Mast cell activation in arthritis: detection of α- and β-tryptase, histamine and eosinophil cationic protein in synovial fluid

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1. Although mast cell hyperplasia is a feature of rheumatoid arthritis and osteoarthritis, the extent and nature of mast cell activation in joint disease have not been clearly established.

2. We have investigated the levels of mast cell tryptase and histamine and also of eosinophil cationic protein in synovial fluid collected from 31 patients with rheumatoid arthritis, 14 with seronegative spondyloarthritis and nine with osteoarthritis.

3. α-Tryptase, which is likely to be released constitutively from mast cells, appeared to be the major form in synovial fluid, as the assay with antibody AAS detected appreciably more tryptase than that with antibody G5. β-Tryptase, which is released on anaphylactic activation of mast cells, was detected in 14 out of 45 synovial fluid samples studied, with concentrations of up to 12 μg/l measured by the G5 assay. The apparent levels of β-tryptase, but not of α-tryptase, were closely correlated with those of histamine in the synovial fluid. Patients with osteoarthritis appeared to have a greater proportion of β-tryptase in the synovial fluid than those with rheumatoid arthritis, as well as higher concentrations of histamine. Eosinophil cationic protein was present at high levels in the synovial fluid, although eosinophil numbers were low, and its concentrations were not correlated with the concentrations of the mast cell products.

4. These data suggest that anaphylactic degranulation of mast cells may have occurred to a greater extent in osteoarthritis than in rheumatoid arthritis, despite the relative lack of synovial inflammation in osteoarthritis. Although the eosinophil cationic protein detected may not reflect eosinophilic inflammation in the joint, the presence in synovial fluid of tryptase of both major forms, and of histamine, appears to indicate that mast cell products are secreted constitutively, as well as by processes of anaphylactic degranulation in rheumatoid arthritis, seronegative spondyloarthritis and osteoarthritis.

INTRODUCTION

The mast cell has the capacity to play a key role in arthritis, and increased numbers of mast cells have been identified in the synovial tissues of patients with rheumatoid arthritis (RA) and osteoarthritis (OA) [1–3]. These cells are distributed throughout the joint as well as being present in the synovial fluid (SF) [3, 4], and their location at the cartilage–pannus junction and in areas of matrix metalloproteinase deposition suggests roles in matrix remodelling [5]. The degree of mast cell hyperplasia in synovial biopsies appears to be related to the degree of clinical synovitis and lymphocytic infiltration, and increases in mast cell numbers have been reported to be associated with flares in disease activity [1, 6]. In contrast, administration of corticosteroids and clinical improvement can be accompanied by a decrease in mast cell numbers [1].

Mast cells can release an array of mediators with the potential to cause inflammation and tissue remodelling in the joint. The major secretory products of these cells are the neutral proteases, accounting for a cumulative total of up to 60 pg/cell [7]. Of these, the most abundant is tryptase (EC 3.4.21.59; 10–35 pg/cell), a tetrameric serine protease of 132 kDa which is found in all mast cells. Several sequences for tryptase have been described, of which the most different, termed α- and β-tryp-
tase, exhibit 90% similarity in amino acid sequence [8]. Tryptase purified from human tissues, which seems to be predominantly of the β type [9], can degrade certain neuropeptides, including vasoactive intestinal peptide and calciton gene-related peptide [10, 11], and activate procollagenase [12]. In addition, tryptase is a potent growth factor for fibroblasts [13, 14] and can stimulate collagen release from these cells [14].

Chymase, a serine protease present in a subpopulation of mast cells that is plentiful in synovial tissues [15, 2], may participate in processes of tissue degradation [16] and by activating the interleukin-1β precursor [17] and degrading interleukin-4 [18], could control cytokine bioavailability. Histamine secretion, or the generation of eicosanoids, may result in increases in vascular permeability [19]. In addition, mast cells may synthesize, store and release a number of cytokines involved in inflammation and fibrosis [19] and the release of basic fibroblast growth factor from synovial mast cells has been postulated to be an important stimulus for fibrotic processes in the joint [20].

