5' Nucleotidase activity in the human synovial lining in rheumatoid arthritis

B. HENDERSON, J. J. JOHNSTONE, AND J. CHAYEN

From the Division of Cellular Biology, Mathilda and Terence Kennedy Institute of Rheumatology, Bute Gardens, London W6 7DW

SUMMARY 5'-Nucleotidase (EC 3.1.3.5), a plasma membrane-bound enzyme, has been assayed in unfixed tissue sections of human synovium, activity being measured by scanning and integrating microdensitometry. Activity was markedly increased in the lining cells of the rheumatoid synovial membranes.

The activity of 5'-nucleotidase (5-NT) is raised in the serum and synovial fluid of patients with rheumatoid arthritis.¹ Farr *et al.*² found that the 5-NT activity in synovial fluid was related to the severity of the disease and polymorphonuclear leucocyte content of the fluid. However, on the basis of the intensity of the histochemical stain for 5-NT in polymorphonuclear leucocytes and in free-floating lining cells in synovial fluid Farr and coworkers suggested that the lining cells were the source of the increased 5-NT in the rheumatoid joint fluids. This suggestion was strengthened by the finding that in unfixed tissue sections rheumatoid synoviocytes stained more intensely with the histochemical test for 5-NT than did the lining cells from osteoarthritic joints. However, because this effect was not quantified, the 5-NT activity in the lining cells of human rheumatoid and nonrheumatoid synovial tissue has now been re-examined by an improved assay system, controlled by a specific inhibitor of the alkaline phosphatase activity that may otherwise contribute to the apparent 5-NT activity. The enzyme activity has been measured by microdensitometry.

Materials and methods

Biopsy specimens of human synovial membranes were taken either at arthrotomy for internal derangement or at synovectomy. The nonrheumatoid specimens were taken from the knee, either from quiescent joints or from otherwise normal joints

after recent mechanical trauma. The rheumatoid specimens were taken mainly from the knee during synovectomy from patients who had 'definite' or 'classical' disease according to the diagnostic criteria of the American Rheumatism Association.³ Pieces of tissue approximately 4 mm³ were chilled by precipitate immersion in n-hexane (BDH 'free from aromatic hydrocarbons' grade, boiling range 67-70°C). After no more than 1 minute each specimen was removed from the hexane and stored at -70°C in a corked dry glass tube. Specimens were sectioned, normally at 10 µm in a Bright's cryostat maintained at a cabinet temperature of -25 to -30°C with the knife cooled to -70°C by packing its haft in solid carbon dioxide. The sections were transferred from the knife to slides which were at the ambient temperature of the laboratory, thus ensuring that the sections became flash-dried.⁴ The method ensures that sections produced are free from ice damage.⁵ For each sample several sections were stained with toluidine blue to establish the precise histology.

ASSAY METHOD FOR 5'-NUCLEOTIDASE

Sections were assayed for 5'-nucleotidase activity by a modification of the calcium-trapping method of Chayen *et al.*⁵ The assay medium consisted of adenosine 5'-monophosphate (disodium salt) (Sigma) 4 mM, calcium chloride (dihydrate) (BDH) 340 mM, manganese chloride (BDH) 0.06 mM, L-p-bromotetramisole oxalate (Aldrich) 0.1 mM.

Substrates were dissolved in 0.1 M Tris-HCl (BDH), pH 8.3. Sections were reacted in the medium at 37°C for various times. At the end of the reaction sections were washed under running tap water for 5 min, rinsed in distilled water, and then transferred

Accepted for publication 6 June 1979
Correspondence to Dr B. Henderson, Division of Cellular Biology, Kennedy Institute of Rheumatology, Bute Gardens, London W6 7DW.

to a bath of 0.1% cobalt nitrate (BDH) for 5 min. Sections were then washed thoroughly in distilled water before being exposed to a 0.1% solution of ammonium sulphide (BDH) in distilled water for 1 min. The sections were then washed, dried, and mounted in Farrant's medium.

