INHIBITOR REGULATION OF TISSUE KALLIKREIN ACTIVITY IN THE SYNOVIAL FLUID OF PATIENTS WITH RHEUMATOID ARTHRITIS

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SUMMARY
Tissue kallikrein (TK) and α1-antitrypsin (AT)/TK complexes can be detected in SF from patients with RA if components of the fluids which interfere with the detection of TK are removed. α2-Macroglobulin (α2-M) in SF was demonstrated to contain trapped proteases which were still active in amidase assays. Removal of α2-M from RA SF reduced their amidase activity. However, at least some of the remaining activity was due to TK because it was soya bean trypsin inhibitor resistant and trasylol sensitive and was partly removed by affinity chromatography on anti-TK sepharose. Removal of RF from the fluids reduced the values obtained for TK levels by ELISA. Addition of SF to human urinary kallikrein (HUK) considerably reduced the levels of TK detected suggesting the presence of a TK ELISA inhibitor in the fluids. Removal of components of >300 kDa from SF markedly reduced the TK ELISA inhibitory activity and increased the values for both the TK and α1-AT/TK levels in fluids as measured by ELISA. It is considered this novel inhibitor does not bind to the active site of TK but rather binds to the site reactive with anti-TK antibodies.

KEY WORDS: Tissue kallikrein, α1-Antitrypsin-tissue kallikrein complexes, α2-Macroglobulin, Neutrophils, Rheumatoid arthritis

Several reports have suggested that pain and swelling in inflammatory joint disease may be mediated by kinins formed by the enzymatic action of kallikreins [1–3]. It has been proposed that in the inflamed joint, inhibitors which regulate the activity of proteases such as tissue kallikrein (TK) and plasma kallikrein (PK) may either become ineffective as a result of degradation by oxygen free radicals or be overwhelmed by an excessive conversion of the kallikrein zymogen into an active form. One consequence would be the increased formation of kinins, and another a rise in the concentration of uninhibited enzymes directly causing tissue damage. Normal endogenous constraint on the enzymic activity of tissue kallikrein is exercised by the serpin inhibitor α1-antitrypsin (α1-AT), and on plasma kallikrein by α2-macroglobulin (α2-M) and C1 esterase inhibitor. Large quantities of these inhibitors are present in the SF of patients with RA [4,5], and the extent to which they control the activity of the two kallikreins may be reflected in the degree to which interferences are observed with assay systems developed to measure free and uncomplexed TK and PK in SF.

Earlier experiments from this laboratory [6] identified a TK in SF which was immunoreactive and possessed enzymic activity both on the synthetic amidase substrate, D-Val-Leu-Arg-pNA, and on human kininogen, the natural substrate. In order to quantify amidase activity in body fluids that could be ascribed to TK, it was necessary to measure samples in the presence of soya bean trypsin inhibitor (SBTI), which does not affect the enzymic activity of TK, but binds to other enzymes in biological fluids like PK that also hydrolyse D-Val-Leu-Arg-pNA [7]. Inactive TK can be measured after activation of samples by thermolysin or by trypsin (which must itself then be inhibited by SBTI, prior to the addition of the peptide substrate in the amidase assay). When we found an inexplicably higher amidase activity following trypsin activation of SF proenzymes, we suspected a possible problem on the determination of precise values for both active and proforms of TK and PK in such fluids.

Studies of enzyme-inhibitor complexes in RA SF have indicated the presence of enzymes bound to either α1-AT or α2-M, including PK-α2-M, elastase-α1-AT and α1-AT/TK [8–10]. A particular characteristic of the α2-M molecule is that because of its selective affinity for trapping PK (88 kDa) and trypsin (23 kDa) and not TK (35–45 kDa), α2-M constrains these enzymes without occluding their active site. Another feature is that once serine proteases become trapped in the α2-M cleft a diffusion barrier is created so that inhibitors like SBTI (about 22 kDa) are prevented from gaining access, whereas inhibitors like trasylol (6.5 kDa) gain access readily. However, problems are created when one attempts to determine precise values for SBTI-sensitive (PK) and -resistant (TK) in SF. Because PK is trapped by α2-M, and retains the capacity to hydrolyse the amidase substrate (0.58 kDa), spuriously high values would be recorded.


