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Kidney International, Vol. 64 (2003), pp. 1817–1821

Urinary neopterin concentrations in patients with Balkan endemic nephropathy (BEN)

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Background. Balkan endemic nephropathy (BEN) is of great clinical importance in restricted areas of Bulgaria, Serbia, Croatia, Bosnia, Herzegovina, and Romania, since the etiology of BEN is still unknown.

Methods. In urine samples from 48 patients (41 females and 7 males, aged 65.6 ± 6.87 years) with BEN living in an endemic area of Vratza district, Bulgaria, neopterin concentrations were measured by high-pressure liquid chromatography (HPLC) and compared with other clinical and laboratory investigations, including creatinine, hemoglobin, and erythrocyte sedimentation rates (ESRs).

Results. Urinary neopterin concentrations were 263 ± 128 (mean ± SD; range, 78 to 786 μmol/mol creatinine), 24 (50%) of BEN patients presented with increased concentrations as compared to the established normal ranges. Average ESRs were increased (1 hour, 29.0 ± 14.7 mm/hour) and hemoglobin was decreased (109.3 ± 16.4 g/L). Hemoglobin correlated inversely with ESRs (rs = −0.787 and –0.780) and creatinine concentrations ($r = -0.690$, all $P < 0.001$), but not with neopterin concentrations. Neopterin concentrations also did not correlate with serum creatinine levels. There existed an age relationship of ESR, creatinine, and hemoglobin, but not of neopterin. Neopterin concentrations were slightly lower in five females with low titers of antibodies against local B1 hantavirus strain ($P < 0.05$).

Conclusion. The findings can support an immune-mediated inflammatory process in the pathogenesis of BEN only in a subgroup of patients.

Balkan endemic nephropathy (BEN) is of great clinical importance in restricted areas of the Balkany. In these countries, BEN, a chronic tubulointerstitial kidney disease, is regarded as a national problem of majority [1]. The onset is in childhood with a latent period of several decades and it is clinically expressed usually only at an advanced stage of renal failure. A female predominance was established. BEN is characterized by anemia, absence of hypertension, gradually destroyed renal function, a low-degree proteinuria and poor urinary sediment. The pathologic process progresses to intense fibrosis and tubular atrophy, and at the end stage of the disease, renal failure develops. About 30% to 48% of BEN patients develop transitional-cell tumors of the upper urothelium. Tumors are usually malignant, multiple, and two thirds have bilateral localization in the renal pelvis, one third in the urethra and in the bladder [2].

The etiology of BEN is unknown. Different hypotheses try to explain the striking endemic distribution of the disease with an etiologic role of genetic factors, exogenous factors (i.e., heavy metals, fungal toxins as ochratoxin A, inorganic compounds), or infectious agents [3–11].

Concepts of the possible role of hereditary factors and infectious agents as well as predisposition to tumor development suggest an involvement of immune mechanisms in the pathogenesis of the disease.

In this study, urine samples of BEN patients were collected and tested for the concentration of neopterin. Neopterin is produced in large amounts by monocyte-derived macrophages upon activation with Th1-type cytokine interferon-γ, and increased neopterin concentrations are characteristic for cellular immune activation [12, 13].

METHODS

Five endemic villages from Vratza district (Gorno Peshtene, Tishevitza, Vesletz, Beli Izvor, and Pudria), all in Bulgaria, were screened for BEN. First-morning urine samples from 48 patients (41 women and seven men; age, 65.5 ± 6.9 years; range, 52 to 79 years) with

Key words: Balkan endemic nephropathy, neopterin, urinary.

Received for publication January 24, 2003
and in revised form March 21, 2003, and June 20, 2003
Accepted for publication July 2, 2003

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BEN living in the endemic area were collected. Specimens were kept at –20°C until measured.

Clinical investigations comprised blood tests [hemoglobin concentrations, red blood cells, leukocytes, and erythrocyte sedimentation rate (ESR), blood urea, creatinine, and uric acid] and urine analyses (pH, protein, glucose, bilirubin, urobilinogene, blood, nitrite, specific urine weight, ketons, and leukocytes). None of the patients showed symptomatology of any acute viral infection.

