Metabolism of Complex Carbohydrates by Fibroblasts from Rheumatic and Normal Human Subjects

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SUMMARY We used a double-labeling technique to compare the metabolism of complex carbohydrates by skin fibroblasts from patients with rheumatic heart disease and from age- and sex-matched normal controls. Fibroblasts were subcultured through a similar number of passages prior to their growth in the presence of \(^{13}C\) or \(^{3}H\)-labeled glucosamine, a precursor of complex carbohydrates. At the preconfluent, confluent, and postconfluent stages of growth, the culture medium and trypsin digest of the \(^{3}H\)- or \(^{13}C\)-labeled fibroblasts were combined, incubated with Protease, then successively fractionated on Biogel P-2, Biogel P-100, and DE-52 cellulose. Individual fractions were assayed for radioactivity, and the radioactive elution profiles of labeled complex carbohydrates were compared. These profiles showed that trypsin digestion releases quantitatively more high molecular weight complex carbohydrates and proportionately less heterogeneous glycopeptides from normal fibroblasts, as compared to fibroblasts from rheumatic patients. Chemical analysis of the components of these complex carbohydrates revealed that complex carbohydrates of fibroblasts from rheumatic patients contained more glycoproteins and proportionately less hyaluronic acid than complex carbohydrates of normal fibroblasts. In addition, normal fibroblasts secreted significantly more high molecular weight complex carbohydrates into the medium than fibroblasts from rheumatic patients. These differences were confined to the medium recovered from fibroblast cultures grown to post confluency but not to earlier stages of growth. These findings suggest the possible presence of a biochemical alteration in the synthesis of complex carbohydrates by fibroblasts of rheumatic individuals. The basis and exact nature of this alteration remain to be defined. Circ Res 46: 651-659, 1980

THE SPECIFIC lesions of acute rheumatic fever which occur in the heart, joints, and, to a lesser degree, in other organs are swelling and hyalinization of the collagenous ground substance of the fibrous tissue (Küinge, 1933). The susceptibility of certain individuals to this complication of group A streptococcal infection has raised the possibility of an inherent abnormality in the reaction of the tissue of rheumatic individuals to injury (Baig et al., 1978). Because acid mucopolysaccharides (AMPS) are primary components of the ground substance of valvular tissue, we undertook in a previous study to determine whether differences were present in the AMPS composition of rheumatic and non-rheumatic valvular tissue (Baig et al., 1978). The results of that study revealed that the total AMPS content of mitral valvular tissue from young patients with rheumatic valvular disease was significantly higher than that of nonrheumatic valvular tissue (Baig et al., 1978). Differences in AMPS composition of valvular tissue also were present in rheumatic and nonrheumatic individuals of all ages. However, the differences were significant only in the youngest group of patients studied, mainly in patients 20 years of age or less. These findings suggested the possible presence of an inherent abnormality in the synthesis of tissue AMPS of patients with rheumatic valvular disease.

The present study was undertaken to determine whether the presence of such an abnormality could be reflected in the metabolism of AMPS of other tissues. Skin fibroblasts from rheumatic patients (rheumatic fibroblasts) and age-matched controls (normal or non-rheumatic fibroblasts) were grown in tissue culture, and the metabolism of AMPS and other complex carbohydrates, which include glycosaminoglycan, glycoproteins, and other polysaccharide polymers, was examined. Although no gross differences in the AMPS content of fibroblasts were observed, significant differences in the amounts of complex carbohydrates formed by fibroblasts from rheumatic and nonrheumatic individuals were encountered in this study.

Methods

Patients

The patients studied included four with chronic inactive rheumatic heart disease and four nonrheumatic controls who were admitted for reparative
surgery or were seen in our clinic for follow-up care. The diagnosis of rheumatic heart disease was based on a history of an acute episode of rheumatic fever associated with carditis, fulfilling the modified Jones Criteria (Committee Report, 1965). None of the patients with rheumatic carditis was receiving any medications other than penicillin prophylaxis at the time of the study. Age-matched controls were selected from individuals presenting to this health center for diseases other than collagen vascular diseases or known inheritable disorders of metabolism. The ages of the patients and controls ranged from 10 to 15 years. Skin biopsies were obtained either from the skin of the forearm under local anesthesia or from the incision site during surgery. Informed consent was obtained from all individuals studied as outlined by the guidelines of the Committee on Human Experimentation of this institution and the Department of Health, Education, and Welfare.

