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EXTENDED REPORTS

Purine enzyme activities in recent onset rheumatoid arthritis: are there differences between patients and healthy controls?

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Abstract

Objective—Purine enzyme activities may predict the effectiveness of azathioprine treatment and be associated with increased deaths from infectious diseases. In rheumatoid arthritis, patients show variable responses to azathioprine and a higher percentage of death is caused by infections. The aim of the study was to investigate possible rheumatoid arthritis associated abnormalities of purine enzyme activities by measuring several of these enzymes in patients with recent onset rheumatoid arthritis before treatment with disease modifying antirheumatic drugs or prednisone.

Methods—23 patients with recent onset rheumatoid arthritis and 28 healthy controls were studied. Activities of the enzymes 5'-nucleotidase, purine nucleoside phosphorylase (PNP), hypoxanthine guanine phosphoribosyltransferase (HGPRT), and thiopurine methyltransferase (TPMT) were measured. Assessment of disease activity and blood sampling for routine measurements and HLA typing were done simultaneously.

Results—Purine enzyme activities did not differ between patients and healthy controls. Enzyme activities had no significant relations with indices of disease activity or rheumatoid factor titre or with the rheumatoid arthritis associated HLA types. Activity of 5'-nucleotidase decreased with age (P = 0.05) and was lower by about 27% (P = 0.007) in males than in females.

Conclusions—In rheumatoid arthritis patients, neither the variability in azathioprine effectiveness nor the increased death rate from infections can be explained by pre-existing abnormalities in the activities of the purine enzymes 5'-nucleotidase, PNP, HGPRT, or TPMT at an early stage of the disease, before disease modifying antirheumatic drugs or prednisone treatment. Besides adjustment for age, results of studies involving purine 5'-nucleotidase activity should also be adjusted for sex.

Azathioprine is used in the treatment of immunological diseases and to prevent graft rejection in transplant recipients. After ingestion the drug is rapidly converted to 6-mercaptopurine (6-MP), a drug which is used in the treatment of leukaemia. 6-MP is a substrate in three metabolic pathways: catabolism to 6-thiouric acid by xanthine oxidase, methylation to methylmercaptopurine metabolites by thiopurine methyltransferase (TPMT), and conversion by hypoxanthine-guanine phosphoribosyltransferase (HGPRT) to 6-thioguanine-monophosphate which consequently results in formation of cytotoxic 6-thioguanine nucleotides (figure 1).

Rheumatoid arthritis patients treated with azathioprine show variable responses ranging from successful reduction of disease activity to occurrence of side effects like leucopenia, pancytopenia, gastrointestinal complaints, hepatotoxicity, or hypersensitivity.

Nowadays four different enzyme disturbances are known to be related to the effect of azathioprine treatment. Patients with the Lesch-Nyhan syndrome (inborn HGPRT deficiency) are resistant to azathioprine and persons with TPMT deficiency or low activity of purine 5'nucleotidase are prone to develop bone marrow toxicity. Finally the simultaneous use of azathioprine and allopurinol, a competitive inhibitor of xanthine oxidase, increases toxicity of azathioprine so that the dosage of azathioprine has to be reduced to approximately 25%.

Rheumatoid arthritis may be connected to purine metabolism not only by the use of azathioprine but also by the disease itself since it involves at very least a dysregulation of the immune system, expressed by autoimmunity and changes in lymphocyte subsets. In rheumatoid arthritis, there is probably also a deficiency of the immune system, since it has been established that death due to infectious diseases occurs more often in rheumatoid arthritis patients than in the general population.

Early death due to infection is a well known consequence of a number of immune deficiency syndromes, which in turn are related to purine enzyme deficiencies, for example T lymphocyte malfunction due to
deficiency of purine nucleoside phosphorylase (PNP) and (adult onset) primary hypogammaglobulinaemia and combined immunodeficiency due to deficiency of 5'-nucleotidase.

Despite the fact that there are no previous data showing that subtle changes in enzyme levels have an impact on azathioprine effectiveness or susceptibility to infections, the above mentioned observations still raise the question of whether the purine enzymatic make up in rheumatoid arthritis patients is also different. In addition, it would be very useful if purine enzyme activities, IgM-RF, and biochemical profiles, serum uric acid, and HLA types, and routine blood tests (patients only) were collected immediately after the clinical assessments.

To determine purine enzyme activities of 5'-nucleotidase, PNP, and HGPRT, blood was collected in 10 ml Vacutainer tubes containing polystyrene granules (Becton and Dickinson). Mononuclear cells were obtained by Ficoll-Hypaque sedimentation and in these cells the enzyme activities were measured by a new high performance liquid chromatography method. Enzyme activities were done in rather heterogeneous groups of patients. The influence of different disease modifying antirheumatic drugs, prednisone, other medications, duration of the disease, age, and sex could not be excluded.

