Determination of Neopterin in Serum and Urine

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Concentrations of neopterin, a product of activated macrophages, in serum from 662 apparently healthy individuals (ages 1 to 97 years, median 22 years) were measured by radioimmunoassay and the results statistically analyzed. Consistent with prior investigations on the urinary excretion of neopterin, we found no significant sex dependence, but values for subjects younger than 18 or older than 75 years were significantly higher. Renal clearance of neopterin in nine healthy individuals was 218 (SD 44) ml/min, which suggests that the kidneys have an active role in neopterin excretion. Results for neopterin concentrations measured in serum by RIA and “high-performance” liquid chromatography (HPLC) are consistent, but for urinary neopterin the concordance between methods was weak. Therefore, the RIA should be used only for measuring neopterin in serum. In comparison of the clinical utility of serum neopterin and urinary neopterin/creatinine concentrations, in patients with gynecological tumors, the latter values (measured by HPLC) discriminated slightly better between patients with favorable and unfavorable prognoses.

Additional Keyphrases: radioimmunoassay · chromatography, reversed-phase · age-related effects · cancer · immune system · reference interval · drug abuse

Measurement of neopterin concentrations provides an immediate, sensitive, reliable means to monitor activation of T-cells and macrophages. In vitro, major amounts of neopterin are released exclusively by macrophages, particularly when stimulated with gamma interferon derived from activated T-cells (1, 2). Thus, an increase in neopterin concentrations supplies information on the activation state of cell-mediated immunity that is not otherwise readily obtainable. This information is particularly useful for the follow-up of allograft recipients (3) for the prognosis of patients with cervical cancer (4), for staging patients with urological tumors (5), and for monitoring autoimmune diseases and diseases caused by mycobacteria, protozoa, and viruses (6). These clinical studies have involved measurement of urinary neopterin by “high-performance” liquid chromatography (HPLC) (7, 8). More recently, a radioimmunoassay (RIA) for neopterin in serum and another one for determinations in urine have become available (9).

Our aim in this study was to assist clinicians by determining normal values for neopterin in serum for different age groups as well as the renal clearance of neopterin. In addition, we compared the RIA and the established HPLC method for use with urine and serum specimens. Finally, we examined whether concentrations of neopterin in serum and urine are similarly suitable for discriminating patients with favorable and unfavorable clinical course of disease. For this assessment we used patients with gynecological tumors and compared the results with those for concentrations of neopterin in serum and urine of parenteral drug abusers. The latter subgroup was chosen because their neopterin concentrations are expected to be less influenced by changes in renal function than for patients with malignant disorders.

Materials and Methods

Instrumentation. For HPLC analyses we used an LC 5500 (Varian, Palo Alto, CA) controlled by a Vista 402 data system (Varian), and equipped with a RF 530 fluorescence detector (Shimadzu, Tokyo, Japan). Radioactivity was measured with a Gamma Szint BF 5300 (Berthold Lab., Wildbad, F.R.G.).

Reagents. Neopterin RIA kits for serum and urine were obtained from Henning, Berlin, F.R.G. All other reagents (analytical grade) were products from Merck, Darmstadt, F.R.G. Neopterin was a gift of Prof. W. Pfeiderer, Konstanz, F.R.G.

Procedures

Serum creatinine was assessed by a modified Jaffé method (10).

HPLC. We determined urinary neopterin and creatinine by reversed-phase HPLC as described elsewhere (7, 8). Briefly, the procedure was as follows. We used a C18 reversed-phase column (4 × 125 mm, 7-µm-diameter packing, Merck) protected with a 4 × 4 mm guard column of similar material. The mobile phase is potassium phosphate buffer (15 mmol/L, pH 6.4), the flow rate 0.8 ml/min. After diluting 100 µl of untreated urine specimens with 1.0 ml of mobile phase containing 2 g of disodium-EDTA per liter, we inject a 10-µl sample. Neopterin is quantified by its native fluorescence (353 nm excitation, 438 nm emission), the simultaneously determined creatinine is measured by ultraviolet absorbance at 235 nm. The detection limit of the method for neopterin was 40 nmol/L. Intra- and interassay CVs for the neopterin/creatinine ratio were 4.7% and 5.8%, respectively (7). Thirty-five urinary components have been tested for possible interferences; furthermore, during a five-year period, 200,000 specimens from various groups of patients have been assayed and no interfering substances or drug metabolites have been encountered.