Cell Cultures

Skin biopsies were processed for culture as described by Martin (1973). The explants were grown in 25-cm² plastic tissue culture flasks (Falcon) at 37°C in an atmosphere of 5% CO₂ using Eagle's minimal essential medium (MEM) (Grand Island Biological Laboratory) supplemented with 20% heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The medium was changed every 3-4 days until fibroblast confluence was achieved. The first monolayer of tissue fibroblasts was trypsinized and subcultured into 75-cm² plastic tissue culture flasks. Subsequent subcultures were processed in a similar fashion. Labeling experiments were performed on fibroblast subcultures that had undergone 14-15 passages.

Labeling Experiments

Double-labeling techniques (Baig and Roberts, 1973; Baig et al., 1975) were used to compare the metabolism of AMPS and other complex carbohydrates in normal and rheumatic fibroblasts. Fibroblasts from age- and sex-matched rheumatics and controls were subcultured through the same number of passages. The matched sets of fibroblasts were grown in the presence of labeled glucosamine in the medium to serve as a precursor of AMPS, glycoproteins, and other complex carbohydrates containing amino sugars and/or sialic acid (Marshall and Neuberger, 1968). Initially, each flask of normal fibroblasts was supplied with 20 μCi of ¹⁴C-labeled glucosamine (59 mCi/mmol), whereas rheumatic fibroblasts were supplied with 50 μCi of ³H-labeled glucosamine (3.2 Ci/mmol). This was followed by reverse labeling studies, with the normal fibroblasts grown in the presence of ³H-labeled glucosamine and the rheumatic fibroblasts grown with ¹⁴C-labeled glucosamine. At confluence, the radioactive medium was poured off and saved. The monolayer of fibroblasts adhering to the surface of the flask was rinsed twice with Hanks' balanced salt solution (HBSS) to wash off traces of the unused labeled precursor and the complex carbohydrates released into the medium by the fibroblasts. These washes were combined with the saved medium and labeled as the "culture medium." The fibroblast monolayer was detached from the surface of culture dishes by the addition of 2 ml of 0.25% wt/vol trypsin solution in HBSS. The excess trypsin solution was decanted and retained. The Falcon dishes then were incubated at 37°C for 5 minutes, and the pelleted monolayer of fibroblasts was suspended in 5 ml of HBSS. The suspension was centrifuged at 4°C for 5 minutes at 500 g and the supernatant of this first wash was decanted and retained. The pelleted fibroblasts were washed twice more with 2 ml of HBSS. The three washes were combined with the trypsin solution and labeled as the "trypsin digest."

The three separate preparations resulting from processing each cell culture in the above manner, i.e., the culture medium, the trypsin digest, and the washed fibroblasts were stored at −20°C until they were fractionated as described below.

Fractionation of Trypsin Digest

The trypsin digest obtained from ¹⁴C-labeled normal fibroblasts and ³H-labeled rheumatic fibroblasts were combined. A tenth volume of 0.1 M disodium-EDTA was added to chelate divalent cations to prevent the precipitation of polyanionic AMPS by Ca²⁺. The combined trypsin digest were digested further with Pronase according to the conditions described by Spiro (1965). To the combined trypsin digest, 5 mg of Pronase were added, and the mixture was incubated at 37°C in the presence of tolenue to prevent bacterial growth. After 24 hours of incubation, an additional 2.5 mg of Pronase were added to the mixture, and incubation was continued for another 24 hours. The latter process was repeated every 24 hours for a total of 96 hours. After digestion with Pronase, the particulate matter was removed by centrifugation, and the supernatant was reduced in volume to 2–3 ml by evaporation under vacuum. The concentrated supernatant then was loaded onto a Biogel P-2 column (2.5 × 30 cm) previously equilibrated with 0.01 M ammonium acetate, pH 7.0. The column was eluted with the equilibrating buffer, and 5-ml fractions were collected. Individual fractions were assayed for radioactivity, and the radioactive material was eluted. The void front of the column was pooled and reduced to a small volume (2–3 ml) by evaporation under vacuum. This material was further fractionated through a Biogel P-100 column (2.5 × 60 cm) using the same method as described for the Biogel P-2 column.