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for the SBTI-resistant TK which has no affinity for, and therefore forms no complexes with α2-M [4].

The initial aim of this work was thus to determine whether or not α2-M interfered with TK measurements. Additional studies were directed towards identifying the activity due to TK in functional assays as judged by adsorption with solid-phase anti-TK IgG. Further, attempts to develop an ELISA for the determination of TK and α1-AT/TK levels in SF, indicated the presence of substances interfering in these assays. Therefore, the second aim was to identify the interfering substances.

PATIENTS AND METHODS

Patients

For these studies two pools of SF were used consisting of fluid collected from the knees of 14 patients (Table I) with RA treated at the Rheumatology Department of Bristol Royal Infirmary.

Sample collection

Therapeutically aspirated SF from inflamed joints was centrifuged at 5000 rpm at room temperature (RT) for 7 min to remove cells and debris. The supernatants were thoroughly mixed, aliquoted and stored at -20°C. Prior to use, the SF were pooled, treated with hyaluronidase (22.5 IU/ml SF for 30 min at 37°C) to reduce viscosity and centrifuged at 5000 rpm for 7 min.

Measurement of TK enzymic activity

1. Amidase assay. The amount of functionally active TK was measured by assessing the activity of the enzyme on the synthetic substrate, D-Val-Leu-Arg-pNA (S2266, Kabivirum) [11] in the presence of SBTI as modified by Figueroa et al. [12]. A further development of the method allowed measurement of samples in a microtitre plate (end point assay) rather than in a spectrophotometer (rate assay), thereby increasing sensitivity. Briefly, 50 μl standard human urinary kalirein (12.5–400 ng/ml HUK; Protogen) was added to 100 μl of assay buffer (0.2 M Tris/Hcl, pH 8.2), or 50 μl sample was added to 50 μl of buffer containing SBTI (300 μg) and EDTA (375 μg). The mixtures were incubated at 37°C for 30 min and made up to 150 μl with buffer. For activation of proenzyme, the sample was first incubated for 45 min at 37°C with 30 μg trypsin (Sigma) in a final volume of 100 μl. Fifty microlitres of the substrate (S2266) was then added to all samples and the absorbance at 405 nm measured after a further incubation for 3 h at 37°C. Non-specific absorbance due to the samples was determined by incubating the samples in the absence of the substrate. Values were corrected for the non-specific absorbance. Values in test samples were calculated by reference to the TK standard curve and expressed as ng/ml. A standard curve with an identical concentration range was determined using the synthetic substrate S2266 in order to convert the values into mU/mg protein [12].

2. Kininogenase assay. Freeze-dried SF samples were reconstituted to their original volume with distilled water, and assayed with kininogen, prepared according to the method of Marin-Grez and Carretero [13], as the substrate. A 50 μl sample was incubated with 50 μl of kininogen (2 mg/ml in 0.1M NaH2PO4, 3 μM 1, 10-phenanthroline, 30 μM EDTA, pH 8.3) for 30 min at 37°C. The reaction was terminated with 1 ml 98% ethanol, and each sample heated to 70°C for 10 min. The mixture was centrifuged, and the supernatant transferred to a new micropipette; the precipitate washed once with 500 μl of 98% ethanol, centrifuged, the two supernatants combined and then freeze-dried. The residue was dissolved in 0.5 ml radioimmunoassay

<table>
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<tr>
<th>No.</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Joint</th>
<th>Duration (yr)</th>
<th>Pain</th>
<th>Swelling</th>
<th>Volume (ml)</th>
<th>Therapy</th>
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TABLE I

Clinical details of patients with RA at the time of aspiration (RS)

Abbreviations: SH, Shoulder; KN, knee.
Second-line drugs: auranofil; azathioprine; gold (i.m.); methotrexate; penicillamine; salazopyrine.
(RIA) buffer. The kinin concentration was determined by RIA [14], and activity expressed as fmole/min/ml of the original SF volume.

Removal of α2-M

α2-M was removed from SF by adsorption with anti-α2-M IgG (Dakopatts) coupled to CnBr Sepharose. SF was incubated with an equal volume of anti-α2-M Sepharose and 0.2 vol 0.1 M Tris/0.5 M NaCl pH 8.5 for 30 min at room temperature. The mixture was centrifuged and the supernatant collected.

