Effect of thrombin inhibition on synovial inflammation in antigen induced arthritis

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Abstract

Objective—To determine the effect of the thrombin inhibitor, hirudin, on the pathogenesis of murine antigen induced arthritis (AIA).

Methods—AIA was induced by intra-articular injection of methylated bovine serum albumin in the knee joints of previously immunised mice. Hirudin (injected subcutaneously 3 × 200 µg/mouse/day) was given over 13 days, starting three days before arthritis onset, and its anticoagulant effect monitored by clotting times. Arthritis severity was evaluated by technetium-99m (⁹⁹mTc) uptake in the knee joints and by histological scoring. In addition, intra-articular fibrin deposition was examined by immunohistochemistry, and synovial cytokine mRNA expression measured by RNase protection.

Results—Joint inflammation, measured by ⁹⁹mTc uptake, was significantly reduced in hirudin treated mice at days 7 and 10 after arthritis onset. Histologically, synovial thickness was markedly decreased in hirudin treated mice compared with untreated ones. By contrast, no difference in articular cartilage proteoglycan content was found between both groups. Intra-articular fibrin deposition and synovial interleukin 1β mRNA levels, were slightly reduced (~20%) in arthritic joints from hirudin treated mice compared with untreated ones at day 10 of AIA.

Conclusion—Hirudin reduces joint inflammation associated with AIA by fibrin-dependent and independent mechanisms.

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease characterised by synovial inflammation and hyperplasia, leading to progressive cartilage and bone destruction. Although immunological processes clearly play a part in RA, they do not account for all the features of chronic inflammation found in rheumatoid joints, in particular for the abundant fibrin deposits in RA synovia.

The formation of extravascular fibrin within the arthritic joints probably results from activation of the coagulation pathway during inflammation. Increased permeability of the inflamed synovial microvasculature leads to extravasation of plasma fibrinogen. This latter could be rapidly converted to fibrin by thrombin, formed through activation of the tissue factor mediated coagulation pathway. Besides fibrin deposition, evidence for increased extravascular coagulation within the rheumatoid joint includes the demonstration of an intact coagulation pathway within the rheumatoid synovium and, in particular, the existence of increased expression of the tissue factor and thrombin. Moreover, in RA synovial fluids, reduced levels of coagulation factors with concomitantly increased concentrations of thrombin activity and thrombin-antithrombin (TAT) complexes have been found, suggesting continuing coagulation. The persistence of fibrin in the inflamed joint may have deleterious effects. As intra-articular fibrin reflects the balance between local fibrinolysis and coagulation, we proposed that efficient fibrinolysis or reduced coagulation, or both, are necessary for the resolution of arthritis. For fibrinolysis, we have shown that in mice which were deficient in urokinase-type plasminogen activator or plasminogen, arthritis was exacerbated, but this exacerbation was attenuated by defibrinogenation with ancrd. Taken together, these results emphasise the linkage between inflammation and fibrin deposition in arthritis.

Another important player in the coagulation cascade, which also sustains inflammation, seems to be thrombin. Besides its well known activity in the coagulation pathway, thrombin generation in the joint may also affect synovial cell proliferation and inflammatory cell recruitment during arthritis. These effects are independent of the formation of fibrin, and are mediated through the cell surface thrombin receptor PAR-1 (protease activated receptor 1). PAR-1 is a member of the family of G coupled proteins with seven transmembrane domains. This multifunctional receptor is activated by proteolytic cleavage of an N-terminus extramembrane fragment by thrombin, such that the newly created N-terminus can then act as a “tethered ligand”, leading to receptor activation.

In light of these results we wished to determine if inhibition of thrombin may be therapeutic in experimental arthritis. Hirudin, a highly specific thrombin inhibitor, inhibits thrombin activity directly by non-covalent binding to the substrate recognition and catalytic binding sites of the enzyme. Hirudin itself is a naturally occurring molecule produced by the salivary glands of the leech, Hirudo medicinalis, but in clinical studies a number of synthetic hirudin derivatives have been used. Lepirudin, the recombinant hirudin which was employed in this study, has already been used in the treatment of heparin induced thrombocytopenia, deep vein thrombosis, and
for 30 minutes at room temperature. Bound antibody was visualised using the avidin-biotin-peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA). The colour was developed by 3,3'-diaminobenzidine (Sigma Chemical Company) containing 0.01% H₂O₂. After extensive washing in water, slides were counterstained with Pan- nicoalou and mounted in Merckoglass. Staining specificity was confirmed using, as primary antibodies, non-immune rabbit serum or fibrinogen preadsorbed immune serum. An incubation without the first antibody served as a negative control. Fibrin immunostaining in the synovial membrane was graded independently by two observers unaware of the animal treatment on a scale of 0 (no fibrin at all) to 6 (maximum fibrin staining).