To measure serum neopterin by HPLC, we used a modification of the method of Huber et al. (11): inject 20 µL of undiluted serum onto a 4 × 125 mm C18 reversed-phase column (as above), with distilled water as mobile phase (flow rate, 0.7 ml/min). The portion of effluent that contains all the neopterin (which is eluted within 2 min) is then switched onto a second similar C18 column and eluted with sodium dodecyl sulfate, 0.1 g/L in distilled water (adjusted to pH 4.0 with trifluoroacetic acid) at a flow rate of 0.7 ml/min. The detection limit of this method is 1 nmol/L. Intra- and interassay CVs were 3.7% and 9.3%, respectively.

RIA. The RIA procedures were as follows: Incubate 50 µL
of serum with 100 μL of neopterin antiserum for 1 h at room temperature, then add 100 μL of 125I-labeled neopterin. Incubate again for 1 h, then add 2 mL of aqueous polyethylene glycol (PEG) solution (60 g/L). Centrifuge at 2000 × g for 10 min, discard the supernate, and count the radioactivity of the pellet with a scintillation counter. The detection limit of the method is 1 nmol/L. The intra-assay CV (the mean of the percentage deviations of the duplicates from their means, for 25 randomly selected duplicates derived from five different assays) was 1.2%. The interassay CVs, determined by analyzing two control serum specimens in 14 different assays, was 12% for a mean concentration of 4.7 nmol/L, 7.1% for 14.1 nmol/L.

Urine specimens were diluted 10-fold with buffer, then centrifuged (5 min, 2000 × g). We used 20 μL of the diluted urine in the RIA procedure without extraction or purification, proceeding as for the serum method described above. The detection limit was 4 nmol/L. Intra-assay variation, defined as above, was 1.2%. We could not assess interassay variation because we used only two kits.

Specimens

To assess the normal concentrations of neopterin in serum, we used the serum RIA and serum specimens from 662 apparently healthy individuals, ages 1 to 97 years (median 22 years). Of these, 263 were children, ages 1 to 18 years (median six years).

We determined the renal clearance of neopterin in nine apparently healthy individuals, five women and four men. For measurements in serum we used RIA; for urine, HPLC.

For comparison of concentrations of neopterin in serum as measured by RIA and HPLC, we measured neopterin in 19 serum specimens obtained from some of the children in the apparently healthy group. In these specimens, neopterin concentrations ranged from 2.7 to 28.0 nmol/L (median 11 nmol/L).

To compare the concentrations of urinary neopterin measured by RIA and by HPLC, we analyzed 86 urinary specimens from unselected apparently healthy individuals.

To compare the clinical significance of serum and urinary neopterin concentrations, we collected 83 serum and urine specimens from 72 consecutive patients with gynecological tumors. We determined the neopterin in serum by RIA and the neopterin/creatinine ratio in urine by HPLC. We also measured serum creatinine, serum neopterin (RIA), and urinary neopterin/creatinine (HPLC) in another 19 patients with gynecological tumors. Serum neopterin and urinary neopterin/creatinine values were recorded from 35 apparently otherwise-healthy drug abusers studied elsewhere (12).

Specimens were protected from light and stored at −20 °C until analyzed. We were careful not to freeze and thaw serum samples more than twice.

Statistical Analyses

Differences of neopterin values in different groups were assessed by the Kruskal–Wallis test, linear regression analysis, and Spearman rank correlation analysis. The reference ranges were estimated by a nonparametric percentile method (13). For calculations we used the Cyber 74 computer (Control Data Corp., Minneapolis, MN) with the BMDP program package (University of California Press, 1983 ed.).

Results

Correlations in Healthy Subjects

Age and sex dependence of neopterin concentrations in serum. Figure 1 illustrates the concentrations of neopterin in serum from the healthy individuals. Because nonparametric statistics were used, we did not exclude possible outliers. Three age groups were identified as showing significantly different values for neopterin (Kruskal–Wallis test, p < 0.0001, Table 1), but there was no statistically significant sex dependence (Kruskal–Wallis, p > 0.05). Subjects between ages 18 and 75 years showed no significant age dependence of the serum concentrations, but children (<18 years) and elderly subjects (>75 years) had significantly higher neopterin concentrations than did the middle group. We chose the 95th percentiles as the upper normal limits.

Renal clearance of neopterin. The mean renal clearance of neopterin in nine healthy subjects (Table 2) was 218 (SD 44) mL/min.