Neopterin and creatinine concentrations in urine samples were determined by high-pressure liquid chromatography (HPLC) [12]. Briefly 100 mL of urine was diluted in 1 mL 15 mmol/L Soerensen potassium phosphate buffer, pH 6.4 containing 5 mmol/L ethylenediaminetetraacetic acid (EDTA). Neopterin and creatinine were separated by reversed-phase HPLC and expressed as μmol neopterin per mol creatinine ratio to take into account variations of urine dilution [12]. As stationary phase, a LiChroCART 125-4 column (RP-18 endcapped, grain size 5 μm) (Merck, Darmstadt, Germany) was used, the mobile phase was a 15 mmol/L potassium phosphate buffer, pH 6.4 (Soerensen). Neopterin was quantified by its natural fluorescence (excitation at 353 nm wavelength; emission at 438 nm wavelength) and creatinine was simultaneously quantified by ultraviolet absorption at 235 nm wavelength in one single run [12]. HPLC measurements were performed in subsequent runs within 1 day. Intra-assay variation of the HPLC method is ≤6% [12].

Preliminary virus analyses could be performed only in a subgroup of 34 individuals. By indirect immunofluorescence, antibodies against nephrotopic viruses with endemic areas of circulation [e.g., hantaviruses (hantavirus strains, standard Hantaan and F3955, local B1)] and West Nile flavivirus virus were analyzed.

Because not all laboratory results showed normal distribution, for comparisons of groups nonparametric Mann Whitney U-test was employed. Associations between neopterin concentrations, age, and other laboratory results of patients were calculated by Spearman rank correlation analysis.

RESULTS

Demographic data and neopterin concentrations in the individual urine samples are shown in Table 1. Increased neopterin concentrations were observed in 50% of the BEN patients, as compared to the 95th percentile of healthy controls [12] (Table 1). Mean neopterin concentration was 263.1 μmol/mol creatinine (see Table 2), which exceeds the upper limits of normal for men and women of all age groups. Elevated ESRs, as well as decreased hemoglobin concentrations, were also evident in the patients (Table 2).

There was no difference of neopterin concentrations between men and women, and there existed no correlations between neopterin and age and also not to other available laboratory parameters (Table 3). Creatinine concentrations correlated positively with ESRs and inversely with hemoglobin levels. In addition, lower hemoglobin levels were associated with higher ESRs (Table 2). When splitting the patients into two groups at the median creatinine concentration (=132 μmol/L), the group with higher creatinine concentrations presented with lower hemoglobin (mean ± SD, 101.5 ± 15.7 g/L; $P < 0.001$) and higher ESRs (ESR 1 hour, 33.8 ± 16.0 mm/hour; $P = 0.01$) as compared with the group with lower creatinine (hemoglobin, 118.9 ± 10.6 g/L; ESR 1 hour, 22.9 ± 10.4 mm/hour). No such differences were found for age and urinary neopterin concentrations. Of the two patients on hemodialysis, one presented with the highest (=786 μmol neopterin/mol creatinine), the other with the lowest neopterin concentration (=78 μmol neopterin/mol creatinine) of all patients. Age correlated only weakly and positively with creatinine and ESRs and negatively with hemoglobin, and when patients were split in two groups at the median of their age (=65 years), there was no difference between any of the parameters (all $P > 0.05$).

There was no difference between any of the measured parameters in cases with and without BEN family history. BEN patients with diagnosed malignancy were older (73.0 ± 3.67 years; $N = 5$) than those without such diagnosis (64.6 ± 6.63 years, $P < 0.01$), but again, none of the laboratory parameters differed between the two groups of BEN patients. All the results of statistical analyses did not change when two patients on hemodialysis were excluded.

Preliminary data in a subgroup of 34 patients revealed comparatively low levels of antibodies against hantavirus strains (standard Hantaan and F3955, local B1) in nine patients (27%) (Table 4). Besides, in two cases (two out of 34) (6%) anti-West Nile virus antibodies have been recorded (Table 4). There was no association between the serologic findings and any of the laboratory measurements with the exception of neopterin: neopterin concentrations were lower in the 5 females seropositive for local hantavirus strain B1 (168.2 + 66.1 μmol neopterin/mol creatinine) compared to residual seronegative BEN patients (279.3 + 144.3 μmol neopterin/mol creatinine; $U = 1.98$, $P < 0.05$).

