Characterisation of soluble and cell associated phospholipase A₂ from rheumatoid synovial fluid*

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SUMMARY The hydrolysis of radiolabelled *Escherichia coli* phospholipids, and micellar dispersions of phosphatidylethanolamine and phosphatidylcholine, were used to characterise the phospholipase A₂ activity in synovial fluid from patients with rheumatoid arthritis. Cell free fractions of synovial fluid contain a phospholipase A₂ enzyme that preferentially releases [¹⁴C]oleic acid from *E coli* biomembranes (specific activity 291.3 (SEM 27.6) pmol/min/mg). This enzyme requires calcium and is optimally active at neutral pH. Purified dispersions of phosphatidylethanolamine are also readily degraded by the soluble enzyme, but it is not active against phosphatidylcholine. The substitution of [¹⁴C]oleic acid by [³H]arachidonic acid for the labelling of *E coli* allowed differentiation between the soluble phospholipase A₂ and the cell associated phospholipase A₂ present in sonicates of mononuclear cells and neutrophils from peripheral blood and synovial fluid. The cell associated phospholipase A₂ preferentially releases [³H]arachidonic acid from *E coli* cardiolipin. In this paper the phospholipid substrate specificity of phospholipase A₂ from rheumatoid synovial fluid, the optimal assay conditions for its detection, and a standardised expression of activity in terms of pmol per minute per mg of protein are established.

Key words: arthritis, inflammation, arachidonic acid, phospholipid metabolism, eicosanoid, neutrophil, mononuclear cell, synovial cell.

Prostaglandins and hydroxyl compounds of arachidonic acid are potent mediators of the inflammatory response identified in inflammatory sites in patients with rheumatoid arthritis. For example, prostaglandin E₂ and leucotriene B₄ have been found in the synovial fluid^{1,2}; while 5-hydroxyeicosatetraenoic acid has been identified in synovial tissues.³ Prostaglandin E₂ as a vasodilator and hyperalgesic agent may cause erythema, oedema, and pain.⁴ Leucotriene B₄ provokes a variety of responses in neutrophils, including chemotaxis, mobilisation of calcium, aggregation, release of lysosomal enzymes, and generation of superoxide anions.⁵ 5-Hydroxyeicosatetraenoic acid is also chemotactic for neutro-

phils. It is possible that these agents act sequentially and in combination to produce local oedema and cellular migration into the rheumatic joint. The release of arachidonic acid from membrane phospholipids, presumably by phospholipase A₂, is believed to be the rate limiting step for the generation of this family of biologically active metabolites of arachidonic acid. Recently, high concentrations of phospholipase A₂ have been detected in synovial fluid,^{6,7} peripheral blood neutrophils,⁸ and peripheral blood mononuclear cells⁹ from patients with rheumatoid arthritis. Although no correlation has been found between the concentrations of phospholipase A₂ in rheumatoid synovial fluid and the clinical activity of the disease, it has been suggested that proinflammatory phospholipase A₂ may have a contributory role in the pathogenesis of rheumatoid arthritis.⁶⁻⁹ This paper reports the biochemical properties of a soluble phospholipase A₂ present in cell free fractions of synovial fluid from patients with rheumatoid arthritis. We determined the substrate

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specificity of this enzyme based on the hydrolysis of phospholipids from radiolabelled *E coli* biomembranes, and purified micellar dispersions of radiolabelled phosphatidylethanolamine and phosphatidylcholine. The possibility of two different enzymes with phospholipase A₂ activity in the rheumatoid synovial fluid is suggested.