RATIONALE OF ASSAY

Phosphate, liberated from adenosine 5'-monophosphate (AMP) by the action of 5-NT, is trapped by the calcium ions in the assay medium to form an insoluble colourless precipitate of calcium phosphate. In order to visualise this reaction product the calcium phosphate is first converted to the colourless cobalt phosphate by immersion of sections in a solution of cobalt nitrate; the cobalt phosphate is then converted into the grey-black cobalt sulphide by exposing sections to a solution of ammonium sulphide. Manganese chloride is included in the assay medium as an activator of the enzyme⁶ and L-p-bromotetramisole oxalate as a specific inhibitor of alkaline phosphatase.

SPECIFICITY OF THE ASSAY

Enzymes other than 5-NT, and present in most cells, are capable of dephosphorylating adenosine monophosphate (AMP). The most active of these enzymes are acid phosphatase and alkaline phosphatase. The former acts optimally at acidic pH values and was unlikely to contribute to the present assay, done at pH 8.3. This was confirmed by the fact that fluoride, a potent inhibitor of acid phosphatase, had no effect on the 5-NT activity even when added to the assay-medium at a concentration of 10 mM. Alkaline phosphatase in most tissues is specifically inhibited by L-p-bromotetramisole.⁷ It also inhibits this enzyme activity in the synovium.⁸ Hence its routine inclusion in the assay medium ensured that alkaline phosphatase could not have contributed to the final activity seen in the sections tested for 5-NT activity. Omission of the substrate (AMP) from the assay medium resulted in only a slight residual 'activity' possibly due to metallophilicity (i.e., non-specific metal binding). Nickel chloride, a known and often used inhibitor of 5-NT,⁹ when added to the assay medium at 10^{-2} M caused almost complete inhibition of activity.

MEASUREMENT

Fifteen to 20 synovial lining cells in each of 2 duplicate sections were measured, by means of a Vickers M85 scanning and integrating microdensitometer, at 550 nm; a $\times 40$ objective was used with the smallest size of scanning spot (0.5 μ m diameter in the plane of the section) and with the mask size adjusted to accommodate one selected

cell at a time.¹⁰ Linearity of the response was checked by measuring the activity in serial sections of most of the specimens reacted for various periods of time up to 30 min. As all but one specimen showed linear kinetics between 15 and 30 min of reaction, all activities were reported in terms of a 30 min assay period. For most purposes the relative absorption, recorded by the microdensitometer, was converted to integrated extinction by means of suitable calibration.

Student's *t* test was used to evaluate the results.

Results

REPRODUCIBILITY

Four blocks were taken from one specimen of non-rheumatoid synovial tissue, and the 5-NT activity in the lining cells was measured in duplicate sections taken from each block. The mean and standard

(a)

Fig. 1 The difference in the activity of 5-NT in the synoviocytes from (a) a nonrheumatoid and (b) a rheumatoid synovial lining. $\times 145$.

(b)

error of the mean of all the readings (relative absorption/unit area) made from each of these blocks was 170 ± 17 , 202 ± 14 , 208 ± 14 , and 215 ± 19 . Thus the percentage deviation of the mean value for each block from the overall mean value was 17, 1.5, 4, and 7% respectively; the differences between blocks were not statistically significant. Thus sampling error, whether within a block or between different parts of a specimen of synovium, was only slight.

5-NT ACTIVITY IN SYNOVIAL LINING CELLS

5-NT activity has been assayed in the lining cells of 10 nonrheumatoid and 6 rheumatoid synovial

specimens. The typical appearance of the nonrheumatoid and rheumatoid sections after 30 min of reaction can be seen in Fig. 1. The range of activities in the 2 populations of lining cells can be seen in Fig. 2. The mean activity and standard error of the mean (in terms of integrated extinction per 30 min) for the nonrheumatoid specimens was 0.14 ± 0.02 ; that for the rheumatoid specimens was 0.69 ± 0.06 . This difference was highly significant ($P < 0.001$). There was no overlap between the 2 populations. This marked difference in 5-NT activity between nonrheumatoid and rheumatoid synovial lining cells was seen more clearly when all readings from each specimen were plotted in the form of a population histogram. Thus Fig. 3 shows the population histograms of 5-NT activity in synoviocytes from 2 nonrheumatoid and 2 rheumatoid synovial linings reacted at the same time under identical conditions. There is virtually no overlap of these readings.