ANTICOAGULATION WITH HIRUDIN

(Telu1, Thr2)-63-desulphohirudin (Refludan, Hoechst Marion Roussel SA, Zurich, Switzerland) was used. The solution was reconstituted from the supplied powder in a concentration of 200 µg/100 µl with 0.9% NaCl. The reconstituted solution is stable for 24 hours at room temperature. The mice were injected subcutaneously with 100 µl of this solution three times a day, every eight hours. The treatment lasted 13 days, starting three days before induction of arthritis and during the 10 days of AIA. PBS injected mice were used as controls.

CLOTTING TIMES

To obtain plasma, blood was collected from the tail vein or from the inferior vena cava in anaesthetised animals in 0.12 M trisodium citrate (one volume of citrate to nine volumes of blood). Blood samples were centrifuged at 1500 g for 15 minutes at 4°C and plasmas stored at −20°C until used. All clotting times, performed on citrated plasma diluted in Owren’s buffer (sodium diethylbarbiturate buffer pH 7.35) prewarmed at 37°C, were obtained with an Amelung microcoagulometer (Dialine, Itingen, Switzerland). For determination of the prothrombin time (PT) 50 µl of fivefold diluted plasma was used. After addition of 100 µl of a thromboplastin reagent (Recombiplastin Ortho, Almedica, Galmiz, Switzerland), time to thrombus formation was recorded. For activated partial thrombin time (aPTT) 50 µl of a twofold diluted plasma was incubated with 50 µl of activated cephalin reagent (Actin, Dade-Behring, Düdingen, Switzerland) for two minutes at 37°C. After addition of 50 µl of 0.025 M CaCl₂, time to thrombus formation was recorded. For thrombin time (TT) measurement, 50 µl of twofold diluted plasma was used. After addition of 50 µl of a twofold diluted thrombin reagent (Thromboclotin, Dade-Behring, Düdingen, Switzerland), time to thrombus formation was recorded.

THROMBIN-ANTITHROMBIN III (TAT) DETERMINATIONS

TAT complex concentration in plasma was measured by a commercially available enzyme linked immunosorbent assay (ELISA) kit.
Table 1 Coagulation times and plasma thrombin-antithrombin (TAT) levels of hirudin treated mice with antigen induced arthritis (AIA). Activated partial thrombin time (aPTT), thrombin time (TT), prothrombin time (PT), and plasma TAT levels were measured in untreated and hirudin treated mice with AIA, 10 days after arthritis onset. Citrated plasmas were prepared from blood collected 1.5 h, 3 h, and 5 h after the last subcutaneous injection of hirudin. Results are expressed as means (SEM).

<table>
<thead>
<tr>
<th></th>
<th>aPTT (s)</th>
<th>Thrombin time (s)</th>
<th>Prothrombin time (s)</th>
<th>Plasma TAT (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice with AIA</td>
<td>36.7 (1.3)</td>
<td>27.3 (0.6)</td>
<td>12.9 (0.4)</td>
<td>8.38 (108)</td>
</tr>
<tr>
<td>Mice with AIA + hirudin:</td>
<td>116.1 (6.1)</td>
<td>&gt;250 (0)</td>
<td>217.9 (32.1)</td>
<td>3.99 (0.84)</td>
</tr>
<tr>
<td>1.5 h after last injection</td>
<td>54.6 (5.5)</td>
<td>28.4 (0.8)</td>
<td>4.94 (0.96)</td>
<td>14.3 (0.5)</td>
</tr>
<tr>
<td>3 h after last injection</td>
<td>36.1 (1.1)</td>
<td>28.4 (0.8)</td>
<td>4.94 (0.96)</td>
<td>14.3 (0.5)</td>
</tr>
<tr>
<td>5 h after last injection</td>
<td>36.1 (1.1)</td>
<td>28.4 (0.8)</td>
<td>4.94 (0.96)</td>
<td>14.3 (0.5)</td>
</tr>
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Statistical significance was tested by Student’s t test: *p<0.05; †p<0.01.

