Correlation between synovial neopterin and inflammatory activity in rheumatoid arthritis*

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SUMMARY According to recent investigations neopterin (a pyrazinopyrimidine derivative) is a biochemical marker that reflects the activity of the proinflammatory immunocellular system of the synovial tissue in rheumatoid arthritis (RA). Interferon gamma, derived from antigen activated T lymphocytes, stimulates macrophages to synthesise and release neopterin into the culture supernatant in vitro. To extend this in vitro model to a clinical level a sensitive new radioimmunoassay technique was used to measure neopterin concentrations in the synovial fluid (SF) of 17 patients with active RA, nine with osteoarthritis, and six with acute gout, and in that of 12 controls undergoing meniscectomy. The SF neopterin concentrations were significantly higher in patients with RA than in the other groups of patients, particularly the controls. Multivariate analysis showed that SF neopterin concentrations correspond better with the systemic inflammatory activity of RA than with the local disease activity of the knee joints. Thus the study strengthens the hypothesis that neopterin reflects the essential role of the activated immunocellular reaction in the pathogenesis of RA.

Neopterin (a pyrazinopyrimidine compound) is synthesised from guanosine triphosphate by macrophages.¹ In vitro, it has been shown that its release is stimulated by interferon gamma derived from antigen activated T lymphocytes.² Thus neopterin is a specific marker for the activation of the cellular immune system. Its specific role in the cellular immune response is still unknown, however, as it is only an intermediate product in the synthesis of the cofactor biotin. As mononuclear cells accumulate in the synovia of inflamed joints in patients with rheumatoid arthritis (RA),³ and as the pathogenesis of RA depends on the central immunoregulating effect of antigen activated T lymphocytes,⁴ neopterin should be released into the synovial fluid (SF) in such patients. Therefore, in an attempt to extend the in vitro experiments to a clinical level we made a comparative study of the diagnostic significance of SF neopterin in patients with RA and with other rheumatoid diseases and tried to correlate the SF neopterin concentrations in

RA with the degree of systemic and local inflammatory activity.

Patients and methods

PATIENTS, CLINICAL ASSESSMENT, AND SF COLLECTION

From three groups of ambulatory outpatients with persistent knee effusion, who regularly attended the rheumatic disease service of the University Hospital of Marburg, West Germany, we selected 32, who, according to the 1987 modified American Rheumatism Association criteria⁵ had classical or definite RA (n=17), osteoarthritis (n=9), or acute gout (n=6). Table 1 shows their demographic and clinical characteristics. All patients were receiving treatment with non-steroidal anti-inflammatory agents, but not prednisolone, and six of 17 patients with RA were receiving gold treatment. The criteria for exclusion included a history of RA of less than two months; associated psoriasis, seronegative spondyloarthropathy, or Reiter's disease; and recent or anticipated treatment with systemically or intra-articularly administered corticosteroids. All patients with osteoarthritis had radiologically proved degenerative joint disease and at least one of the following criteria of disease activity: swelling, pain with

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motion, and pain at rest. To obtain normal mean control values we also measured the neopterin concentrations in the non-inflammatory SFs of 12 patients undergoing meniscectomy. The grading of RA in individual patients was performed according to radiographic criteria of Steinbrocker, modified by Larsen *et al.*⁶ Six patients had stage I disease, four had stage II, five had stage III, and two had stage IV. The local disease activity of RA was clinically assessed on the basis of the following subjective and objective findings and scored from 0 to 5: pain at rest or with motion (from 'no pain' to 'most severe'), the difference in joint circumference (from 0.5 cm to 4.0 cm in 0.7 cm steps), and difference in skin temperature of the knee joint (from 0.5°C to 2.4°C in 0.5°C steps).^{7, 8} The total volume of knee joint SF and the echogenic areas of the pannus and the inflamed knee joint capsule were assessed with ultrasonography.⁸ Ultrasonographic scans were made transversely and longitudinally on both knees using a commercial contact high resolution real time scanner, with a 7.5 MHz transducer. The sodium pertechnetate ^{99m}Tc uptake in the knee joint (^{99m}Tc index) was measured as a percentage of the total injected activity, also according to a previously reported method.⁹ Radiographs of the knee joints were scored from 0 to 3 according to the degree of destruction, as in a previous study.¹⁰ Indicators of systemic inflammatory disease activity were scored from 0 to 5 and included the Ritchie index,¹¹ the degree of anaemia and thrombocytosis, and the erythrocyte sedimentation rate. Finally, the individual total index was calculated by adding the scores of all subjective and objective tests and then dividing them by the number of tests to give a final score that indicated mild (class I), moderate (class II), or severe (class III) local or systemic disease activity, according to previously described methods.^{8, 12}

