5'-Nucleotidase as a marker of both general and local inflammation in rheumatoid arthritis patients

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Abstract

Objectives. To evaluate measurements of serum and synovial fluid 5'-nucleotidase (5'N) activity as a marker of general and local inflammation in arthritis, and to resolve a contradiction in the literature as to whether or not the activity of 5'N in the synovial fluids of rheumatoid arthritis (RA) patients is raised in comparison with that in the synovial fluids of other arthritis patients.

Methods. Assays for 5'N were carried out in the presence of inhibitors of other phosphatases, AMP deaminase and of 5'N itself.

Results. The 5'N activity in the synovial fluid of RA patients was both significantly higher (mean 1.7-fold) and had a greater variance than that in the synovial fluids of other arthritis patients, and the contradiction in the literature was resolved. There was a strong correlation between the 5'N activity in the sera of RA patients and their erythrocyte sedimentation rate. There was no significant correlation between the 5'N in the serum and synovial fluid for the RA patients, in marked contrast to the strong correlation between the two 5'N activities shown by the osteoarthritis patients. The 5'N activity was greater in the synovial fluid than in the serum for virtually all the patients, showing that it was being made locally.

Conclusions. The 5'N activity in the serum (which came mostly from the liver) could be used as a marker of general inflammation, whereas the 5'N in the synovial fluid was mostly produced locally, and could be used as a marker of joint inflammation, particularly for the RA patients.

Key words: 5'-Nucleotidase, Rheumatoid arthritis, Synovial fluid, Blood sedimentation.

Ecto-5'-nucleotidase (5'N), EC 3.1.3.5, is a glycosyl phosphatidylinositol-linked enzyme [1–3] found in the outer layer of the plasma membranes of many cells. It can be released by treatment with some phospholipases. Monocytes do not possess the enzyme, but develop it as they mature to macrophages, which lose the enzyme when they become activated [4]. It is also present on mature B cells [5] and cytotoxic T cells, where it is reported to be involved in T-cell killing [6], but not on neutrophils or other circulating blood cells, although it is present on hepatocytes [7] and in serum. Since the mass of the liver in a normal adult is ~1.4 kg, and the synovial mass is ~1.6 g [8], it is likely that the serum 5'N level is dominated by release from non-articular tissues.

Early reports by Kendall et al. [9] and Farr et al. [10] found a mean value for the 5'N activity in the synovial fluid (SF) of rheumatoid arthritis (RA) patients of 35.5 IU, compared to a value of 11.53 IU for non-RA patients. However, Wortmann et al. [11] found that the 5'N activity was higher in the SF from osteoarthritis (OA) patients, as opposed to those with RA, gout or pseudogout. They attributed the discrepancy between their results and those of the previous group to an error in the latter's assay caused by adenosine deaminase, which Wortmann et al. [11] found in the SF of RA patients. However, AMP deaminase (adenylate deaminase) is an enzyme more likely to distort the measurements. It is found in a relatively high concentration in neutrophils (and muscle cells) [12], and would therefore be expected to be present in the SF from the RA patients only. Investigations by two different groups [10, 13], using assay methods not subject to interference by either adenosine deaminase or AMP deaminase, agreed that the 5'N on the synovial lining cell membranes is raised in RA.

In view of these considerations, it seemed worthwhile to investigate further the discrepancy between the reported values for 5'N in RA and OA since 5'N levels...
in SF could potentially be a measure of the activation of the synovial lining cells, while levels in the serum could reflect activation of the liver, with only a small contribution from the articular tissue.

Patients, materials and methods

Patients

SF samples were collected from 27 patients with RA (mean age 60.3 yr, female:male (F:M) ratio 1.7:1), 10 patients with OA (mean age 65.6 yr, F:M ratio 2.3:1), two male patients with ankylosing spondylitis (AS) (ages 64 and 25 yr), two male patients with gout (ages 70 and 29 yr), one female patient with juvenile-onset chronic arthritis (JCA) now aged 24 yr, one male patient with psoriatic arthritis (PsA) (age 49 yr) and one female patient with reactive arthritis (ReA) (age 28 yr). Samples from both knees were obtained for five RA patients, one ReA and one AS patient. Samples were obtained on two occasions, at least 3 months apart, from the same patient for three RA patients and the JCA patient. When more than one sample was obtained from a patient, the 5’N values usually differed quite markedly, and were plotted independently. All the RA patients fulfilled the ARA criteria for diagnosis [14] and had a mean disease duration of 10.3 yr (range 1–39), only four patients had had the disease for 3 yr or less. The RA patients were treated with non-steroidal anti-inflammatory drugs (NSAIDs) (22/27), disease-modifying anti-rheumatic drugs (DMARDs) (20/27) and/or prednisolone (8/27); only one patient received no medication. The DMARDs consisted of d-penicillamine (7), gold (5), methotrexate (3), sulphasalazine (2), azathioprine (2), cyclosporin (1) and cyclophosphamide (1). The OA patients had a mean disease duration of 5.2 yr (range 0.5–14) and 3/10 were treated with NSAIDs. Five patients had a disease duration of 3 yr or less. The JCA patient had the disease for 22 yr, and was treated with prednisolone, sulphasalazine, methotrexate and NSAID. The AS patients had the disease for 1.8 and 6 yr, and were both treated with NSAID; one was also on prednisolone. The patients with gout were either new or had the disease for 0.25 yr. One was treated with NSAID, the other with colchicine and allopurinol. The patient with ReA had had the disease for 2.6 yr, and was treated with NSAID and sulphasalazine. The patient with PsA had had the disease for 2.8 yr and was treated with NSAID.

