Neopterin as a Predictive Marker for Disease Progression in Human Immunodeficiency Virus Type 1 Infection

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We assessed the value of urinary neopterin concentrations for prognosis of disease progression in HIV-1-infected patients. Sixty-eight anti-HIV-1-seropositive homosexuals with lymphadenopathy syndrome were tested for urinary neopterin and T-cell subset counts in 1982–83, and the incidence rate at which they developed acquired immuno deficiency syndrome (AIDS) between then and May 1988 was evaluated. Overall, 21 of 68 (30.9%) cases progressed to AIDS, with a yearly progression rate of 4–9%. The predictive value of urinary neopterin concentrations was higher (P = 0.0042) than that of CD4+ T-cell counts (P = 0.015) or the CD4+/CD8+ T-cell ratio (P = 0.022). Counts of CD8+ T-cells failed to show predictive significance (P = 0.29). Similarly, multivariate-regression analysis indicated that neopterin concentrations and CD4+ T-cell numbers were significant co-variates. Produced by human macrophages activated by interferon gamma, neopterin is thus a marker of macrophage activation via T cells. We conclude that these data demonstrate a correlation between the amount of T-cell-macrophage activation, as measured by urinary neopterin concentrations, and the progression of the disease.

Human immunodeficiency virus type 1 (HIV-1) is the causative agent for the acquired immunodeficiency syndrome (AIDS), which was first reported in the United States in 1981 (1).

Several reports have addressed the problem of early prediction of HIV-1-related disease progression. Low numbers of CD4+ T-helper/inducer cells and low ratios of CD4+ T cells per CD4+ cytotoxic/suppressor T cells were shown to be associated with more unfavorable disease course (2–9). Additionally, HIV-1 p24 antigenemia (8), increased concentrations of β2-microglobulin in serum (8), and increased urinary concentrations of neopterin (10) indicated more rapid progression of the disease.

In 1982–83, we measured urinary neopterin concentrations in a group of patients with generalized lymphadenopathy. Early results of that study have been published elsewhere (11, 12). Now we have analyzed the association between urinary neopterin concentrations and the likelihood of progression towards AIDS in the period since the initial neopterin determinations. For comparison, we performed analogous analyses for quantifying CD4+ and CD8+ T cells and determining the CD4+/CD8+ T-cell ratios, concomitantly with measurements of neopterin.

Materials and Methods

Patients: The study population of 68 patients was enrolled in 1982 and the first half of 1983. These patients are a randomly chosen subset of an entire cohort described in detail earlier (2, 9). All had HIV-1-associated lymphadenopathy syndrome and some had various constitutional symptoms. All were homosexual or bisexual men and were shown retrospectively to have been anti-HIV-1 seropositive already at their initial visit by ELISA (Abbott Laboratories, North Chicago, IL) confirmed by Western blot.4

Assay methods: Neopterin was determined in first morning urine specimens by a reversed-phase "high-performance" liquid chromatography (HPLC) method described elsewhere (13), which allows simultaneous determination of urinary creatinine. Neopterin concentrations were related to creatinine values to compensate for physiological variations in the analyte concentrations in urine.

4 Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Dept. of Health and Human Services.
Percentages of T-helper/inducer cells (CD4+) and T-suppressor/cytotoxic cells (CD8+) were measured by indirect immunofluorescence after reaction with OKT4 and OKT8 monoclonal antibodies, respectively (Ortho Diagnostic System, Inc., Raritan, NJ), followed by reaction with a fluorescein-conjugated anti-mouse immunoglobulin. All specimens were analyzed with a fluorescence-activated cell sorter (FACS-IV; Becton-Dickinson FACS Systems, Sunnyvale, CA) as described (14).

Absolute numbers of these lymphocyte subsets were determined by multiplying the percentages of each subset by the absolute lymphocyte count. The ratio of T-helper/inducer cells to T-suppressor/cytotoxic cells (CD4+/CD8+) is the percentage of the former divided by the latter.

