Mucopolysaccharide Sulfation in Normal and Leukemic Leukocytes

By Peter Lau, Arlan J. Gottlieb, and William J. Williams

Mucopolysaccharide sulfation was demonstrated in leukemic granulocytic precursors in short-term suspension culture by incorporation of $^{35}$SO$_4$ into a compound identified chromato-graphically as chondroitin sulfate. The gel filtration pattern of sulfated mucopolysaccharide obtained from leukemic leukocytes was qualitatively similar to that found with normal granulocytic precursors. Sulfation of mucopolysaccharide was about 50\% of the normal level in cells from chronic granulocytic leukemia, and approximately 15\% of the normal level in cells from acute granulocytic leukemia or chronic granulocytic leukemia in blastic crisis. Lymphocytes from acute and chronic lymphocytic leukemia showed only traces of sulfate incorporation. Leukocyte mucopolysaccharide sulfation was studied in cultured cells from patients with acute leukemia to determine whether this reaction could aid in distinguishing acute granulocytic leukemia from acute lymphocytic leukemia. Significant levels of sulfation were obtained in cells from all leukemias judged to be acute granulocytic, while virtually no incorporation was found in cells from acute lymphocytic leukemia when a 24-hr incubation period was employed. In studies employing a 3-hr incubation, agreement as to the cell of origin of the leukemia and the degree of sulfation in the intracellular fraction was obtained in 16 of 18 determinations performed on 16 patients with acute leukemia. It is proposed that determination of $^{35}$SO$_4$ incorporation in the intracellular fraction may help to differentiate acute granulocytic and acute lymphocytic leukemia.

INORGANIC $^{35}$SO$_4$ is useful in studies of the biosynthesis of mucopolysaccharide because of its limited incorporation into compounds other than sulfate esters.\(^1\) In the hematopoietic system, inorganic sulfate incorporation occurs primarily in cells of the myeloid series and in megakaryocytes.\(^2\)\(^-\)\(^7\) In short-term suspension cultures of bone marrow, virtually all of $^{35}$SO$_4$ is incorporated into myeloblasts, promyelocytes, and myelocytes.\(^7\) The present

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report extends our previous observations on normal cells to a study of the incorporation of inorganic sulfate into neoplastic hematopoietic cells. It was found that cells from the patients with acute granulocytic leukemia (AGL) and chronic granulocytic leukemia (CGL) are capable of synthesis of mucopolysaccharide, although to a lesser extent than normal granulocytic precursors. Cells from patients with acute lymphocytic leukemia (ALL) or chronic lymphocytic leukemia (CLL) do not incorporate significant amounts of radioactive sulfate. The difference between $^{35}$SO$_4$ incorporation into cells from patients with AGL and ALL may provide an additional means of differentiating these types of acute leukemia.

MATERIALS AND METHODS

Patients

Complete studies were performed on blood or bone marrow from 24 patients with leukemia. Five patients with anemia secondary to systemic disease served as controls. Data from these patients are summarized in Table 1. Studies to evaluate the clinical applications of SO$_4$ incorporation were carried out on 16 patients with acute leukemia. Details on these patients are presented in the text and in Fig. 2.

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<th>Table 1. Mucopolysaccharide Synthesis in Normal and Leukemic Leukocytes</th>
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<td><strong>Category</strong></td>
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<td><strong>1. Control group</strong></td>
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<td><strong>2. Chronic granulocytic leukemia</strong></td>
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<td><strong>3. Chronic granulocytic leukemia in Blastic crisis</strong></td>
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<td><strong>5. Acute lymphocytic leukemia</strong></td>
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* Precursor cells—incuding myeloblasts, promyelocytes, and myelocytes; lymphoblasts and lymphocytes in cases of ALL and CLL.
† Busulfan.
‡ Methotrexate.
§ Methotrexate, vincristine, prednisone, folinic acid, and hydroxyurea.
|| Mean of four experiments.
¶ Vincristine, prednisone, methotrexate, and others.
** Chlorambucil.
Suspension Culture Technique

Cultures were prepared as described previously.7 Marrow (1 ml) or blood 5–10 ml) was centrifuged for 5 min at 50 g. The nucleated cell-rich supernatant fluid was removed and recentrifuged for 10 min at 800 g. If patients were receiving cytotoxic medications at the time of the study, the cells were washed with 10 ml of 0.15 M NaCl. The cells were resuspended in 5 ml of Eagle's minimum essential (Spinner) medium (Hyland Lab., Los Angeles, Calif.). Duplicate cell counts were performed on aliquots of the final cell suspension using a Fisher Autocytometer. The volume of cell suspension used was based on these counts.