Materials and methods

SYNOVIAL FLUIDS AND PERIPHERAL BLOOD CELLS

Synovial fluid from 11 patients with classical or definite rheumatoid arthritis as defined by the American Rheumatism Association criteria¹⁰ were investigated. All patients were taking a non-steroidal anti-inflammatory drug, two patients were also receiving 2.5 mg of prednisone daily. Synovial fluid was obtained from the knee joint during a flare up of the disease. Peripheral blood cells from three patients were obtained at the same time as the synovial fluid aspiration. Cells were removed from the synovial fluid by immediate centrifugation at 4°C, 900 rpm (280 g) for 10 minutes. Polymorphonuclear neutrophils were separated from mononuclear cells by Lymphoprep (Gallard-Schlesinger, NY) gradient centrifugation at 1400 rpm (380 g) for 30 minutes at room temperature as previously described.^{11 12} Contaminating erythrocytes were removed by a brief hypotonic lysis, and the cells were resuspended in Dulbecco's phosphate buffered saline pH 7.4 (Gifco, Grand Island, NY). Cell suspensions were sonicated for 30 seconds (setting 4) using a Branson ultrasonic probe. The cell free fraction of synovial fluid was diluted with 0.9% saline to bring the protein to within the linear range of the assay. Peripheral blood neutrophils and peripheral blood mononuclear cells were isolated from whole heparinised blood (10 units/ml) after plasmagel sedimentation of erythrocytes and Lymphoprep gradient centrifugation as described above for synovial fluid cells. Peripheral blood neutrophils and peripheral blood mononuclear cells were separately resuspended in phosphate buffered saline and sonicated. The protein content was determined by the method of Lowry *et al*,¹³ using bovine serum albumin as standard.

CALCIUM DETERMINATION IN CELL FREE SYNOVIAL FLUID

Freshly isolated cell free fractions of synovial fluid were used for calcium determination by atomic absorption spectrophotometry. Samples were treated with 10% trichloroacetic acid to precipitate proteins, and free calcium was measured on a Perkin-Elmer atomic absorption spectrophotometer, model 280.

LABELLED SUBSTRATES

Phospholipids of *E coli* biomembranes were labelled with [¹⁴C]oleic acid or [³H]arachidonic acid as previously described.^{14 15} The biosynthetic preparation of [³H]arachidonic acid labelled phosphatidylethanolamine and [³H]arachidonic acid labelled phosphatidylcholine was carried out as previously described.¹⁶ Briefly, human neutrophils from normal volunteers were incubated with [³H]arachidonic acid (18.5 kBq, 0.1 μmol/3.5×10⁷ cells) for two hours at 37°C. Approximately 55% of the total [³H]arachidonic acid radioactivity added to the cell suspensions was incorporated into the neutrophil phospholipids. Lipids were extracted by the method of Bligh and Dyer,¹⁷ and the phospholipids were separated by thin layer chromatography using a solvent consisting of chloroform:methanol:acetic acid:water, 75:50:10:6 (by vol). Fractions corresponding to phosphatidylethanolamine and phosphatidylcholine were scraped, eluted, and the specific activity [³H] dpm/nmol phospholipid-phosphate determined.

PHOSPHOLIPASE ASSAY

Phospholipase A₂ specifically hydrolyses the ester linkage in the C-2 position of phospholipids to yield free fatty acid and lysophospholipid. When [¹⁴C]oleic acid labelled *E coli* was used as substrate the reaction mixtures contained approximately 50 nmol of phospholipid (specific activity 6090 dpm/nmol), 100 mM trometamol (TRIS)/HCl buffer pH 7.0, 5 mM CaCl₂, and 0.05–0.10 mg of protein in a total volume of 0.5 ml. Incubations were carried out at 37°C in a shaking water bath for 30 minutes. Reactions were stopped by the addition of two volumes of methanol, and the products of hydrolysis were extracted by the method of Bligh and Dyer. Samples were vortexed, and the organic and aqueous phases were separated by centrifugation at 900 rpm for five minutes. The organic layer was removed, dried under N₂ and lipids were spotted onto thin layer chromatography plates with unlabelled standards (Serdary Lipids, Ontario, Canada). Thin layer chromatography plates were developed in a neutral lipid solvent system, consisting of hexane:diethyl ether:90% formic acid, 90:60:6 (by vol). Lipids were visualised by brief exposure to iodine vapours and identified by comparison with cochromatographed standards. After sublimation of the iodine the lipid fractions were scraped into scintillation vials and the radioactivity determined using 3 ml of Bray's solution scintillation cocktail (RPI, Mount Prospect, IL).

When [³H]arachidonic acid labelled *E coli* was used as substrate the reaction mixtures contained approximately 8 nmol of phospholipid (specific

activity 404.1 dpm/nmol), 100 mM trometamol-HCl buffer pH 7.0, 5 mM CaCl₂, and 0.05–0.10 mg of protein in a total volume of 0.5 ml. Reaction mixtures were incubated and further processed as for [¹⁴C]oleic acid labelled *E. coli*.

