THE EVOLUTION OF ACID MUCOPOLYSACCHARIDES IN CARRAGEENIN GRANULOMATA

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AN APPROACH to the understanding of the role of acid mucopolysaccharides (MPS) in inflammation and in the development of granulation tissue, still far from being cleared, can be represented by the analysis of MPS present in experimental granulomata, and by the analysis of their changes, in relation to the evolution of both cellular and structural components. Slack (1957, 1958), in carrageenin granulomata, indentified hyaluronic acid (HA) and chondroitinsulphuric acid (CSA), without excluding the presence of other MPS; Berenson and Dalfares (1960) identified in turpentine granulomata HA, heparitinsulphuric acid (Hep-S), chondroitinsulphuric acid A (CSA-A) and chondroitinsulphuric acid B (CSA-B). In the same tissue Fishkin and Berenson (1961) observed the presence of an electrophoretically homogeneous glycoprotein, containing glucosamine, galactosamine and sialic acid, besides several aminoacids. HA and CSA were observed also by White, Shetlar and Schilling (1961) in the newly formed connective tissue around and inside s.c. implanted wire mesh cylinders: these authors also posed the important question of the evolution of MPS in tissue which undergoes transformation, pointing out that the glycoproteins appear early, followed by HA and, later, by CSA.

The data concerning the MPS evolution in granulomata, so important to clarify their role in fibrillogenesis, are nevertheless scanty: in the present work we aimed at studying the MPS of carrageenin granuloma and their changes during tissue development.

MATERIALS AND METHODS

Production of carrageenin granulomata.—A batch of 120 rats weighing 230–270 g. was divided into 3 groups of 40 rats. In each animal 2 granulomata sidewise in the back, were produced injecting in the subcutaneous tissue 4 ml. of air and 2 ml. of a 1 per cent solution of carrageenin in sterile saline. The animals were treated with penicillin for 3 days.

In a preliminary experiment on 120 rats the course of granulomata development and the procedures of MPS extraction were determined. The first group was killed after 4, the second after 7, and the last one after 11 days; this period allows the granulomata to be easily dissected from the skin and surrounding tissues; a more prolonged period was not advisable because after 16–18 days the size of granulomata is very reduced and they are more adherent to tissue: in this case a valueless portion of subcutaneous tissue must be removed at the same time.

All the granulomata belonging to the same group were dissected, collected, minced and stored for 7 days in acetone replaced 3 times during this period.

Extraction of MPS.—MPS extraction was carried out according to a method similar to that of Scott (1960) and of Schiller, Slover and Dorfman (1961): the dried material was digested with papain at 65° for 15 hr. in phosphate buffer (pH 6,25) containing cysteine and EDTA, and filtered through celite; the filtrate was added with trichloroacetic acid up to 7·5 per cent concentration; after a 5 hr. rest at 2°, it was filtered again, dialyzed 15 hr. at

2° against 2 changes of distilled water, filtered and precipitated with 2 volumes of ethanol containing sodium acetate and acetic acid (Meyer 1956). After 48 hr. at 2° the precipitate was collected by centrifugation, washed with ethanol, ether ethanol (1 : 3), ether, and dried. The subsequent analyses were performed on this material (designated as "crude MPS").

Analyses on extracted MPS.

Chromatography on Dowex 50W × 8 (200–400 mesh, column 0·8 × 40 cm, 0·3 N HCl eluent, 1·5 ml. fractions) of the hydrolysate of crude MPS, according to the method of Gardell (1953). The hydrolysis was carried out with 5 N HCl for 7 hr., at 100°. The amount of hexosamines was evaluated according to Gardell.

Extraction of MPS.–CPC (Cetylpyridinium chloride) complexes at increasing NaCl concentrations, according to Schiller *et al.*, (1961). When the extraction was performed on a large scale (100 mg. of crude MPS; Prodi, 1963) the fractions extracted by a given NaCl concentration were collected together and precipitated with ethanol. When the amount of the precipitate was sufficient, hexosamines (Cessi and Piliego 1960) and uronic acids (Dische 1947) determination, glucosamine-galactosamine separation (Gardell 1953), electrophoresis on cellulose acetate, tests for enzymic digestion (Prodi 1963) and electrophoresis on Hyflo Super-Cel were performed. When the precipitate was scarce it was found suitable to perform an electrophoresis on cellulose acetate, to dye the strips in an alcoholic solution of toluidine blue, and, after washing in alcohol and drying, to cut out the died spots, hydrolyse them in 5 N HCl for 7 hr. at 100°, and determine the amount of hexosamines as above: this technique offers good and reproducible results for amounts of crude MPS as small as 300 μg.