Each culture contained: (1) 30–60 million nucleated cells in 8 ml of culture medium, or in 5 ml of culture medium and 3 ml autologous plasma; (2) penicillin, 60 U/culture, and streptomycin sulfate, 60 μg/culture; (3) Na35SO4, 10–30 μCi (specific activity 447–488 mCi/m mole). The cultures were incubated at 37°C with constant magnetic stirring. Tripletate cells counts were obtained at the start and after 24 hr of incubation. Differential cell counts were performed on Wright-stained smears of the zero and 24-hr samples by the method previously described.7

Measurement of Incorporation of 35SO4

After 24 hr in culture, the cells were collected by centrifugation at 800 g for 10 min, and 35SO4 incorporation was assayed as previously described.7 Because a large amount of sulfated mucopolysaccharide is secreted into the medium of cultures of normal bone marrow,7 the growth medium from the cultures of leukemic cells was also processed for determination of radioactivity.7 This fraction will be referred to as the extracellular fraction. Since only myeloblasts, promyelocytes, and myelocytes are capable of sulfate incorporation in this system,7 the incorporation of 35SO4 in both intracellular and extra-cellular fractions was expressed per 10^7 of these cells when lymphocytic leukemia was studied, sulfate incorporation was expressed per 10^7 lymphoblasts (acute) or lymphocytes (chronic).

Inhibition of Sulfation by Puromycin: The effect of puromycin on sulfated mucopolysaccharide synthesis was evaluated in paired cultures as described previously,7 using a final concentration of 25 μg/ml.

Characterization of Sulfated Compound in Leukemic Leukocytes

Gel Filtration: The procedure was performed as described previously,7 using material from 60 to 100 million nucleated cells from each of three control patients, five patients with CGL, two patients with CGL in blastic crisis, two patients with AGL, and three patients with CLL.

Paper Chromatography: The mucopolysaccharides of both the intracellular and extra-cellular fractions of 100 million cells were chromatographed as described previously.7 Three control patients, two patients each with AGL and CGL, one patient with ALL, and one patient with CLL were studied.

Clinical Application

To permit more rapid determination of the ability of leukemic cells to sulfate mucopolysaccharide, cultures containing 24–32 million nucleated cells and 100 μCi of Na35SO4 in 8 ml of medium were incubated at 37°C for 3 hr. The cells were collected by centrifugation, washed once with 10 ml of 0.15 M NaCl, and suspended in 0.5 ml of distilled water. Following mechanical agitation (Vortex Agitator) for 1 min, the mixture was applied to a column of Sephadex G-200 (0.9 × 60 cm), and gel filtration effected as previously described.7 The growth media from these cultures were lyophilized, and the residues were subjected to gel filtration after they were redissolved in distilled water.7 Fractions of 1 ml were collected and assayed for radioactivity.7 As expected, two peaks were found. The fractions constituting the first peak were pooled and then dialyzed in a
heated cellophane bag against four changes of 4000 ml distilled water for 24 hr. The radioactivity present in these peaks was determined before and after dialysis. The dialysate was studied by two-dimensional paper chromatography, according to Vestermark and Bostrom, and by chromatography on Dowex 1-x8 resin equilibrated in 0.5 M NH₄Cl. Radioactivity on the paper chromatograms was detected with a Packard Radiochromatogram Scanner.

RESULTS

The cell count after 24 hr in culture was between 80%–90% of the initial value in nine cultures and 90%–100% in the remaining 20 cultures (Table 1). Cell morphology at the end of incubation was unchanged, except for occasional cytoplasmic vacuolization. No differences in cell morphology or preservation were noted when plasma was added to the culture media. After 3 hr in culture, less than 1% of the cells were stained by incubation with 0.1% trypan blue for 5 min and after 24 hr, only 3%–5% of the cells were stained after similar treatment.

Incorporation of Inorganic Sulfate

Sulfate incorporation was highest in control marrow cultures (Table 1). Cells from patients with CGL incorporated only half as much ³⁵SO₄ as the normal cells. Cells from patients with AGL and CGL in blastic crisis incorporated 12%–20% of the normal levels. Cells from ALL and CLL incorporated only small amounts of sulfate.

