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Serum 27E10 antigen: a new potential marker for staging HIV disease

N. LÜGERING, R. STOLL, T. KUCHARZIK, G. BURMEISTER†, C. SORG* & W. DOMSCHKE
Department of Medicine B and Institute of Experimental Dermatology, University of Münster, Münster, Germany, and †BMA Biomedicals AG, Augst, Switzerland*

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SUMMARY

MRP8 and MRP14 are myeloic related proteins expressed by most circulating and emigrated neutrophils and monocytes. Their composite molecule MRP8/14 (27E10 antigen) was shown to exhibit striking antimicrobial properties. The aim of the present study was to assess the value of MRPs as markers for detection of the different stages of HIV infection (Centres for Disease Control and Prevention, 1993). By employing the ELISA technique we measured serum concentrations of these proteins in samples from 122 HIV patients at the various stages of disease, and the results were compared with those for healthy controls. Serum levels of the heterodimeric molecule 27E10 were significantly increased ($P < 0.001$) in patients with CDC stages II and III, with the highest levels being in patients with stage III and acute ongoing opportunistic infections. For the single component MRP14, significantly raised levels ($P < 0.05$) were only found in HIV stage III individuals with acute clinical events. Similar associations were not found for MRP8 alone. Increase was not related to $CD4^+$ cell count. There was a significant correlation between 27E10 antigen serum concentrations and levels of neopterin in patients with HIV stages II and III without acute concurrent illness. Patients being treated with Zidovudine showed no statistically significant variation in levels of 27E10 and its single components MRP8 and MRP14 compared with untreated patients. These findings suggest that elevation of MRP14 levels occurs in HIV^+ individuals at later stages post-HIV infection, after the onset of opportunistic infections. 27E10 antigen is concluded to be a potential marker for the different stages of HIV disease.

Keywords MRP8 MRP14 27E10 neopterin Zidovudine HIV infection

INTRODUCTION

Monitoring of disease progression by serological markers in HIV-infected patients is important if therapeutic interventions are to be implemented and evaluated. Among the various cellular and serological markers investigated to date, the CD4 lymphocyte count is generally accepted as the mainstay for monitoring HIV disease [1–3], whereas the other indices are still controversial and not sufficiently characterized [4]. However, individual CD4 lymphocyte counts have been found to be highly variable over time, and considerable overlap in CD4 cell counts among the clinically defined stages of HIV infection has been found [1,5]. Thus, there is a need for other clinical markers [6,7], especially those that might be useful in monitoring antiviral treatment and in detection of opportunistic infections.

The two proteins MRP8 and MRP14 have recently been isolated and molecularly cloned [8,9]. Both proteins are expressed during myelomonocytic differentiation [10–12]. They belong to the steadily growing S-100 family of relatively small Ca^{2+} binding proteins [13]. Although the function of MRPs is largely unknown, their role in the regulation of cell growth and differentiation is the focus of great interest. Some of the proteins have been suggested as modulators of cell cycle progression, cell differentiation and cytoskeletal-membrane interactions [14]. MRP8 and MRP14 are present at high concentrations in granulocytes and monocytes, but are absent in lymphocytes [15]. As 27E10 complex was shown to exhibit striking antimicrobial activity at biological levels, it is also called calprotectin [16]. Other synonyms for MRP8/MRP14 are calgranulin A/B, cystic fibrosis antigen and L1 antigen light/heavy chain [9,17]. Recently, Bhardwaj *et al.* showed association of a MRP8/MRP14 heterodimer to the cell membrane by flow cytometric surface analysis which is recognized by the MoAb 27E10 [18]. Elevated plasma levels of 27E10 antigen can be found in different kinds of inflammatory diseases, such as cystic fibrosis [19,20] and inflammatory bowel disease [21].

In this study we measured serum levels of MRP8, MRP14 and their heterodimer 27E10 in HIV patients at the different

Correspondence: N. Lügering MD, Department of Medicine B, University of Münster, Albert-Schweitzer-Str. 33, D-48129 Münster, Germany.

stages of disease. Serum MRP levels of AIDS patients with different types of opportunistic infections were assessed at the acute stage of illness and compared with 'clinically stable AIDS patients'. We demonstrate for the first time that levels of 27E10 are markedly elevated in clinical stages II and III, those for MRP14 in HIV^+ stage III individuals after clinical onset of opportunistic infection.