LABORATORY TESTS ON SF

As much SF as possible was aspirated and then immediately analysed for total and differential leucocyte counts. Biochemical tests and immunological studies included total protein, C reactive protein, and immune complex determinations, performed by immunoturbidimetric assay¹³ using an automated micropipette nephelometer system (Beckman Immunochemistry Systems, Munich). The prostaglandin E₂ concentration was measured by radioimmunoassay.¹⁴ The neopterin concentration was determined with a new, highly sensitive radioimmunoassay, according to a previously reported method,¹⁵ in which aliquots (150 µl) of the unextracted samples are analysed in triplicate at a 1:10 dilution in the standard diluent of the test:

Table 1 Patients' demographic and clinical characteristics

	RA* (n=17)	Osteoarthritis (n=9)	Gout (n=6)
Women	11	2	0
Men	6	7	6
Age (years)†	48.1 (2.7)	63.0 (2.3)	41.8 (2.3)
Duration of disease (years)†	6.5 (1.5)	12.5 (6.5)	0.29 (0.06)
Treatment			
NSAIDs*	17	9	6
Gold	6	—	—
Steroids	—	—	—

*RA=rheumatoid arthritis, NSAIDs=non-steroidal anti-inflammatory drugs.

†Values are means (SEM).

details of the binding affinity and the immunological specificity of antineopterin antibody have been described elsewhere.¹⁵

Briefly, before radioimmunoassay of the SF neopterin each sample was generally submitted to iodine oxidation in 0.2 M HCl to obtain the total concentration of neopterin and its didydro and tetrahydro derivatives. The radioimmunoassay used was highly specific for D-erythro-neopterin with cross reacting antibodies to various structurally related pteridines. Thus N²-carboxypropylneopterin was used as actual hapten, with its radioiodinated tyramine derivative as the tracer, a D-erythro-neopterin equivalent was read off from a standard curve, and then the value was divided by the amount of each pterin, multiplied by 100 to obtain the percentage of the cross reaction. For radioimmunoassay of the SF neopterin a mixture of 150 µl of sample or standard compound in 0.02 M phosphate buffer pH 7.5 containing 0.1% bovine serum albumin, 100 µl of 8000-fold diluted antiserum, and 100 µl of the above buffer was incubated at 37°C for 30 minutes. A solution of the tracer (100 µl, 20 000 cpm) was added, and the mixture was kept at 4°C for one hour. Antigen-antibody complex was precipitated by a second antibody method¹⁵ using antirabbit IgG and dextran T-70, and the radioactivity of the precipitate was counted.

With 6000 to 8000 disintegrations per minute of tritiated neopterin (Henning Radiochemical Centre, West Berlin) in a volume of 1.5 ml the lowest detectable concentration of the compound was 20 pg/ml of SF.