Fractionation through Biogel P-2 and P-100 resulted in the separation of large molecular weight AMPS and other complex carbohydrates resistant to digestion by Pronase from small molecular...
weight glycopeptides. The labeled large molecular weight complex carbohydrates eluting within the void front of the Biogel P-100 column were fractionated further to resolve individual AMPS by employing DE-52 cellulose ion exchange chromatography as described by Kraemer (1971a, 1971b). The DE-52 cellulose column (1 X 10 cm) was equilibrated with 0.01 M ammonium acetate. Labeled large molecular weight complex carbohydrates that eluted within the void volume of the Biogel P-100 column were loaded onto the DE-52 cellulose column, and the column was washed with 50 ml of equilibrating buffer. The ion exchange column then was eluted with a linear salt gradient ranging from 0.01 to 2.0 M ammonium acetate (200 ml of each) to resolve various labeled complex carbohydrates. Five-milliliter fractions were collected, and individual fractions were assayed for radioactivity.

Fractionation of the Culture Medium

As with the trypsin digests, the culture media from 14C-labeled normal and 3H-labeled rheumatic fibroblasts also were combined. The digestion of the combined media with Pronase was carried out using 20 mg of Pronase initially, with the subsequent addition of 10 mg of Pronase at 24-hour intervals. Pronase digestion was carried out for 96 hours under incubation conditions similar to those described for the Pronase digestion of the trypsin digest. The fractionation procedures used to separate labeled complex carbohydrates secreted by the fibroblast cell cultures into the culture medium were similar to those described above for the trypsin digests.

Identification of Individual AMPS

High molecular weight AMPS and other complex carbohydrates resolved following DE-52 cellulose ion exchange chromatography of trypsin digest and culture medium were identified by using the following criteria.

Cellulose Acetate Electrophoresis

Labeled AMPS were subjected to coelectrophoresis in parallel with known standard AMPS. Electrophoresis was carried out in 0.1 M barium acetate, pH 8.0, or in 0.1 M pyridine-0.5 M formic acid buffer, pH 3.0, as suggested by Hata and Nagai (1972), using cellulose acetate strips at 140 V and a current of 0.5 mA/cm for 45 minutes. After electrophoresis, the cellulose acetate strip was cut into two halves longitudinally. The portion containing the standard AMPS was stained with 0.1% toluidine blue to visualize the individual AMPS, whereas the other half, containing the unknown labeled AMPS, was cut transversely into 1-mm pieces. Individual pieces were transferred into separate scintillation vials and soaked in 0.5 ml of 0.15 M NaCl. After soaking for 24 hours, 5 ml of Aquasol (Amersham/Searle) were added. The vials were counted to estimate the electrophoretic mobility of labeled AMPS which were then compared with the mobilities of the standards.

Hyaluronidase Digestion

Labeled AMPS were subjected to digestion with bovine testicular hyaluronidase (Sigma) at pH 5.3, in 0.1 M sodium phosphate buffer containing 0.15 M NaCl, for 48 hours at 37°C. The hyaluronidase-digested AMPS fraction then was loaded onto a Biogel P-100 column. The column was equilibrated and eluted as described above. The susceptibility of labeled AMPS to hyaluronidase was assessed by the shift in the elution volume of the digested labeled AMPS from the void volume toward the salt volume.

Secretion Into the Medium of Labeled Complex Carbohydrates by Normal and Rheumatic Fibroblasts

Passage-matched normal and rheumatic fibroblasts were grown in culture in the presence of labeled glucosamine. During growth, 1-ml aliquots were removed from the medium at predetermined intervals covering preconfluent, confluent, and postconfluent stages of growth. These aliquots were assayed for radioactivity and then dialyzed extensively against distilled water. The nondialyzable material was assayed for radioactivity to estimate the amount of labeled complex carbohydrates secreted by fibroblasts into the medium at various stages of growth. These nondialyzable complex carbohydrates were fractionated further into their respective glycoprotein and AMPS components. An equal volume of 25% trichloroacetic acid was added to dialyzed aliquots and allowed to stand for 48 hours at 4°C to resolve the trichloroacetic acid-soluble AMPS from trichloroacetic acid-insoluble glycoproteins and proteins. These fractions were then assayed for radioactivity.