When purified phosphatidylethanolamine or phosphatidylcholine were used as substrate the reaction mixtures in a total volume of 0.5 ml contained approximately 12 nmol of [³H]arachidonic acid labelled phosphatidylethanolamine (specific activity 1780 dpm/nmol) or 15 nmol of [³H]arachidonic acid labelled phosphatidylcholine (specific activity 1243 dpm/nmol), 100 mM trometamol-HCl buffer pH 7.0, 5 mM CaCl₂, and protein as previously indicated. Mixtures with either [³H]arachidonic acid labelled phosphatidylethanolamine or [³H]arachidonic acid labelled phosphatidylcholine were incubated for 60 minutes, during which the hydrolysis of labelled *E. coli* was related linearly to time. The reactions were stopped with two volumes of methanol, and lipids were extracted by the method of Bligh and Dyer.¹⁷ Radioactive lipids were separated by thin layer chromatography and isolated for scintillation as described before. Phospholipase A₂ activity is expressed in terms of specific activity as pmol of phospholipid hydrolysed per minute per mg of protein (pmol/min/mg).

STATISTICAL ANALYSIS

Differences in phospholipase A₂ activity between the various sources of enzyme were evaluated by Student's *t* test. The percentage distribution of radiolabel on *E. coli* between control and cell free synovial fluid was compared statistically by Student's *t* test. Differences in phospholipase A₂ activity between assays with and without bovine serum albumin were evaluated by paired *t* test. All data are reported as the arithmetic mean (SEM).

Results

PHOSPHOLIPASE A₂ ASSAY

Incubation of [¹⁴C]oleic acid or [³H]arachidonic acid labelled *E. coli* with a cell free fraction of synovial fluid or cell sonicates from synovial fluid and peripheral blood resulted in the release of radioactivity into the reaction mixture. Thin layer chromatography of the total lipid extract from the reaction mixture allowed separation of the free fatty acids from the phospholipids of *E. coli*. Table 1 compares phospholipase A₂ activity from different sources using *E. coli* biomembranes labelled with [¹⁴C]oleic acid or [³H]arachidonic acid. Phospholipase A₂ activities in cell free fractions of synovial fluid ranged from 96.6 to 410 pmol/min/mg with a mean value of 291.3 (27.6) pmol/min/mg ([¹⁴C]oleic acid) and 93.6 (12.6) pmol/min/mg ([³H]arachidonic acid). Peripheral blood neutrophils had very low activity against either substrate: 18.6 (1.6) pmol/min/mg ([¹⁴C]oleic acid) and 10.6 (0.1) pmol/min/mg ([³H]arachidonic acid). By contrast, synovial fluid neutrophils expressed greater activity against both substrates (171.3 (57.6) and 66.8 (46.3) pmol/min/mg for [¹⁴C]oleic acid and [³H]arachidonic acid labelled *E. coli* respectively). There was also greater phospholipase A₂ activity in mononuclear cells from synovial fluid than in those from peripheral blood (356.6 (36.3) and 490 (150) v 221.3 (46) and 225.1 (63.3) pmol/min/mg). We also tested the enzyme against purified micellar dispersions of radiolabelled phosphatidylethanolamine and phosphatidylcholine. When [³H]arachidonic acid labelled phosphatidylethanolamine was used the mean specific activity was 91 (44) pmol/min/mg with a range from 13.3 to 146.6 pmol/min/mg. We were not able to detect enzymatic activity against [³H]arachidonic acid labelled phosphatidylcholine; furthermore, addition

Table 1 Phospholipase A₂ activity (pmol/min/mg) in synovial fluid and cells from patients with rheumatoid arthritis. Values are mean (SEM)

Source of phospholipase A ₂	[¹⁴ C]oleic acid- <i>E. coli</i> (pmol/min/mg)	[³ H]arachidonic acid- <i>E. coli</i> (pmol/min/mg)
Cell-free SF* (n=11)	291.3 (27.6)†	93.6 (12.6)
PB* neutrophils (n=3)	18.6 (1.6)‡	10.6 (0.1)
SF neutrophils (n=3)	171.3 (57.6)	66.8 (46.3)
PB mononuclear cells (n=3)	221.3 (46)§	225.1 (63.3)
SF mononuclear cells (n=3)	356.6 (36.3)	490 (150)

*SF=synovial fluid; PB=peripheral blood.