Preparative electrophoresis on Hyflo Super-Cel (Prodi 1963), performed by a modification of the methods of Gardell, Gordon and Aqvist (1950) and Schiller, Mathews, Jefferson, Ludowieg and Dorfman (1954). The MPS fractions, obtained by elution with water of the Hyflo blocks, were precipitated with alcohol and analysed as previously indicated. This electrophoretic method, with which Berenson and Dalfares (1960) separated the MPS of turpentine granulomata, proved to be useful only if the amount of various fractions of MPS do not differ to a great extent : otherwise the poorest fractions of MPS are not satisfactorily recovered after elution and precipitation.

It should be noted that the carrageenin still present in the tissue and extracted together with tissue MPS does not interfere in the analyses ; as it has been previously observed, they are based mainly on hexosamine estimations : moreover, data reported in the literature concerning MPS–CPC complex extractions indicate that the carrageenin is extracted at NaCl concentrations higher than those we used.

RESULTS

Amount of extracted MPS

The average weight of dry and fat-free material for each granuloma is 273 mg (4 days) ; 253 mg (7 days) ; 344 mg (11 days). The extracted MPS have a hexosamine content ranging between 18 and 23 per cent ; their amount for each gram of starting material slightly decreases with the increasing age of granuloma : given as hexosamine content, it is mg 1·77 (4 days) ; mg 1·69 (7 days) ; mg 1·57 (11 days).

Glucosamine/galactosamine ratio

The percent ratio between the 2 hexosamines in the crude MPS, as determined by column chromatography, is given in Table I.

TABLE I.—*Percent Ratio of Hexosamine in Crude MPS*

	4 days	7 days	11 days
Glucosamine (per cent)	84·5	89·3	72
Galactosamine (per cent)	15·5	10·7	28

Thus it appears that the prevailing hexosamine is glucosamine ; in the last period a remarkable increase of galactosamine content is observed.

Separation of MPS with CPC

The extraction of complexes obtained by precipitating the aminosugar containing compounds with CPC at 0·04 N NaCl was carried out with 0·4 N, 1·2 N, and 2·1 N NaCl. Remarkable quantities of material are not precipitated by CPC and remain in solution in 0·04 N NaCl. Only traces of hexosamine containing materials are extracted at 2·1 N NaCl. Therefore three groups can be taken into account : (a) Supernatant fraction ; (b) MPS extracted by 0·4 N NaCl (or 0·4 N NaCl MPS) ; (c) MPS extracted by 1·2 N NaCl (or 1·2 N NaCl MPS). The quantitative changes of these three fractions during the course of the experiment are shown in table II. As usual, all the data are referred as hexosamine content.

TABLE II.—*Quantitative Changes in CPC Treated Fractions*

	4 days	7 days	11 days
Supernatant fraction (per cent)	5·7	8·7	24
0·4 N NaCl MPS (per cent)	82·5	82·5	55·4
1·2 N NaCl MPS (per cent)	11·8	8·8	20·6

The most relevant changes take place between the 7–11th day : the percentage of MPS in 0·4 N NaCl fraction decreases, that of 1·2 N NaCl fraction increasing at the same time. These changes have to be considered together with the above mentioned changes in glucosamine/galactosamine ratio. It can be remembered that with a 0·4 N NaCl solution, glucosamine containing hyaluronic acid (HA) and, possibly, galactosamine containing chondroitin are extracted from MPS—CPC complexes while 1·2 N NaCl extracts galactosamine containing chondroitinsulphuric acid (CSA) and glucosamine containing heparitinsulphuric acid (Hep-S). Between 7–11 days also the hexosamine containing supernatant fraction is increasing, at a rate similar to that of MPS in 1·2 N NaCl fraction.

Column chromatography of hydrolysates of 0·4 N NaCl fractions obtained from large scale separations of MPS–CPC complexes have shown only one peak, revealed as glucosamine by paper chromatography (descending system with amyl alcohol, pyridine, water 7 : 7 : 6 on Whatman n. 1, 3 day run ; ninhydrin staining). Hexuronic acid and hexosamine are in equimolecular amount. Cellulose acetate electrophoresis (20 × 2·5 cm. strips ; veronal buffer pH 8·6 ; 130 V ; 3 hr run, alcoholic toluidine blue staining) of 0·4 N NaCl MPS shows a slowly migrating spot, with characteristic lateral indentations scarcely methachromatic, quite similar to the spots obtained with HA from umbilical cord. A faster migrating spot was also evidenced : but a Hyflo Super-Cel preparative electrophoresis of 0·4 N NaCl MPS, carried out for isolating this fraction, has shown only one hexosamine and hexuronic acid containing peak, corresponding to HA : the fast migrating spot in cellulose acetate electrophoresis, therefore, contains neither hexosamines nor hexuronic acid. The 0·4 N NaCl fraction is sensitive to hyaluronidase. So it can be concluded that the 0·4 N NaCl fraction is constituted of HA, without differences during the evolution of the granuloma.