Assay for Radioactivity

Radioactivity of the combined 14C and 3H samples was determined using a Beckman LS-250 liquid scintillation counter with settings adjusted so that no 3H spilled into the 14C channel, while the overlap of 14C into the 3H channel was held at 10%. Counting efficiency for 3H was 27.2% and that for 14C was 62.7%. The total 3H or 14C radioactivity in the eluates of each column was determined, and the percentage of the radioactivity present in each 5-ml fraction was calculated. The radioactive elution profiles for the various preparations then were plotted based on this calculation.

Chemicals and Radiochemicals

D-1-14C-glucosamine (59 mCi/mmol) and D-1-3H-glucosamine (3.2 Ci/mmol) were obtained from Amersham-Searle Corporation. Hyaluronic acid, heparin sulfate, chondroitin sulfates, A, B, and C standards, were a gift from Dr. B. Mathews of the University of Chicago.
Analytical Techniques
Uronic acid content of the samples was determined by the carbazole method of Dische (1947) with glucuronic acid as standard. The protein content of samples was determined by a modification of the Lowry technique described by Campbell and Sargent (1967), with bovine serum albumin as the standard.

Statistical Analysis
Significance of differences in the percentage of labeled high molecular weight complex carbohydrate secreted into the medium by normal and rheumatic fibroblasts at various stages of growth was analyzed using a t-test for small samples (Bancroft, 1963).

Results
AMPS Content of Normal and Rheumatic Fibroblasts.
Skin fibroblasts from four patients with rheumatic heart disease and from four normal controls were harvested after the 15th subculture. The confluent monolayer was washed three times with 10 ml of HBSS and treated with trypsin, and the recovered cell suspension was centrifuged for 5 minutes at 500 g, at 4°C. The cells were washed twice in HBSS, and the supernatant was combined with the trypsin digest. The supernatant of the trypsin digest and the cell washes were recovered, concentrated by evaporation to 1 ml, and 10- and 250-μl samples were analyzed for protein and AMPS contents, respectively. The cell pellet was solubilized by the addition of 1 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 2% sodium dodecyl sulfate, 5 mM cysteine hydrochloride, and 0.25 mM EDTA followed by boiling for 1 minute. Samples of the dissolved material (20 μl of a 1:10 dilution) were analyzed for protein content, and 200 μl were analyzed for AMPS content.

Results of the protein and AMPS analyses of the trypsin digest of the cells and of the solubilized fibroblasts for each individual cell line are summarized in Table 1. No salient differences were observed in the protein and AMPS contents of either the trypsin digest or fibroblasts solutions between rheumatic and nonrheumatic fibroblasts. There was also no apparent difference in the AMPS content per milligram protein of the digest of rheumatic and nonrheumatic fibroblasts.

The absence of gross differences in the AMPS content of rheumatic and nonrheumatic fibroblasts prompted subsequent studies with radiolabeled precursors to determine whether differences were present in the metabolism of complex carbohydrates of these fibroblasts. An analysis of the elution profiles of the complex carbohydrates released by trypsin digestion of the fibroblasts, as well as the complex carbohydrates present in the culture medium, was undertaken, using the double-radiolabeling and fractionation techniques described above.

Complex Carbohydrates Released from Fibroblasts by Trypsin
A typical radioactive elution profile obtained after Biogel-P2 filtration of the trypsin digests of 14C-labeled normal cells and 3H-labeled rheumatic fibroblasts is shown in Figure 1A. This first fractionation step resulted in the separation of high molecular weight components, including the complex carbohydrates solubilized by trypsin from the cell surface of the fibroblasts, from the low molecular weight labeled precursors of these complex carbohydrates. A major peak, containing the large molecular weight complex carbohydrates eluted with the void volume, and two minor peaks were eluted in the retention volume of the column. As can be seen, the elution profiles of 14C- and 3H-labeled trypsin digests were identical.

