Culture from Mouse Bone Marrow of a Subclass of Mast Cells Possessing a Distinct Chondroitin Sulfate Proteoglycan with Glycosaminoglycans Rich in N-Acetylgalactosamine-4,6-disulfate*

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Ehud Razin‡, Richard L. Stevens‡, Fumiko Akiyama[§], Karl Schmid[§], and K. Frank Austen‡

From the ‡Department of Medicine, Harvard Medical School, and the Department of Rheumatology and Immunology, Brigham and Women's Hospital, Boston, Massachusetts 02115 and the §Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118

A differentiated population of cells with metachromatically staining granules and surface IgE receptors was obtained from mouse bone marrow cultured for 2 weeks in the presence of conditioned medium derived from concanavalin A-stimulated splenocytes. The cells were found to incorporate large amounts of \[^{35}S\]sulfate into an intracellular \[^{35}S\]labeled proteoglycan of \(M_r \sim 200,000 \) containing a maximum of seven glycosaminoglycan side chains \((M_r = 25,000)\). After chondroitinase ABC treatment of density gradient-purified \(^{125}\)I-serine-labeled proteoglycan, the resulting core was \(M_r \sim 26,000\) as assessed by gel filtration. Two-dimensional cellulose acetate electrophoresis of \[^{35}S\]labeled glycosaminoglycan revealed a single type of glycosaminoglycan that migrated at the position of oversulfated chondroitin sulfate E from squid cartilage. Chondroitinase ABC degradation of the \[^{35}S\]labeled glycosaminoglycan yielded two cleavage products in approximately equal molar amounts which co-migrated in both descending paper chromatography and high voltage paper electrophoresis with a monosulfated disaccharide, 2-acetamido-2-deoxy-3-O-(\(\beta\)-d-glucuronic acid)-4-O-sulfod-galactose, and a disulfated disaccharide, 2-acetamido-2-deoxy-3-O-(\(\beta\)-d-glucuronic acid)-4,6-di-O-sulfod-galactose. The release of some free \[^{35}S\]sulfate from the oversulfated disaccharide with either chondro-4-sulfatase or chondro-4-sulfatase and the complete desulfation by their combined action established that the oversulfated disaccharide contained N-acetylgalactosamine-4,6-disulfate. The \[^{35}S\]labeled proteoglycan of these unique IgE receptor-bearing and histamine-containing cells, therefore, is composed of chondroitin sulfate with a relatively homogeneous population of cells resembling mast cells as assessed by their morphologic characteristics has recently been described (1-3). These cultured cells manifest granules that stain metachromatically with cationic dyes at acid pH (1-3), contain 100–450 ng of histamine/10^6 cells (1-2), and possess IgE-Fc cell surface receptors which have been quantitated by radioligand binding and which have been recognized functionally by the release of histamine in response to anti-IgE (1). The differentiation and maintenance of these mouse bone marrow-derived mast cells in culture requires the presence of conditioned medium containing growth factors derived from splenocytic lymphocytes undergoing mitosis in response to concanavalin A. The differentiation factor has been separated from both T-cell growth factor (4) and granulocyte-macrophage stimulating factor present in the same conditioned medium (5), and is physicochemically and functionally similar to a glycoprotein produced by monoclonal T-cell hybridomas (5). As defined by their metabolism, histamine content, and ultrastructure (2, 3), mast cells have also been differentiated in vitro from mouse bone marrow with mouse myelo-monocytic leukemia-derived conditioned medium (6) and from mouse fetal liver with concanavalin A-stimulated mouse spleenocyte-derived conditioned medium (7). The cultured mouse mast cells have been phenotyped as negative for Thy-1 (3, 7-9), Ly-1 (7-9), Ly-2 (7-9), and Mac-1 (3), and positive for Ly-5 (3, 7) and H-2 (8, 9) whereas the status of the fa determinant is unresolved.