To detect possible differences present at the beginning of the disease (rheumatoid arthritis associated) and to obviate several confounding variables from the two previous reports, we have studied a homogeneous group of rheumatoid arthritis patients within the first year of diagnosis and before treatment with a disease modifying antirheumatic drug or prednisone.

The study protocol was approved by the ethics committee of the hospital.

**Methods**

**Patients and Controls**

In the period from February 1993 to April 1994, 23 rheumatoid arthritis patients and 28 healthy controls entered the study. They were all Caucasian. Patients had to meet the following criteria: the 1987 ARA criteria, disease duration less than one year and no previous treatment with disease modifying antirheumatic drugs or prednisone. The use of non-steroidal antiinflammatory drugs (NSAID) or paracetamol was allowed. Patients with other coexisting diseases were excluded, as were persons using allopurinol.

To avoid influences of possible circadian rhythms, clinical assessments and blood sampling were done between 9 and 11 am for all participants.

**Laboratory Assessments**

Indices of disease activity included patients assessments of pain and general health (GH) on visual analogue scales (VAS) of 10 cm (0 = best possible, 10 = worst possible), the Ritchie articular index (RAI), the number of swollen joints, and the degree of morning stiffness. The disease activity score (DAS) was calculated by using the RAI, the number of swollen joints, the VAS-GH, and the erythrocyte sedimentation rate (ESR).

**Clinical Assessments**

Venous blood samples for determination of purine enzyme activities, IgM-RF (HIISA, positive if > 10 IU ml⁻¹), HLA types, and routine blood tests (patients only) were collected immediately after the clinical assessments. Routine blood analyses included haematological and biochemical profiles, serum uric acid, the ESR (Westergren, mm in 1 h), and C-reactive protein (mg ml⁻¹). Tissue typing of all patients for HLA class I and II (DR/DQ) was performed by standard techniques.

To determine purine enzyme activities of 5'-nucleotidase, PNP, and HGPRT, blood was collected in 10 ml Vacutainer tubes containing polystyrene granules (Becton and Dickinson). Mononuclear cells were obtained by Ficoll-Isoaque (density 1.077 g ml⁻¹). Nycomed) gradient centrifugation of defibrinated blood and in these cells the enzyme activities were measured by a new high performance liquid chromatography method. Enzyme activities
are expressed in nmol 10^9 mononuclear cells h^{-1} of incubation. To measure the TPMT activity in red cell lysate as described by Weinstulboum et al., peripheral blood was collected in 10 ml Monoject tubes containing 150 USP units of lithium heparin (Sherwood Medical). TPMT activity is expressed in pmol 10^6 erythrocytes h^{-1} of incubation. All enzyme assays were carried out in quadruplicate.

STATISTICAL ANALYSIS
Pearson's correlation analyses were used to study relations between purine enzyme activities and indices of disease activity, between indices of disease activity and age, and between enzyme activities and rheumatoid factor. Since the distributions of 5'-nucleotidase (patients and controls) and HGPRT (patients) were not quite normal, Spearman's rank correlation analyses were also performed. Two sample Student t tests were used to compare enzyme activities in HLA positive and HLA negative patients (and additionally Mann-Whitney tests with respect to 5'-nucleotidase and HGPRT). Influence of age on enzyme activities was analysed by linear regression analysis. Differences between patients and healthy controls and between the two sexes with respect to enzyme activities (and additionally with respect to the square root of 5'-nucleotidase) were examined by a two way analysis of covariance adjusting for age.

The statistics were processed by SAS computer software (SAS Institute, Cary, NC, USA) and by NCSS computer software (version 5.1, 1988, Dr J L Hintze, North Kaysville, Utah, USA). Test results were considered to be significant at P ≤ 0.05.

Results
STUDY POPULATION
Characteristics of patients and healthy controls are given in table 1. All patients had normal laboratory values for kidney and liver function and a normal serum urate. Only minor abnormalities were found in haematological variables (low haemoglobin concentration, raised platelet count) and were considered to be attributable to activity of the disease (data not shown).

RELATIONS BETWEEN ENZYME ACTIVITY, DISEASE ACTIVITY, RHEUMATOID FACTOR, AND HLA TYPE
Pearson's coefficients of correlation for the relations between enzyme activities and the indices of disease activity (including the DAS_{28}) are presented in table 2. They varied from -0.34 to 0.46. None of these correlation coefficients reached significance except the one between 5'-nucleotidase activity and ESR. However, checking the latter correlation by a Spearman analysis which in this case is probably better in view of the not quite normal distribution, no significance was found. Furthermore, no relevant correlations were found between indices of disease activity and age. A relation between the rheumatoid factor titre and the activity of the tested enzymes could not be demonstrated (data not shown).