ANTI-BSA ANTIBODIES MEASUREMENT
Ninety six well plates were coated for two hours with 1% BSA in PBS. Plasma (100 µl), serially diluted in PBS/1% gelatin (final dilutions 1/50, 1/100, 1/200, 1/400) was incubated for two hours at room temperature. Wells were washed four times with TTBS (50 mM Tris pH 7.4, 140 mM NaCl, containing 0.05% Tween 20). Then, 100 µl/well of alkaline phosphatase conjugated antimouse IgG (Sigma Chemical Company) diluted 1/500 in TTBS was added. After four washings with TTBS, color was developed with 100 µl/well of p-nitrophenylphosphate (Sigma Chemical Company) and the reaction stopped by adding 25 µl/well of 3 M NaOH. Plate reading was performed at 405 nm, and the results expressed as the percentage of absorbance units of control mice.

RNASE PROTECTION ASSAY
Total RNA was extracted from cryostat sections of synovial tissues from knee joints using the Trizol reagent (Gibco BRL, Switzerland).

RNase protection assay was performed with the mCK-2 mutiprobe set (Pharmingen, Basel, Switzerland), according to the manufacturer’s instructions. Briefly, antisense [32P]UTP labelled riboprobes were synthesised by in vitro transcription of the mCK-2 DNA template set. DNase I treatment was performed to remove the DNA templates and riboprobes were purified by phenol/chloroform extraction, followed by ethanol precipitation with glycogen as carrier. For each sample about 5 µg of total RNA was hybridised overnight at 52°C with 3 x 10^6 cpm of the labelled multiprobe. Samples were first treated with an RNase cocktail (Ambion), then RNase was removed by proteinase K treatment, and samples purified by phenol/chloroform extraction, followed by ethanol precipitation with glycogen as carrier. Protected fragments were resolved through a 5% sequencing gel. Precise quantification was determined by analysing the gel with an InstantImager apparatus and software (Packard Instruments). For each protected band analysed, a cpm/mm² value was obtained. This value was corrected for sample loading errors by normalising with the respective cpm/mm² value calculated for the constitutively expressed glyceraldehyde phosphate dehydrogenase (GAPDH) gene which was also included in the template set.
STATISTICAL ANALYSIS
The Wilcoxon/Kruskal-Wallis (rank sum test) for unpaired variables was used to compare differences between groups with non-Gaussian distribution. The unpaired Student’s t test was used to compare means for normally distributed values. A level of p<0.05 was considered significant.

Results
EFFICACY OF ANTICOAGULATION BY HIRUDIN IN MICE
In a pilot experiment (with six mice) we found that one daily subcutaneous injection of 200 µg of hirudin led to prolonged aPTT and TT only for up to four hours (results not shown). On the basis of these preliminary results, we decided to treat mice with subcutaneous injections of hirudin every eight hours. To confirm that hirudin, given three times a day over 13 days (three days before and 10 days after AIA onset), was efficient, the aPTT, TT, and PT were measured at different times after the last subcutaneous injection of 200 µg of hirudin. There was a significant prolongation of the aPTT and TT, which attenuated with time. An anticoagulant effect was observed at three hours after injection, with a return to normal coagulation times at five hours after injection (table 1). As already noted by others,18 no prolongation of the PT was seen with this treatment.

Inhibition of thrombin by hirudin was also documented by plasma TAT levels. Plasma TAT levels were reduced in hirudin treated mice, though this decrease reach significance only at 1.5 hours after the hirudin injection (see table 1).

ANTI-MBSA ANTIBODIES LEVELS IN HIRUDIN TREATED AND CONTROL MICE
To verify that hirudin treated mice generated an adequate immune response to mBSA, anti-BSA antibody levels were measured by ELISA.

