Epidemiology Units, Institut de recherches médicales Malardé, Papeete 98713 Tahiti (French Polynesia); *** Pharmaceutic Faculty, Lyon, France; **** Danish Bilharziasis Laboratory, Charlottenlund, Denmark; ***** Institut de biologie et chimie des protéines, UMR 5086 CNRS-UCBL, Lyon, France.
Correspondence: Dr Philippe Esterre, Institut Pasteur de Guyane Française, 97306 Cayenne Cedex (French Guiana).
Tel.: 594 594 292612 – Fax: 594 594 318083.
E-mail: pesterre@pasteur-cayenne.fr

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 beetween 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 hydrocoele 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 (CrossLapsH™, 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

Endemic controls & patients groups	No.	Age (mean ± SD) in years	Sex (no. of males/ no. of females)	Inclusion criteria	Immunologic criteria
Endemic controls	22	29.9 ± 13.2	13/9	Resident, MF = 0, No disease	IgG-CA-
Patients					
FilL group	17	48.7 ± 18.3	8/9	ADL, Mf+	IgG+CA+
EleP group	7	38.2 ± 9.8	4/3	Typical elephantiasis	IgG+CA±
Hyd group	4	33.4 ± 10.9	2/2	Hydrocele	IgG+CA±
ChyL group	2	ND	1/1	Chyluria	IgG+CA±
Microbial lymphangitis	4	ND	2/2	Typical erysipela, MF = 0	IgG-CA- ASLO+ASDB+

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.

assay (HA test, Pharmacia Diagnostics, Les Ulis, France) and reference interval was 7-90 µg/Lin healthy adults.

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 refence 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 FILARIASIS 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), hydrocoeles (181.8 ± 132.9 ng/mL) and elephantiasis (153.8 ± 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 hydrocoeles group (0.60 ± 0.5 U/mL) than in the controls (0.95 ± 0.21 U/mL), the microbial (1.10 ±
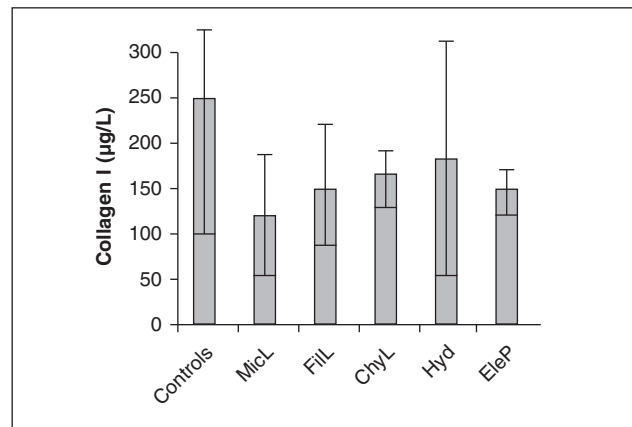

Fig. 1. – Collagen I levels, as determined by ELISA, in urine of patients with lymphatic filariasis according to their clinical status. FilL: filarial lymphangitis; ChyL: chyluria; Hyd: hydrocoele; EleP: elephantiasis, in comparison with controls and bacterial lymphangitis (MicL).
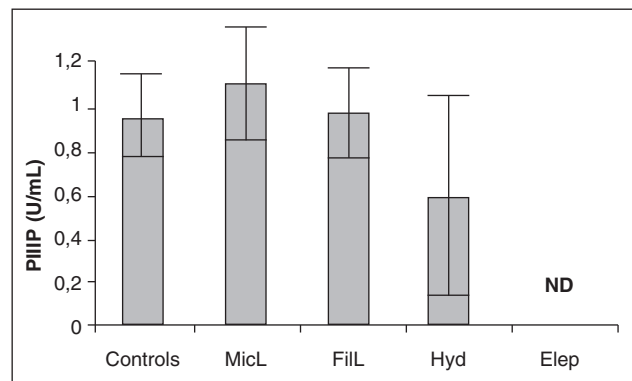

Fig. 2. – Serum levels of N-terminal propeptide of type III collagen, determined by RIA, in patients with lymphatic filariasis according to their clinical status.
FilL: filarial lymphangitis; ChyL: chyluria; Hyd: hydrocoele; EleP: elephantiasis, in comparison with controls and bacterial lymphangitis (MicL).

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 hydrocoeles 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 'hydrocoeles' levels (t = 3.42, P = 0.003 and t = 3.58, P = 0.009, respectively).
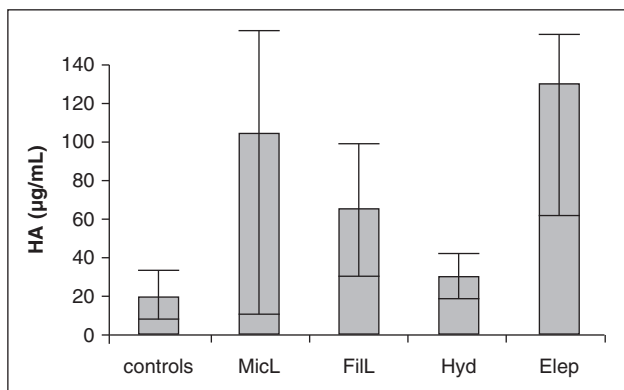
Fig. 3. – Serum levels of hyaluronan (HA), determined by radiometry, in patients with lymphatic filariasis according to their clinical status. FilL: filarial lymphangitis; ChyL: chyluria; Hyd: hydrocoele; EleP: elephantiasis, in comparison with controls and bacterial lymphangitis (MicL).
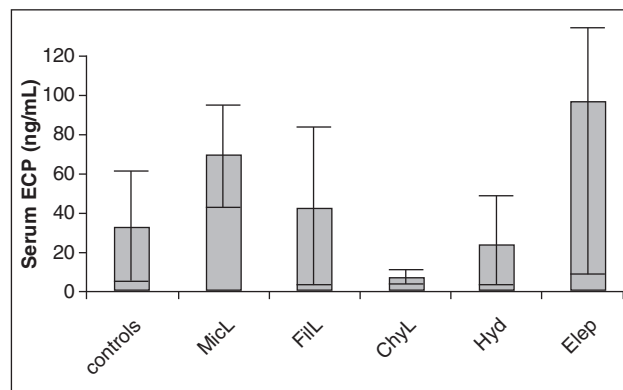

Fig. 4. – Eosinophil Cationic Protein (ECP) levels, determined by ELISA, in sera of patients with lymphatic filariasis according to their clinical status.
FilL: filarial lymphangitis; ChyL: chyluria; Hyd: hydrocoele; EleP: elephantiasis, in comparison with controls and bacterial lymphangitis (MicL).

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 ± 7.4 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 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 (9.7 ± 8.3 ng/mL), hydrocoeles (3.3 ± 1.1 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 EPX levels were within the normal range for chyluria (731.3 ± 238.9 ng/mL) and hydrocoeles (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 = 0.163), 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 potentiel 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 degradated 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 pionnering study in Tanzania (Fleming-Hubertz *et al.*, 1997). An intriguing finding was that significant correlations were found beetween serum HA level and urine level of collagen I (negative correlation) or serum level of PIIIP (positive correlation). Serum PIIIP 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.

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