The appearance of mast cells in gastrointestinal tissue and skin of a mutant mouse strain deficient in this cell type after administration of bone marrow from a congenic mouse strain with a sufficient number of mast cells supports a bone marrow origin for the mast cell (10). However, neither the metachromatically staining bone marrow-derived cells appearing in the cultures (1-9) nor the transferred bone marrow cells that differentiate in vitro in the mast cell-deficient recipient strain (10) have been characterized as mast cells by the chemical criterion of a secretory granule rich in heparin proteoglycan. A predominant chemical difference between blood basophils and peritoneal or tissue mast cells resides in the nature of the proteoglycan in their respective secretory granules. Heparin proteoglycan is synthesized by rat peritoneal (11) and human tissue (12, 13) mast cells, while chondroitin sulfate-dermatan sulfate glycosaminoglycans are the major biosynthetic products of normal guinea pig (14) and rat leukemic (15) basophils. The recent findings that purified rat peritoneal mast cells polymerize chondroitin sulfate rather than heparin onto the exogenous acceptor, \(\beta\)-D-xylulose (16), and that a proportion of the chondroitin sulfate glycosaminoglycans contain an uncharacterized oversulfated disaccharide indicate that in the mature peritoneal mast cell the type of glycosaminoglycan synthesized may vary depending upon whether the acceptor
is β-D-xyllose or the peptide core of heparin proteoglycan. In the present study, dividing mast cells differentiated in vitro from bone marrow by hormone treatment were used. Isolated, usual intracellular proteoglycan with chondroitin sulfate side chains possessing large amounts of N-acetylgalactosamine-4,6-disulfate. Since the proteoglycan of the bone marrow-derived cultured mast cells is distinct from that synthesized by either blood basophil or mature tissue mast cells, these in vitro differentiated cells are considered to represent a subclass of mast cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Male BALB/c mice, C3H mice, C57Bl/6J mice, and Sprague-Dawley rats were from Jackson Laboratories, Bar Harbor, ME; RPMI 1640, fetal calf serum, 2-mercaptoethanol, L-glutamine, nonessential amino acids, penicillin, streptomycin, and Dulbecco's modified Eagle's medium were from Grand Island Biological Co.; garcamycin was from Schering; PD-10 gel filtration columns, Sephadex G-25, Sephadex G-200, Sephadex G-75, and Sepharose CL-4B were from Pharcma; [G-H]heparin (0.6 mg/ml), [methyl-3H]carbolic anhydride (1.2 Ci/mmol), [3H]HCl (M = 69,000), [3H]labeled mast cells (M = 60,000), [Cl-35S]labeled cellobiose (M = 150,000), [L-3H]serine (3 Ci/ml), [L-3H]isoctylene (3 Ci/ml), and [35S]labeled cellobiose (100 Ci/ml) were from New England Nuclear. Hydrofluor was from National Diagnostica, Somerville, NJ; Alcian blue, 1,2-dimethoxyethane, butyl nitrate, and XAR-5 x-ray film were from Eastman Kodak; Whatman No. 1 chromatography cellulose sheets were from EM Laboratories, Elmsford, NY; Spectrum No. 3 dialysis tubing was from Fisher; human umbilical cord hyaluronic acid, pig mucosa heparin, and bovine testicular hyaluronidase were from Sigma; whale cartilage chondroitin sulfate A, shark cartilage chondroitin sulfate C (M = 60,000), Proteus vulgaris chondroitinase ABC, Antherobacter aurescens chondroitinase AC, Proteus vulgaris chondro-6-sulfatase, Proteus vulgaris chondro-4-sulfatase, MDI-4S, MDI-6S, and MDI-OS were from Miles, MDI-diS0, and MDI-diSO were prepared by chondroitin ABC digestion of squid cartilage and shark cartilage chondroitin sulfates, respectively. Squid cartilage chondroitin sulfate E was a gift from Dr. N. Seno, Department of Chemistry, Ochanomizu University, Tokyo, Japan. Sturgeon cartilage chondroitin sulfate C, sturgeon notochord chondroitin sulfate A, shark cartilage chondroitin sulfate C, and [3H]labeled proteoglycan, [3H]labeled proteoglycan (M = 2 x 10^6) and rat [3H]heparin proteoglycan (M = 750,000) and [3H]heparin glycosaminoglycans (M = 80,000-100,000) (18) were prepared as previously described (16, 19).