**Statistical analyses:** The cumulative incidence of AIDS was computed by the Product Limit approach (15) for all patients and separately for subgroups of patients. These subgroups were defined by splitting the group in two at the median for either neopterin or one of the T-cell variables. Differences of incidences between subgroups of patients were assessed by the generalized Wilcoxon test (16). Whether combinations of neopterin concentrations and T-cell data were significant co-predictors for progression to AIDS was tested by the multivariate Proportional Hazards model (17). In this analysis the data were again treated as binary variables, dichotomized at the median values.

**Results**

Neopterin was measured and T cells were differentiated and counted for the patients with lymphadenopathy syndrome in 1982–83 (Table 1). Neopterin concentrations were not correlated with either of the T-cell data (P > 0.1, Spearman rank correlation coefficients).

Of the 68 patients enrolled in this part of the study, 21 (31%) progressed towards AIDS from the initial testing to the end of May 1988. The cumulative frequency of cases developing overt AIDS was 7.4% (SE 2.9%) by one year after collection of specimens, 11.8% (SE 3.9%) after two years, 20.6% (SE 4.9%) after three years, and 27.9% (SE 5.3%) after four years.

Figure 1 shows the cumulative incidences of AIDS cases among the subgroups of patients defined by T-cell variables and by neopterin concentrations. As illustrated, neopterin was the best single predictor for disease progression. An even slightly better discrimination was obtained by relating neopterin to the CD4+/CD8+ ratio or to the CD4+ T-cell number alone. CD4+ T-cell number and the CD4+/CD8+ T-cell ratio are individually of similar but less discriminating power, whereas the number of CD8+ T cells does not discriminate significantly between cases progressing towards AIDS and those that do not. The suitability of the proportionality assumption has been checked graphically by making use of the "log minus log survival plot" (not shown).

A stepwise multivariate-regression analysis by the Proportional Hazards method shows that—among the variables CD8+ T-cell number, CD4+ T-cell number, CD4+/CD8+ T-cell ratio, and neopterin—only the combination CD4+ T-cell number and neopterin is jointly significant. The regression coefficients obtained are 1.58 (SE 0.52, P = 0.0008) for neopterin and 1.14 (SE 0.47, P = 0.0111) for CD4+ T-cell number. From these coefficients we conclude that the risk for disease progression towards AIDS, adjusted for the CD4+ T-cell count, for a patient with a neopterin concentration higher than the median value is 4.9 times greater (95% confidence interval, 1.8 to 13.4) than for a patient with neopterin concentration below the median value. Similarly, the relative risk associated with low numbers of CD4+ T cells, adjusted for neopterin, is 3.1 (95% confidence interval: 1.3 to 7.8).

**Discussion**

The overall progression rate towards AIDS in the group of homosexuals studied was 21/68 (30.9%) during the five-year period, somewhat lower than the rates observed in other homosexual cohorts, e.g., from New York (18). Most of our study patients were enrolled relatively early in their HIV infection, according to the date of onset of lymphadenopathy noted by the patient or his physician, whereas patients in the early cohorts from New York had been infected longer at the time of enrollment (the rate of progression appears to increase with increasing time since infection). The rate of progression to AIDS in our study is similar to those of cohorts in San Francisco and other cities except New York City.