†p<0.001 v peripheral blood neutrophils.

‡p<0.001 v synovial fluid neutrophils.

§p<0.05 v synovial fluid mononuclear cells.

Table 2 Effect of cell free synovial fluid on the percentage distribution of [14 C]oleic acid and [3 H]arachidonic acid in *E coli* phospholipids

Compound	[14 C]oleic acid- <i>E coli</i>		[3 H]arachidonic acid- <i>E coli</i>	
	Control	Cell free synovial fluid	Control	Cell free synovial fluid
Triglyceride	0.5	0.6	2.4	2.4
Free fatty acid	3.4	8.0*	10.2	12.8*
Diglyceride	0.9	0.9	6.1	7.6
Monoglyceride	0.5	0.4	13.9	13.7
Cardiolipin	28.1	36.3	36.8	23.4*
Phosphatidylethanolamine, phosphatidylglycerol	61.5	49.9*	27.1	29.8
Phosphatidylserine, phosphatidylinositol	3.0	1.7	1.7	8.2
Phosphatidylcholine	1.5	1.8	0.9	1.2
Sphingomyelin, lysophosphatidylcholine	0.5	0.2	1.1	1.1

* $p < 0.001$ v control.

of either the ionic detergent deoxycholic acid or the non-ionic detergent Triton X-100 to the reaction mixture did not affect phospholipase A_2 activity. Several variables of phospholipid hydrolysis by cell free synovial fluid phospholipase A_2 were examined using labelled *E coli* substrate. Table 2 shows the distribution of radioactivity among lipids of *E coli* labelled with [14 C]oleic acid and [3 H]arachidonic acid. Approximately 95% of the total lipid radioactivity was associated with phospholipids labelled with [14 C]oleic acid, whereas phospholipids labelled with [3 H]arachidonic acid represented only 68% of the total lipid radioactivity. The most heavily labelled compounds were phosphatidylethanolamine/phosphatidylglycerol (61.5% and 27.1% for [14 C]oleic acid and [3 H]arachidonic acid respectively) and cardiolipin (28.1% and 36.8% for [14 C]oleic acid and [3 H]arachidonic acid respectively).

Incubation of [14 C]oleic acid labelled *E coli* with cell free synovial fluid resulted in a significant increase of radioactivity in the free fatty acid fraction from 3.4% to 8.0% ($p < 0.001$). Degradation of phosphatidylethanolamine may account for this increase in the fatty acid fraction as there was a corresponding decrease in the phosphatidylethanolamine fraction from 61.5% to 49.9% ($p < 0.001$). When [3 H]arachidonic acid labelled *E coli* was used cardiolipin was the main phospholipid degraded, radioactivity decreasing from 36.8% to 23.4% ($p < 0.001$); a corresponding increase in the fatty acid fraction from 10.2% to 12.8% ($p < 0.001$) was observed.

Substitution of [3 H]arachidonic acid for [14 C]oleic acid as the radioactive labelled fatty acid allowed differentiation of phospholipase A_2 from two different sources—that is, cell free and cell associated synovial fluid. We found that soluble phospholipase

A_2 present in cell free fractions of synovial fluid preferentially released [14 C]oleic acid rather than [3 H]arachidonic acid from *E coli* biomembranes. The phospholipids hydrolysed were phosphatidylethanolamine and phosphatidylglycerol. When sonicates of mononuclear cells or neutrophils from synovial fluid were tested a preference for [3 H]arachidonic acid was observed. Cardiolipin was the main phospholipid hydrolysed by the cell associated enzyme. No correlation was found between the phospholipase A_2 activity in the synovial fluid and the activity of the disease as evaluated by a modified rheumatoid activity index. The white blood cell count in the synovial fluid did not correlate with phospholipase A_2 activity, and we were unable to show any effect of treatment on the levels of phospholipase A_2 activity.

