RELATIONSHIPS BETWEEN SEVERAL MARKERS OF EXTRACELLULAR MATRIX TURN-OVER AND ULTRASONOGRAPHY IN HUMAN SCHISTOSOMIASIS MANSONI

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Abstract. We measured the concentrations of several serum and urinary fibrosis markers, which are metabolites of extracellular matrix, in schistosomiasis patients to investigate their relationship with the ultrasonographic scoring system and with parasitologic data. This study was conducted in patients with various stages of the disease evaluated by ultrasonography (intestinal disease with no organ involvement, with minor hepatosplenic involvement and with severe disease) and in endemic controls. The level of hyaluronan, which were increased in infected patients compared with controls ($P < 0.01$), was the only fibrosis marker that correlated with the ultrasonographic score ($P = 0.003$) and is thus a potential serum marker of schistosomiasis-associated morbidity. Urinary free pyridinoline levels were lower ($P < 0.001$) in infected patients with fibrosis (score $\geq 1$) than in nonfibrotic patients. A two-year follow-up of the patients treated with praziquantel showed that type I collagen and hyaluronan decreased during the first year post-treatment, whereas free pyridinolines peaked after 12 months and decreased thereafter.

With more than 200 million people affected worldwide and probably 500,000 deaths per year, schistosomiasis is the second major parasitic disease after malaria.1-3 Five species of digenic trematodes affect humans, with Schistosoma mansoni, S. japonicum, and S. haematobium being the most important from an epidemiologic point of view. Progress towards the control of schistosomiasis has been recently made, but the development of appropriate tools is still required for the assessment of worm burden and of specific morbidity during the monitoring of the effects of chemotherapy and/or vaccination.4-6

The recent application of ultrasonography in field conditions, including the Malagasy focus on which the present work is based, allowed the determination of a score that reflects S. mansoni-induced morbidity.7-8 The ultrasonographic scoring of schistosome-induced hepatic injury, including perportal pipe-stem fibrosis, correlates well with the pathologic evidence of fibrosis in liver biopsies and to a lesser degree with the intensity of infection.7-9 Although ultrasonography is a noninvasive technique, it is based on sophisticated tools, requires well-trained physicians, and its standardization is still a matter of debate after two World Health Organization (WHO)-sponsored workshops (Cairo, Egypt in 1990 and Niamey, Niger in 1996). More easy-to-use indicators of morbidity, such as the reagent strip test for hematuria in S. haematobium infection, are clearly needed to monitor S. mansoni and S. japonicum infections and their reversal after chemotherapy.10 This is particularly true for the planning and monitoring of control programs in areas of different endemicity, where public health decisions have to be made by existing basic health care services.10

Several markers reflecting the turn-over of extracellular matrix (ECM), have been reported for the monitoring of fibrosis in a noninvasive way.11-12 In the present study, conducted during a two-year follow-up of S. mansoni-infected patients treated with the anti-helminthic praziquantel, we measured the concentration of serum and urinary markers of fibrosis first to investigate their relationship with the score established by ultrasonography and then to monitor the effects of praziquantel treatment on their levels. Indeed, only a few studies reported changes in circulating ECM markers following chemotherapy and these were for short periods.13,14

The metabolism of the major liver collagen, type I collagen, was assessed by two immunoassays: one using an antibody directed against the fully processed molecule (CI) that detects mainly a degradation product of type I collagen in serum, and the other measuring the C-terminal cross-linked telopeptide (ICTP) that is released in serum as a degradation product of type I collagen.15-17 The synthesis of type III collagen was monitored by the measurement in serum of the whole molecule (CIII) and of the N-terminal propeptide of type III procollagen (PIIINP). The degradation of mature collagen was evaluated by the urinary excretion of free pyridinolines, which are cross-linked amino acids formed through post-translational modifications of collagen. The level of pyridinoline increases in the liver during fibrogenesis and is related to the degree of reversibility of the fibrotic process.18-21 In murine schistosomiasis, its urinary level is correlated to the duration of infection and to the collagen content of hepatic granulomas.22 We also measured the serum levels of laminin, the major noncollagenous glycoprotein of basement membranes which is correlated with portal hypertension, and ubiquitous glycosaminoglycan hyaluronan (HA) since its level correlates with the histologic stage of liver fibrosis in primary biliary cirrhosis and chronic viral hepatitis C and with collagen content in chronic hepatitis.23-25