Treatment of samples with solid phase anti-TK

After removal of α2-M, RA SF samples were incubated with 0.1 vol of anti-TK IgG coupled to CnBr Sepharose in the presence of 0.5 M NaCl and pH 8.5 for 30 min at room temperature. The samples were treated similarly after activation with trypsin.

Measurement of TK by ELISA

Incubations were for 60 min at 37°C with 100 μl of solution added to each well of a microtitre plate (Immulon), unless otherwise indicated. Rabbit anti-human TK IgG raised by immunizing a rabbit with purified HUK (KDB1) [12] (5 μg/ml in carbonate buffer consisting of 15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) was used to coat plates. After blocking the plates (30 min at RT) at first with 5% bovine serum albumin (BSA) but later with 5% low fat bovine milk (Marvel original, Premier Brands, UK) in Tris (0.02 M)-NaCl (0.14 M) 0.05% Tween 20 (Sigma) pH 7.2, the plates were incubated sequentially at 37°C with: sample or standard HUK, goat anti-HUK (Protogen: 10 μg/ml), alkaline-phosphatase-labelled rabbit anti-goat IgG (Sigma Chemicals; 1:250) and substrate solution (1 mg/ml p-nitrophenyl phosphate in carbonate buffer). Between each incubation, the wells were rinsed 3× for 10 sec with excess wash (0.05% Tween 20 in PBS pH 7.4); all reagents were made up, initially in 0.1% BSA/PBS but later in 5% Marvel. The hydrolysis of substrate was assessed by measuring absorbance at 405 nm in a Biorad (UK) colorimetric plate reader initially after 60 min but later 240 min (to increase sensitivity) at 24°C. Non-specific absorbance was determined by performing incubations in the absence of the sample, and sample values corrected for the non-specific absorbance. For each experiment, values for test samples were calculated by reference to a simultaneously performed TK standard curve (range: 20 to 0.01 ng/ml), and expressed as ng/ml and later as ng/mg protein (as judged by the Bradford Coomassie blue method). The minimum detection value for TK in the 240 min incubation experiments was 0.014 ng/ml.

Fig. 1.—Amidase activity of an RA SF pool, before and after incubation of active and activated proenzymes with inhibitors. 1, No inhibitor; 2, soya bean trypsin inhibitor (SBTI); 3, trasylol and SBTI; 4, activated proenzyme with no inhibitor; 5, activated proenzyme with SBTI; 6, activated proenzyme with trasylol; 7, activated proenzyme with trasylol and SBTI. SBTI inhibits plasma kallikrein and trypsin, but not tissue kallikrein; whereas trasylol inhibits all three enzymes. The results are the mean and s.d. of three experiments.
FIG. 2.—Double immunodiffusion against anti-α2-M, showing the presence of α2-M in SF, and its absence after filtration through 300 kDa filter. 1, RA SF; 2, RA SF absorbed IgG sepharose; 3, fraction of RA SF retarded by 300 kDa filter; 4, RA SF centrifuged through 300 kDa filter; 5, saline.

α1-AT/TK ELISA

α1-AT/TK complexes were prepared by incubating 25 μl of 0.8 μg/ml of lyophilized HUK (Green Cross Corporation, Osaka, Japan) with 25 μl of 100 μg/ml of α1-antitrypsin (Sigma Chemicals, UK). Both reagents were diluted in 0.2 M Tris-HCl buffer (pH 8.2). For the complex ELISA, the Immulon microtitre plate was coated with the polyclonal rabbit anti-TK IgG (KDB1), diluted in coating buffer (see TK ELISA) to give a concentration of 5 μg/ml. Next, the microtitre plate was incubated for 30 min at room temperature, with 5% Marvel to block the unoccupied sites. Subsequently, the plate was incubated either with test sample or with α1-AT/TK complexes to generate curves with concentrations of complexes that ranged from 0.05 to 50 ng/ml. At the end of this period sheep antihuman α1-AT conjugated with alkaline phosphatase (Serotec, UK) was added in a dilution of 1.250. Finally, the well contents were incubated (overnight) with the substrate (disodium p-nitrophenol phosphate, 1 mg/ml) for the alkaline phosphatase to develop the chromophore. The absorbance values were read on the Biorad plate reader. For each step, unless otherwise stated, the plate was incubated for 1 h at 37°C and washed three times (for 10-sec periods) between steps with PBS-Tween 20 buffer (pH 7.4). Values for test samples were calculated from the standard curve generated for each experiment, and expressed as α1-AT/TK ng/mg protein.