Synovial fluid collection

SF samples were taken from the knees of the patients when indicated as part of routine clinical practice. The samples were handled aseptically and were centrifuged at 500 g for 30 min in a MSE Chilspin centrifuge. Samples (20 µl) of the supernatant were transferred to sterile 1.8 ml Eppendorf tubes and frozen at −70 °C; the rest of the supernatant was stored in 1 ml aliquots at −70 °C.

Serum

With the patient’s consent, 10 ml of serum were taken in addition to routine blood samples at the time of joint aspiration and stored in aliquots at −70 °C. Some patients refused to give a serum sample.

Materials

Coformycin was obtained from Calbiochem. [3H]AMP from Amersham International and AMP, α,β-methylene adenosine diphosphate (AMPCP), levamisole and β-glycerophosphate from Sigma.

5’N assay

The 5’N assay used was adapted from that of Rowe et al. [15], and was not subject to interference from adenosine deaminase or AMP deaminase. A mixture containing 0.08 μM AMP with 0.4 μCi (1.48 × 10⁴ Bq)/ml [2-3H]AMP, 20 mM MgCl₂ and 200 mM glycine/NaOH (pH 8.5) was diluted 1:1 with 0.145 mM NaCl. Acid phosphatase was inhibited by the pH, alkaline phosphatase by 1 mM levamisole [16], and any other non-specific phosphatases by 0.06 mM β-glycerophosphate [17]. Adenosine deaminase and AMP deaminase (EC 3.5.4.6) were inhibited by 10 μg/ml coformycin [18]. 5’N was inhibited by AMPCP, the non-metabolizable analogue of ADP [19]. Samples (20 μl) of SF or serum in conical 1.8 ml Eppendorf tubes were incubated for 15 min at 37 °C with the above mixture, with or without inhibitors. Except for special experiments, the inhibitors of other phosphatases (levamisole and β-glycerophosphate) were present. After the incubation, the tubes were transferred to melting ice, and unreacted substrate precipitated with 0.15 ml of 0.145 mM ZnSO₄, followed by 0.5 ml of 0.15 M Ba(OH)₂ solution. The tubes were centrifuged at 8500 g for 1 min, then 0.5 ml supernatant was added to 5 ml Cytoscin, and counted for 3H on a scintillation counter. Results were expressed as micromoles of substrate hydrolysed per minute per litre of sample (IU). The samples with and without coformycin were kept on ice for 1 h before the assay, as coformycin is a slow-acting inhibitor [18].

Statistical analysis

The 5’N values of the SF for the three groups of patients were compared using the non-parametric Kruskal–Wallis many-group test for the equality of populations and further multiple comparison tests [20]. The variances were compared using Bartlett’s test. Regression analysis was used to study the relationships between the serum 5’N and the erythrocyte sedimentation rate (ESR) for the RA patients, and between the 5’N in the SF and serum for the RA and OA patients.

Results

In view of the discrepancies previously reported in the 5’N results, the assay method used in this work was tested as shown in Fig. 1. This compares the specific inhibition of 5’N activity in the serum and SF of patients by AMPCP, the non-metabolizable analogue of ADP,
in the presence of inhibitors of alkaline and non-specific phosphatases. Three OA and three RA patients were studied. It will be seen that there is a smooth curve for the inhibition of activity in the serum of the RA patient, and in the serum and SF of the OA patient, but that there is a bump in the SF curve for the RA patient, which is partially (and in some cases completely) removed by coformycin, an inhibitor of both adenosine deaminase and AMP deaminase. The effect disappears at concentrations of AMPCP $\geq 100$ μM. The inhibition produced by 200 μM AMPCP varied between $\sim 80$–90%, and depended on the patient rather than the disease or the type of fluid investigated. The assay method used in this work was therefore deemed to measure $5'$-N nucleotidase satisfactorily, without significant interference from other enzymes.