PATIENTS AND METHODS

Patients and controls

The controls were 30 HIV^- laboratory workers and hospital personnel (CDC 0), matched with the patients for age and sex (mean age 37·32 years; range 22–56 years) and 38 HIV^- subjects (mean age 36·45 years; range 19–54 years) belonging to groups at risk for HIV infection (26 i.v. drug users (IVDUs) and 12 homosexual men). Among the IVDUs, 15 subjects had a virus C and/or B-related chronic hepatitis; four homosexual men had serological evidence of previous hepatitis virus B and/or C-infection. All of them had no serum antibodies to HIV as determined by enzyme immunoassay and confirmed by Western blot. Study participants ($n = 122$) were staged according to the Centres for Disease Control and Prevention (CDC) classification [22], which categorizes individuals on the basis of clinical conditions associated with HIV infection and $CD4^+$ T lymphocyte counts. It includes three main categories of patients: A (1-2-3), B (1-2-3) and C (1-2-3). Stage I included A1,A2 and B1, stage II A3,B2 and B3, and stage III C1,C2,C3. Fifteen patients belonged to class I (mean age 34·50 years; range 22–53 years; Karnofsky performance score 85 ± 11), 30 patients to class II (mean age 37·67 years; range 24–62 years; Karnofsky performance score 80 ± 14), and 77 to class III (mean age 22·10 years; range 18–51 years; Karnofsky performance score 60 ± 18). Of the last group, 36 patients had well defined acute clinical events at the time of this study. *Pneumocystis carinii* pneumonia ($n = 10$), cytomegalovirus retinitis ($n = 9$) cerebral toxoplasmosis ($n = 10$), and disseminated *Mycobacterium avium* infection ($n = 7$). Serum samples were obtained from April 1993 to December 1994. Patients were carefully examined in order to exclude the presence of other diseases such as diabetes mellitus and diseases of kidney, as well as active infection with hepatitis viruses, which can give rise to serum elevation of MRPs. Diabetes mellitus was excluded on the basis of results of an oral glucose tolerance test. Kidney disease and active infection with hepatitis viruses were excluded by means of serological examinations, including the determination of creatinine, hepatitis B virus marker (hepatitis surface antigen, hepatitis Be antigen), hepatitis C virus marker (hepatitis C RNA anti-HCV), and serum aspartate aminotransferase. None of the patients with CDC stage II had been treated with Zidovudine or dideoxyinosine (DDI) before blood was withdrawn, nor were any undergoing treatment with systemic medical agents. Forty patients with CDC stage III had been treated with Zidovudine.

ELISAs of MRP8, MRP14 and MRP/14

Sera were separated from clotted blood samples by centrifugation at 7000 *g* for 15 min after allowing them to settle for 4 h at 4°C. If not analysed on the same day, serum samples were stored at −20°C and were thawed only once.

Three different sandwich ELISAs were performed in order to determine different complexes of the MRP family according to the manufacturer's instructions (BMA Biomedicals AG, Augst, Switzerland). The basis of the test is the differentiation of three different complex-families of MRP molecules: MRP8 monomers and homopolymers, MRP14 monomers and homopolymers, and MRP8/MRP14 heterodimers and heterpolymers (G. Burmeister & G. Gallacchi, manuscript submitted). The pairs of MoAbs used in the three different assays are specific either for MRP8 or for MRP14. To make sure that heterocomplexes between MRP8 and MRP14 were determined, MoAb 27E10 was used as a captive antibody. The epitope of this antibody is generated by complex formation of both MRPs [18]. Maxi-Sorb Immunoplates (96-well; Nunc, Wiesbaden, Germany) were coated with affinity chromatography-purified MoAbs specific either for recombinant MRP8 or for recombinant MRP14 or specific for the heterocomplex of MRP8 and MRP14 in carbonate buffer pH 9·5, overnight at 4°C or for 1 h at 37°C. Unspecific binding of MRPs to the plate was blocked by 1% bovine serum albumin (BSA; Sigma A7030), 0·2% gelatine (BioRad, München, Germany) and 0·1% Tween 20 (BioRad) in PBS solution for 1 h at 37°C. Washing was carried out between each different incubation with PBS pH 7·2 containing 0·1% Tween 20. Recombination MRP8, MRP14 and reconstituted recombinant MRP8 and MRP14 spontaneously forming MRP8/14 heterocomplexes were used as standards and incubated in serial dilutions from 1000 to 0·98 ng/ml on the appropriate plates for 1 h at 37°C. The corresponding biotinylated monoclonals were incubated for 1 h at 37°C. Further incubation of Extravidin HRPO (Sigma, E-2886) was carried out for 30 min at 37°C. After adding 2.2′ azino-di-(3-ethylbenzthiazoline-6-sulfonate) dissolved (10 mg/25 ml) in citrate-phosphate buffer pH 4·0 and addition of 10 ml H_2O_2 as substrate for the peroxidase, the assay could be measured after 20 min at room temperature in an ELISA reader for 96-well plates. The results of the test samples were calculated by standard curves. The detection ranges of the assays were: 4–125 ng/ml for MRP8, 4–62·5 ng/ml for MRP14, and 15·6–250 ng/ml for the MRP8/14 heterocomplexes. Most of the test samples had to be diluted as follows to fall within the detection ranges: MRP8 1/5–1/10, MRP14 1/10–1/50, MRP8/14 heterocomplexes 1/100–1/1000 or greater.