Despite the potential roles of mast cells in mediating inflammation and tissue remodelling, there is little information available on the extent of mast cell activation in the synovial tissues of patients with arthritis. Histamine has been detected in SF [4], but the usefulness of this amine as a clinical marker of mast cell activation is limited by its rapid rate of degradation in vivo and by its presence in basophils and other cell types. Tryptase offers distinct advantages as a clinical marker of mast cell activation, being restricted almost entirely to this cell type [21], with only negligible quantities present in basophils [22]. Moreover, immunoreactive tryp
tase is relatively stable in biological fluids [23].

Elevated concentrations of tryptase are rare in the serum except in cases of systemic anaphylaxis or mastocytosis [24], but important information on the contribution of mast cells in a number of more localized inflammatory conditions has been obtained by measuring the levels of this unique mast cell protease in other body fluids. Thus, the detection of elevated concentrations of tryptase in bronchoalveolar lavage fluid has provided evidence for the involvement of mast cells in bronchial asthma, and similar conclusions have been drawn from tryptase determinations in nasal lavage fluids from rhinitics and skin blister fluid from atopic patients [23]. In SF from patients with arthritis, there have been brief reports indicating the presence of tryptase [25, 26]. However, a systematic study of tryptase levels in various forms of arthritis has not yet been reported.

In the present study we have employed RIAs incorporating antibodies with different affinities for tryptase isomers to determine tryptase levels in SF collected from patients with RA, seronegative spondyloarthritis (SpA) and OA. We have examined relative concentrations of the different forms of mast cell tryptase, as well as the levels of histamine, albumin (a marker of microvascular permeability) and the extent of inflammatory cell accumulation. In addition, as mast cell activation in allergic disease is frequently associated with the infiltration and activation of eosinophils, we have investigated whether the concentrations of mast cell products are associated with those of eosinophil cationic protein (ECP). We provide evidence that mast-cell-derived mediators are present in elevated concentrations in the SF in diverse forms of joint disease, and that this is a consequence of both constitutive release and an increased degree of mast cell degranulation.

METHODS

Patients

Patients had active RA, SpA or OA involving at least one knee and were attending a routine rheumatology outpatient clinic for therapeutic aspiration of SF and steroid injection. Numbers of patients in each of these categories and their medication are shown in Table 1. Seven SF samples from OA patients were analysed for the presence of crystals, but none was found. No joint had been injected within the preceding 3 months. SF was collected from both knees of some patients. Unless otherwise indicated, for the sake of consistency, the data presented here relate to SF from the left knee only where both were aspirated. The erythrocyte sedimentation rate and the volume of SF collected were noted.

All patients gave their written informed consent, and the approval of the local ethics committee was obtained.

Collection and processing of SF

After subcutaneous injection of lignocaine into the skin surrounding the knee joint, SF was aspirated and placed immediately on ice. The SF was centrifuged (350 g, 10 min, 4°C) and aliquots of the cell supernatant were frozen rapidly by placing on dry ice. Both the supernatants and aliquots of the cell suspension were stored at −70°C before the assay of mediators. Cells were washed once in PBS (pH 7.6) and were resuspended in 1 ml of PBS for cytocentrifuge preparations.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No.</th>
<th>Median age (range)</th>
<th>DMARD</th>
<th>Corticosteroids</th>
<th>NSAID</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>31</td>
<td>61 (36-85)</td>
<td>2†</td>
<td>6†</td>
<td>31</td>
</tr>
<tr>
<td>SpA</td>
<td>14</td>
<td>37 (24-63)</td>
<td>3</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>OA</td>
<td>9</td>
<td>68 (36-82)</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
</tbody>
</table>

†One patient was taking both a DMARD and corticosteroids.
Cell counts

Cell suspensions were stained with Kimura stain and nucleated cells were enumerated using an improved Neubauer haemocytometer. Cytocentrifuge preparations were air-dried, fixed in acetone for 1 min, and stained by the Giemsa procedure. Differential cell counts were performed by a blinded observer, who counted at least 500 cells.