Resolution of the large molecular weight complex carbohydrates from the glycopeptides that eluted together with these carbohydrates as a major peak in the void volume of the Biogel P-2 column was achieved by subjecting this peak to filtration through a Biogel P-100 column (Fig. 1B). Although

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<th>Table 1</th>
<th>Total Protein and AMPS Content of Trypsin Digest of Rheumatic and Nonrheumatic Skin Fibroblast and of the Same Fibroblasts Solubilized with Sodium Dodecyl Sulfate</th>
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COMPLEX CARBOHYDRATES OF RHEUMATIC FIBROBLASTS/Baig and Ayoub

30 20 10
Rheumatic - H
Rheumatic - H

30 20 10

10 20 30 40 50 60
FRACTION NUMBER

FIGURE 1 Biogel P-2 (panel A), Biogel P-100 (panel B), and DE-52 diethylaminoethyl cellulose (panel C) column chromatography of combined trypsin digests from radiolabeled normal and rheumatic fibroblasts. Total radioactivity eluted, panel A: $^3$H = 354 x 10^3 dpm and $^{14}$C = 125 x 10^3 dpm; panel B: $^3$H = 301 x 10^3 dpm and $^{14}$C = 107 x 10^3 dpm; panel C: $^3$H = 84 x 10^3 dpm and $^{14}$C = 48 x 10^3 dpm. The total radioactivity recovered averaged to 95% of that applied.

The radioactive elution profile of $^{14}$C, representing the trypsin digest from normal fibroblasts, is not different from the radioactive elution profile of the $^3$H, representing the trypsin digest from rheumatic fibroblasts, quantitative differences in the peaks eluted are present. The first peak eluted within the void volume of the column represents high molecular weight complex carbohydrates, and the retained two peaks represent a heterogeneous population of lower molecular weight glycopeptides. Trypsin appears to have released more of the high molecular weight complex carbohydrates and proportionately less of the low molecular weight heterogeneous glycopeptides from normal than from rheumatic fibroblasts.

The high molecular weight complex carbohydrates eluted with the void volume of Biogel P-100 column then were subjected to fractionation on DE-52-cellulose ion exchange column. This fractionation step resulted in the elution of two major peaks (Fig. 1C). Quantitative differences in the elution profiles also were encountered in this fractionation step. Compared to normal fibroblasts, rheumatic fibroblast digests yielded proportionately more label eluting in the first peak.

Although differences similar to those described above were observed between each of the four rheumatic fibroblast cultures and their matched controls, additional evidence supporting the consistency of the observed differences in radioactive elution profiles between rheumatic and normal fibroblast trypsin digests was obtained. Matched skin fibroblasts from normal individuals were labeled with either $^{14}$C or $^3$H, such that one group of normal fibroblasts received $^3$H and the other group of normal fibroblasts received $^{14}$C-labeled glucosamine. The cells were trypsinized at confluence, and the trypsin digests were recovered and mixed, digested with Pronase, and subjected to the fractionation steps described above. Results of the radioactive elution profiles of $^{14}$C- and $^3$H-labeled trypsin digests are shown in Figure 2. As can be seen, the elution profiles of the $^3$H- and $^{14}$C-labeled normal fibroblasts were identical. Similar results were ob-

30 20 10

PERCENT OF TOTAL RADIOACTIVITY ELUTED

10 20 30

FRACTION NUMBER

FIGURE 2 Biogel P-2 (panel A), Biogel P-100 (panel B), and DE-52 diethylaminoethyl cellulose (panel C) column chromatography of trypsin digests from radiolabeled normal fibroblasts.
tained when mixed trypsin digests of the $^3$H- and $^{14}$C-labeled rheumatic fibroblasts were subjected to the fractionation steps described earlier.

The above results suggested that differences in the elution profile of $^3$H- and $^{14}$C-labeled trypsin digests are observed only when the trypsin digest of normal fibroblasts is compared with the trypsin digest of rheumatic skin fibroblasts. These data also suggested that the digestion of fibroblasts with trypsin in a similar manner resulted in the release of different quantities of complex carbohydrates from the surface of normal cells as compared to rheumatic fibroblasts.