A second α1-AT/TK-complex ELISA was developed using biotin-extravidin conjugates. After applying the primary antibody (KDB1), samples and the secondary antibody (sheep anti-human α1-AT, 8 μg/ml), biotin conjugated anti-sheep IgG (1.2000) was added, and thereafter extravidin labelled alkaline phosphatase. All further steps were performed as already described except that the incubation time with the substrate was 2 h. Overall these modifications increased the sensitivity of detection fourfold.

Removal of high molecular weight components from SF

SF were filtered through 300 kDa filtration membranes (Ultrafree, Millipore, Japan) by centrifugation at 5000 rpm at 4°C for 12 h.

Removal of RF from SF

SF, either pooled or individual samples, were incubated with 0.3 vol of heat aggregated rabbit IgG coupled to CnBr Sepharose and 0.2 vol of 0.1 M Tris/0.5 m NaCl pH 8.5 for 30 min at room temperature. Removal of RF was verified by assaying for the presence human IgM bound to IgG coated microtitre plates. In addition, TK standards (0.63, 2.5, 10 ng/ml HUK) were measured in the presence of SF following adsorption of RF.

Spiking experiments

Known amounts of RA SF were added to varying concentrations of HUK and TK measured.

Attempts to remove or destroy TK inhibitors in SF

The treatments included: (a) pre-incubation with 5 mg of SBTI/ml for 30 min at 37°C, (b) acid-heat treatment: acidification with HCl to pH 4.0 for 30 min at room temperature, followed by heating at pH 7.0 at 60°C for 45 min, (c) centrifugation through membranes with either 10, 100 or 300 kDa cut off (Ultrafree, Millipore).

RESULTS

The initial experiments which alerted us to a possible problem with α2-M are shown in Fig. 1. They were designed to determine the amidase activity profile of an RA SF pool. Note particularly the high amidase activity of SF after trypsin activation of precursor enzymes (column 4) which was only partly inhibited by SBTI (column 5) but reduced substantially by trasylol (column 6). Since SBTI inhibits only PK and trypsin but not TK whereas trasylol inhibits all three enzymes these results could indicate that there are large amounts particularly of precursor TK in RA SF. On the other hand, as trasylol has access to a2-M trapped proteases while SBTI has no access, they could suggest that trypsin and other enzymes were bound by a2-M and were still available to cleave the amidase substrate.

To distinguish between these possibilities experiments were set up to determine the effect of removing α2-M amidase activity in RA SF. The first approach to remove α2-M was by centrifugation through millipore filters with an exclusion value of 300 kDa. The efficacy of this procedure was confirmed by Ouchterlony gel diffusion (Fig. 2). The amidase activity in the retarded and filtered fractions of an RA SF pool is shown in Figs 3(a) and 3(b). It can be seen that the amounts of active and proenzyme in the filtered fraction were considerably reduced as compared with the retarded fraction. Comparison of the amidase activity in the filtered
FIG. 3.—Amidase activity of (a) the retarded and (b) filtered fractions of RA SF, after centrifugation through 300 kDa millipore filters. The protein concentration of the samples was; 45.1 mg/ml for untreated RA SF; 31.67 mg/ml for fraction of RA SF retarded by 300 kDa millipore filter; and 9.04 mg/ml for fraction filtered through 300 kDa millipore filter. For the inhibitor profiles consult Fig. 1. The results are the mean and s.d. of three experiments.
TABLE II
Effect of removing α-2 macroglobulin on RA SF amidase and kininogenase activity

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<th>Amidase (mU/ml)</th>
<th>Kininogenase (fmole/ml)</th>
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<tr>
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<td>Plus α2-macroglobulin</td>
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<tr>
<td>SF</td>
<td>57.00</td>
<td>100.00</td>
</tr>
<tr>
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<td>5.60</td>
</tr>
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<td>445.00</td>
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<tr>
<td>SF</td>
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<td>1.26</td>
</tr>
<tr>
<td>SF + SBTI</td>
<td>4.25</td>
<td>7.40</td>
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</tbody>
</table>

Abbreviation: SBTI, soya bean trypsin inhibitor.