Enzyme activities in HLA positive and HLA negative patients were examined for HLA-DR1, HLA-DR2, HLA-DR3, and HLA-DR4. There were no differences (data not shown).

ENZYME ACTIVITY—INFLUENCE OF AGE
In rheumatoid arthritis patients as well as in healthy controls only 5'-nucleotidase activity showed a significant decrease with increase of age (P = 0.05 and P = 0.009 respectively) (table 3). The estimates of the yearly decrease ranges from 0.21 nmol 10^6 cells h^{-1} in rheumatoid arthritis patients to 0.37 nmol 10^6 cells h^{-1} in the healthy controls.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Characteristics of patients and controls. Values are mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA patients (n=23)</td>
<td>Controls (n=26)</td>
</tr>
<tr>
<td>Age in years [range]</td>
<td>54.0 (14.0)</td>
</tr>
<tr>
<td>Male/female (n)</td>
<td>13/10</td>
</tr>
<tr>
<td>Disease duration in months [range]</td>
<td>4.3 (3.5)</td>
</tr>
<tr>
<td>RF positive patients (n)</td>
<td>20</td>
</tr>
<tr>
<td>Disease activity indices</td>
<td></td>
</tr>
<tr>
<td>ESR (Westergren)</td>
<td>31.2 (22.4)</td>
</tr>
<tr>
<td>Ritchie articular index</td>
<td>11.8 (6.7)</td>
</tr>
<tr>
<td>No of swollen joints</td>
<td>16.3 (9.2)</td>
</tr>
<tr>
<td>VAS general health</td>
<td>42.0 (23.5)</td>
</tr>
<tr>
<td>DAS_{28}</td>
<td>4.1 (1.3)</td>
</tr>
<tr>
<td>HLA type:</td>
<td></td>
</tr>
<tr>
<td>positive/negative (n)</td>
<td></td>
</tr>
<tr>
<td>DR1</td>
<td>5/15</td>
</tr>
<tr>
<td>DR2</td>
<td>2/18</td>
</tr>
<tr>
<td>DR3</td>
<td>0/4</td>
</tr>
<tr>
<td>DR4</td>
<td>10/9</td>
</tr>
</tbody>
</table>

RA, rheumatoid arthritis; RF, rheumatoid factor; ESR, erythrocyte sedimentation rate; VAS, visual analogue scale; DAS_{28}, disease activity score; HLA, human lymphocyte antigen.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Coefficients of correlation according to Pearson (and Spearman) between indices of disease activity and purine enzyme activity and between indices of disease activity and age in rheumatoid arthritis patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>Swollen joints</td>
</tr>
<tr>
<td>No</td>
<td>ESR</td>
</tr>
<tr>
<td>RAI</td>
<td>0.02</td>
</tr>
<tr>
<td>(0.12)</td>
<td>(0.15)</td>
</tr>
<tr>
<td>PNP</td>
<td>0.25</td>
</tr>
<tr>
<td>(0.19)</td>
<td>(0.22)</td>
</tr>
<tr>
<td>HGPRT</td>
<td>-0.19</td>
</tr>
<tr>
<td>(0.10)</td>
<td>(0.21)</td>
</tr>
<tr>
<td>TPMT</td>
<td>-0.33</td>
</tr>
<tr>
<td>Age</td>
<td>0.02</td>
</tr>
</tbody>
</table>

RAI, Ritchie articular index; ESR, erythrocyte sedimentation rate; DAS_{28}, disease activity score; Swollen joints; VAS-GH, visual analogue scale general health; 5'-NT, 5'-nucleotidase; PNP, purine nucleoside phosphorylase; HGPRT, hypoxanthine guanine phosphoribosyltransferase; TPMT, thioruridine methyltransferase.

* Critical value (n=23) for Pearson (and Spearman): 0.41 (0.42); n=22. Mean (SD) of healthy controls.
ENZYME ACTIVITY—INFLUENCES OF GROUP, SEX, AND AGE

Results of the two way analysis of covariance examining the influences of group, sex, and age on enzyme activities are shown in the tables 4 and 5. No significant differences between rheumatoid arthritis patients and controls, averaged over both sexes, could be established. Interaction tests showed that mean patient-control differences were not clearly different in the two sexes.