Fig. 2 5'-nucleotidase activity in rheumatoid and nonrheumatoid synoviocytes

Fig. 3 Population histograms showing 5-NT activity in rheumatoid and nonrheumatoid synoviocytes. The histograms record the frequency with which particular levels of activity (relative absorption) are found in these specimens

5-NT ACTIVITY IN BLOOD VESSELS

5-NT activity was present in the blood vessels in both the rheumatoid and nonrheumatoid synovial specimens. Within any one synovial specimen there was a considerable range of activity in different vessels, the activity being highest in the smaller vessels. This wide range of activity and also the heterogeneity of blood vessels made it difficult to quantify this activity. However, it seemed clear that the marked changes that were found in the rheumatoid synovial lining cells did not occur in the blood vessels in the rheumatoid synovial tissue.

TIME COURSE OF THE 5-NT ACTIVITY IN LINING CELLS

In preliminary studies designed to optimise assay conditions for this enzyme it was found that the time course of the reaction in lining cells was different in nonrheumatoid and rheumatoid tissue. Thus in nonrheumatoid and recently traumatised tissue the activity in lining cells showed a latent period of 5–10

min before the appearance of reaction product. In rheumatoid specimens this lag period was small or absent (Fig. 4). This finding was confirmed in 4 rheumatoid and 5 nonrheumatoid specimens. When nonrheumatoid sections were treated with agents, such as benzyl alcohol, that are known to perturb membrane structure and increase 5-NT activity in purified plasma membranes, no increase in enzyme activity was found.

Discussion

Farr *et al.*² have shown a high correlation between the severity of inflammation in the rheumatoid joint and the level of 5-NT activity in the synovial fluid and suggested that the enzyme was derived from the lining cells. Raised levels of 5-NT activity in homogenates of the outer layers of rheumatoid synovial tissue have been reported by Kar *et al.*¹¹ and Takala *et al.*¹²

With the use of an improved cytochemical assay for 5-NT activity, which includes the use of an inhibitor of alkaline phosphatase activity, the 5-NT activity in nonrheumatoid synovium was found mainly in the blood vessels and to some extent in small linearly orientated structures suggestive of small capillaries or lymphatics. Activity in the lining cells was low. In contrast, rheumatoid synovial lining tissue showed a marked rise in 5-NT activity in the lining cells but only a moderate increase in the blood vessels. There was more activity in the stroma of the rheumatoid tissue, but this probably merely reflected the increased cellularity of this tissue. In quantitative terms (of extinction per unit area per unit time) activity in lining cells rose from a mean value ($\pm$ SEM) of $0.14 (\pm 0.02)$ to $0.7 (\pm 0.06)$, that is, a 5-fold rise. This difference was highly significant ($P < 0.001$).

The presence of what appeared to be a lag phase in the 5-NT activity in nonrheumatoid synoviocytes might indicate that the state of the enzyme in these cells is characteristically different from that in rheumatoid synoviocytes. It could have been argued that the 'lag-phase' was due to the fact that the low rate of liberation of phosphate, by the weakly active enzyme, was inadequate to cause precipitation of the calcium phosphate, as required by this technique. However, this cannot be the explanation of the change in rate of the reaction in the specimen from the recently traumatised, nonrheumatoid joint.