**Radiolabeling of Cell Cultures and Isolation of Intracellular Proteoglycans and Glycosaminoglycans**—Approximately 1 x 10^6 bone marrow cells, obtained from femurs of 2-month-old male BALB/c mice, were cultured in 50% (v/v) RPMI 1640 supplemented with 10% (v/v) fetal calf serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 0.1% nonessential amino acids, 100 units/ml penicillin, and 100 units/ml streptomycin, pH 7.2 (enriched medium), and 50% (v/v) conditioned medium (1) for 14 days at 37 °C. Conditioned medium was obtained from the co-culture of C57Bl/6J and C3H mouse spleen cells (10^6/ml) for 2 days in enriched medium containing 2 μg/ml concanavalin A. The mouse bone marrow cells differentiated into mast cells during 2 weeks of culture in a humidified atmosphere containing 5% (v/v) CO_2; the culture medium was changed every 7 days.

Serosal mast cells were isolated from BALB/c mice and Sprague-Dawley rats by peritoneal lavage with Tyrode's buffer containing 0.1% (w/v) gelatin and 0.005% (w/v) pig mucosa heparin. Rat and mouse peritoneal mast cells were concentrated by bopyknic and isoosmotic sedimentations (11, 20) to greater than 97% and 90%, respectively, as assessed by metachromasia after staining with toluidine blue. Approximately 3 x 10^6 mast cells were obtained per mouse and 1 x 10^6 mast cells were obtained per rat. Swarm rat chondrosarcoma chondrocytes, isolated by trypsin-collagenase treatment of the tumor, were cultured for 4 days in Dulbecco's high glucose modified Eagle's medium containing 15% (v/v) fetal calf serum, as described (21). The rat basophilic leukemia cell line RBL-2H3 was cultured at isoelectric point. Cells density ranging from 0.5 x 10^6 to 1.0 x 10^6 cells/ml in fresh enriched medium containing 50-100 μg of [35S]sulfate/ml, 100 μCi of [3H]isoleucine/ml, or 100 μCi of [3H]isoleucine/ml and [35S]sulfate (22) plus 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 units/ml penicillin, and 0.1 ml of 1% (w/v) Zwittergent 3-12 detergent containing 0.1 M 6-amino-hexanoic acid, 0.1 M sodium EDTA, 0.005 M benzamidine HCl, 0.001 M sodium iodoacetamide, 0.1 M sodium acetate, pH 6.0, followed 30-60 s later by the addition of 1 ml of 4 M GnHCl containing the same protease inhibitors (16). [3H]-labeled glycosaminoglycans were obtained by sonication of labeled cells (0.5 x 10^6 NaCl at 1°C) plus 50 μM 2-mercaptoethanol cycle power, 3 with a Branson Sonifier) and alkali cleavage for 17 h at 4 °C; the cell extract was neutralized with 5 μM acetic acid and made 4 ml in GnHCl. Pig mucosa heparin glycosaminoglycan (100 μg) was added to the preparations of [3H]-labeled proteoglycans, [3S]-labeled proteoglycan, or [3S]-labeled glycosaminoglycans to prevent quantitation of [3H]sulfate into macromolecules. Solid cesium chloride was added to samples of the cell extracts to give final densities ranging from 1.4 to 1.6 g/ml. After centrifugation at 55,000 x 7 for 40 h (23), the dissociative cesium chloride gradients were divided into four approximately equal fractions differing in their buoyant densities. The fractions containing the [3S]-labeled proteoglycans and [3S]-labeled glycosaminoglycans were dialyzed against water and lyophilized.

**Physicochemical and Biochemical Characterization of Proteoglycans and Glycosaminoglycans**—250 μl samples of each detergent/ GnHCl cell extract were applied directly or applied after cesium chloride density gradient centrifugation to replicate Sepharose CL-4B columns (0.5 x 120 cm) to determine the relative hydrodynamic size of the [3S]-labeled proteoglycan. Each column was equilibrated and eluted with 4 ml GnHCl containing 50 μg/ml of pig mucosa heparin glycosaminoglycan, 0.1 M sodium sulfate, 0.1 M Tris-HCl, pH 7.5, at a flow rate of 1.5 μl/min. The 0.5-ml column fractions were mixed with an equal volume of 70% ethanol and were analyzed for radioactivity. In addition to Sephadex CL-4B columns, [3H]-labeled proteoglycans, rat mast cell [3H]-labeled proteoglycan, rat mast cell [3H]heparin glycosaminoglycan, pig dermam sulfate, and [3S] sulfate were used to standardize the gel filtration column.