Males showed a significant lower (P < 0.007) mean 5'-nucleotidase activity than females (averaged over both groups: ±6 activity units or 27% lower activity). Mean male 5'-nucleotidase activity was 80% and 68% of the female activity respectively for the rheumatoid arthritis group and the control group.

A significant (P = 0.005) decrease of 5'-nucleotidase activity with age was again found. This decrease amounted about 0.23 activity units per year. Activities of PNP and HGPRT showed a tendency to increase with age (P = 0.10 and P = 0.07, respectively) about 1.1 and 0.09 activity units per year, respectively. (For 5'-nucleotidase activity, similar results were found after a square root transformation.)

Discussion

Our previous pilot study indicates that activities of some purine enzymes in rheumatoid arthritis differ from those in healthy controls and are associated with the outcome of azathioprine treatment, whereas there is no clear relation between enzyme activity and disease activity. However, the group of patients in that study is heterogeneous and the influence of disease modifying antirheumatic drugs, prednisone, other medications, disease duration, age, and sex cannot be excluded. It was to determine if the differences in enzyme activities between rheumatoid arthritis and controls are related to the disease itself, the drugs used, or other confounding variables that we started the present study. In contrast to the other studies, the enzyme activities are measured solely in recent onset rheumatoid arthritis patients not previously treated with a disease modifying antirheumatic drug or prednisone.

One of the pitfalls may be that enzyme activity reflects disease activity. Our tests are reading on this issue. Most correlation coefficients show little or no relation between enzyme activities and indices of disease activity.

In accordance with a previous study, 5'-nucleotidase activity is significantly age dependent, showing decreasing values with increasing age. The degree of change is comparable for patients and controls. A combined analysis with adjustment for sex does not change this finding. It may be that age is also a factor influencing PNP and HGPRT activities. Although the change was not significant, both enzyme activities tended to increase with age.

Activity of 5'-nucleotidase is also subject to a substantial influence of sex and as far as we know this has not been reported before. A combined analysis of patients and controls with adjustment for age shows a 27% lower 5'-nucleotidase activity in males than in females. The underlying mechanism is not known but the difference in hormonal status may possibly play a role.

Susceptibility, prognosis, and clinical course of rheumatoid arthritis are related to the rheumatoid factor status and some HLA types. 1 5 '1 The same report shows that chronic disease in general is associated with somewhat higher TPMT levels and that NSAID are associated with lower levels of TPMT, while others do not. 3 '4 '5 The report shows that chronic disease in general is associated with somewhat higher TPMT levels and that NSAID are associated with lower levels of TPMT. Although a gender difference was not significant, it is important to be aware of this difference when interpreting the results of our study.
found in our study, the possibility that opposite influences of NSAID and chronic disease mask a difference with healthy controls cannot be entirely excluded. However, based on our results we conclude, with some reservation about TPMT activity, that the variability in response to azathioprine treatment and the increased death rate from infectious diseases cannot be explained by pre-existing (rheumatoid arthritis-associated) differences in the enzyme activities tested.

The previous reports about rheumatoid arthritis patients, as we mentioned above, were based on patient groups characterised by heterogeneity in disease duration and medication. In addition, the enzyme activities were determined by different enzyme assays, the type of $5'$-nucleotidase investigated differed (membrane bound $5'$-nucleotidase) versus total $5'$-nucleotidase (cytoplasmic and $5'$-nucleotidase) and in neither report were enzyme activities adjusted for age and sex. For these reasons a comparison of previous data and the data presented in this study is hardly possible. Furthermore, it is known that activities of some purine enzymes are not evenly distributed among the several mononuclear cells or cell types or different stages of maturity. In that and with age percentile changes occur in lymphocytic subunits and maturity, it is conceivable that age, disease duration, or disease modifying antirheumatic drug use may cause a shift in the subtype or maturity of peripheral blood mononuclear cells, and that different disease modifying antirheumatic drugs may have different induction effects on enzyme activities. Further analyses of these assumptions are needed and some are under investigation now.

In summary, no differences were found in purine enzyme activities of PNP, HGPRT, or TPMT between patients with early rheumatoid arthritis before disease modifying antirheumatic drug or prednisone treatment and healthy controls. The tested enzyme activities have no associations with disease activity, nor with the rheumatoid factor titre, nor with the rheumatoid arthritis associated HLA types. Our study confirms the significant decrease of $5'$-nucleotidase activity with age. It also shows a tendency, although not significant, towards increasing activities of PNP and HGPRT with age. It also shows a tendency, although not significant, towards increasing activities of PNP and HGPRT with age. A new finding is that 5-nucleotidase activity in males is about $27\%$ lower than in females. Moreover, it was found that 5-nucleotidase activity is about $27\%$ lower than in females.

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