The results for the $5'$-N assays on the SF from the RA, OA and ‘Other’ patients are shown in Fig. 2, together with the means $\pm$ s.d. and the medians. The range is shown by the highest and lowest points in the three groups. The medians and means are reasonably close, showing that the distributions for the three groups are not particularly skew; however, the variance for the RA patients is significantly greater than that for the other groups: $P < 0.0001$. The Kruskal–Wallis test for the equality of populations gives $P < 0.0001$ for the groups being equal, and further multiple comparison tests show that RA is significantly different both from OA and ‘Other’ ($P < 0.05$), but that OA and ‘Other’ do not differ significantly.

Cholesterol crystals were noted in the SF of the RA patient with the highest $5'$-N activity.

Table 1 compares the present mean activities for $5'$-N in the SF of RA, OA and all non-RA patients with those published previously, some of which have been recalculated in IU. For OA, or non-RA patients, the present results and those obtained by all the previous groups are comparable, particularly if the presence of alkaline phosphatase in Kendall et al.’s [9] result is noted. This suggests that all three methods are of comparable sensitivity. However, there is a discrepancy in the results for the RA patients, which can be attributed largely to AMP deaminase.

The relationship between the serum $5'$-N and the ESR for the RA patients is shown in Fig. 3. The correlation between the two variables is highly significant ($P < 10^{-5}$), indicating that the serum $5'$-N could be taken as a general measure of inflammation. As mentioned earlier, the serum level of $5'$-N is likely to be dominated by the liver. The JCA patient also showed a high value for the serum $5'$-N and a high ESR (22.6 IU, and 46).

Figure 4 compares the contrasting relationship between the $5'$-N in the serum and the SF for the RA

Table 1. Mean values for $5'$-nucleotidase in synovial fluid

<table>
<thead>
<tr>
<th>Reference</th>
<th>RA</th>
<th>OA</th>
<th>All non-RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kendall et al. [9]</td>
<td>35.5</td>
<td>–</td>
<td>11.5</td>
</tr>
<tr>
<td>Farr et al. [10]</td>
<td>44.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Wortmann et al. [11]</td>
<td>3.2 (12.0)</td>
<td>3.2 (6.3)</td>
<td>–</td>
</tr>
<tr>
<td>Present work</td>
<td>14.0 ± 6.2</td>
<td>8.9 ± 2.1</td>
<td>8.1 ± 2.0</td>
</tr>
</tbody>
</table>

The $5'$-N values are given in IU, which is μmol AMP hydrolysed/min/l.

Wortmann et al.’s results show the $5'$-N inhibited by 25 μM AMPCP; the results in parentheses are calculated for all the $5'$-N, using data from Fig. 1.

Fig. 1. Inhibition of $5'$-N by AMPCP. ■, SF; △, serum; □, SF+coformycin; ▽, serum + coformycin.

Fig. 2. Comparison between the $5'$-N activity in the synovial fluids of RA, OA and other patients. ●, RA; ▲, AS; ○, JCA; ○, ReA; ▽, OA; △, gout; ▣, PsA. Means are shown $\pm$ s.d. and the median is also shown. The RA group is significantly higher, and has a significantly greater variance, than the OA or ‘Other’ groups.

Fig. 3. The $5'$-N in the serum of RA patients is proportional to the ESR.

Fig. 4. Inhibition of $5'$-N by AMPCP. □, SF; △, serum; □, SF+coformycin; ▽, serum + coformycin.
patients and the OA patients. For the RA patients, there is no significant correlation between the 5’N in the serum and SF, but the OA patients show a good correlation. Levick [21] showed that the ratio of albumin (from the liver) in the SF/serum for RA patients was 0.65. The size of 5’N (71 kDa) [3] is comparable to albumin (69 kDa) so that it would be expected to show the same ratio if all the 5’N was coming from the serum. However, 21/26 of the RA points show a 5’N SF/serum ratio which is greater than this, showing that the 5’N in the SF must be dominated by local production and not by transfer from the plasma. For all the OA patients, the SF/serum ratio is >1, so 5’N was still being produced locally, but it is clearly influenced by the same factors affecting the serum 5’N production. Data for the two AS and one PsA patients resembled those for the OA patients. The ratio of the mean values for 5’N in the SF/serum was 1.4 for the RA patients and 1.2 for the others. However, in the JCA patient, the serum value was 22.6 IU and the SF value 7.3 IU, giving a SF/serum ratio of 0.32. Three of the RA patients also showed SF/serum values which were <0.65. All these patients had a very pronounced inflammatory oedema with 20–40 ml of SF, and the capillary walls would act as a partial barrier to 5’N entry from the plasma. No serum samples were obtained from the gout and ReA patients.