Because the above differences were quantitative, the possibility that these differences may have been a consequence of variations in the growth stages of the fibroblast cultures at the time of harvest had to be examined. Skin fibroblasts from one normal and one rheumatic individual were grown in the presence of $^{14}$C-labeled glucosamine and $^3$H-labeled glucosamine, respectively. The cells were harvested at the preconfluent (3 days), confluent (5 days), and postconfluent (7 days) stages of growth. The trypsin digests obtained from these $^3$H-labeled and $^{14}$C-labeled fibroblasts at each stage of growth were combined and processed through the fractionation steps described above. Differences in elution profiles similar to those encountered in the initial study, and illustrated in Figure 1, were observed regardless of the stage of fibroblast growth. These studies indicated that the quantitative differences in complex carbohydrates found in the trypsin digests of normal and rheumatic fibroblasts were not a consequence of differences in the growth stage of the fibroblast cultures and suggested that these differences may reflect an abnormal metabolism of complex carbohydrates by rheumatic skin fibroblasts.

**Identification of High Molecular Weight Complex Carbohydrates**

Studies were conducted to characterize the nature of the complex carbohydrates found in the trypsin digests of the fibroblast cultures. The two major peaks eluted at the start of the gradient from the DE-52 cellulose column (Fig. 1C) were analyzed. Not enough material was available for chemical analysis from the minor peak which eluted at the end of the gradient. The first major peak was eluted at the beginning of the salt gradient, and, therefore, represents a weakly acidic complex carbohydrate. The material was resistant to digestion with hyaluronidase. These results suggest that this may be a glycoprotein, but its actual chemical nature remains to be determined. The second major peak was characterized as hyaluronic acid. Coselectrophoresis of this peak with standard hyaluronic acid, using two buffer systems, resulted in a migration pattern similar to standard hyaluronic acid. Treatment of this peak with hyaluronidase followed by filtration through the Biogel P-100 column resulted in a shift of the hyaluronidase-treated material toward the salt volume.

Because of the low amounts of radioactivity eluted in the minor peak, it was not chemically characterized. However, based on results obtained by Kraemer (1971a, 1971b) on the resolution of known AMPS under similar ion exchange chromatographic conditions, this peak most likely represents heparin sulfate and/or dermatan sulfate, since it is exchanged from the column at the same ion concentrations required to exchange these AMPS.

**Complex Carbohydrates Secreted into the Medium**

Representative elution profiles obtained after column chromatographic separation of complex carbohydrates secreted into the medium from $^{14}$C-labeled normal and $^3$H-labeled rheumatic fibroblasts are shown in Figure 3. These profiles are similar to those previously obtained with the trypsin digests. However, unlike the $^{14}$C and $^3$H elution profiles obtained with the trypsin digest, of normal and rheumatic media were found to be identical. Studies to identify the chemical nature of the various peaks eluted from the DE-52 cellulose column (Fig. 3C) revealed an initial peak eluting with the neutral

![Figure 3 Biogel P-2 (panel A), Biogel P-100 (panel B), and DE-52 diethylaminoethyl cellulose (panel C) column chromatography of combined media from rheumatic and normal fibroblasts.](http://circres.ahajournals.org/.../Downloaded-from-http://circres.ahajournals.org/.../by-guest-on-October-19,2017)
buffer, followed by two acid peaks (peak I and peak II) that eluted at the onset of the ammonium acetate gradient. These two peaks were similar to the initial two peaks of the trypsin digest which eluted under identical ion exchange column chromatographic conditions (Fig. 1C). The third, peak III, which eluted at the middle of the ammonium acetate gradient, was identified by the methods described above as dermatan sulfate (chondroitin sulfate B).

The above results suggested that the relative proportions of the various components of complex carbohydrates secreted into the culture medium by normal and rheumatic fibroblasts were similar. The possibility of finding differences in the absolute amounts of complex carbohydrates secreted into the medium by the normal and rheumatic fibroblasts was investigated. The methodology used to investigate this possibility is described in detail in Methods. Results obtained from these studies are summarized in Figure 4. As can be seen, the percent of nondialyzable radioactivity associated with complex carbohydrates present in the culture medium of normal and rheumatic fibroblasts increased with growth of cells. No significant differences were observed between the percent of labeled high molecular weight complex carbohydrate secreted into the medium by normal and rheumatic fibroblasts at their preconfluent and confluent stages of growth (2nd and 4th days, respectively). However, during the postconfluent stage of growth (6th and 8th day), the percent of radioactivity associated with high molecular weight complex carbohydrates secreted into the medium by normal fibroblasts was significantly higher than rheumatic fibroblasts (mean of percent for the four determinations \( \pm SD = 10.6 \pm 2.1 \) vs. \( 7.38 \pm 1.21, P < 0.04 \) for 6 days; and \( 13.98 \pm 0.72 \) vs. \( 10.25 \pm 1.08, P < 0.002 \) for 8 days). The nondialyzable high molecular weight complex carbohydrates secreted into the medium were fractionated into trichloroacetic acid-soluble AMPS and trichloroacetic-insoluble glycoprotein fractions. No significant differences in the relative proportions of these components were found throughout the entire growth period of normal and rheumatic fibroblasts.