DISCUSSION

The monitoring of neopterin concentrations in body fluids is a sensitive way to detect Th1-type immune response [12, 13] initiated by various causes. Measurements of neopterin concentrations in urine, serum or cerebrospinal fluid are employed as a laboratory diagnostic tool (e.g., to earlier detect immunologic complica-

Table 1. Demographic characteristics and neopterin concentrations in urine samples of Balkan endemic nephropathy (BEN) patients

Patient	Gender	Age *years*	Diagnosis	Creatinine *μmol/L*	Neopterin *μmol/mol creatinine*
8	F	71	CRI, family history	187	190
9	F	65	CRI (hemodialysis)	1243	78
16	F	75	Primary RT, nephrectomy (right)	175	252
44	F	62		66	259
69	F	76	Family history	150	388
70	F	74	Family history	142	314
108	F	57	Family history	126	220
139	F	64	Family history	118	162
162	M	62	CRI	240	212
187	F	69	Family history	134	255
192	F	67	Obstructive RT, hematuria	119	302
207	F	61	Family history	106	348
212	F	76	Primary bladder tumor, cystostoma	147	186
236	F	57	Family history	140	171
253	F	61	Family history (sister of 306)	141	379
306	F	65	Family history (sister of 253)	132	350
419	F	72	Primary RT, nephrectomy (left)	87	426
464	M	52		95	163
546	F	61	Family history (sister of 20)	136	200
563	F	67	Family history (sister of 19)	126	166
620	F	55		136	252
638	M	75	RT (right)	612	205
640	F	66		136	250
660	F	60		116	143
675	F	63		135	205
686	F	78	Family history	124	547
695	F	58	Family history	100	174
720	F	62		89	203
824	F	58	Family history (two brothers dead)	106	176
826	M	69		128	242
833	F	59		86	396
848	F	67	Family history (two brothers dead)	132	469
929	F	69	Family history (sister of 930)	130	262
930	M	65	Family history (brother of 929)	108	412
935	F	77		79	148
959	F	59	Family history	136	490
968	F	62	Family history	128	248
969	F	61	Family history (parents dead)	156	292
970	F	75	Anemia	420	214
1047	F	69	Family history (husband dead)	138	204
1059	F	69		126	164
1069	F	79		182	235
1087	F	69		144	144
1130	F	58		120	231
1164	M	68		116	215
1168	F	57	Family history (parents dead)	156	130
1254	M	66	Family history, CRI	Hemodialysis	786
1325	F	56		96	171

Abbreviations are: CRI, chronic renal insufficiency; RT, renal tumor. Age-related upper limits of normal, 95th percentiles in healthy individuals [12]: females 46 to 55 years, 229; 56 to 65 years, 249; >65 years, 251; males 46 to 55 years, 197; 56 to 65 years, 218; >65 years, 229 (all concentrations in μmol neopterin/mol creatinine).

Table 2. Neopterin concentrations and erythrocyte sedimentation rates (ESR) in 48 patients with Balkan endemic nephropathy (BEN)

	Mean	SD	Range
Urinary neopterin *μmol/mol creatinine*	263	128	78–786
Hemoglobin *g/L*	109	16.4	55–140
ESR			
1 hour	29.0	14.7	7–68
2 hours	52.7	25.2	15–110
Creatinine *μmol/L*	169	182	66–1243

tions in transplant recipients or to predict prognosis in HIV infection and malignancy) [12]. Neopterin concentrations are also increased with high frequency during acute virus infections [12, 14]. Neopterin concentrations were found to be increased during episodes of viral infection, including acute hepatitis A and B [15], Epstein-Barr virus (mononucleosis), cytomegalovirus [14, 16], and measles [17]. In cytomegalovirus infections [16], increased neopterin concentrations are observed in the majority of patients before antibody seroconversion becomes detectable. Usually neopterin concentrations nor-

Table 3. Associations between age and laboratory characteristics of patients with Balkan endemic nephropathy (BEN)

	Hemoglobin	ESR (1 hour)	ESR (2 hours)	Creatinine	Age
Neopterin	−0.032 NS	0.226 NS	0.221 NS	−0.055 NS	0.174 NS
Hemoglobin		−0.787 <0.001	−0.780 <0.001	−0.690 <0.001	−0.261 <0.05
ESR (1 hour)			0.972 <0.001	0.487 <0.001	0.313 <0.05
ESR (2 hours)				0.476 <0.001	0.339 0.01
Creatinine					0.316 0.02

r and *P* values in brackets are shown; NS is not significant.