Figure 3  Histologies and immunohistologies of whole knee joint sections of control and of hirudin treated mice with antigen induced arthritis. Figures A and C show safranin O stained sections of arthritic knee joints at day 10 after arthritis induction. Note the difference of thickness of synovial membrane (S) which is thicker in the control arthritic mice than in the hirudin treated ones. Figures B and C show fibrin(ogen) immunostaining (indicated by (F) on Figure 3B) of adjacent sections.
on day 10 after arthritis onset. No significant differences in antibody levels were seen between immunised mice treated with hirudin or PBS treated mice (fig 1).

EFFECT OF HIRUDIN TREATMENT ON 
TECHNETIUM UPTAKE IN AFFECTED KNEE JOINTS
To explore whether the anticoagulation treatment had an effect on the course of AIA, we measured knee joint inflammation in control and hirudin treated mice by the ratio of $^{99mTc}$ uptake in the inflamed arthritic joint and that of the non-arthritic contralateral knee joint at different times up to day 10 (fig 2). In PBS treated animals, inflammation developed rapidly so that by day 3, the uptake ratio reached 1.85. Inflammation declined subsequently, and by day 10, the uptake ratio was 1.4. In hirudin treated mice, we observed an attenuation of the inflammatory response throughout the duration of the experiment (day 3: 17% decrease; days 7 and 10: 13% decrease). A significant difference in $^{99mTc}$ uptake was seen on days 7 and 10 in comparison with control animals.

EFFECT OF HIRUDIN TREATMENT ON 
HISTOLOGICAL FEATURES OF ARTHRITIS
We compared the histological features of arthritic knee joints from untreated control and hirudin treated mice at day 10 after arthritis onset (figs 3A and C). In both groups some signs of synovitis were seen in all mBSA injected joints, which were absent in contralateral knees injected with PBS only (results not shown). In the untreated group, synovial membrane thickness (indicated by S on fig 3A) was greater than in the hirudin treated group (fig 3C). The effect of AIA on articular cartilage was also evaluated. Induction of arthritis led to a decrease in proteoglycan content in both groups, as shown by the partial loss of safranin O staining (figs 3A and C).

Semi-quantitative scoring was performed on histological sections using a four point (0–3) grading system to evaluate synovial thickness and cartilage damage. Each joint was scored by two independent assessors. The reduction in synovial thickness in hirudin treated animals at day 10 was statistically significant (fig 4A), but cartilage damage, though diminished, was not statistically different (fig 4B).

EFFECT OF HIRUDIN TREATMENT ON FIBRIN DEPOSITION IN ARTHRITIC JOINTS
We wanted next to evaluate if hirudin treatment reduces intra-articular fibrin deposition in AIA. Fibrin content was analysed by fibrin immunohistochemistry—a brown colour indicating positivity (figs 3B and D)—and by visual scoring (fig 4C). The antibody specificity was shown by the absence of staining when sections were incubated with normal rabbit serum or when the polyclonal antibody was preincubated with an excess of purified murine fibrinogen (results not shown). In arthritic knee joints of untreated mice, fibrin was found in the synovium, the synovial fluid, and on the surface of the articular cartilage (fig 3B). Decreased amounts of fibrin were detected in hirudin treated mice (fig 3D). When immunohistological scoring was used, mice treated with hirudin showed a 20% reduction in the level of fibrin compared with non-treated animals, though this did not reach statistical significance.

EFFECT OF HIRUDIN TREATMENT ON IL1β mRNA
To establish whether hirudin treatment might alter the pattern of inflammatory cytokine expression in the synovium we analysed, after 10 days of AIA, synovial mRNA expression of different molecules (interleukin 1β (IL1β); interleukin 1 receptor antagonist (IL1Ra); macrophage migration inhibitory factor (MIF)), by RNase protection (fig 5). In synovial tissues from non-arthritic knees, only MIF mRNA could be detected, whereas in arthritic knees, there was a marked induction of IL1β and IL1Ra mRNAs. Quantitative results were obtained by Phosphoimager analysis after normalisation by GAPDH mRNA levels. In hirudin treated mice, at day 10 of AIA, synovial IL1β mRNA levels were reduced by 20% compared with sham treated mice (sham v hirudin, 100 (16)% v 80 (13)%), four mice per group, mean (SEM), but this reduction did not reach significance. In the same conditions, IL1Ra and MIF mRNA levels were unchanged (results not shown).
Discussion

In RA, fibrin deposition within the joint is prominent, suggesting that it may have a pathogenetic role in the disease. Based on our prior observations that mice deficient in urokinase showed more severe synovitis and more destructive arthritis during AIA, we proposed that increased fibrin deposition in these animals may have a deleterious effect on arthritis. As thrombin generation is essential for fibrin deposition, and also because thrombin receptor activation may mediate some of the inflammatory cellular responses found during AIA, we decided to investigate if thrombin inhibition might reduce synovial inflammation in this animal model of RA. We started hirudin treatment before the joint injection of mBSA to give the best chance of observing if hirudin can modulate joint inflammation.