Discussion

Our initial approach to the investigation of the presence of differences in the metabolism of AMPS between rheumatic and nonrheumatic cells consisted of assaying chemically the content of these components in skin fibroblasts grown in tissue culture. Our inability to detect, by this technique, any differences in the AMPS composition of material released from cell membranes by trypsin digestion, or even in the solubilized rheumatic and non-rheumatic fibroblasts, suggested that these differences may be subtle and that a more sensitive and exacting approach was required for the detection of these differences. Therefore, we resorted to the use of radiolabeled precursors to compare the metabolism of these components by rheumatic and nonrheumatic skin fibroblasts and to assess the presence of differences in their incorporation, as well as in their excretion by fibroblasts into the culture medium.

To maintain valid comparisons, we attempted throughout the course of this study to use fibroblasts from closely matched individuals, fibroblasts which had undergone an identical number of subcultures and which were grown under identical conditions. The use of double-labeling techniques allowed the simultaneous processing of samples from rheumatic and nonrheumatic cells and enabled us to assess the presence of even minor differences in the metabolism of complex carbohydrates by these fibroblasts. In addition, by using reverse labeling in these procedures, we were able to achieve valid analytical comparisons uninfluenced by experimental variations in the techniques used. This approach was first used to perform comparative studies on the nature and relative proportions of various complex carbohydrates solubilized by trypsin from the cell surface of normal and rheumatic fibroblasts at preconfluent, confluent, and postconfluent stages of growth. The same approach also was used in studying the nature and relative proportions of various complex carbohydrates secreted into the medium by normal and rheumatic fibroblasts and in determining the absolute amounts of nondialyzable large molecular weight complex carbohydrates secreted into the medium by normal and rheumatic fibroblasts.

These differences are reflected by the quantitative differences in the elution profiles of large molecular weight complex carbohydrates from non-
rheumatic fibroblasts as compared to rheumatic fibroblasts that have been subjected to mild trypsin digestion. Analysis of these large molecular weight complex carbohydrates released from the cell revealed that they were primarily composed of glycoproteins and AMPS, with hyaluronic acid being the major component of the mucopolysaccharides. Further analysis of the proportions of these components in the trypsin digest of the fibroblasts showed that more hyaluronic acid was present in the complex carbohydrates derived from nonrheumatic fibroblasts, whereas more glycoproteins were present in digests of rheumatic fibroblasts. These differences were encountered only when trypsin digests from rheumatic and nonrheumatic fibroblasts were eluted simultaneously. The reproducibility of these differences in all four sets of rheumatic and nonrheumatic cultures attested to the validity of these differences. Additional confirmation of these findings was procured by examining two factors that may have engendered such differences. The possible influence of isotope effect on the labeling of complex carbohydrates of fibroblasts was excluded by labeling fibroblasts from the same individual with $^{14}$C or $^{3}$H. This procedure yielded similar profiles regardless of the isotope used. The other factor, that of the possible influence of stage of growth of the cells in culture, was excluded as a variable by the finding of similar differences at all stages of cell growth, i.e., during the preconfluent, confluent, or postconfluent stages of growth.

Studies of the amounts of complex carbohydrates secreted into the culture medium by normal and rheumatic fibroblasts revealed significantly lower concentrations of these carbohydrates in the postconfluent stage of growth, but not at earlier stages of growth. This difference became insignificant when the fibroblasts were allowed to remain in culture for several days beyond the postconfluent stage. The appearance of significant differences in cellular products at certain stages of fibroblast growth, and their subsequent disappearance with further growth, is reminiscent of the report of abnormal elevation of phosphoribosylphosphate synthetase in fibroblasts of patients with Lesch-Nyhan syndrome (Kooyman et al., 1976). As depicted in that report, the fibroblasts of three patients demonstrated significant elevation of levels of this enzyme in the subconfluent stage of growth, with subsequent decline of these levels toward normal following the 8th day of culture. Of interest in this report is that purine synthesis by these fibroblasts was maximal on the 5th and 7th days of culture and declined sharply to near normal levels on the 9th day. This latter pattern is similar to our observations on the secretion of complex carbohydrates into the culture medium.