This is, to our knowledge, the first study comparing ultrasonographic data and the circulating levels of ECM metabolites for the assessment of S. mansoni-induced morbidity in patients treated with praziquantel. In addition, a previous study of circulating adhesion molecules (soluble intercellular adhesion molecule-1 [ICAM-1] and various selectins) provided us the opportunity to investigate the relationship between the serum concentrations of these adhesion molecules and ECM metabolites.26

PATIENTS AND METHODS

Patients. The individuals included in this study were residents from the rural village of Belagera, Madagascar, an hyperendemic focus of schistosomiasis mansoni. The overall prevalence was 62%, with 34% of the individuals excreting more than 400 eggs/gram of feces and a 69% frequency of splenomegaly.2 According to parasitologic data (prevalence
and intensity of infection, we distinguished two groups of patients: those with a high egg count (> 800) and those with a low egg count (< 160). The patients were treated with praziquantel (every six months during two years) and followed for two years.8,26

The morbidity staging was evaluated by ultrasonography according to a modified classification first established by WHO.9 We used a scoring system from 0 (corresponding to the intestinal form of the disease in infected patients) to 3 (corresponding to severe hepatosplenic injury). About 30% of the inhabitants presented definite schistosomiasis-associated liver lesions.8,26 Endemic controls were inhabitants of the same village who were uninfected by S. mansoni (no eggs detected by two Kato-Katz tests) and who had an ultrasonographic score of 0. Nonendemic controls were members of the laboratory staff of the Institut de Pasteur de Madagascar who had never lived in areas endemic for schistosomiasis.26 All subjects were screened for the serologic detection of hepatitis B and C.27

Blood and urine sampling. The protocol for the entire study8,26 was reviewed and approved by the Ethical Committee of the Institut Pasteur de Madagascar and the Malagasy Ministry of Health (Antananarivo, Madagascar). Informed consent was obtained from each patient before blood collection.8 Blood samples were collected by venipuncture. The serum was separated by centrifugation after clotting. The serum and urine samples were stored at 4°C during the 3-hr transport from the villages to the laboratory and were then stored at −20°C until analysis.

Serum metabolites of extracellular matrix. The concentration of the C-terminal telopeptide of type I collagen was measured by equilibrium radioimmunoassay (Telopeptide ICTP 

\[ \text{ICP} \] radioimmunoassay kit; Orion Diagnostica, Espoo, Finland).17 The reference range in healthy adults was 1.31–4.31 μg/L for ICTP.28 Two radioimmunoassays developed in our laboratory were used to measure the concentration of CI and CII in serum.15,16 The upper values for the CI and CII assays were 200 μg/L and 40 μg/L, respectively, in healthy adults. The N-terminal propeptide of type III procollagen and laminin P1 (the major protease-resistant fragment of laminin) were also analyzed by radioimmunoassays (RIA-

\[ \text{PAP} \] PIIP and RIA-gnost®-laminin P1; CIS Bio International, Gif-sur-Yvette, France). The reference intervals were

\[ 0.3–0.8 \text{ U/ml for PIIPN and 1.04–1.64 U/ml for laminin. Serum HA was measured by a radiometric assay based on HA high-affinity binding proteins (HA test; Pharmacia Diagnostics, Les Ulis, France) and varied between 7 and 90 μg/L in healthy adults.} \]

Urinary excretion of free pyridinolines. Free pyridinolines (pyridinoline and its deoxy analog) in urine were measured by a competitive enzyme immunoassay (Pyrilinks; Metra Biosystems Inc, Palo Alto, CA). Urinary excretion was expressed as nM/nM of creatinine. Urinary creatinine was quantified by the Jaffé procedure29 using a reagent kit (Sigma Diagnostics, St. Louis, MO). The mean ± SD excretion of free pyridinolines was found to be 34.18 ± 15.76 nM/nM of creatinine in 28 healthy nonendemic controls.

Soluble adhesion molecules. Serum concentrations of soluble E- and soluble P-selectins were measured by ELISAs using commercially available kits (R & D Systems Europe, Abingdon, United Kingdom) as previously described.26

Statistical analysis. Data are presented as the mean ± SD. The statistical significance of the differences between two groups was assessed by the nonparametric Mann-Whitney U-test. The relationship between two parameters was assessed by the calculation of the Spearman rank correlation coefficient. The significance level was defined as \( P < 0.05 \) for all statistical tests. The statistical analysis was performed with a dedicated software (StatView II; Abacus Concepts, Berkeley, CA).