The results of our experiments showed that hirudin did indeed significantly attenuate the severity of AIA, as measured by both $^{99m}$Tc uptake and synovial histology. We were able to exclude an effect of hirudin on the immune response to BSA, as plasma anti-BSA antibody titres were equivalent in the two groups of mice. In parallel with the reduction in synovitis, we also found a trend towards diminished synovial IL1β mRNA production in hirudin treated animals, further suggesting that the treatment attenuated the proinflammatory state within the knee.

It has been previously reported that thrombin may also directly damage cartilage, as it induces proteoglycan release in vitro. In our study we found a trend, though not statistically significant, towards reduction of cartilage degradation in hirudin treated mice. The absence of a significant effect may be explained by the relatively insensitive histological grading system used to evaluate cartilage damage.

Intra-articular fibrin staining was reduced by hirudin treatment, though only by 20%. This small reduction is most probably accounted for by the dosing schedule used in our study, which only provided partial anticoagulation during the period of study. For practical reasons, we chose to give hirudin to mice subcutaneously three times daily, as it was impossible to give it by continuous infusion (as in man). Overall, no adverse effects were seen, and no animals developed signs of spontaneous bleeding. Lepirudin, the form of hirudin given, has a half life of 0.8–1.4 hours in man when given subcutaneously, with a Cmax of 1.7–2.6 hours. In our preliminary experiments we showed that after a 200 µg dose an anticoagulant effect was seen three hours after subcutaneous injection, but was no longer evident at five hours. If it is assumed that an anticoagulant effect was present for up to four hours after each injection, the animals were therefore anticoagulated for only around 12 hours a day. We have also done pilots experiments using more conventional anticoagulants, such as antivitamin K. In these experiments, anticoagulation was maintained over 24 hours, but some mice had severe bleeding problems. Nevertheless, in the non-bleeding mice we also obtained decreased synovial inflammation (as measured by $^{99m}$Tc uptake and synovial histology), thus reinforcing the linkage between anticoagulation and reduced joint inflammation.

Despite the partial anticoagulation induced by hirudin treatment, and the moderate reduction of intra-articular fibrin content, a clear reduction was seen in synovial inflammation. This suggests that hirudin may inhibit more than the coagulative function of thrombin. Thrombin can promote the chemotaxis of neutrophils and monocytes and their adhesion to endothelial cells. It can also induce mitogenesis of synoviocytes, and can act on endothelial cells to increase vascular permeability and the synthesis of various proinflammatory molecules (see review). These cellular events of thrombin are mediated by the PAR-1 thrombin receptor.

![Synovial cytokine mRNA levels in knee joints from untreated or hirudin treated mice.](image-url)
receptor. In arthritis, PAR-1 is abundantly expressed in inflamed rheumatoid synovial tissues. In this respect, it is interesting to note that a single injection of a low dose of thrombin can induce an inflammatory response in the injected rat knee or oedema in the injected rat hind paw. Moreover, thrombin receptor-activating peptides, which are synthetic peptides corresponding to the "tethered ligand" of PAR-1, mimicked the effect of thrombin in inducing paw oedema, further underlying the importance of thrombin receptor activation in inflammation. The availability of PAR-1 deficient mice will in the future allow a more precise investigation into these cellular actions of thrombin in vivo.

In conclusion, we have shown that thrombin inhibition by hirudin attenuates synovial inflammation and the histological changes of AIA. Although the roles of thrombin in synovial inflammation associated with AIA are not totally explained, the ability of thrombin to participate in both haemostatic and inflammatory responses suggests that selective and direct inhibition of thrombin by hirudin based reagents may represent an additional treatment strategy in RA.

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