Measurement of tryptase

Two different RIAs were adapted to measure tryptase in SF: one of these was a commercially available RIA with monoclonal antibody G5 [27] coated on plastic tubes (Pharmacia, Uppsala, Sweden), while the other was a RIA with monoclonal antibody AA5 [28] coupled to an agarose solid support (see below). In preliminary experiments, the commercially available assay was performed according to the manufacturer's instructions, adding 50 µl of undiluted SF sample per tube. However, investigation of the parallelism of standard curves after serial dilution or spiking of samples revealed the presence of an interfering factor in certain SF samples. The interference was overcome by adding an equal volume of high-salt assay buffer (4 mol/l NaCl, 0.8 mol/l Tris, 8 mmol/l EDTA, pH 7.6) as sample diluent. SF samples with low levels of tryptase were incubated with 1 mg of hyaluronidase/ml (EC 3.2.1.35; Sigma, Poole, Dorset, U.K.) for 5 min at room temperature in order to reduce viscosity, then freeze-dried and reconstituted in a fourfold lower volume of buffer before assay for the second time. In control experiments, neither the hyaluronidase treatment nor concentration by freeze-drying affected the assay for tryptase (data not shown). Concentrations of tryptase below the effective lower limit of detection of 0.25 µg/l were expressed as 0 µg/l.

The RIA with monoclonal antibody AA5 employed antibody purified from mouse ascites fluid by protein G chromatography (Pharmacia), coupled to cyanogen bromide-activated agarose (Sepharose 4B, Pharmacia) according to the manufacturer's instructions and stored in Tris buffer (0.2 mol/l Tris, 10 mmol/l EDTA, pH 7.6). Aliquots of 50 µl SF or standard (Pharmacia) were added to 50 µl high-salt buffer and 100 µl of 20% AA5-agarose suspension, and mixed overnight at room temperature. The agarose was washed three times with 2 ml of Tris buffer, centrifuging each time at 350 g for 10 min, the supernatant was removed and the agarose beads were incubated with 50 µl of 125I-labelled monoclonal antibody G4 against tryptase (Pharmacia; approximately 100 000 d.p.m./50 µl) overnight at room temperature. After three washes with 2 ml of normal saline (154 mmol/l NaCl), the radioactivity in the pellet was measured in a gamma counter. The limit of detection for this assay was 1 µg/l (calculated as three SDs above the mean value for the zero standard).

Western blotting with recombinant α- and β-tryptase

It was noted in preliminary experiments that more tryptase was detected when substituting G5 with AA5 as the capture antibody in the RIA. As G5 antibody has recently been found to have a high affinity for the recombinant β isoform of tryptase, but not for recombinant α-tryptase [27], we considered the possibility that discrepancies between these assays might relate to different degrees of binding to these isoforms. Therefore, the affinities of AA5 antibody for recombinant α- and β-tryptase were investigated on Western blots.

Recombinant human tryptases were expressed and purified by immunoaffinity chromatography from culture supernatants of sf-9 cells infected with baculovirus constructs containing either α- or β-tryptase [29]. Both α- and β-tryptases were subjected to SDS/PAGE with 12% gels (Novex, San Diego, CA, U.S.A.), transferred to nitrocellulose, and probed with AA5 antibody using the procedure reported previously [27], except that the AA5 antibody was not biotinylated and a 1 h incubation with alkaline phosphatase-conjugated goat IgG anti-mouse immunoglobulin G (Fc specific) antibody (1/2000; Sigma) was included. The blot was developed with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Measurement of other markers

Histamine levels were determined by RIA (Immunotech, Marseilles, France), and ECP was measured by fluorimetric enzyme immunoassay (Pharmacia) using the Pharmacia CAPS® system. The limits of detection were 0.1 µg/l and 2 µg/l, respectively. Albumin in the SF was assayed by an immunoturbidometric procedure using a rabbit anti-albumin antibody (Dako, High Wycombe, Bucks, U.K.) in a Multistat centrifugal analyser (Instrumental Laboratories, Lexington, MA, U.S.A.).