RESULTS

Extracellular matrix metabolites in S. mansoni-infected patients before chemotherapy. The serum metabolites of type I and III collagens (CI, ICTP, and CII) and laminin showed no significantly correlation with either the parasitological data or the fibrosis score established by ultrasonography in Malagasy patients infected with S. mansoni (Table 1), although the serum level of CI showed a progressive, but statistically nonsignificant increase as a function of the morbidity score evaluated by ultrasonography (Figure 1). In contrast, the concentration of HA in the serum of schistosomiasis patients was correlated with this score (\( r = 0.505, P = 0.003 \)).

When only parasitologic data were considered (i.e., in-

\[ \text{Table 1} \]

Mean ± SD levels of serum (ICTP, CI, CII, PIIINP, HA, and laminin) and urinary (PYDs) metabolites of extracellular matrix in schistosomiasis patients before chemotherapy

<table>
<thead>
<tr>
<th>Groups</th>
<th>ICTP (μg/L)</th>
<th>CI (μg/L)</th>
<th>CII (μg/L)</th>
<th>PIIINP (U/ml)</th>
<th>HA (μg/L)</th>
<th>Laminin (U/ml)</th>
<th>PYDs nM/nM of creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected (Parasitology +)</td>
<td>6.6 ± 13.5</td>
<td>158.7 ± 160.2</td>
<td>10.3 ± 13.4</td>
<td>1.04 ± 0.4</td>
<td>45.1 ± 63.5</td>
<td>1.64 ± 0.6</td>
<td>63.2 ± 43.0</td>
</tr>
<tr>
<td>Noninfected (Parasitology −)</td>
<td>5.3 ± 3.2</td>
<td>114.9 ± 55.6</td>
<td>9.2 ± 8.8</td>
<td>1.05 ± 0.3</td>
<td>43.6 ± 51.5</td>
<td>1.31 ± 0.3</td>
<td>66.5 ± 50.9</td>
</tr>
<tr>
<td>Severe fibrosis (score 2–3)</td>
<td>6.6 ± 16.8</td>
<td>174.1 ± 187.1</td>
<td>10.9 ± 14.3</td>
<td>0.9 ± 0.3</td>
<td>79.1 ± 81.9</td>
<td>1.54 ± 0.5</td>
<td>44.5 ± 27.2</td>
</tr>
<tr>
<td>Fibrosis (score ≥1)</td>
<td>5.9 ± 14.7</td>
<td>145.8 ± 152.5</td>
<td>10.4 ± 12.7</td>
<td>0.9 ± 0.2</td>
<td>58.4 ± 69.0</td>
<td>1.48 ± 0.5</td>
<td>44.2 ± 25.3</td>
</tr>
<tr>
<td>No fibrosis (score = 0)</td>
<td>6.3 ± 3.5</td>
<td>122.9 ± 57.4</td>
<td>8.8 ± 8.9</td>
<td>1.2 ± 0.4</td>
<td>25.0 ± 16.9</td>
<td>1.41 ± 0.4</td>
<td>72.1 ± 50.9</td>
</tr>
</tbody>
</table>

* ICTP = C-terminal cross-linked telopeptide; CI = fully processed molecule; CII = whole molecule; PIIINP = N-terminal propeptide of type III procollagen; HA = hyaluronan; PYDs = pyridinolines.
Affected versus noninfected), it appeared that the mean concentrations of serum markers were similar in infected patients and uninfected endemic controls, except for CI and laminin levels, which were somewhat lower in endemic controls than in infected patients (Table 1). The level of free pyridinolines was within the same range in the endemic noninfected controls and infected patients with no detectable liver injury (ultrasonographic score = 0). Both groups had a higher urinary excretion level of free pyridinolines than nonendemic Malagasy subjects (Figure 2). However, the mean level of pyridinolines was significantly lower (P < 0.001) in patients with fibrosis (score ≥ 1) than in patients showing no ultrasonographic sign of fibrosis (Table 1), although there was no difference between patients with mild fibrosis (score = 1) and those with severe fibrosis (score ≥ 2, Figure 2).

