MUCOPOLYSACCHARIDES IN SYNOVIAL FLUID

DETECTION OF CHONDROITIN SULPHATE

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Hyaluronic acid is usually the only polysaccharide present in human synovial fluid. However, the finding of abnormally high amounts of galactosamine suggested that other polysaccharides, perhaps originating from ground substance, might also occur (Laurent, 1957; Smith, Crowley, and Giles, 1960). Their presence has hitherto been only surmised, for galactosamine could have arisen from the carbohydrate moiety of mucoproteins and not from chondroitin sulphate itself; but we did detect a sulphated mucopolysaccharide in synovial fluid obtained from a patient with osteo-arthritis, the only instance among nineteen patients with various forms of arthritis (Barker, Bayyuk, Brimacombe, Hawkins, and Stacey, 1963). We now report a study in which a particular search has been made for such polysaccharides. At the same time, techniques for separating synovial hyaluronic acid and for isolating the hyaluronic acid-protein complexes have been examined further.

Material and Methods

Material

Synovial fluid was aspirated from the knee joints of twelve patients, of whom the clinical details are listed in the Table. Diagnosis of rheumatoid arthritis, made by one of us (C.F.H.), was established according to the criteria set forth by the American Rheumatism Association (Ropes, Bennett, Cobb, Jacox, and Jessar, 1957). We accepted as normal fluid obtained from apparently normal joints during the operation for meniscectomy. Fluids were stored in sterile bottles at -25°C .

Methods

(1) *Separation of Hyaluronic Acid-Protein Complexes from Free Proteins by Fractionation of Synovial Fluid on Diethylaminoethyl (DEAE) Sephadex.*—The gel filtration media, Sephadex, is synthesized by cross-linking the polysaccharide, dextran, to give a three-dimensional network. A swollen Sephadex gel excludes molecules which exceed a certain size, smaller molecules penetrating the gel to an extent dependent upon their size. A mixture of macromolecules is eluted from the gel in order of decreasing

TABLE
CLINICAL PARTICULARS OF TWELVE PATIENTS

Case No.	Sex	Age (yrs)	Diagnosis	Waalser-Rose Test	Erythrocyte Sedimentation Rate (mm./1 hr Westergren)	Hb (g./100 ml.)
1	M	30	Traumatic synovitis	—	—	—
2	M	35	Traumatic synovitis	—	—	—
3	M	60	Rheumatoid arthritis (definite)	Negative	11	14.6
4	F	60	Rheumatoid arthritis (definite)	Negative	38	11.1
5	M	58	Rheumatoid arthritis (classical)	1/1,024	20	12.3
6	F	55	Rheumatoid arthritis (classical)	Negative	9	11.7
7	F	58	Rheumatoid arthritis (classical)	1/1,024	44	11
8	M		Osteo-arthritis	Negative		
9	M	65	Osteo-arthritis of knees	1/32	74	12.9
			Rheumatoid arthritis (classical)			
10	F	61	Osteo-arthritis of knees	Negative	32	12.3
			Rheumatoid arthritis (definite)			
11	F	60	Rheumatoid arthritis (classical)	1/128	115	10.8
12	F	67	Rheumatoid arthritis (classical)	1/128	48	12.9

molecular weight. Chemical attachment of diethylaminoethyl (DEAE) residues to Sephadex produces a high capacity anion exchanger with low non-specific adsorption.

Aliquots (3 to 6 ml.) of each fluid were diluted with buffer and passed down columns of DEAE-Sephadex (30×2.8 cm.), equilibrated with 0.1M-phosphate buffer, pH 7, and eluted (500 ml.) with a linear gradient (0.02→0.7M) of NaCl in the same buffer. Thereafter the column was washed with 2M NaCl in phosphate buffer (25 ml.) followed by a linear gradient (0.2M→2M) of NaCl hydrochloric acid (100 ml.) in equimolar proportions. The fractions (5 ml.) were scanned for proteins using the optical density at $279 m\mu$, and for hexuronic acid by a modification (Gregory, 1960) of the Dische (1947) carbazole reaction. A reference curve was prepared, using D-glucuronic acid as the standard.