Statistics

Data were analysed using the Kruskal–Wallis one-way analysis of variance test for multiple comparisons. If a significant difference was found between the groups, further analysis was performed with the Mann–Whitney U-test. For a comparison of the tryptase levels measured using the two RIAs, the Wilcoxon signed rank test was employed. Values for P of less than 0.05 were deemed statistically significant. The measure of association used was Spearman's coefficient of rank correlation (rS). Since a number of possible correlations were investigated...
for this test, only values for \( P \) of less than 0.01 were taken as significant.

RESULTS

Cellular constituents and albumin levels

In SF from patients with RA and SpA, neutrophils predominated, whereas macrophages were the most numerous cell type in those patients with OA (Table 2). Eosinophils were detected in only a few samples. While albumin concentrations, taken as a marker of microvascular permeability, tended to be lower in the SF samples from OA patients than those from the patients with RA or SpA, the difference did not reach statistical significance.

Tryptase

Using the RIA procedure with G5 antibody, with an effective lower limit of detection of 0.25 \( \mu g/l \), tryptase could be detected in SF from four out of 23 patients with RA, three out of thirteen with SpA and four out of six with OA (Fig. 1a). Using the RIA with monoclonal antibody AA5 (Fig. 1b), tryptase was detected in all SF samples, and levels recorded were higher than those using the RIA with G5 antibody \(( P < 0.0001, n = 37 )\). However, considering all disease categories together, tryptase concentrations measured by the AA5 RIA were significantly correlated with those determined by the G5 RIA \(( P < 0.01, r = 0.444, n = 37 )\). The ratio of tryptase measured by the G5 RIA compared with that measured by the AA5 RIA was significantly greater for SF from OA patients (median 0.128) than that from those with RA (median 0.00; \( P < 0.05 )\).

There was no association between tryptase levels (however measured) and the numbers of any cell type or with the patient’s erythrocyte sedimentation rate or with the volume of SF collected. No differences in SF tryptase levels were observed between the RA patients taking disease-modifying anti-rheumatic drugs or corticosteroids and those on other therapy.

Antibody binding to \( \alpha \)- and \( \beta \)-tryptase

AA5 antibody bound to both isoforms of recombinant human tryptase on Western blots. The antibody had comparable affinity for \( \alpha \)- and \( \beta \)-tryptases over a range of dilutions (Fig. 2). The binding pattern seen with AA5 differs from that found previously with antibody G5 which binds with much higher affinity to the \( \beta \) form than the \( \alpha \) form of tryptase [27]. Thus, the AA5 RIA is likely to have detected both \( \alpha \) - and \( \beta \)-tryptase, whereas the G5 RIA will have detected predominantly the \( \beta \) form.

Histamine

Histamine was detected in almost all SF samples investigated (Fig. 3). The SF from patients with OA contained significantly more histamine than that from the patients with RA \(( P < 0.01 )\), but there were no other significant differences between disease categories. For the SF samples as a whole, the histamine content of SF samples was correlated with tryptase concentrations measured using the G5 RIA \(( P < 0.005, r = 0.486, n = 38 )\), but not with the levels determined by the AA5 RIA. When the values obtained by the G5 RIA were subtracted from those obtained by the AA5 RIA to give an indication of the concentrations of \( \alpha \)-tryptase, no association with histamine concentrations was found. Histamine concentrations were not correlated with numbers of neutrophils, macrophages or lymphocytes or with the patient’s erythrocyte sedimentation rate or the volume of SF collected.

The histamine content of cell lysates prepared from the SF of certain RA patients ranged from 0.07 to 42 ng/10^6 cells (median 2.39 ng/10^6 cells; \( n = 12 )\), indicating the presence of mast cells or basophils free in the fluid. There was no correlation between the quantity of histamine in the cell lysate and levels of either histamine or tryptase free in the SF.