Trams and Lauter¹³ showed that 5-NT, present in the plasma membrane, has its active site outside the cell. The enzyme is enclosed within a phospholipid milieu termed the 'phospholipid annulus'; depending on its precise composition and physicochemical state, the annulus can exert a

Fig. 4 Time-course of 5-NT activity in lining cells
— Rheumatoid synoviocytes.
--- Nonrheumatoid synoviocytes
... Synoviocytes in a recently traumatised joint

marked influence on the catalytic activity of 5-NT.¹⁴ Thus agents that increase the fluidity of phospholipids increase the 5-NT activity of purified preparations of isolated plasma membranes.¹⁴

Previous work¹⁵ showed that the state of the phospholipid-protein associations in rheumatoid synoviocytes was very different from that in the nonrheumatoid counterparts. This could account for the increased 5-NT activity reported in the present communication, although attempts to modify the activity in sections of nonrheumatoid tissue by means of benzyl alcohol failed to modulate this activity. However, although this agent increases phospholipid fluidity in isolated plasma membranes it is conceivable that more rigorous methods would be necessary to achieve the same result in the relatively intact cells in the sections used in the present study.

We are grateful to Mr B. Cashman, FRCS, Mr A. Catterall, FRCS, and Dr B. Bourke for their interest in this work and for supplying the specimens. We are glad to acknowledge a grant towards this work from the Medical Research Council, and the support of the Arthritis and Rheumatism Council for Research.

References

- ¹ Kendall M J, Cockel R, Becker J, Hawkins C F. Raised serum alkaline phosphatase in rheumatoid disease—an index of liver dysfunction? *Ann Rheum Dis* 1970; **29**: 537–540.
- ² Farr M, Kendall M J, Shuttleworth R, Meynell M J, Hawkins C F. Source and significance of 5-nucleotidase in synovial fluid. *Ann Rheum Dis* 1973; **32**: 326–330.
- ³ Ropes M W, Bennet G A, Cobb S, Jessar R A. Diagnostic criteria for rheumatoid arthritis. *Ann Rheum Dis* 1959; **18**: 49.
- ⁴ Chayen J, Bitensky L. Multiphase chemistry of cell injury. In: Bittar E E, Bittar N, eds. *The Biological Basis of Medicine*. London and New York: Academic Press, 1968; 337–368.
- ⁵ Chayen J, Bitensky L, Butcher R G. *Practical Histochemistry*. New York and London: Wiley, 1973.
- ⁶ Hardonk M J. 5'-Nucleotidase I. Distribution of 5'-nucleotidase in tissues of rat and mouse. *Histochemie* 1968; **12**: 1–17.
- ⁷ Borgers M, Thoné F. The inhibition of alkaline phosphatase by L-p-bromotetramisole. *Histochemistry* 1975; **44**: 277–280.
- ⁸ Johnstone J J, Bitensky L, Cashman B, Chayen J. The effect of zinc on alkaline phosphatase activity in rheumatoid synovial tissue. *Ann Rheum Dis* 1978; **37**: 552–556.
- ⁹ Bodansky O, Schwartz M K. 5'-Nucleotidase. *Adv Clin Chem* 1968; **11**: 277–328.
- ¹⁰ Chayen J. Microdensitometry. In Slater T F, ed. *Biochemical Mechanisms of Liver Injury*, London and New York: Academic Press 1978; 257–291.
- ¹¹ Kar N C, Cracchiolo A, Mirra J, Pearson C M. Acid, neutral, and alkaline hydrolases in arthritic synovium. *Am J Clin Path* 1976; **65**: 220–228.
- ¹² Takala I, Mäkisara P, Kulonen E. The activities of plasma-membrane marker enzymes in rheumatoid synovial tissues and fluids. *Scand J Rheumatol* 1977; **6**: 33–36.
- ¹³ Trams E G, Lauter C J. On the sidedness of plasma membrane enzymes. *Biochim Biophys Acta* 1974; **345**: 180–197.
- ¹⁴ Dipple I, Houslay M D. The activity of glucagon-stimulated adenylate cyclase from rat liver plasma membranes is modified by the fluidity of its lipid environment. *Biochem J* 1978; **174**: 179–190.
- ¹⁵ Henderson B, Bitensky L, Chayen J. Altered phospholipids in human rheumatoid synoviocytes. *Ann Rheum Dis* 1978; **37**: 24–29.