EFFECT OF CALCIUM AND EDTIC ACID

As several soluble phospholipase A_2 s have been shown to be calcium requiring enzymes it was of interest to investigate the divalent cation requirement of this synovial fluid phospholipase A_2 from patients with rheumatoid arthritis. Figure 1 shows that when [14 C]oleic acid labelled *E coli* was used as substrate the phospholipase A_2 activity increased with calcium concentration up to 5 mmol/l, with no further increase at higher concentrations of calcium. Maximal activity against [3 H]arachidonic acid labelled *E coli* was found at 0.05 mmol/l calcium concentration. In both cases the addition of edetic acid caused a progressive loss of activity and total inhibition at concentrations of edetic acid higher than 0.05 mmol/l. Our determination of free calcium in cell free synovial fluid ranged from 61 to 68 mg/l with a mean concentration of 65.3 mg/l.

Fig. 1 Effect of Ca⁺⁺ (○—○) and edetic acid (△—△) on phospholipase A₂ activity in cell free synovial fluid from patients with rheumatoid arthritis. Reaction mixture composition is described in 'Materials and methods'. Incubations were carried out for 30 minutes. Lipids were extracted and separated by thin layer chromatography. Results are the mean of two separate experiments, each performed in duplicate. AA=arachidonic acid.

Fig. 2 Effect of pH on phospholipase A₂ activity in synovial fluid from patients with rheumatoid arthritis. Reaction mixtures were incubated in the presence of buffers over the range pH 4–10. Results are the mean of two separate experiments, each performed in duplicate. AA=arachidonic acid.

EFFECT OF pH

The pH dependence of cell free synovial fluid phospholipase A₂ activity was studied comparatively using both [14C]oleic acid and [3H]arachidonic acid labelled *E. coli* over the range pH 4 to 10 (Fig. 2). When [14C]oleic acid labelled *E. coli* was used as substrate the maximal activity was found at pH 7.5. With [3H]arachidonic acid labelled *E. coli* we found optimal activity at pH 7.0.

EFFECT OF TIME

Incubation of the reaction mixture for varying

lengths of time resulted in a time dependent hydrolysis of *E. coli* phospholipids (Fig. 3). The time course of hydrolysis of labelled *E. coli* indicates that the reaction was linear during the first 30 minutes of incubation. When [3H]arachidonic acid labelled phosphatidylethanolamine was used as substrate the reaction was linear during the first 60 minutes of incubation.

EFFECT OF PROTEIN

Phospholipase A₂ activity of cell free synovial fluid was consistently linear with respect to time and

Fig. 3 Effect of time on phospholipase A₂ activity in synovial fluid from patients with rheumatoid arthritis. Reaction mixture composition is described in 'Materials and methods'. Incubations were stopped at indicated times. Results are the mean of two separate experiments, each performed in duplicate.

protein content when phospholipid hydrolysis was less than 20%. As most recent reports measure synovial fluid phospholipase A₂ activity in assay mixtures containing bovine serum albumin^{6,7} the effect of albumin was determined using both substrates. The presence of bovine serum albumin in the reaction mixture at concentration of 100 mg/l resulted in an increase of phospholipase A₂ of 183% for [¹⁴C]oleic acid labelled *E coli* and 191% for [³H]arachidonic acid labelled *E coli*.

Discussion

This is the first report on phospholipid substrate specificity for a phospholipase A₂ present in the synovial fluid from patients with rheumatoid arthritis. We established standard assay conditions and characterised the phospholipase A₂ activity in cell free synovial fluid from rheumatoid patients. In previous publications^{6,7} phospholipase A₂ activity has been expressed in units/ml, which does not allow for easy comparison because of the variability in synovial fluid protein concentrations. We suggest, therefore, the measurement of specific activity in pmol of substrate hydrolysed per minute per mg of protein as the appropriate units for expression of enzymatic activity. Cell free synovial fluid phospholipase A₂ hydrolysed phospholipids from *E coli* biomembranes, and purified micellar dispersions of