Serum and urinary neopterin concentrations determined by both RIA and HPLC. Nineteen serum samples for which neopterin was measured by RIA (y) were also analyzed by HPLC (x). Linear regression analysis of the results yielded: y = 1.02x + 1.04 nmol/L (r = 0.98). We also measured neopterin in 86 urine samples by RIA and by HPLC; results by the two methods were less well correlated (Figure 2).

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**Table 1. Nonparametric Percentile Estimates of Neopterin Concentrations in Serum of Apparently Healthy Individuals**

<table>
<thead>
<tr>
<th>Age, years</th>
<th>Median, nmol/L</th>
<th>S.D.</th>
<th>5th</th>
<th>95th</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>Group</td>
<td>Median ± SE</td>
<td>Mean ± SE</td>
<td>Range</td>
</tr>
<tr>
<td>263</td>
<td>0–18</td>
<td>6.00 ± 0.15</td>
<td>6.78 ± 0.22</td>
<td>2.7–28.0</td>
</tr>
<tr>
<td>359</td>
<td>19–75</td>
<td>4.90 ± 0.09</td>
<td>5.34 ± 0.14</td>
<td>1.0–33.6</td>
</tr>
<tr>
<td>40</td>
<td>75–97</td>
<td>8.50 ± 0.81</td>
<td>9.67 ± 0.79</td>
<td>2.9–30.0</td>
</tr>
</tbody>
</table>

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Table 2. Renal Clearance of Neopterin in Nine Apparently Healthy Individuals

<table>
<thead>
<tr>
<th>Age, years</th>
<th>Neopterin concn, nmol/L</th>
<th>Vol of 24-h urine, mL</th>
<th>Glomerular filtr. rate for neopterin, mL/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>5.6</td>
<td>950</td>
<td>1560</td>
</tr>
<tr>
<td>30</td>
<td>4.5</td>
<td>1096</td>
<td>1720</td>
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<tr>
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<td>6.4</td>
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<td>26</td>
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<td>20</td>
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<td>1953</td>
<td>700</td>
</tr>
<tr>
<td>23</td>
<td>9.0</td>
<td>1756</td>
<td>1400</td>
</tr>
<tr>
<td>24</td>
<td>4.9</td>
<td>2315</td>
<td>700</td>
</tr>
<tr>
<td>25</td>
<td>4.5</td>
<td>1272</td>
<td>900</td>
</tr>
</tbody>
</table>

Fig. 2. Concentrations of urinary neopterin (μmol/L) in urine of 86 apparently healthy subjects, as measured by RIA and by HPLC. Line of identity (solid line) shown for comparison.

Correlations in Cancer Patients and Drug Abusers

For sample from 72 patients with gynecological tumors we determined concurrently neopterin values for serum (by RIA) and urinary neopterin/creatinine values (by HPLC). The patients were classified into two groups according to their clinical status: favorable clinical course (no evidence of disease or partial or complete remission) and unfavorable course (evidence of disease, progression, or relapse).

The clinical status was compared with the frequencies of neopterin concentrations above the upper limits of normal (95th percentiles for serum; Table 1; for urine values see ref. 6). Results for serum neopterin were in concordance with the clinical diagnosis in 22/32 (69%) patients without evidence of disease and in 32/40 (80%) patients with evidence of disease. The corresponding comparisons were 24/32 (75%) and 35/40 (88%) for the urinary neopterin/creatinine results. In 52/72 (72%) of patients, the urinary neopterin/creatinine and the serum neopterin results yielded the same classification (data not shown). In all, 54/72 (75%) patients are correctly classified by the results for serum neopterin (RIA) and 59/72 (82%) by the urinary neopterin/creatinine findings (HPLC). Thus, the discriminatory power of urinary neopterin/creatinine values appears to be slightly superior (chi square value 28.87 vs 17.38). To assess whether this observation represents physiological differences, we collected another set of urine and serum specimens from 13 of these patients a few days later. Results were identical to the first observations.

The correlation of serum neopterin (RIA) and urinary neopterin/creatinine (HPLC) results was analyzed by linear regression and Spearman’s rank correlation method. Including all values, the linear and the Spearman’s rank correlation coefficients were 0.92 and 0.65, respectively. If three “off-scale” values (4372, 160; 783, 65; 1370, 43) are excluded, the remaining 80 values (shown in Figure 3) show similar linear and Spearman’s rank correlation coefficients: 0.52 and 0.60, respectively. Thus, a moderate correlation was found, which is significantly different from zero (p < 0.001).