Radioactivity was equally distributed between the extracellular and intracellular fractions in the control group and in CGL in blastic crisis. Less radioactivity was found in the extracellular than intracellular fractions in CGL not in blastic crisis. Radioactivity was greater in the extracellular fraction in AGL.

No consistent differences were found between incorporation of radioactivity into cells from blood and cells from bone marrow from patients with CGL, CGL in blastic crisis, or AGL. The ratio of extracellular to intracellular radioactivity varied from culture to culture using either marrow or blood as the source of the cells.

Since there was considerable variation of ³⁵SO₄ incorporation from one culture to another, the reproducibility of the assay system was evaluated by studying ten specimens in duplicate cultures. Extracts of the cells were compared in all ten duplicate incubations, while extracellular extracts were compared in eight duplicate cultures. The amount of radioactivity incorporated into both cellular and extracellular fractions of the paired cultures agreed closely. Thus, in 11 of the 18 studies done on the ten duplicate cultures, variation was within 5% of the mean value for each pair.

Puromycin inhibited sulfate incorporation by 94%–100% in cultures from two patients with CGL and one with CGL in blastic crisis, as well as in three control cultures.

Characterization of Intracellular and Extracellular Sulfated Compound

Gel Filtration Study: The elution pattern of the radioactivity in the intracellular and extracellular fractions was similar for both control and leukemic
MUCOPOLYSACCHARIDE SULFATION IN LEUKOCYTES

Fig. 1. Elution patterns of intracellular and extracellular mucopolysaccharide from Sephadex G-200 (0.9 × 60 cm) column, equilibrated in 0.15 M NaCl-0.01 M Tris buffer at pH 7.5. Solid line indicates intracellular fraction, and dotted line the extracellular fraction.

granulocytes (Fig. 1). The radioactive peak from leukemic lymphocytes was very small but was eluted in the same volume as that required to elute the peak from the granulocyte cultures. After papain digestion, the radioactive peaks from both the intracellular and extracellular fractions of leukemic granulocytes were eluted more slowly, as was previously demonstrated for cultures of normal marrow.7

The sulfated compound in both the intracellular and extracellular fractions from control and leukemic leukocytes was completely precipitable by trichloroacetic acid (TCA). After digestion with papain, less than 1% of the radioactivity was associated with TCA-precipitable material. Between 80% and 100% of the radioactivity originally present was recovered after the material was digested with papain and was gel filtered.

Descending Paper Chromatography: The radioactivity from both the intracellular and extracellular fractions migrated identically with chondroitin sulfate as a single spot with \( R_f 0.9 \). The \( R_f \) of heparin in this system is 0.76. The radioactive spot stained metachromatically with azure A.

Clinical Application

The incorporation of \(^{35}\text{SO}_4\) into cells from 16 patients with acute leukemia after 3 hr in culture was compared with the classification according to the clinical history, cell morphology, and cytochemical characteristics.9 Seven of the patients were considered to have AGL; three, CGL in blastic crisis; four, ALL; and two, lymphosarcoma cell leukemia. All except five patients with AGL were receiving chemotherapy at the time of the study. More than 70% of the leukocytes in the blood or bone marrow of each of these patients were blasts. Blood and bone marrow were studied simultaneously in two patients with AGL.

The patterns of elution of the sulfated mucopolysaccharides from the
Fig. 2. $^{35}$SO$_4$ incorporation in acute leukemia after 3-hr incubation. Open circle with black dot, acute granulocytic leukemia, marrow, untreated; black circle, acute granulocytic leukemia, blood, untreated; open circle, acute granulocytic leukemia, blood, treated; open triangle, chronic granulocytic leukemia in blastic crisis, blood, treated; open square, acute lymphocytic leukemia, blood, treated; cross, lymphosarcoma cell leukemia, blood, treated.

Sephadex G-200 columns in these experiments were the same as those observed following the 24-hr incubation period (Fig. 1), except that a second peak of radioactivity, due to sodium sulfate (see below), was eluted after 30–45 ml of effluent with the highest level at 37–38 ml.