FIG. 4(a).—Effect of removing RF from an RA SF pool on the measurement of TK by ELISA. 1, Untreated SF; 2, elute from IgG Sepharose; 3, fraction of RA SF retarded by 300 kDa millipore filter; 4, fraction of RA SF filtered through 300 kDa millipore filter. The results are the mean and S.D. of three experiments. (b) Inhibitory effect of RF absorbed (IgG Sepharose) RA SF pool on the measurement of a tissue kallikrein (HUK) standard curve by ELISA. Continuous line, HUK standard curve. ---, HUK standard curve in the presence of neat RF factor absorbed SF. The results are the mean and s.d. of three experiments.

FIG. 5.—Effect of various treatments on the ability of RF absorbed RA SF to inhibit the measurement of TK by ELISA. 1, None; 2, addition of soya bean trypsin inhibitor (SBTI); 3, acid/heat; 4, removal of <10 kDa components; 5, removal of α2-M; 6, removal of >100 kDa components; 7, removal of >300 kDa components.

Fractions following incubation with the inhibitors, SBTI (inhibits PK) and trasylol (inhibits both kallikreins), provided true estimates of the relative proportions of both active and pro tissue and PK in RA SF.

In the second approach, a SF pool from RA patients was preadsorbed with solid-phase anti-α2-M. The successful removal of immunoreactive α2-M from the fluid was verified by Ouchterlony immunoprecipitation and Western blotting (results not shown). In these experiments the amidase and kininogenase activities in the pool were compared, before and after removal of α2-M (Table II). The results showed that the SBTI-resistant activity was reduced by removal of α2-M (which contained the α2-M trapped proteases), whereas the kininogenase activity was not affected. Prokallikreins (tissue and plasma) in SF treated with trypsin and converted into their respective active forms resulted in high activities, both of which were reduced after the removal of α2-M.

In order to verify the presence of TK in α2-M depleted RA SF, the amidase activity was measured before and after adsorption with anti-TK IgG. Additionally, the amount of tissue prokallikrein (following trypsin activation) was measured similarly after adsorption with anti-TK IgG. The results showed that solid phase anti-TK removed 25% (n = 3) of active and 31% (n = 6) of pro enzyme TK.

Immunoreactivity of TK in SF
Experiments were designed to determine the levels of immunoreactive TK in SF by ELISA. As RF (mainly IgM anti-IgG) is known to interfere with such immunoassays, the effect of removing RF on the ELISA measurements of TK was examined. Removal of the RF from pooled SF reduced the apparent TK value
Studies on inhibitors in RA SF

The ELISA results suggested that an inhibitor might be present in RA SF. To examine this possibility, a RF-absorbed SF pool was treated in several ways, and the effect of these treatment on the recovery of added TK was determined. As illustrated in Fig. 5, only about 6% was recovered when TK was added to the untreated pool or that in which protease activity had been inhibited by SBTI or acid/heat treatment. Removal of components of less than 10 kDa or removal of α2-M failed to improve recovery substantially. By contrast, if SF components greater than 100 kDa or 300 kDa were removed prior to spiking, there was a considerable improvement of recovery.

One possibility was that TK had become complexed to α1-AT. We therefore examined RA SF for the presence of α1-AT/TK complexes. Fig. 6 shows that such complexes can be detected in RA SF. Whereas removal of RF reduced the values, filtration through 300 kDa exclusion membrane resulted in a fourfold increase in the specific activity of the α1-AT/TK complexes. Similar results were obtained with individual RA SF (results not shown).

Comparison of TK functional activity and immunoreactivity in SF

In order to determine whether the inhibition observed in the ELISA experiments affected the functional activity of TK we compared in both assays recovery of TK added to SF. Whereas in the TK ELISA, the markedly reduced recovery of added TK was seen with both untreated and α2M-free SF, this was not the case when similar experiments were performed to examine the functional activity of the enzyme in the amidase assay as shown in Fig. 7.