As this study shows, reliable predictive information on HIV-1-related disease can be obtained by measuring neopterin without counting lymphocyte subsets. Univariate and multivariate analyses of AIDS incidence rates in subgroups of patients defined by neopterin or T-cell variables show

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**Table 1. Neopterin Measurements and T-Cell Quantification in 68 Patients with Lymphadenopathy Syndrome, 1982–83**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Median</th>
<th>Range Values</th>
<th>Normal values*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ T cells, per μL</td>
<td>480</td>
<td>34–1068</td>
<td>389–1942</td>
</tr>
<tr>
<td>CD8+ T cells, per μL</td>
<td>641</td>
<td>165–1526</td>
<td>161–1079</td>
</tr>
<tr>
<td>CD4+/CD8+ T-cell ratio</td>
<td>0.73</td>
<td>0.21–2.27</td>
<td>0.99–4.26</td>
</tr>
<tr>
<td>Neopterin, μmol/mol creatinine</td>
<td>307</td>
<td>124–1360</td>
<td>187</td>
</tr>
<tr>
<td>Months since onset of lymphadenopathy syndrome</td>
<td>15.5</td>
<td>1–47</td>
<td></td>
</tr>
</tbody>
</table>

* 95% confidence limits based on 289 normal blood donors. * 97.5% confidence limit (13).
that the predictive value of neopterin concentrations in the studied patients was even higher than that of CD4+ T-cell counts or CD4+/CD8+ T-cell ratios. This agrees with results obtained in other groups of HIV-1-infected patients, e.g., in homosexual men in Vienna, Austria (19), and in heterosexual intravenous drug addicts incarcerated in Innsbruck, Austria (10). Interestingly, the predictive power of two composite variables, the ratios between neopterin concentrations and CD4+/CD8+ ratio, or CD4+ T-cell numbers, was even better, in agreement with a proposal of Crocchiolo et al. (20). Thus, for these two combinations the high-risk group showed an overall progression rate towards AIDS of 17/34 (50.0%), in contrast to 4/34 (11.8%) in the low-risk group.

Neopterin is produced by human monocyte/macrophages upon stimulation with interferon gamma (21). In various clinical conditions generally characterized by the involvement of T cells and macrophages, the measurement of neopterin provides a simple, noninvasive tool for assessing the activation state of the afferent side of cell-mediated immunity (22). Recently, increased concentrations of circulating interferon gamma and a close correlation with neopterin concentrations were established in patients with HIV-1 infection (23).

The application of neopterin measurement in HIV-1-related disease has recently been summarized (24). In brief, increased concentrations of neopterin are seen early in the course of disease, usually before CD4+ T cells begin to decrease (25). Anti-HIV-1 seropositive subjects usually show neopterin concentrations increased to a similar degree in patients with or without persistent generalized lymphadenopathy, whereas concentrations are significantly higher in patients with AIDS-related complex: the highest concentrations are seen in patients with full-blown AIDS.

We conclude from these results, and also from several previous studies (10, 14, 24, 25) on neopterin in HIV-1 infection, that activation of cell-mediated immune mechanisms appears to be common in infected patients and is significantly associated with more rapid progression towards the fully expressed disease. This agrees with several other reports that have shown the importance of immunological activation in vivo (11, 24) as well as in vitro (26).

From a practical point of view, neopterin measurement is easily done by HPLC or by commercially available radioimmunoassay (e.g., Neopterin RIA; Henning-Berlin, Berlin, F.R.G.) in urine, serum (27), and other biological fluids such as cerebrospinal fluid (28). Thus measurement of neopterin offers a reliable alternative to more invasive methods for frequent judgment of the clinical status of HIV-1-infected patients.

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References
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Radioimmunoassay of 5-Methoxytryptophol in Plasma

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5-Methoxytryptophol (ML) is synthesized by the pineal gland, but no radioimmunoassay has been described for its routine measurement in human plasma. We have developed and validated such an RIA. The assay is sensitive (detecting as little as 8 ng/L) and specific, and requires no extraction stage. A preliminary study of healthy volunteers showed an intra-individual variation in plasma ML that was independent of the sex of the subject and the time of daytime collection. Investigation of the 24-h pattern of ML in seven men revealed a low-amplitude daily rhythm (P <0.03). Mean concentrations of ML between noon and midnight significantly exceeded those between 0030 and 1130 hours in each individual (P <0.05). This assay is practical and convenient, and it should greatly assist in investigation of factors affecting concentrations of ML in human plasma.