For estimation of the molecular weight of the proteoglycan core, the bottom density gradient centrifugation fraction from 10° (1°) serum-labeled bone marrow-derived mast cells was applied to a Sephadex G-200 (superfine) column (0.6 x 110 cm) equilibrated and eluted with 0.5 M sodium acetate, pH 6.0, containing 1 mg/ml of bovine serum albumin at a flow rate of 1.5 μl/min. A 50-μl sample of each 0.5-ml column fraction was analyzed for radioactivity. The excluded volume of the column, which contained 65% of the radioactivity in the D1 fraction, was pooled, concentrated, and incubated for 4 h with 0.002 unit of chondroitinase ABC (24). The digestion mixture was filtered on the same Sephadex G-200 column under identical chromatographic conditions and each 0.5-ml fraction was analyzed for radioactivity. The column was calibrated with rat chondrosarcoma [3H]-labeled proteoglycan, [1^3]Cl-globulin, [3C]carbonic anhydrase, [3^1]albumin, and [3S]sulfate.

The lysophosphatidic acid fraction from the cesium chloride gradient (starting density, 1.4-1.5 g/ml) from alkali-treated cells was suspended in water and added to a Sephadex G-200 column (0.6 x 120 cm) to determine the relative hydrodynamic size of the β-eliminated [3S]-labeled glycosaminoglycans. The column was equilibrated and eluted with 0.5 M sodium acetate, pH 6.0, containing 50 μg/ml of pig mucosa heparin glycosaminoglycan at a flow rate of 1.5 μl/min; each fraction was analyzed for radioactivity. The molecular weight of the liberated mast cell [3S]-labeled glycosaminoglycans was estimated by the gel filtration method of Wasteson (25) using rat...
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The 35S-labeled glycosaminoglycans were also analyzed by two-dimensional electrophoresis (26, 27). The first direction of electrophoresis was carried out in 0.1 M pyridine, 0.46 M formic acid, pH 3.1, for 90 min at 180 V and the second direction was in 0.1 M barium acetate, pH 8.0, for 3 h at 110 V. Human hyaluronic acid, sturgeon chondroitin sulfate C, pig heparin, squid chondroitin sulfate E, and pig dermatan sulfate were used for reference. Reference glycosaminoglycans were visualized by staining with toluidine blue or by autoradiography using Kodak XAR-5 film. The digestion products were localized by autoradiography. The 35S-labeled proteoglycan, shark chondroitin sulfate C, pig heparin, squid chondroitin sulfate E, and pig dermatan sulfate were used for reference. Reference glycosaminoglycans were visualized by staining with toluidine blue or by autoradiography using Kodak XAR-5 film. The digestion products were localized by autoradiography.

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RESULTS

The mouse bone marrow-derived mast cells incorporated [35S]sulfate into macromolecules as assessed by PD-10 chromatography under dissociative conditions at a rate of 2 to 12 times greater than that observed for mouse or rat peritoneal mast cells or rat basophilic leukemia cells radiolabeled in enriched medium without the addition of concanavalin A-stimulated splenocyte conditioned medium (Table I). The addition of conditioned medium during the 4-h radiolabeling had no effect on the incorporation of [35S]sulfate into macromolecules by the rat basophils. The [35S]sulfate incorporation by rat peritoneal mast cells in enriched medium also was not altered by the presence of conditioned medium, and was not significantly different from that obtained previously in Dulbecco's high glucose modified Eagle's medium containing 15% (v/v) fetal calf serum (16). The incorporation of [35S]sulfate into macromolecules by rat chondrocytes in fetal calf serum containing medium exceeded that of the mouse bone marrow mast cells, but more than 95% of the chondrocyte biosynthetic product was exported to the extracellular matrix while more than 95% of the mast cell product remained cell-associated.