ECP

High concentrations of ECP were measured in many SF samples, particularly in the RA and SpA groups (Fig. 4). There was no significant correlation between ECP levels and tryptase or histamine concentrations, the numbers of eosinophils or other cell

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>( 10^3 \times ) Nucleated cells (ml^-1)</th>
<th>( 10^3 \times ) Neutrophils (ml^-1)</th>
<th>( 10^3 \times ) Macrophages (ml^-1)</th>
<th>( 10^3 \times ) Lymphocytes (ml^-1)</th>
<th>( 10^3 \times ) Eosinophils (ml^-1)</th>
<th>Albumin (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>1700 (0-81 000)</td>
<td>1230 (0-5700)</td>
<td>257 (0-80 000)</td>
<td>4 (0-1540)</td>
<td>0 (0-6)</td>
<td>23.8</td>
</tr>
<tr>
<td>SpA</td>
<td>1620 (35-9780)</td>
<td>1320 (0-8730)</td>
<td>258 (0-4360)</td>
<td>7 (0-183)</td>
<td>0 (0-13)</td>
<td>24.9</td>
</tr>
<tr>
<td>OA</td>
<td>117 (0-6380)</td>
<td>5 (0-5800)</td>
<td>45 (0-458)</td>
<td>1 (0-101)</td>
<td>0 (0-13)</td>
<td>18.6</td>
</tr>
</tbody>
</table>
types present, or with the erythrocyte sedimentation rate or volume of SF collected.

Comparison of mediator content in SF from different knees

For ten patients, SF was aspirated from both knees on the same visit to the clinic. Levels of histamine and ECP in the samples obtained from left and right knees were often quite similar (Fig. 5). There was some degree of correlation between ECP concentrations in left and right knees, although not when applying our more stringent criteria for statistical significance ($r_s = 0.720, n = 9, P = 0.029$). However, when SF was aspirated more than once from the knees of six patients after an interval of between 2 and 30 months, the concentrations of tryptase (AA5 assay), histamine and ECP differed markedly between samples collected at different times with tenfold variations observed in some cases (data not shown).

**DISCUSSION**

Our findings show that mast cell activation occurs in synovial tissues in quite different forms of joint disease. Mast cell tryptase measured by different assays and also histamine were detected not only in the SF of patients with inflammatory arthritides, but also in samples collected from those with OA.
Indeed, the histamine levels and the relative proportions of $\beta$-tryptase measured by the immunoassay with G5 antibody were significantly higher for the OA group than for those with RA. The observation of mast cell activation in the absence of marked inflammatory changes seems surprising, even though mast cell hyperplasia is a feature of the synovial tissues of patients with OA [3, 15].

The choice of capture antibody had a major bearing on the amount of tryptase measured. Considerably more tryptase was detected in SF samples using AA5 rather than G5 monoclonal antibody. A parallel situation has been found in serum samples from normal subjects and patients with mastocytosis, in which lower values for serum tryptase concentrations were determined by the G5 assay than with an assay with another capture antibody, B12 [27]. The differences in assay performance were related to differences in the specificity of the capture antibody, for whereas B12 bound recombinant $\alpha$- and $\beta$-tryptase with equal affinity, G5 had an affinity for the $\beta$ form which was much greater than that for $\alpha$-tryptase. The explanation for the different findings with the G5 and AA5 assays in the present study appears to be similar, for AA5 reacted equally well with both $\alpha$- and $\beta$-tryptase on Western blots. Thus with AA5 as the capture antibody, both $\alpha$ and $\beta$ forms will have been detected in the SF, while with the G5 assay, the tryptase measured is likely to be predominantly that of the $\beta$ form.

The apparent detection of high levels of $\alpha$-tryptase in the serum of patients with mastocytosis, but not those with anaphylactic shock, has prompted the suggestion that $\alpha$-tryptase is synthesized and released constitutively from mast cells [27], possibly on account of an amino acid substitution which precludes intracellular processing of the pro-enzyme [29]. On the other hand, $\beta$-tryptase appears to be the stored form which is released on anaphylactic degranulation as the G5 assay detects high levels of tryptase in the serum of patients with anaphylaxis [27]. These contentions are supported in the present study by the finding that SF levels of histamine correlated closely with levels of $\beta$-tryptase (as determined by the G5 RIA), but not with levels of both $\alpha$- and $\beta$-tryptase (determined by the AA5 RIA) or the apparent concentration of $\alpha$-tryptase (estimated by subtracting values obtained by the G5 RIA from those of the AA5 RIA).