The additional finding in our study of significantly greater amounts of large molecular weight complex carbohydrates in the culture medium of normal fibroblasts as compared to rheumatic fibroblasts is in line with the differences noted in the trypsin digest of these cells. However, the fact that these differences were observed only at the postconfluent stage of growth may argue that they do not denote differences in an active secretory process, but that they reflect degradative changes in the cell membrane. Such changes could have occurred during late stages of culture with leakage of the complex polysaccharides into the medium. This phenomenon would be similar to the one described for "leakage" of intracellular enzymes from fibroblasts of patients with "I-cell disease" (Wiesmann et al., 1971; Leroy et al., 1972). In the present study, however, the situation is reversed with "leakage" of complex carbohydrates occurring from normal rather than the rheumatic fibroblasts. Although this may be a possible explanation, the findings would still suggest that rheumatic skin fibroblasts behave differently than nonrheumatic skin fibroblasts under similar culture conditions.

Earlier studies on the AMPS content of mitral valvular tissue obtained from normal and rheumatic individuals revealed that valvular tissue from young rheumatic individuals contained significantly more AMPS than age-matched normal controls (Baig et al., 1978). The results of the studies reported here did not reveal similar difference in the AMPS content of skin fibroblasts of normal and rheumatic individuals. However, the finding of consistent differences in the relative proportion of various AMPS and other complex carbohydrates found in the trypsin digest, as well as the culture medium of normal and rheumatic fibroblasts, suggests the possible presence of a biochemical alteration in the metabolism of these complex carbohydrates by the cells of rheumatic individuals. A determination of the exact nature of this alteration, and of whether it reflects an inborn defect or is acquired through the disease process, awaits further investigation.

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Hata R, Nagai Y (1972) A rapid and micromethod for separation of growth, and the subsequent decline of these levels toward normal following the 8th day of culture. Of interest in this report is that purine synthesis by these fibroblasts was maximal on the 5th and 7th days of culture and declined sharply to near normal levels on the 9th day. This latter pattern is similar to our observations on the secretion of complex carbohydrates into the culture medium. The additional finding in our study of significantly greater amounts of large molecular weight complex carbohydrates in the culture medium of normal fibroblasts as compared to rheumatic fibroblasts is in line with the differences noted in the trypsin digest of these cells. However, the fact that these differences were observed only at the postconfluent stage of growth may argue that they do not denote differences in an active secretory process, but that they reflect degradative changes in the cell membrane. Such changes could have occurred during late stages of culture with leakage of the complex polysaccharides into the medium. This phenomenon would be similar to the one described for "leakage" of intracellular enzymes from fibroblasts of patients with "I-cell disease" (Wiesmann et al., 1971; Leroy et al., 1972). In the present study, however, the situation is reversed with "leakage" of complex carbohydrates occurring from normal rather than the rheumatic fibroblasts. Although this may be a possible explanation, the findings would still suggest that rheumatic skin fibroblasts behave differently than nonrheumatic skin fibroblasts under similar culture conditions.

Earlier studies on the AMPS content of mitral valvular tissue obtained from normal and rheumatic individuals revealed that valvular tissue from young rheumatic individuals contained significantly more AMPS than age-matched normal controls (Baig et al., 1978). The results of the studies reported here did not reveal similar difference in the AMPS content of skin fibroblasts of normal and rheumatic individuals. However, the finding of consistent differences in the relative proportion of various AMPS and other complex carbohydrates found in the trypsin digest, as well as the culture medium of normal and rheumatic fibroblasts, suggests the possible presence of a biochemical alteration in the metabolism of these complex carbohydrates by the cells of rheumatic individuals. A determination of the exact nature of this alteration, and of whether it reflects an inborn defect or is acquired through the disease process, awaits further investigation.

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