Table 4. Antibodies against West Nile virus (WNV) and hantavirus strains in serum samples of 34 patients with Balkan endemic nephropathy (BEN) (indirect immunofluorescence test)

	Antibodies (reciprocal titer values) against			
Patient	WNV	Egypt	Hantavirus Hantaan	F3955 B1
8	—	—	—	10
9	—	—	—	10
16	—	—	—	—
44	—	—	—	10
69	20	—	—	—
70	—	—	—	—
546	—	—	10	—
563	—	—	—	—
620	—	—	—	—
638	—	—	—	—
640	—	—	10	—
660	—	40	80	80
675	—	—	—	—
686	—	—	—	—
695	—	—	—	—
720	—	—	—	—
824	—	—	—	—
826	—	—	—	—
833	—	—	—	—
848	—	—	—	—
929	—	—	—	—
930	—	—	—	—
935	—	—	—	—
959	—	—	—	—
968	—	—	10	—
969	—	—	—	—
970	—	—	—	—
1047	—	—	—	—
1059	—	—	—	—
1087	—	—	—	—
1130	—	20	20	—
1164	—	80	—	—
1254	—	—	—	—
1325	20	—	—	20

malize after seroconversion and when clinical symptomatology has ceased. However, elevated serum and urine neopterin concentrations levels cannot only be detected in acute and late stage of HIV infection, they are also found in more than 75% of patients with asymptomatic HIV-1 infection [18, 19].

Renal impairment may increase blood neopterin concentrations [18]. Therefore, we decided to determine urinary neopterin concentrations expressed as the neopterin per creatinine ratio. Data of this study show that there was no relationship between serum creatinine and urine neopterin concentrations when expressed as μmol neopterin/mol creatinine. The lacking association between creatinine and urinary neopterin concentrations is further strengthened by the observation that patients on hemodialysis ($N = 2$) presented, on the one hand, with the lowest and, on the other, with the highest neopterin concentrations of the study population.

Because, among other reasons, viral infection is discussed as a possible cause for BEN [8–11], it was of interest to examine neopterin concentrations in urine samples from BEN patients. However, only 50% of BEN patients presented with elevated neopterin concentrations compared to healthy controls. There was no difference between BEN patients with and without family history. Survival of BEN cases with family history was found longer when compared with BEN cases without family history [20]. Again, these subgroups in our study did not differ regarding their neopterin concentrations. Only a few patients tested weakly positive for antibodies against hantavirus strains or anti-West Nile virus. Five females seropositive for local hantavirus strain B1 had lower neopterin concentrations than seronegative BEN patients. The number of patients tested was limited and the titers measured were low, still the result would fit to the earlier observation that higher neopterin concentrations, indicating cellular (=Th1-type) immune activation, are related to lower Th2-type markers such as IgE or antibody titers [21, 22] in the sense of a cross-regulatory influence of Th1- versus Th2-type cytokines. Nevertheless, our observations would fit the notion that at least half of the patients examined in this study do not comprise patients suffering from acute virus infections.

Earlier studies show that malignant tumor diseases are frequently associated with increased neopterin concentrations [23]. Thereby, higher neopterin concentrations are associated with shortened survival. Half of the BEN patients presented with increased neopterin concentrations, which is similar to the frequencies with elevated neopterin observed earlier in patients with solid cancer such as gynecologic neoplasias or cancer of the prostate. However, only a minority of our BEN patients presented with tumors, and their neopterin concentrations did not differ from patients without such diagnosis. A possible predictive value of neopterin concentrations still has to be demonstrated.

In several malignant and infectious diseases, an association between higher neopterin concentrations and lower hemoglobin concentrations was observed [24]. This was not found in our study population. In addition, in chronic or protracted infections very often an associa-

tion exists between neopterin concentrations and ESR [24–26], again this was not true in our individuals. With this respect, patients with BEN differ considerably from observations made in patients with infections or malignant diseases.

The lack of a strong increase of neopterin concentrations may reflect the very slow progression of BEN, the pauci-cellular infiltrates, marked interstitial fibrosis without prominent inflammation. The absence of elevated neopterin concentrations in patients with malignant tumors might be due to the characteristically superficial, epithelial type of tumors seen in BEN patients. Overall, the absence of increased neopterin in half of the patients might support the hypothesis of a toxic nephropathy with a genetic predisposition and may argue against an ongoing viral infection.

CONCLUSION

Approximately 50% of BEN patients present with increased urinary neopterin levels. The patients with elevated neopterin levels did not have a higher degree of renal insufficiency, and there were no differences in other variables studied between the patients with increased neopterin and those with normal values. The background of this elevation, whether it is infectious or of other origin, remains unresolved. Also, an association between higher neopterin and ESR or lower hemoglobin concentrations, which is commonly observed in infections and malignant processes, is obviously missing in patients with BEN.

ACKNOWLEDGMENTS

This work was supported by the Austrian Federal Ministry of Social Affairs and Generations, and by INCO-COPERNICUS project ERB IC15-CT98-0318.

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