Column chromatography of hydrolysates of 1·2 N NaCl fractions has revealed the presence of both glucosamine and galactosamine, showing the occurrence of a

glucosamine containing MPS (likely heparitinsulphuric acid), besides galactosamine containing CSA. Electrophoresis on cellulose acetate of the same fraction has revealed a fast migrating metachromatic spot corresponding to CSA, and a second little slower migrating metachromatic spot : the ratio of hexosamine amounts in the two spots is the same as that of the ratio glucosamine/galactosamine as determined by column chromatography : it can be inferred that the faster spot is given by CSA and the slower one by Hep-S. The electrophoretic mobility of Hep-S is much higher than that of HA, different from what was stated by Berenson and Dalfares (1960) in MPS extracted from turpentine granulomata. The ratio between glucosamine and glactosamine of 1·2 N NaCl fraction after 4, 7 and 11 days is respectively 0·28, 0·40 and 0·25 ; therefore the ratios of Hep-S and CSA do not change to a great extent, in spite of the remarkable change of their total amount between 7–11 days : only a small increase in CSA content in the last period can be noted. The fraction 1·2 N NaCl at 11 days was shown almost completely resistant to hyaluronidase action.

On the ground of the above mentioned data, the evolution of the relative amount of HA, CSA and Hep-S between 4–11 days are summarized in Table III.

TABLE III.—*Changes in HA, CSA and Hep-S between* 4 *and* 11 *Days.*

	4 days	7 days	11 days
HA	87·3	90	73
CSA	9·9	7·7	21·6
Hep-S	2·8	2.3	5.4

In Table III reference to the aminosugar containing compounds not precipitated with CPC (supernatant fraction) is omitted : this fraction contains hexuronic acids and both glucosamine and galactosamine (36 per cent glucosamine and 64 per cent galactosamine at 7 days, and 63 per cent glucosamine and 77 per cent galactosamine at 11 days). The electrophoretic pattern of this fraction is rather complex, showing 3 spots, after chromatography on cellulose acetate, of which the faster is metachromatic and does not contain hexosamines, the last is poorly metachromatic and contains hexosamine : its mobility is intermediate between HA and CSA. A more satisfactory study of this fraction is necessary.

DISCUSSION

The data reported above show an increase of sulphated MPS relative to HA in the evolution of carrageenin granuloma, in agreement with the results obtained by White *et al.*, (1961). These data can be related also with previous observations such as the increasing galactosamine content in the granulomatous tissue developed in subcutaneously implanted polyvinyl sponge (Noble and Boucek, 1958); the evolution of carbazol/orcinol ratio in the hexuronic acids of the same kind of tissue (Bollet, Goodwin, Simpson and Anderson, 1958); the presence of CSA in the regenerating tissue 6 days after tenotomy (Kodicek and Loewi 1956). They can also be related to the histochemical data given by Campani and Reggianini (1950) and the chemical ones given by Campani, Zonta and Ugazio, (1959) which refer to granulation tissue from skin wounding. A relationship between the increase of CSA and perhaps of Hep-S and the fibrous organization of newly formed connective tissue is possible. So far the evidence of a possible role of CSA and perhaps other MPS in the fibrogenesis is poor ; many quantitative data,

however, are meanwhile being gathered which can be used in order to explain the significance of the morphological and histochemical changes of experimental granulomatous tissue. Great care must be taken in the interpretation of the data collected because this technique is only apparently unique, as can be inferred from different proportions of MPS isolated in each kind of granuloma. Nevertheless the comparison of the few available data offers a common aspect, that is the progressive evolution of fibroblast activity towards sulphated MPS synthesis, while at the beginning only HA is produced; this change runs parallel with the structural organization of fibrous connective tissue. A similar pattern during the structural organization of the dermis in the first weeks of life takes place (Prodi 1963).

SUMMARY

Acid mucopolysaccharides extracted from carrageenin granulomata obtained in the rat, after 4, 7 and 11 days from the injection, have been analyzed. Considerable changes in the ratio of the various fractions have been observed: during the evolution of the granulomatous tissue from the seventh to the eleventh day hyaluronic acid, which in the first period accounts for almost all mucopolysaccharides, decreases, whereas the sulphated mucopolysaccharides (chondroitinsulphuric and heparitinsulphuric acids) increase. Between 7–11 days a remarkable rise of an unidentified hexosamine containing a fraction not precipitable with cetylpyridinium chloride was observed.

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