Measurement of other immunologic and virologic parameters

Blood was also taken for measurement of $CD4^+$ lymphocyte count, thrombo count, aspartate aminotransferase, lactate dehydrogenase concentration and neopterin. $CD4^+$ lymphocytes were counted by direct and indirect immunofluorescence, using MoAbs and flow cytometry. Serum levels of neopterin were determined by a commercially available radioimmunoassay (RIA; IMMUtest Neopterin, Henning Berlin GMBH, Berlin, Germany) following manufacturer's recommendations. The limit for detection was 1·23 nmol/l neopterin.

Statistical analysis

Wilcoxon's two-sample test and Kruskal–Wallis test combined with the Nemenyi test were used for comparison between groups. Correlation coefficients were calculated by Spearman's rank correlation test.

RESULTS

Serum levels of MRP8, MRP14 and 27E10

27E10 antigen was detectable in the sera of all subjects. The lowest levels were found in the healthy HIV$^-$ controls (CDC 0). 27E10 was significantly increased in patients with HIV stages II (A3,B2,B3) and III (C1,C2,C3), with the highest concentrations being in patients with stage III (Fig 1). Serum concentrations of 27E10 were 2300 ± 600 ng/ml for healthy controls (CDC 0), 3200 ± 500 ng/ml for patients with HIV stage I (A1,A2,B1), 8900 ± 3200 ng/ml for patients with stage II ($P < 0.001$ versus CDC 0 and stage I) and 25 600 ± 6900 ng/ml in patients with stage III ($P < 0.001$ versus CDC 0, stage I and stage II). Mean MRP8/14 levels in HIV$^-$ i.v. drug users were also elevated (4520 ± 800 ng/ml), reaching no statistical significance. Homosexual men showed normal concentrations of the heterodimeric protein compared with HIV$^-$ healthy controls (2250 ± 610 ng/ml). In all groups, no difference was found for MRP14 and MRP8 alone, respectively. There was no correlation to counts of CD4$^+$ lymphocytes, thrombocytes, leucocytes and lactate dehydrogenase concentration (data not shown).

As the patients with stage II in this study were not receiving systemic agents, we exclude the possibility that the elevated 27E10 concentrations in sera from HIV-infected individuals were due to medication.

Serum levels of neopterin

Serum concentrations of neopterin were 6·4 ± 2·3 nM for the healthy controls, 7·1 ± 3·4 nM for patients with HIV stage I, 21·3 ± 8·6 nM for patients with stage II ($P < 0.05$ *versus* CDC 0 and stage I), and 35·8 ± 10·3 nM for patients with stage III ($P < 0.001$ *versus* CDC 0, stage I and stage II). AIDS patients who suffered from acute clinical events at the time of this study, had neopterin levels similar to the remaining AIDS patients. When comparing serum levels of neopterin and MRP8/14, a significant correlation was found in patients with HIV CDC stages II and III without acute ongoing opportunistic infections. Correlation data for neopterin and 27E10 were as follows: $r = 0.62$; $P < 0.05$ for stage II; $r = 0.068$; $P < 0.001$ for stage III without acute clinical events.

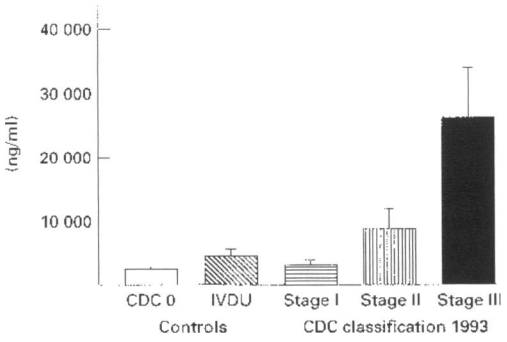

Fig. 1. 27E10 antigen serum levels in sera of 122 HIV-infected individuals, in 26 intravenous drug users (IVDUs), and in 30 healthy controls (CDC 0). Patients were grouped according to the new CDC classification 1993: A(1-2-3), B(1-2-3) and C(1-2-3). Stage I included A1, A2 and B1 ($n = 15$), stage II A3, B2 and B3 ($n = 30$), and stage III C1, C2 and C3 ($n = 77$). Means ± s.d. are given in all cases.