Extracellular matrix metabolites during the follow-up of *S. mansoni*-infected patients treated with praziquantel. No significant variation in ICTP, PIIINP, and CHI levels was found during the follow-up of infected patients either in all patients or the fibrotic patients. The mean serum levels of these markers were similar before treatment and after a 24-month follow-up period in the infected patients with or without fibrosis. We observed a trend towards a decrease, more pronounced during the first 12 months, in serum laminin levels at the end of the follow-up in the infected patients and those with fibrosis. In contrast, the mean ± SD level of CI collagen decreased by half in the infected group (220.00 ± 185.96 μg/L, n = 34 versus 109.47 ± 40.24, n = 19; P = 0.016) and in the subgroup of patients with fibrosis (245.00 ± 239.07, n = 19 versus 108.75 ± 39.89, n = 8; P = 0.136) during the first year of follow-up. No further variations were observed during the second year of follow-up, at the end of which the CI level was significantly less than the pretreatment levels in the infected patients (P = 0.012) and in patients with fibrosis (P = 0.026). Some variations in serum levels of CI occurred during the 2-year follow-up in patients without fibrosis, but they were not statistically significant. The concentrations of serum HA (Figure 3) remained higher in hepatosplenic patients than in intestinal patients during the follow-up period. However levels of HA decreased markedly during the first year of follow-up. In patients with fibrosis, the urinary excretion of free pyridinolines remained below the mean level found in patients without fibrosis. Pyridinolines peaked in both groups after 12 months of follow-up and decreased thereafter (Figure 4).

We failed to detect any significant variation in the serum or urinary ECM metabolites in the three patients who exhibited an increase in the ultrasonographic score despite the anti-parasitic treatment with praziquantel.

Relationships of ECM metabolites with the serum level of adhesion molecules. It was interesting to note that the levels of some serum ECM metabolites were correlated with the serum concentrations of two adhesion molecules (soluble ICAM-1 and E-selectin) measured in a parallel study that established the correlation of soluble ICAM-1 levels with the ultrasonographic score. 26 Indeed, we previously reported a positive correlation between the serum levels of HA and ICAM-1 (r = 0.677, P = 0.02) that were both correlated with disease severity evaluated by ultrasonography. 26 In the
present study, we found that the serum levels of PIIINP and E-selectin were significantly correlated ($r = 0.68, P = 0.016$; Figure 5), whereas the correlation between urinary free pyridinolines and serum L-selectin was of borderline statistical significance ($r = 0.43, P = 0.08$; Figure 6), which might be due to the limited size of the group of patients ($n = 20$).

**DISCUSSION**

We have compared the levels of several markers of liver fibrosis with ultrasonographic and parasitologic data in schistosomiasis patients living in a hyperendemic focus of *S. mansoni* to evaluate the dynamic process occurring in the course of the disease and during its treatment with praziquantel. Indeed, it is particularly important to detect the recurrence of liver injury and the poor efficiency of the antihelminthic drug to define the timing of drug administration in large-scale schistosomiasis control programs.

Serum HA was the only serum marker of those assayed in this study that was correlated with the ultrasonographic score of fibrosis. Thus, it is a potential serum marker of morbidity in human schistosomiasis and is likely associated with the extent of the granulomatous reaction in the liver. The lack of a correlation between urinary free pyridinolines and the ultrasonographic score for evaluating fibrosis was not unexpected since we did not find any difference in pyridinoline excretion levels between patients with portal fibrosis and those with bridging fibrosis in a study that included patients with alcoholic and viral liver diseases. Furthermore, it should be stressed that ultrasonography, although clearly adapted to field studies, does not reflect the kinetics of fibrogenesis, but rather a bulk lesion that may or may not be active, while urinary pyridinoline levels should reflect mostly collagen cross-linking activities and, at least in the murine model of schistosomiasis, the deposition of mature collagen in liver granulomas.