(2) *Fractionation of Deproteinized Synovial Fluid by Sephadex Gel-200 to isolate Mucopolysaccharide Fractions.*—Pronase P (5 mg.) in 0.1M phosphate buffer, pH 7 (5 ml.), containing a trace of calcium chloride was sterilized by passage through a Hemming filter and incubated at 37°C . for 24 hours with synovial fluid (3 to 6 ml.) in the dialysis bag of a sterile, aseptic dialysis apparatus (Chelton and James, 1963) containing the same buffer in its outer compartment. The digest was fractionated on a column (30×2.8 cm.) of Sephadex gel-200, equilibrated with 0.1M-phosphate buffer, pH 7, and eluted with the same buffer at 7 to 10 ml. per hour. The fractions (5 ml.) were assayed for peptide debris, using the ninhydrin assay of Moore and Stein (1948), and for uronic acid (Gregory, 1960).

(3) *Other Studies of the Mucopolysaccharide Fractions*

(a) Protein content was assayed by the method of Lowry, Rosebrough, Farr, and Randall (1951) calibrated against serum albumin.

(b) The ester sulphate content was determined by the method of Jones and Letham (1956).

(c) Where desirable the mucopolysaccharide was further fractionated on a calibrated column of Deacidite FF (Chloride form) as described by Barker, Cruickshank, and Webb (1965). This procedure separates protein debris, hyaluronic acid, chondroitin 4- and 6-sulphates, dermatan sulphate, heparin, and keratan sulphate. Column eluates were scanned with orcinol (Svennerholm, 1956).

Results

Fractionation on DEAE-Sephadex and elution with a solution of NaCl (—0.32M) separated free protein from protein-polysaccharide complexes in synovial fluids from twelve patients.

“Normal” fluid, obtained during operation for meniscectomy, was unique in showing a protein peak at 0.0M NaCl, together with one at 0.04M NaCl and the hyaluronic acid-protein complexes were eluted in a series of peaks between 0.27 and 0.55M

—NaCl (Fig. 1, opposite lower diagram). Fluids from Patients 1 and 2 with traumatic synovitis showed a remarkable similarity in the elution pattern (Fig. 1, upper diagrams) of their hyaluronic acid-protein complexes, namely a multiplicity of peaks above 0.48M NaCl, some requiring acidic elution. Further examination of the polysaccharide-protein complexes of one of these fluids revealed the presence of hexosamine and hexuronic acid, though there was no combined sulphate; the protein contents were 23, 22.9, 20.4, and 49 per cent., respectively, in order of elution.

Fluids from three cases of rheumatoid arthritis (Nos 3, 4, and 5) also showed only complexes of the hyaluronic acid-protein type, eluted at about 0.3M NaCl, which sometimes required acidic elution. The protein contents of mucopolysaccharide fractions from two of them were 31.5, 12, 8, 9.7, 13.2, and 7.7 per cent. for No. 3 and 35, 5.7, 9.6, and 23.8 per cent. for No. 4, in order of elution. Two other cases of rheumatoid arthritis (Nos 6 and 7), in whom the disease was complicated by peripheral neuropathy, had synovial fluid of particular interest for both contained only traces of hyaluronic acid eluted in the range 0.32—0.45M NaCl. In No. 6 the remaining mucopolysaccharide complexes were sulphated (12 and 14.5 per cent. sulphate) and eluted only in the acid range, and persisted following treatment with intra-articular prednisolone. However, after 3 days' treatment, the polysaccharide (0-sulphate 14 per cent.) was eluted at 0.42—0.5M NaCl, and after 7 days' treatment it was eluted in two fractions (0-sulphate 12 and 13 per cent. respectively) in the range 0.43—0.51M NaCl and 0.51—0.55M NaCl.

No such sulphated polysaccharide was found in a patient with osteo-arthritis (No. 8) or in one with osteo-arthritis and rheumatoid arthritis (No. 9). In No. 8 protein contents of the mucopolysaccharide fractions were 10.8, 24.8, and 19.6 per cent. respectively, in order of elution.

In order to examine more closely those cases in which infiltration of sulphated mucopolysaccharides into synovial fluid had occurred, a series of fluids were deproteinized with Pronase under aseptic conditions. It was then possible to separate the polysaccharide component from the protein debris by fractionation on Sephadex gel-200, only the polysaccharide being excluded from the gel. The nature of the polysaccharide present was ascertained by passage down a calibrated column of Deacidite FF (Chloride form). Extensive investigation of separations of human cartilage (Barker, Guy, and Cruickshank, 1965) and skin constituents (Barker,

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Fig. 1.—Fractionation of the synovial fluids of two patients with traumatic synovitis on DEAE-Sephadex. Lower large diagram is that of control normal fluid obtained at meniscectomy.