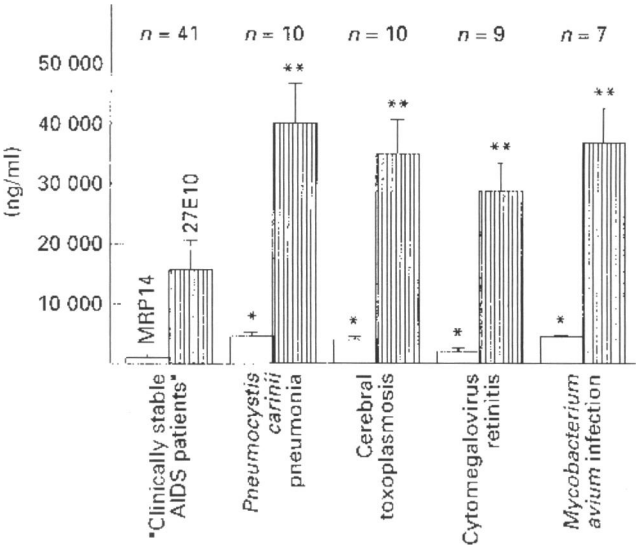

Fig. 2. MRP14 and 27E10 antigen serum levels in 'clinically stable AIDS patients' (patients without acute ongoing AIDS-defining events) and in sera of AIDS patients with acute ongoing secondary opportunistic infections. Means ± s.d. are given. Statistical significance of AIDS patients with acute ongoing secondary opportunistic infections *versus* 'clinically stable AIDS patients': *$P < 0.05$; **$P < 0.0001$.

Relationship between serum levels of MRPs and clinical parameters

Thirty-six patients with stage III had well defined acute clinical events at the time of the study. *P. carinii* pneumonia ($n = 10$), cerebral toxoplasmosis ($n = 10$), disseminated *Myco. avium* infection ($n = 7$), and cytomegalovirus retinitis ($n = 9$). These patients with ongoing opportunistic infections had significantly ($P < 0.05$) raised serum levels of MRP14 (except patients with cytomegalovirus retinitis) and the heterodimer 27E10 ($P < 0.0001$) compared with AIDS patients without acute concurrent illness at the time of this study (Fig. 2). As shown in four representative patients (Fig. 3), serum 27E10 levels increased after the start of acute clinical events.

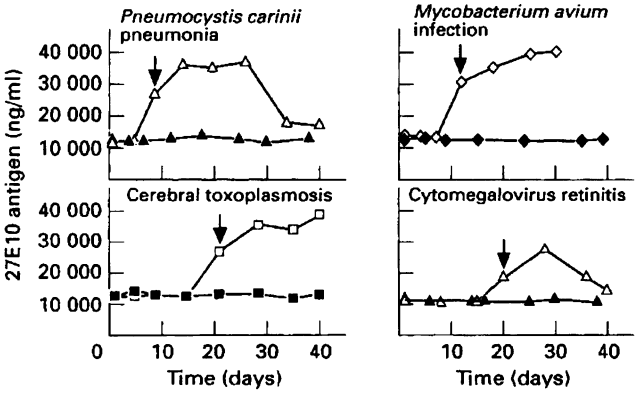

Fig. 3. Longitudinal study of 27E10 antigen serum levels in four representative patients who did actually show (open symbols) or did not show (but had history of the corresponding opportunistic infections) evidence of acute ongoing concurrent illness (closed symbols). The arrow indicates the start of acute clinical event.