The mean levels of ECM metabolites were similar in schistosomiasis patients and endemic controls, except for CI and laminin, which were higher in infected patients. There was no significant change in PIIINP levels in infected subjects with intestinal schistosomiasis mansoni when compared with endemic controls. No patients showed an increase in serum PIIINP levels, regardless of the stage of the disease, although we reported in a previous study that the level of serum PIIINP was elevated in most (16 of 19) patients with an active stage of schistosomal liver fibrosis. This apparent discrepancy might arise from the fact that we included endemic controls in this study, whereas the previous study included the staff of a research institute located in an urban area. Similarly, another study reported a significant increase in PIIINP levels in schistosomiasis patients, even in those with an early active infection and no organ involvement, but it was not indicated if the controls were endemic or nonendemic ones. This point stressed the needs for appropriate controls in field studies since the basal serum and/or urinary values of fibrosis markers might differ significantly in endemic and nonendemic areas, as illustrated by the variations in pyridinoline levels. Indeed, the urinary level of free pyridinolines was higher in endemic controls than in nonendemic ones, and this might have been associated to the prevalence of viral hepatitis in endemic controls since viral hepatitis leads to an increase in urinary pyridinolines. However, this increased background due to infection by hepatitis B or C virus was not an interfering factor in the schistosomiasis patients because we found no relationship between a
positive serology for viral hepatitis B (nine patients) or C (four patients) and an increase in urinary pyridinoline in S. mansoni-infected subjects.

The serum levels of ICTP, laminin, CIII, and PIIINP did not change significantly during the follow-up period. A significant increase in serum PIIINP levels has been reported previously after praziquantel treatment, but it occurred early in the follow-up (four and eight weeks), whereas in our study we assayed the markers at six, 12, and 24 months.\textsuperscript{13,14}

The major changes in serum parameters during the first year of the follow-up of praziquantel chemotherapy are the decrease below pretreatment levels in the mean concentrations of CI and HA. The pronounced decrease in the HA level that occurs during the first 12 months of chemotherapy is consistent with that observed early in the follow-up of infected children and adolescents with active intestinal schistosomiasis.\textsuperscript{15} It likely reflects the improvement of hepatic lesions, as suggested by the correlation with the ultrasonographic score. Indeed, in patients with chronic hepatitis C receiving α-interferon therapy, serum HA levels decreased significantly in patients whose fibrosis improved and were correlated after treatment with the extent of fibrosis scored using the Knodell system.\textsuperscript{33,34} The moderate increase in HA levels observed between 12 and 24 months of follow-up in patients with fibrosis, as in those with no detectable liver injury, was not statistically significant.

The mean excretion of urinary pyridinolines increased during the first year of follow-up and decreased thereafter. This is consistent with our finding that the urinary level of pyridinoline is negatively correlated with the length of the follow-up after praziquantel treatment in murine schistosomiasis.\textsuperscript{22} Although the baseline level (i.e., before chemotherapy) of urinary pyridinolines in patients with no detectable liver injury could be considered normal when compared with that of endemic controls, we have further investigated this group because it served as an internal reference for the highly fibrotic patients. Indeed, despite praziquantel therapy, urinary pyridinoline level of patients with severe fibrosis did not reach that found in patients with no detectable fibrosis. This difference in the level of free pyridinolines suggests that the reversal of severe fibrosis is not complete, which may be explained by a less pronounced effect of praziquantel on fibrotic lesions composed of extensively cross-linked, and not easily degraded, collagen.

New insights into the biologic role of adhesion molecules in immunity against S. mansoni, including a significant role in egg granuloma formation have been recently provided.\textsuperscript{35,36} A previous hospital-based study on 41 patients from the French West Indies compared with nonendemic European controls showed a limited increase in soluble ICAM-1 levels that were not correlated with serology.\textsuperscript{37} Similar to the present analysis of fibrosis markers, we conducted a study of a large panel of circulating adhesion molecules (soluble ICAM-1 and various selectins) in the same groups of schistosomiasis patients.\textsuperscript{26} Our data confirmed the previously reported correlation of disease severity with sICAM-1, but not with the selectins.\textsuperscript{38} The correlation between PIIINP and soluble E-selectin levels was thus unexpected. In contrast, the relationship between HA and soluble ICAM-1 levels was not unexpected since both parameters were independently correlated with the ultrasonographic scoring system.\textsuperscript{26}

In conclusion, simple assays for ECM fibrosis markers could provide new tools for the assessment of morbidity in schistosomiasis mansoni and japonicum and for the evaluation of chemotherapy or vaccination effects. In this regard, serum HA levels, which are correlated with ultrasonographic data, might be used to identify high-risk patients for liver fibrosis. It will be also of particular interest to see how the ultrasonographic lesions regressed in patients with high and low levels of urinary pyridinolines. This will be useful for the long term follow-up of praziquantel efficiency in primary health care services that are in charge of schistosomiasis control in endemic countries.

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