STATISTICAL ANALYSIS

Sensitivity was defined as the proportion of patients with a given diagnosis in whom the neopterin test was positive, and specificity as the proportion of patients without the diagnosis in whom the test was

Fig. 1 Neopterin concentrations in synovial fluid of patients with rheumatoid arthritis (RA) ($n=17$), osteoarthritis (OA) ($n=9$), and gout (Go) ($n=6$) and in 12 controls (Co) undergoing meniscectomy. $p<0.001$ for patients with RA ν controls; $p<0.05$ for patients with RA ν those with osteoarthritis and those with gout.

negative. The results for individual patients (expressed as arithmetic means (SEM)) were compared by analysis of variance using Wilcoxon's signed rank test for paired data. A rank test was applied to the rheumatoid activity index because the paired clinical differences were not normally distributed by Student's t test. Analysis with the Fischer-Yates's correction formula was used for pairs of discrete variables to enable comparison with other data. The significance of all statistical tests was set at the 5% level (0.05). Stepwise logistic regression was

used to determine the simultaneous effect of systemic and local variables on neopterin concentrations. The respective three way contingency tables were analysed with a log-linear model in the BMDP4F computer program.¹⁶

Results

Neopterin was detected in all SF specimens obtained from patients with rheumatic diseases because of the high sensitivity of the radioimmunoassay used in this study. The neopterin concentrations of patients with RA were significantly higher than those of controls ($p<0.001$), and also higher than those with osteoarthritis and gout, though to a lesser degree (both $p<0.05$) (Fig. 1). Neopterin concentrations above 32 nmol/l showed a sensitivity of 65% and a specificity of 92% in differentiating RA from osteoarthritis; a neopterin concentration above 32 nmol/l had a specificity of 96%. In most patients with RA more than half of the SF cells were polymorphonuclear leucocytes (Table 2). Further, SF neopterin values obtained from patients with RA underwent a rank correlation with diverse SF variables (Table 2). The results of these rank correlations showed: SF neopterin ν SF leucocytes (NS); SF neopterin ν SF lactate dehydrogenase (NS); SF neopterin ν SF C reactive protein (NS); but SF neopterin ν SF immune complexes ($r=0.48$, $p<0.02$). We also obtained a correlation between SF neopterin concentrations and the local and systemic disease activity in patients with RA. Local disease activity, as indicated by the ^{99m}Tc indexes and the radiographic scores of the knee joints from which

Table 2 Synovial fluid findings and indicators of local disease activity in controls and patients with rheumatoid arthritis (RA). Values are given as means (SEM)

Finding	Controls ($n=12$)	Patients with RA ($n=17$)	p Value
Neopterin (nmol/l)	10.3 (7.3)	41 (9.0)	<0.001
White blood cell count ($\times 10^9/l$)	1.64 (0.5)	11.3 (3.2)	<0.01
Polymorphonuclear leucocytes (%)	24.4 (5.2)	59.6 (9.4)	<0.01
Protein (g/l)	2.9 (0.6)	5.7 (1.4)	<0.05
Lactate dehydrogenase (U/l)	197 (38)	304 (51)	<0.05
C reactive protein (mg/l)	4.3 (0.7)	12.4 (3.2)	<0.01
Immune complexes (mg/l)	5 (0.7)	12 (2)	<0.05
Prostaglandin E_2 ($\mu\text{g/l}$)	4.47 (2.09)	1.01 (0.46)	NS*
Local disease activity score	3.4 (0.4)	8.4 (1.1)	<0.001
^{99m}Tc index (%)	ND*	12.4 (4.8)	—
Radiographic score	ND	2.4 (0.8)	—

Regression analysis showed no significant correlation ($r=0.18$) between synovial fluid neopterin concentrations and local inflammatory activity of the knee joints, as indicated by the local disease activity score, the ^{99m}Tc index, and the radiographic score, considered together. (See text for explanation of how these were measured.)

*ND=not determined; NS=not significant.

Fig. 2 Multivariate analysis of correlation between neopterin concentrations in synovial fluid and clinical indicators of systemic disease activity in patients with rheumatoid arthritis ($n=17$) ($p<0.03$, $r=0.46$).

the SF had been aspirated, was not significantly correlated with SF neopterin concentrations (Table 2). In contrast, there was a low but significant correlation between neopterin concentrations and the overall severity of systemic inflammatory disease in RA ($r=0.46$, $p<0.03$) (Fig. 2).