Table 1. Serum concentrations of MRP8, MRP14 and 27E10 (ng/ml) antigen in HIV patients with CDC stage III in relation to Zidovudine therapy (mean ± s.d.)

	n	MRP8	MRP14	27E10
CDC stage III without acute AIDS-defining events				
Total	41	29 ± 11	1400 ± 570	15 500 ± 5900
+Zidovudine	19	36 ± 13	1250 ± 390	13 500 ± 4100
−Zidovudine	22	20 ± 8	1690 ± 700	17 100 ± 7100
CDC stage III with acute AIDS-defining events				
Total	36	39 ± 20	3390 ± 900	35 600 ± 8000
+Zidovudine	21	27 ± 17	2600 ± 780	32 200 ± 6500
−Zidovudine	15	48 ± 24	3900 ± 980	37 100 ± 9700

Serum correlation of MRPs in relation to Zidovudine therapy

As Zidovudine therapy might have an important impact on the synthesis of MRPs, we compared mean MRP concentrations in patients at the different stages of HIV infection with and without Zidovudine therapy. As shown in Table 1, there were no statistically significant differences in the different patient groups.

DISCUSSION

This study reports that there are markedly raised serum levels of the composite molecule 27E10 in HIV^+ stage II and III individuals, being significantly higher in stage III than in stage II. Therefore, we suggest that this antigen is a useful parameter of the disease, and can be utilized to provide more precise assessment of the immune status.

In the study, we examined HIV^+ stage III individuals who were asymptomatic ('clinically stable patients with AIDS') or symptomatic with regard to secondary opportunistic infection. Serum levels of 27E10 antigen were markedly elevated in both groups, with the highest concentrations being in AIDS patients with ongoing opportunistic infections. For MRP14 alone, significantly raised levels were only found in HIV^+ stage III individuals with acute clinical events. No significant correlation to $CD4^+$ lymphocyte counts was found. Thus, the high secretion of the single component MRP14 seems not merely to reflect progressive immunodeficiency, but to be associated with ongoing opportunistic infections and other clinical events characteristic of HIV infection. Whether measurement of MRP14 is superior to MRP8/14 in monitoring opportunistic infections in patients with HIV stage III remains to be determined in longitudinal studies.

The mechanisms leading to the high secretion of these proteins in HIV disease have yet to be elucidated. A coherent model of disease development has not yet been established. Mononuclear phagocytes and lymphocytes have been shown to be the cellular targets for the HIV virus [23,24], and high secretion of 27E10 may therefore be a direct or indirect consequence of the infection of blood cells. Hence the increased secretion may be a consequence of increasing viral abundance. Since the elevated levels of 27E10 antigen are not accompanied by an elevation of lactic dehydrogenase and aspartate aminotransferase, gross cellular injury appears not to be the cause of the abundant occurrence of this protein in HIV patients. Nevertheless, at present we can only speculate about modulation of MRP trafficking by the HIV virus.

In this study we were able to assess simultaneously the potential value of other laboratory markers, such as neopterin and $CD4^+$ lymphocyte count, which have been found to correlate with disease progression [1,4]. Serum levels of neopterin were elevated in patients with HIV stages II and III. Our results showed a significant correlation between serum levels of 27E10 and neopterin in patients with HIV stages II and III without acute ongoing opportunistic infections, and we failed to show any correlation between 27E10 antigen and number of $CD4^+$ cells.

The heterodimer 27E10 is a fascinating molecule with strikingly abundant expression in neutrophils, monocytes, certain macrophages and mucosal and squamous epithelia [10,11]. This might hint at an innate defence function of this protein. It was, therefore, of considerable interest that Steinbakk *et al.* described *in vitro* an antimicrobial action of the MRP8 and MRP14 containing L1-protein complex [16]. On the other hand, recombinant monomers of MRP8 and MRP14 did not exhibit antimicrobial activity at all, indicating that the biological function of these proteins is dependent on the complexed form. Similar results were subsequently reported by Sohnle *et al.* [25]. According to the high serum levels of the heterodimer MRP8/MRP14 with enhancement of the single monomer MRP14 in patients with HIV CDC III and acute concurrent illness, both complexes of these proteins and the single component MRP14 may be carriers of biological functions in HIV disease.

As investigated in the present study, we also found enhanced 27E10 serum levels in a proportion of HIV^- individuals at risk, possibly caused by a non-HIV-1 virally induced cell activation. Indeed, viral infections frequently occur in subjects belonging to categories at risk for HIV infection [26].

This is the first report of serological analysis of MRP8, MRP14 and their heterodimeric complex 27E10 in patients with HIV disease. Activation of the MRP system seems to be associated with both different clinical stages and secondary opportunistic infections of HIV infection, thus emphasizing the possible usefulness of repeated assessment of MRP levels over time for monitoring HIV-infected patients. A major challenge for the future will be to elucidate the pathophysiologic mechanisms involved in the release and function of the 27E10 antigen and its single components MRP8 and MRP14 in HIV-induced disease.

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