The mammalian pineal gland synthesizes several 5-methoxyindoles in addition to melatonin (1). One of these, 5-methoxytryptophol (ML), has been shown to be biologically active in some animal species, with both stimulatory and inhibitory effects on the gonadal axis, depending upon the experimental model (2). A role for ML in the control of rhythmic photoreceptor metabolism has also been suggested, ML having been found to be a 50-fold more potent activator of intraretinal photoreceptor disk shedding than melatonin (3).

Investigation of the physiological role of ML, however, has been hampered by lack of a simple assay for it. Pineal ML has been measured with fluorometry (4), gas chromatography/mass spectrometry (GC/MS) (5–7), “high-performance” liquid chromatography (HPLC) (8, 9), and radioimmunoassay (RIA) (10, 11). The RIA developed by Skene et al. (11) has some advantages over the previously published ML assays. It is sensitive enough to assay single pineal glands of hamsters (12); it requires no prior extraction of pineal tissue; and it is specific, rapid, and inexpensive.

To date, only a GC/MS assay of ML in human plasma has been reported (13). It has been used to measure ML in healthy volunteers (14), during the menstrual cycle (15), and during insulin-induced hypoglycemic stress (16). Although sensitive (4 ng/L) and specific, GC/MS is expensive, requires sample extraction, and has a low sample throughput, making it not ideal for studies of circadian rhythms involving a large number of samples.

In this study we have adapted the RIA for pineal ML (11) to measure ML in human plasma and have validated it by HPLC. We have also used it to determine daytime and 24-h ML profiles in healthy volunteers.

Materials and Methods

Materials

Standard ML, “Tricine” [N-tris(hydroxymethyl)methylglycine], and activated charcoal were from Sigma Chemical Co., Poole, Dorset, U.K. Dextran T70 was from Pharmacia Fine Chemicals, Upsala, Sweden. All other chemicals for the RIA (sodium chloride, gelatin) were of analytical grade, from BDH Chemicals Ltd., Poole, Dorset, U.K.

For the HPLC analysis we used sodium dihydrogen orthophosphate (Merck, Darmstadt, F.R.G.); citric acid, triethylamine, and EDTA (Fluka, Buchs, Switzerland); acetonitrile (Baker Chemical Co., Phillipsburg, NJ); and dichloromethane (Prolabo, Vitry/Seine, France).

For the RIA the assay buffer consisted of 17.9 g of Tricine, 9.0 g of sodium chloride, and 1.0 g of gelatin per liter. The charcoal suspension contained 20.0 g of activated charcoal and 0.2 g of Dextran T70 per liter of assay buffer. Both the assay buffer and charcoal suspension were stable for as long as 10 days at 4 °C. Standard ML was dissolved in 50 mL/L absolute ethanol to produce a stock solution of 1 g/L. Stored at 4 °C, this solution was stable for as long as three months. For each assay the standard solution was diluted in assay buffer to provide standards over the range of 1 to 64 pg per tube. Pooled heparinized plasma was charcoal-stripped to remove ML as follows: mix charcoal (100 g/L) with plasma for 2 h at 4 °C, then centrifuge (2500 × g, 15 min) and filter (Seitz filters, Erban Ltd., Suffolk, U.K.).

Procedures

Synthesis of 125I-labeled ML. The procedure has been previously described by Skene et al. (11). For each assay the stock 125I-labeled ML was diluted with Tricine buffer to produce 4000 counts/min per tube.

Production of ML antiserum. A specific antiserum 1320 (batch no. 1312), raised in sheep against ML-bovine serum albumin conjugate (17), was stored lyophilized at −20 °C. The initial antiserum dilution was 1:5000, in assay buffer. ML RIA. We modified the previously published RIA (11) as follows: plasma samples, quality controls, and standards in charcoal-stripped plasma (500 µL each) were incubated

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