**Physicochemical Characteristics of the Intracellular 35S-labeled Proteoglycan from Bone Marrow-Derived Mast Cells**—Cesium chloride density gradient centrifugation under dissociative conditions revealed that the detergent-GnHCl-15% (v/v) sulfate incorporation by rat peritoneal mast cells did not sediment to the bottom D1 fraction at a starting density of 1.6 g/ml, whereas those from the mouse peritoneal mast cells fully sedimented at 1.5 g/ml (Fig. 1). At a starting density of 1.4 g/ml, there was an enrichment of 35S-labeled macromolecules from bone marrow-derived mast cells at the bottom half of the gradient with partial separation from [3H]isoleucine-radiolabeled macromolecules, considered representative of the total proteins synthesized.

The 35S-labeled proteoglycans were isolated from differentiated mouse bone marrow mast cell cultures by cesium chloride density gradient centrifugation under dissociative conditions (fractions D1 and D2 at starting density of 1.4 g/ml) and filtered on Sepharose CL-4B under dissociative conditions as a predominant polydispersed peak with an apparent hydrodynamic size of M, = 150,000 to 250,000 (Fig. 2A). In contrast, the mouse peritoneal mast cell 35S-labeled proteoglycan was filtered with an apparent hydrodynamic size of M, = 750,000, coincident with the [3H]heparin proteoglycan synthesized by isolated rat peritoneal mast cells (11, 16). Thus, the 35S-labeled proteoglycan produced by the bone marrow-derived mast cell cultures was distinct in its size and sedimentation properties.

**Table 1**

**Incorporation of [35S]Sulfate in vitro into proteoglycan by different cell types**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>[35S]Sulfate incorporation</th>
<th>%-associated 35S-labeled macromolecules cell-associated</th>
</tr>
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<tbody>
<tr>
<td>Mouse bone marrow-derived mast cell</td>
<td>57,800 ± 14,500 (n = 7)</td>
<td>&gt;95</td>
</tr>
<tr>
<td>Mouse peritoneal mast cell</td>
<td>7,900 ± 100 (n = 2)</td>
<td>98</td>
</tr>
<tr>
<td>Rat peritoneal mast cell</td>
<td>29,700 ± 400 (n = 4)</td>
<td>73</td>
</tr>
<tr>
<td>Rat basophil leukemia cell</td>
<td>4,800 ± 200 (n = 5)</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Rat chondrosarcoma chondrocyte</td>
<td>450,000 ± 51,000 (n = 27)</td>
<td></td>
</tr>
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</table>

*Mouse peritoneal mast cells, rat peritoneal mast cells, and rat basophilic leukemia cells were incubated for 4 h at 37 °C in a concentration of 100 cells/ml in enriched medium containing 90 μCi of [35S]sulfate/ml. The cultured chondrocytes were radiolabeled for 4 h at 37 °C in Dulbecco's high glucose modified Eagle's medium containing 15% (v/v) fetal calf serum with 50 μCi of [35S]sulfate/ml.*
from that of mouse and rat peritoneal mast cells radiolabeled in vitro.

The \[^{3}H\]serine-labeled proteoglycan from the bone marrow-derived mast cell, purified by cesium chloride density gradient centrifugation and Sephadex G-200 chromatography, was subjected to chondroitinase ABC digestion to remove the chondroitin side chains. Digestion was carried out in the presence of 0.1 mg/ml of bovine serum albumin to minimize proteolysis of the radiolabeled peptide core. Gel filtration on Sephadex G-200 of the remaining \[^{3}H\]serine-labeled peptide core containing any chondroitinase-resistant carbohydrate chains yielded a broad peak of included radioactivity with \(M_r \approx 26,000\), with a range from \(M_r \approx 15,000\) to 58,000. The recovery in four consecutive experiments of \[^{3}H\]serine- or \[^{3}H\]glycine-labeled core was greater than 95% when albumin and heparin carriers were added to the column elution buffer.