Our data suggest that while both $\alpha$- and $\beta$-tryptase are released, there is preferential secretion of the $\alpha$ form in the arthritic joint. By analogy with the situation reported in anaphylaxis and mastocytosis, it is possible that much of the tryptase present in SF may reflect mast cell hyperplasia rather than anaphylactic degranulation. Nevertheless, the presence of $\beta$-tryptase as revealed by the G5 immunoassay, as well as of histamine, does provide compelling evidence that there is appreciable activation of synovial mast cells in arthritis. The precise mechanisms remain to be determined, but reports of immunoglobulin E rheumatoid factors [30] and immunoglobulin E with specificity for cartilage collagens [31] suggest the potential for immunoglobulin E-dependent processes of mast cell degranulation. Mast cells may also be activated by a host of other factors, including certain neuropeptides [32], histamine-releasing factors or cytokines [33–35].

The observation of differences between patient groups in the concentrations of mast cell mediators may reflect differences in the mechanisms of cell activation. There was considerable variation within each of the disease categories, but the ratio of tryptase concentration determined by the G5 assay compared with that obtained by the AA5 assay was significantly greater for the OA than for the RA group. This would suggest that patients with OA may have had a greater proportion of $\beta$-tryptase in

![Fig. 5. Comparison of mediator levels in SF aspirated from both knees of patients at the same visit. Concentrations of (a) tryptase measured by RIA using AA5 antibody, (b) histamine and (c) ECP in the SF of patients with RA (open symbols), SpA (●) and OA (▲) are shown. Each individual patient has been accorded the same symbol throughout.](image)

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the SF than those with RA. Consistent with this was the observation of higher levels of histamine in the SF of the OA group than of the RA group. Patients were selected purely on the basis of their need for therapeutic aspiration of SF and some caution should be exercised when comparing between the disease groups. Nonetheless, it would seem that anaphylactic degranulation of mast cells has occurred to a greater extent in subjects with OA than with RA.

As mast cells are frequently associated with eosinophils in allergic disease, SF samples were assayed for ECP in order to determine if there was an association between mast cell activation and eosinophil degranulation. Very high levels of ECP were present in some samples of SF. This is in accordance with an earlier report by Hallgren et al. [36], who found a mean level of over 500 µg/l of ECP in the SF of RA patients. However, very few, if any, eosinophils were observed in cyt centrifuge preparations in the present study. This apparent paradox could be explained by the loss of eosinophil staining due to excocytosis of the granule contents, or alternatively, the ECP in SF may have been generated by eosinophils resident in the synovial tissues. Neutrophils have also been reported to store small quantities of ECP in their granules [37] and large numbers of these cells were present in many SF samples. The origin of the ECP is not clear, but no association with tryptase or histamine levels was found.

In conclusion, we have detected both α- and β-tryptase in the SF of patients with arthritis. Our findings suggest that the α form of tryptase was present in all the SF samples studied and could provide an indication of mast cell hyperplasia. The β form was detected in a number of samples of SF and with levels being closely associated with those of histamine, appears to reflect mast cell degranulation. The consequences of mast cell activation in the joint could be of major importance for disease progression. Tryptase, histamine and a host of other mediators likely to be co-released from mast cells can profoundly alter the behaviour of other cell types, stimulating microvascular leakage, tissue remodelling, angiogenesis and the ingress of inflammatory cells [19]. However, the consequences of mast cell activation appear to be different in different forms of arthritis. This raises the possibility that mast cell products may be modulated by disease-specific factors, which could modify or attenuate their proinflammatory actions. Despite inflammatory processes being more evident in RA than in OA, there was evidence for processes of anaphylactic mast cell activation occurring in the joint in both conditions.

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