Cruickshank, and Webb, 1965) has established the reproducibility of the point of elution of a particular polysaccharide of the ground substance, so that the method can now be used with some confidence to establish the constituents of mixtures. Examined in this way the fluid of patient No. 10, who had rheumatoid arthritis and osteo-arthritis of the knees, showed only protein debris and the single peak of

hyaluronic acid with no other polysaccharides or ground substance present. Sulphated mucopolysaccharides were also encountered in the synovial fluid (Fig. 2) of patient No. 11, who had rheumatoid arthritis.

Another sample of fluid from patient No. 12, who had rheumatoid arthritis complicated by peripheral neuropathy, showed unique changes. When the

Fig. 2.—Fluid from patient with rheumatoid arthritis showing evidence of ground substance infiltration.

deproteinized fluid was fractionated on Sephadex gel-200, some polysaccharide was excluded (eluent volume 70 to 140 ml.) by the gel and the rest included (140 to 200 ml.) accompanied by protein debris. This latter feature was not seen in twenty other pathological fluids examined. The two polysaccharide fractions were separately fractionated on columns of Deacidite FF (Fig. 3). The main constituents of the included fraction were hyaluronic acid and chondroitin 6-sulphate, the latter probably present as the normal and oversulphated analogues found by Barker, Guy, and Cruickshank (1965) in human knee cartilage. Keratan sulphate was concentrated in the included fraction while chondroitin 4-sulphate was concentrated in the excluded polysaccharide.

Discussion

Mucopolysaccharides, such as chondroitin sulphate, may occasionally be detected in the synovial fluid of patients with arthritis. These have probably infiltrated into synovial fluid from the ground substance of cartilage, in contrast to hyaluronic acid, which is produced by synovial cells. The fact that chondroitin sulphate was found more commonly in rheumatoid synovial fluid was probably due to the selection of our clinical material and we have found it also in osteo-arthritic synovial fluid. Three samples of fluid from patients with rheumatoid arthritis (Nos 6, 7, and 12) were of particular interest, as they showed an unusual pattern of depolymerized hyaluronic acid and Nos 6 and 12 showed sulphated mucopolysaccharides. It was of interest that in all three patients the arthritis was complicated by peripheral neuropathy. The possibility that loss of sensation might cause greater damage to the joint from loss of protective reflexes, as in Charcot's joints, and so cause liberation of mucopolysaccharides from damaged cartilage was considered unlikely; and, in one bed-ridden patient,

sensation in the knees was normal. Peripheral neuropathy usually occurs in severe cases of rheumatoid disease, so severity alone might explain it; also it is generally a manifestation of arteritis, so that damage from ischaemia of vessels supplying joint surfaces and synovial tissues may have caused liberation of enzymes which depolymerize hyaluronic acid, and may have released ground substance from cartilage, but there is no means of confirming this hypothesis. Alternatively, the neuropathy may have been coincidental. The clinical and radiological examination of the joints revealed nothing which explained this occasional finding of mucopolysaccharides.

Summary

Mucopolysaccharides, originating from ground substance, have been isolated from synovial fluid from three patients with rheumatoid arthritis. Mucopolysaccharide-protein complexes, including chondroitin sulphate, have been studied after separation of free proteins by DEAE-Sephadex and after passage of the deproteinized fluid down Sephadex gel-200.

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Les mucopolysaccharides dans le liquide synovial
La détection du sulfate de chondroïtine

RÉSUMÉ

Des mucopolysaccharides, provenant de la matrice, ont été isolés du liquide synovial de trois malades atteints

d'arthrite rhumatismale. Des complexes mucopolysaccharide-protéine, y compris le sulfate de chondroïtine, ont été étudiés après la séparation des protéines libres par DEAE-Sephadex et après le passage du liquide depro-

Los mucopolisacaridos en el líquido sinovial
El aislamiento del sulfato de condroitina

SUMARIO

Mucopolisacaridos, procedentes de la matriz, fueron aislados del líquido sinovial de tres enfermos con artritis reumatoide. Complejos mucopolisacarido-proteína, incluso con el sulfato de condroitina, fueron estudiados después de la separación de las proteínas libres por DEAE-Sephadex y después de pasar el líquido depro-