**Physicochemical Characteristics of the \(^{35}S\)-labeled Glycosaminoglycans from Bone Marrow-derived Mast Cells**—Sephadex G-200 gel filtration of the \(^{35}S\)-labeled glycosaminoglycans liberated by alkali from bone marrow mast cell \(^{35}S\)-labeled proteoglycans and isolated by cesium chloride density gradient centrifugation revealed chains of \(M_r \approx 25,000\) (Fig. 2B) as determined by the method of Wasteson (25). Two-dimensional cellulose acetate electrophoresis of the \(^{35}S\)-labeled glycosaminoglycans followed by autoradiography (Fig. 3B) revealed a single radiolabeled glycosaminoglycan. In the first direction of electrophoresis, the \(^{35}S\)-labeled glycosaminoglycan was as highly charged at pH 3.0 as pig mucosa heparin; however, in the second direction of electrophoresis, the \(^{35}S\)-labeled glycosaminoglycan possessed a weaker affinity for barium ions than did heparin. By comparison with chondroitin sulfates from different sources, the \(^{35}S\)-labeled glycosaminoglycan from bone marrow-derived mast cells co-migrated electrophoretically in two dimensions with the oversulfated chondroitin sulfate E from squid cartilage (Fig. 3).

The \(^{35}S\)-labeled glycosaminoglycans derived from the differentiated bone marrow mast cell cultures were resistant to nitrous acid degradation as assessed by exclusion on PD-10 gel filtration of more than 90% of the treated material (Fig. 4B). Treatment of equal amounts of the same \(^{35}S\)-labeled glycosaminoglycan fraction with either chondroitinase ABC (Fig. 4C) or chondroitinase AC (Fig. 4D) degraded approximately 95% of the total \(^{35}S\)-labeled macromolecules to oligosaccharides. In contrast, the \(^{35}S\)-labeled glycosaminoglycans released from the mouse peritoneal mast cell \([^{35}S]\)heparin proteoglycan by alkali treatment were completely degraded by nitrous acid and were resistant to digestion by chondroitinase ABC (data not shown). Thus, the highly charged \(^{35}S\)-labeled glycosaminoglycan synthesized by mouse bone marrow-derived mast cells was predominantly a chondroitin sul-

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**Fig. 1.** Cesium chloride density gradient centrifugation under dissociative conditions of detergent GdnHCl-liberated \(^{35}S\)-labeled macromolecules from bone marrow-derived mast cells. Starting density at 1.6 g/ml (●—●), at 1.5 g/ml (△—△), and at 1.4 g/ml (■—■), and of \(^{3}H\)isoleucine-radiolabeled macromolecules at 1.4 g/ml (□—□). The distribution of \(^{35}S\)-labeled macromolecules from radiolabeled mouse peritoneal mast cells was obtained at a starting density of 1.5 g/ml (●—●). The top and bottom of the gradient are indicated.

**Fig. 2.** Molecular weight determination of proteoglycans. A, Sepharose CL-4B gel filtration of intracellular \(^{35}S\)-labeled macromolecules from mouse peritoneal mast cells (---) and mouse bone marrow-derived mast cells (■—■); \(^{35}S\)-labeled glycosaminoglycans liberated from bone marrow-derived mast cells, \(HP-\text{PG}\), rat heparin proteoglycan; \(HP\), rat heparin glycosaminoglycan; \(DS\), pig dermatan sulfate; \(CSC\), chondroitin sulfate C; \(CSA\), chondroitin sulfate A.
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A

FIG. 3. Two-dimensional cellulose acetate electrophoresis of a reference glycosaminoglycan mixture. The mixture contained hyaluronic acid (HA), dermatan sulfate (DS), chondroitin sulfate C (CSC), chondroitin sulfate E (CSE), and heparin (HP) (A), and the \(^{35}\)S-labeled glycosaminoglycans prepared from mouse bone marrow-derived mast cell proteoglycan (B). The reference glycosaminoglycans were visualized by Alcian blue staining, and the \(^{35}\)S-labeled glycosaminoglycans were detected by autoradiography. The position of chondroitin sulfate E is depicted by the dotted line superimposed onto the autoradiograph.

Less than 10\% of the total incorporated radioactivity could represent N-sulfated heparin or heparan sulfate.