The results of these studies are presented in Fig. 2. As expected, less than 500 cpm/10$^7$ blasts were incorporated in the cultures from patients with ALL or lymphosarcoma cell leukemia, while undialyzed extracts from cultures from patients with AGL or CGL in blastic crisis contained large amounts of radioactivity, with two exceptions that will be discussed below. These studies were performed without dialysis of the starting material so that the results could be obtained the same day. To evaluate the possibility that the mucopolysaccharide peak was contaminated with inorganic $^{35}$SO$_4$, aliquots of the eluted materials were dialyzed for 24 hr against four changes of 4 liters of distilled water, and the loss of radioactivity was determined. The data from these studies are presented in Fig. 2. Dialysis resulted in loss of radioactivity from several samples of the intracellular fraction, but in all cases radioactivity of 2000 or more cpm/10$^7$ blast cells remained. In the extracellular fraction only slight losses of radioactivity occurred on dialysis. The source of radioactivity lost only dialysis from all preparations was identified as inorganic $^{35}$SO$_4$ by two-dimensional paper chromatography and by ion-exchange chromatography on Dowex 1-X8 resin.

As noted above, all but one preparation from AGL and one from CGL in blastic crisis incorporated radioisotope into the intracellular fraction in excess of 2000 cpm/10$^7$ blasts cells, after dialysis. In one of these patients a 24-hr incubation was performed, and incorporation of $^{35}$SO$_4$ into the mucopolysaccharide fraction, comparable to that found in other cases of AGL at 24 hr, was found. In the other patient the extended incubation was not performed. No significant difference was noted in incorporation of radioactivity into cells from five untreated and two treated patients with AGL.
MUCOPOLYSACCHARIDE SULFATION IN LEUKOCYTES

DISCUSSION

The major component of mucopolysaccharide synthesized by normal and leukemic leukocytes in peripheral blood has been identified as chondroitin-4-sulfate. Significantly greater amounts of mucopolysaccharide are synthesized by immature myeloid cells than by mature cells. Synthesis and sulfation of mucopolysaccharide has been demonstrated to begin in microsomes, while storage of the completed mucopolysaccharide is mainly in the neutrophilic granules. Therefore, since sulfation is not dependent on specific granulation, it is theoretically possible that measurable differences in sulfation could be apparent even in the most primitive cells.

In human bone marrow in suspension culture, the majority of mucopolysaccharide synthesis and sulfation occurs in myeloblasts, promyelocytes, and myelocytes. Thus, radioautography of normal bone marrow cultures labeled with Na\(^{35}\)SO\(_4\) indicates that only 2% of mature granulocytes contain grains slightly above the background, while 45% of granulocyte precursors are heavily labeled.

The present studies confirm that leukemic granulocytes from blood or marrow synthesize sulfated mucopolysaccharides. The sulfated mucopolysaccharides produced by both normal and leukemic granulocytes behave in a similar manner on gel filtration, and the labeled compound produced by both is chondroitin sulfate. Puromycin inhibits the production of labeled mucopolysaccharides by both types of cells. Despite these qualitative similarities, there are marked quantitative differences in mucopolysaccharide synthesis between normal and leukemic granulocytes, with leukemic cells producing less than half as much labeled mucopolysaccharide as normal cells under comparable conditions.

An even more striking difference was observed between incorporation of radioactive sulfate into cells from patients with AGL and ALL, with essentially no labeled mucopolysaccharide being produced by the latter cells. Cells from lymphosarcoma cell leukemia and CLL also do not incorporate significant amounts of \(^{35}\)SO\(_4\). The failure of lymphocytes to sulfate mucopolysaccharides has previously been noted for normal prolymphocytes isolated by glass-wool chromatography.

There was marked variation in the amount of radioactivity incorporated into granulocytic leukemic cells from different patients, but excellent reproducibility was obtained in ten duplicate cultures incubated by the short-term suspension method employed in these studies. This result suggests that the variation of \(^{35}\)SO\(_4\) incorporation observed is inherent in the cells. Factors as concomitant disease, or drugs administered before and during the time of study could perhaps influence the metabolic function of the cells. For example, \(^{35}\)SO\(_4\) incorporation was less in five treated patients with CGL than in two untreated patients. The data are, however, too few to make any conclusive statement on this point.

Attempts were made to exploit for diagnostic purposes the differences in \(^{35}\)SO\(_4\) incorporation into cells from AGL and ALL. These studies indicate that assessment of mucopolysaccharide sulfation in the intracellular fraction is a
useful adjunct to morphologic and clinical means of identifying the cell of origin in acute leukemia. In most instances the study can be completed in one working day, if dialysis is not employed, and therefore can be conveniently utilized clinically.

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