Discussion

The aetiological factor triggering the pathological chain reaction of RA is still unknown.¹⁷ The normally thin human synovium undergoes a profound alteration when the aggressive inflammatory process of RA develops. In rheumatoid synovitis three features predominate: inflammation, proliferation, and infiltration.¹⁸ During the early stages of inflammation in rheumatoid arthritis polymorphonuclear leucocytes are the predominant cells in the SF, and immune complexes, composed of immunoglobulins, complement clearance products, and acute phase proteins—for example, C reactive protein, can also be detected.¹⁸ Thus SF analysis has been widely recommended as an important diagnostic procedure in patients with arthritic effusions.¹⁹ Although repeated SF analysis may provide diagnostic information that is unavailable from history and physical examination and may enable the clinician to reassess various diagnoses, no pathognomonic diagnostic marker for RA has been discovered.¹⁸ Our results mainly confirm that certain diagnostically non-specific SF findings are typical of RA, as has also been observed for other inflammatory arthritides. Except for neopterin, these findings differed significantly only from those

of normal SF specimens obtained from controls undergoing meniscectomy. The important role of the T lymphocyte-macrophage axis in the pathogenesis of RA is widely accepted.²⁰ Therefore, we tried to determine whether SF neopterin concentrations are more useful than the conventionally accepted diagnostic indicators. Our most important finding was the high sensitivity and specificity of neopterin concentrations above 32 nmol/l. Even at lower concentrations the comparatively higher neopterin concentrations give a differential diagnostic hint. Multivariate analysis showed that SF neopterin concentrations in patients with RA reflect the degree of systemic inflammatory disease activity more than they reflect the local disease activity of the knee joints. Why are these concentrations not better correlated with the inflammatory activity at the site where the neopterin was synthesised?

The release of neopterin into the SF by locally accumulated macrophages is mainly stimulated by interferon gamma from the systemically activated T lymphocyte system.^{21 22} This conclusion was confirmed by the results of a previous study,²³ in which urinary neopterin concentrations were found to be greater in patients with RA who had a high degree of systemic disease activity than in those with less systemic activity or with osteoarthritis. The local inflammatory activity, however, is mainly influenced by—and hence corresponds to—the concentrations of prostaglandins, lysosomal enzymes, superoxide radicals released from polymorphonuclear cells, and interleukins, which mediate the production of acute phase proteins.²⁴ This lack of correlation between high SF neopterin concentrations and local disease activity might also reflect the fact that a decrease in joint function in RA may be influenced more by erosive bone destruction (as indicated in our study by an increased radiographic score) than by the extent of synovitis and its clinical symptoms.⁷ Neopterin itself is probably only an intermediate product of bipterin synthesis, without any biological activity or any effect on local inflammatory changes. It reflects the activation of the cellular immune system in RA as a systemic inflammatory disorder, and these changes do not necessarily correlate with local inflammatory reactions. To determine the influence of non-steroidal anti-inflammatory drug treatment on neopterin concentrations and on the clinical activity of RA, more follow up data are needed from patients with RA or other autoimmune diseases, such as ulcerative colitis²⁵ and Crohn's disease.²⁶ As all of our patients with RA received such drug treatment it is possible that it had an effect on the local synthesis and release into SF of prostaglandin E₂. Non-steroidal anti-inflammatory drug treatment, however, obviously does not affect

the products of the systemic inflammatory process—for example, C reactive protein, immunoglobulins, and immune complexes, because they are mediated primarily by interleukins, which remain unaffected by non-steroidal anti-inflammatory agents.²⁴ Although the SF neopterin concentrations in our patients with RA correlated significantly with the degree of systemic inflammatory activity, they may not serve as a specific marker for any autoimmune disease because various concomitant infections of malignant disorders may provoke similar T lymphocyte mediated immune responses, which are invariably associated with an increased release of neopterin by macrophages.²²⁻²⁷ Thus determination of urinary or SF neopterin concentrations may supplement but should never replace careful clinical evaluation of patients with RA.

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