**Discussion**

The discrepancies between the 5’N activities in the SF of RA patients reported by previous workers have been resolved from the data shown in Figs 1 and 2 and Table 1, and by a comparison of assay methods. RA patients do show a significant 1.7-fold increase, but more variability in their SF 5’N activity compared to the OA and other patients.

To measure 5’N, Kendall et al. [9] and Farr et al. [10] used Persijn et al.’s method [22], in which the adenosine produced by 5’N (and non-specific phosphatases such as alkaline phosphatase) is estimated by adding adenosine deaminase, which produces NH₃ which can be measured spectroscopically. However, if AMP deaminase is present, it will produce falsely high results by producing NH₃ directly from AMP, before it is hydrolysed by 5’N to adenosine. Persijn et al.’s assay measures at least three enzymes, 5’N, AMP deaminase and alkaline or other non-specific phosphatases, so the ‘5’N’ values found in [9] and [10] are naturally higher than those found by more specific assays. Since AMP deaminase is found in neutrophils [12], the relative error will be greater for RA rather than for OA patients. Wortmann et al. [11] tried to avoid the non-specificity of the Persijn assay method by measuring the 5’N inhibited by 25 µM AMPCP. Unfortunately, however, ADP and its analogue AMPCP are known to be allosteric effectors for AMP deaminase [23–25], and for other enzymes, and the unexpectedly poor inhibition of the 5’N activity in the SF of the RA patient by low concentrations of AMPCP only (Fig. 1) may well be due to AMPCP being bound to AMP deaminase and other enzymes rather than being available in solution as a competitive inhibitor of 5’N. The presence of the AMP deaminase inhibitor coformycin at least partially abolishes the anomaly, presumably because it stops AMPCP binding to AMP deaminase. Had Wortmann et al. used a higher concentration of AMPCP (≥100 µM), the problem would have been avoided.

There were too few OA and early RA samples to obtain significant results for the effect of disease duration and treatment on the 5’N activity in the SF; although preliminary findings suggested that the activity was lower in both the OA and RA patients if the disease duration was 3 yr or less (0.1 > P > 0.05). It is worth noting, however, that 5’N activity in the SF is essential to the anti-inflammatory function of sulphasalazine and methotrexate [26].

The correlation between the ESR and the serum 5’N in RA patients shown in Fig. 3 probably involves mainly the enzyme from the liver since even in RA patients the liver mass exceeds the synovial mass by ~100-fold. The acute-phase C-reactive protein, like 5’N, is also found on liver hepatocytes, but can be released by interleukin-8 [27], and it is likely that some similar mechanism might release the glycosyl phosphatidylinositol-linked membrane 5’N. This mechanism could act on the liver enzyme, or independently on the synovial lining cells in a particular joint.

Levick and McDonald [28] estimated that the turnover time for albumin in the SF of a normal human knee was ~1 h, but it is far longer in the rheumatoid joint because of the large volume of effuate present. The arrangement of fenestrated capillaries near the synovial surface is disrupted in RA patients [8], which may slow down both the 5’N and the general protein turnover, and therefore account for the lack of correlation between the 5’N in the SF and serum in these patients. The other factor which has a major effect on marker concentration in the SF, beside local production, is the fluid turnover rate. Since this is increased in RA, and is presumably increased to a variable extent, there...
may be a variable dilution of the 5'N in the joints. It was noteworthy that the JCA patient, and the RA patients with low 5'N SF/serum ratios, all produced large volumes of SF. Thompson et al. [29], using the 5'N data from Kendall et al. [9] and Farr et al. [10], calculated that the mean RA patient SF/serum ratio for 5'N was 7, which is higher than the 1.4 found in the present work, but can be explained by the presence of the neutrophil enzyme AMP deaminase in the RA SF, which would presumably show a distribution ratio similar to lactic dehydrogenase (4.2), and would be included in the Thompson 5'N ratio. These workers attributed the raised serum 5'N activities sometimes observed in RA patients as coming from their SF, but the data in this paper are not consistent with this.

Alkaline phosphatase, another glycosyl phosphatidyl-inositol-linked enzyme [30], is also raised in the serum of RA patients. Webb et al. [31] reported that the total activity of this enzyme in serum showed a rather weak correlation with the ESR (P = 0.01). However, the enzyme has several isoforms, and only that from the liver is raised in RA [32], which would make the total activity a less specific marker of liver activation than 5'N. There was a difficulty in believing that the raised serum 5'N in some RA patients came from the liver because most other enzymes indicative of liver damage in general were not raised [33]. This problem disappears now that it is known that 5'N and alkaline phosphatase are only held in the plasma membrane by a glycosyl phosphatidylinositol link from which they could be released by a suitable phospholipase, without damaging the liver cell. It seems, therefore, that the raised 5'N in either the serum or SF of RA patients can be taken as a biochemical marker of general or local inflammation, respectively.

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