DISCUSSION

The first question asked in this paper is whether α2-M interferes with the measurement of active TK in RA SF. The results show that the SF amidase activity of both active and precursor enzyme was inhibited to a greater degree by trasylol which has access to α2-M trapped proteases than by SBTI which has no access. Similarly, the removal of α2-M by filtration or affinity chromatography substantially reduced the SF amidase activity. Thus much of the SBTI-resistant amidase activity in RA SF is due to α2-M protected proteases.

It may be asked which enzyme(s) is responsible for the activity on the S2266 substrate remaining after removal of α2-M. The results suggest that it is due to the presence of both PK and TK. The remaining activity was reduced by SBTI. As SBTI inhibits PK but not TK then the SBTI inhibitable activity is likely to be due to PK. Trasylol (inhibits both enzymes) reduced the remaining activity further which is consistent with the trasylol sensitive but SBTI resistant activity being attributable to TK. In support of this contention some 25% of the amidase activity of α2-M depleted RA SF was removed by affinity chromatography on solid
phase anti-TK. Since (see below) RA SF contains a factor which interferes with anti-TK binding to TK, then the amount of TK removed from RA SF by solid phase anti-TK is probably an underestimate. It is clear therefore that at least some active and proenzyme TK is present in RA SF. Moreover, these results show that the removal of α2-M from any body fluid containing this inhibitor is essential before attempting to measure functional TK in such fluids.

The addition of RF absorbed RA SF to HUK interfered profoundly with the measurement of this TK by ELISA. It follows that the fluids contain some form of inhibitor. Attempts were made to gain further information about the nature of this inhibitor. One possibility is that the added HUK could be destroyed by proteases present in the SF. The fact that recovery was not improved by SBTI- or acid-heat treatment of SF militates against this hypothesis. The possibility that TK ELISA inhibition was due to the pH or ionic strength of SF was excluded by the fact that removal of low molecular weight components from SF did not abrogate the inhibitory effect. However, removal of components greater than 300 kDa MW was associated with loss of ELISA inhibitory activity on added TK. Additionally, the fact that higher values for immunoreactive TK were measured in SF passed through 300 kDa filters than in RF absorbed SF [cf columns 2 and 4, Fig. 4(a)] suggests that removal of the high molecular weight inhibitor enables TK to be detected by ELISA in RA SF and thereby provides further support for the conclusion already attained that TK is present in such fluids.

In other experiments α1-AT/TK complexes were detected by ELISA in RA SF albeit at low levels. However, removal of components greater than 300 kDa sharply increased the values obtained. This result suggests that the same ELISA inhibitor interferes with the detection of α1-AT/TK complexes. The question arises as to how the inhibitor interferes with the detection of TK. The fact that TK added to SF was more easily detected by the amidase assay than by ELISA suggests that the active site of TK is not blocked. It follows that the substance in RA SF interfering with the TK ELISA could be non-specific or due to an inhibitor which binds to a non-enzymic site on TK and thereby interferes with the access of anti-TK to TK. In support of the latter contention an inhibitor with the same properties has been found in normal human sera [10] and in the supernatants from degranulated neutrophils (unpublished results) but not urine or saliva [10] and RA SF does not interfere with the detection of PK by ELISA (unpublished results).

It is now clear that neutrophils stimulated by soluble IgG aggregates release their granule enzymes into the SF of RA patients [15, 16]. Work from our laboratory [13] has also revealed the presence of TK within the granules of the human neutrophil, and of kininogen substrates on the surface of the same cell [17, 18]. It is therefore possible that TK may act to release kinins locally on the neutrophil surface rather than in the general synovial compartment. This mechanism may provide for a more subtle control mechanism for kinin production within the inflamed joint. Work is in progress to establish whether TK released from granules is bound to be neutrophil surface and whether the kinin moiety is missing from RA synovial PMNs.

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**BHPR ANNOUNCEMENTS AND CALENDAR FOR 1994**

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<tr>
<th>Month</th>
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<td>April</td>
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<td>Spring Meeting, Brighton Conference Centre.</td>
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<td>October</td>
<td>12–13</td>
<td>BHPR/BSR Joint Meeting, Staffordshire.</td>
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For further details please contact Anne Mansfield, BSR, 3 St Andrew's Place, Regent's Park, London NW1 4LB. Tel: 071-224 3739; Fax: 071-224 0156.