phosphatidylethanolamine, but not phosphatidylcholine. The specific activity is proportional to the enzyme concentration in the range 50–100 µg protein per incubation mixture. This enzyme is optimally active at neutral pH and requires calcium. The rate of release of [¹⁴C]oleic acid and [³H]arachidonic acid from *E coli* phospholipids was linear up to 30 minutes. Actual concentrations of free calcium in the rheumatoid synovial fluid are sufficient for the in vitro assay to proceed without adding extra calcium; optimal concentrations of calcium for the standard phospholipase A₂ assay were found to be different, however, depending on the fatty acid esterified into *E coli* phospholipids. As a potentially proinflammatory phospholipase A₂ should be able to release arachidonic acid from cellular lipids we decided to use [³H]arachidonic acid labelled *E coli* biomembranes. Owing to the enzymes' substrate specificity we were able to distinguish two different enzymes—namely, the cell free synovial fluid phospholipase A₂ and the cell associated. Phospholipase A₂ from cell free fractions of rheumatoid synovial fluid preferentially releases [¹⁴C]oleic acid from *E coli* phosphatidylethanolamine and peripheral blood, whereas cell associated phospholipase A₂ present in synovial fluid neutrophils and mononuclear cells preferentially releases [³H]arachidonic acid from *E coli* cardiolipin.

Our studies suggest that synovial fluid neutrophils and mononuclear cells may be important sources of phospholipase A₂. Synovial fluid neutrophils express up to 10 times more activity than those from peripheral blood (Table 1), whereas synovial fluid mononuclear cells express approximately 60% more activity than those from peripheral blood. One possible explanation is the presence of a factor(s) in the rheumatoid synovial effusion that activates cellular phospholipase A₂. It is possible that interleukin 1,^{18,19} rheumatoid factors,^{20,21} or immune complexes,^{22,23} all reported in the rheumatoid synovial fluid, may act as activators of cellular phospholipase A₂. This would then trigger mobilisation of free arachidonic acid and further metabolism, leading to raised concentrations of inflammatory eicosanoids in the synovial fluid. Chang *et al.*²⁴ have recently proposed that the inflammatory actions of interleukin 1 may be mediated through activation of a phospholipase A₂, leading to generation of biologically active metabolites of arachidonic acid.²⁴ In support of this interesting hypothesis they showed that interleukin 1 induces intracellular phospholipase A₂ activity in rabbit articular chondrocytes and stimulates secretion of phospholipase A₂ into the culture medium. Furthermore, they showed a dose related synthesis of prostaglandin E₂ by these interleukin 1 responsive cells. The role of

interleukin 1 in generating prostaglandin E₂ has been shown also by other investigators.²⁵⁻²⁷ For example, Dayer *et al*, using isolated adherent synovial cells obtained from rheumatoid synovectomy preparations, showed secretion of prostaglandin E₂ after stimulation with a soluble factor from mononuclear cells.^{25, 26} This factor turned out to be functionally and chemically related to interleukin 1. Mizel *et al* suggested that interleukin 1 may regulate the inflammatory activity of synovial cells through increased synthesis and secretion of prostaglandin E₂.²⁸ As interleukin 1 is present in rheumatoid synovial fluid it is conceivable that neutrophils and mononuclear cells in the joint fluid may be targets of the phospholipase A₂ stimulatory effect of interleukin 1. It is also possible that neutrophils and mononuclear cells acquire phospholipase A₂ from the synovial fluid through non-specific adsorption; in that event other suitable sources of the enzyme must be considered, such as chondrocytes and hyperproliferative synovial tissues.

The cell free synovial fluid phospholipase A₂ described here, which readily hydrolyses *E. coli* phospholipids, is similar to the enzyme characterised by Vadas *et al*.⁷ Although the results show that *E. coli* cardiolipin and phosphatidylethanolamine/phosphatidylglycerol as presented in these experiments are accepted as substrate by the cell associated phospholipase A₂, further studies are clearly needed to establish its substrate specificity and its relation with the phospholipase A₂ in neutrophils and mononuclear cells from patients with rheumatoid arthritis, as described by Bomalaski *et al*.^{8, 9} Although the relevance of these phospholipase A₂s to the chronic inflammatory process in rheumatoid arthritis is presently unknown, lysoderivatives of phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine have been reported in rheumatoid synovial fluid, suggesting the possibility of active phospholipid hydrolysis occurring in the joint space.²⁹

The above observations taken together with the other reports cited confirm the presence of pro-inflammatory phospholipase A₂ in rheumatoid synovial fluid. Furthermore, cell free fractions of synovial fluid contain an apparently different phospholipase A₂ than the cell associated form present in synovial fluid neutrophils and mononuclear cells. The results of this study may help to explain the abnormal regulation of eicosanoid production observed in rheumatoid arthritis.

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