In another 19 patients with gynecological tumors, the concentrations of creatinine in serum ranged from 6.8 to 15 mg/L (mean 10.8 mg/L). The linear correlation coefficient relating urinary neopterin/creatinine with serum neopterin was 0.40; it was 0.36 for urinary neopterin/creatinine vs serum neopterin/creatinine ratio. Thus relating serum neopterin to serum creatinine concentrations did not change the correlation of serum neopterin to urinary neopterin/creatinine values.

We also measured serum neopterin (RIA) and urinary neopterin/creatinine (HPLC) concurrently in 35 otherwise-healthy parenteral drug abusers. As illustrated in Figure 3 (right) the correlation of both methods for this group is stronger (linear correlation coefficient = 0.73, Spearman’s rank correlation coefficient = 0.72) than for the gynecological cancer patients.

Discussion

Serum Neopterin in Apparently Healthy Individuals

The results obtained in this study agree well with data for 1837 blood donors (ages 18–67 years), who had a mean neopterin concentration of 5.89 nmol/L, SD 1.78 nmol/L, and an upper 99% confidence limit of 10.5 nmol/L, estimated with an assumption of gaussian distribution (14). Except for that abstract presentation, the present work represents the first study on serum concentrations of neopterin in healthy individuals that includes subjects in virtually all age groups. The results agree well with those obtained by urinary neopterin/creatinine measurements in healthy subjects (6, 15) if the known sex- and age-dependence of creatinine excretion is accounted for (data not shown). The differences in the chosen upper limits of normal for serum neopterin—10.5 nmol/L (14) vs 8.7 nmol/L in this work—arise from our use of a nonparametric percentile estimation method vs the parametric procedure that Kern et al. used (14). Further, our upper limits of tolerance are based on the

Fig. 3. Concurrently measured concentrations of serum neopterin (RIA) and urinary neopterin/creatinine (HPLC) for 80 patients with gynecological tumors (left) and 35 parenteral drug abusers (right)
The mean renal clearance of neopterin, which was 216 (SD 44) mL/min, is approximately 1.8 times the standard value of 120 mL/min for the glomerular filtration rate. This might be explained (e.g.) by production of neopterin by macrophages of the kidneys, active secretion of the compound by the kidneys, or oxidation processes occurring in the kidneys. However, the last is not likely because similar ratios of native oxidized neopterin compared with total neopterin after chemical oxidation have been found in serum (43%) and urine (45%) (16). Further, the renal clearance of neopterin measured after chemical oxidation processes in serum and urine was about twice the glomerular filtration rate (17), which is in accordance with our results.

**Comparison of the RIA and the HPLC method for serum and urinary neopterin concentrations.** A good linear correlation was observed between neopterin concentrations in serum as measured by RIA and HPLC, as indicated by the results of the linear regression analysis. In contrast, the compared methods were less well correlated for urinary neopterin values. Particularly, the observed slope of 0.64 reflects a considerable proportional error of the RIA compared with the HPLC method. It should be noted that the established HPLC method (7, 8) is specific, provides good performance characteristics, and has been carried out by several laboratories, yielding clinically reliable results. Therefore, it can be concluded that the RIA should not be used for urinary neopterin determination but yields valid results for serum neopterin.

**Correlations in Cancer Patients and Drug Abusers**

Urinary neopterin/creatinine values were found to be influenced considerably by the functional state of the kidney. This consideration is supported by a study on renal allograft recipients in which the correlation coefficient between serum neopterin concentrations and urinary neopterin/creatinine values was increased from 0.26 to 0.78 by relating serum neopterin to serum creatinine concentrations (Aulitzky W, et al., in preparation).

Because patients with gynecological tumors can be assumed to have more affected renal function than is true of drug abusers generally, we also determined creatinine in the sera of these patients. For our tumor patients, the correlation between serum neopterin concentrations and urinary neopterin/creatinine values, however, was similar to the correlation between serum neopterin/creatinine values and urinary neopterin/creatinine values. Further, the finding that several patients with gynecological tumors showed increased urinary but not increased serum neopterin concentrations cannot be explained by decreased glomerular filtration rate. Thus, variables other than kidney function also influence the correlation between serum concentrations and urinary neopterin values in this case.

The weak correlation between serum neopterin concentrations (by RIA) and urinary neopterin/creatinine values (by HPLC) in gynecological-tumor patients indicates that clinical conclusions derived from the two could differ. For the patient group studied, minor advantages of measuring urinary neopterin concentrations were apparent. The present work and a study on renal allograft recipients (Aulitzky W, et al., in preparation) show that the clinical utility of both methods of neopterin measurement should be compared separately for each patient group.

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**References**


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