Identification of N-Acetylgalactosamine-4,6-disulfate in Oversulfated Chondroitin Sulfate from Bone Marrow-derived Mast Cells—Disaccharides obtained after chondroitinase ABC treatment of shark chondroitin sulfate, squid chondroitin sulfate, and density gradient-purified \(^{35}\)S-labeled glycosaminoglycans from mouse bone marrow-derived mast cells were compared by descending paper chromatography. Approximately one-third of the total applied radioactivity co-migrated with the ADi-4S disaccharides from the reference squid glycosaminoglycans as well as with authentic ADi-4S (Fig. 5). Except for a small amount of radioactivity at the origin, the remainder of the \(^{35}\)S radioactivity co-migrated with the disulfated disaccharide from squid chondroitin sulfate (ADi-diS\(_e\)), but not with the slower moving disulfated disaccharide present in shark cartilage chondroitin sulfate (ADi-diS\(_n\)).

High voltage electrophoresis of chondroitinase ABC-treated shark chondroitin sulfate C, squid chondroitin sulfate E, and mouse bone marrow mast cell \(^{35}\)S]chondroitin sulfate along with authentic markers for monosulfated and disulfated di-
saccharides again demonstrated that the $^{35}$S-labeled disaccharides were either mono- or disulfated (Fig. 6). More rapidly migrating trisulfated or tetrasulfated disaccharides were not detected. After digestion of the $^{35}$S-labeled glycosaminoglycan with chondroitinase ABC and chondro-4-sulfatase, the oversulfated $^{35}$S-labeled disaccharide was no longer detectable and free $[^{35}S]$sulfate was present. The further addition of chondro-6-sulfatase to the digestion mixture increased the free $[^{35}S]$sulfate and markedly depleted the detectable monosulfated $^{35}$S-labeled disaccharide.

As quantitated by PD-10 gel filtration chromatography, chondroitinase ABC-resistant $^{35}$S-labeled glycosaminoglycans plus digestion products larger than disaccharides comprised approximately 5% of the total radioactivity (Fig. 7B). The introduction of either chondro-sulfatase with chondroitinase ABC liberated free $[^{35}S]$sulfate (Fig. 7, C and D). Chondro-4-sulfatase treatment yielded approximately twice as much $[^{35}S]$sulfate as chondro-6-sulfatase, whereas the combined action of both sulfatases released all $[^{35}S]$sulfate from the $^{35}$S-labeled disaccharides (Fig. 7E). After correction for the two $[^{35}S]$sulfate residues in the oversulfated disaccharide, the bone marrow-derived mast cell was calculated to be a hybrid chondroitin sulfate composed of disaccharides with N-acetylgalactosamine-4,6-disulfate and N-acetylgalactosamine-4-sulfate in approximately equal amounts.

**Quantitation of the Oversulfated Chondroitin Sulfate Proteoglycan of Bone Marrow-derived Mast Cells**—Uronic acid analysis of the glycosaminoglycans obtained by cesium chloride density gradient centrifugation revealed 32-48 µg/10⁶ cells ($n = 2$). Assuming an approximate 30% contribution of the uronic acid content to the weight of the proteoglycan molecule, the proteoglycan content/10⁶ bone marrow-derived mast cells was calculated to be approximately 1.5-2.3 µg. Chondroitinase ABC digestion of a glycosaminoglycan fraction obtained from 1.3 x 10⁶ cells after cesium chloride density gradient centrifugation, followed by thin layer chromatography, yielded two digestion products in approximately equal amounts. One product migrated in the position of ΔDi-4S and the other in the same position as the radiolabeled disulfated disaccharide synthesized by these cultured cells.

The presence of the novel oversulfated disaccharide was assessed in undifferentiated, freshly isolated mouse bone marrow cells pulsed with $[^{35}S]$sulfate for 4 h. Radiolabeled cells were treated with NaOH to liberate free $^{35}$S-labeled glycosaminoglycans from $^{35}$S-labeled proteoglycan, and chains were purified by cesium chloride density gradient centrifugation. As analyzed by ascending chromatography of chondroitinase ABC-treated $^{35}$S-labeled glycosaminoglycans, more than 90% of the total incorporated radioactivity from these undifferentiated cells was digested to disaccharides. Compared to reference standards, 3% of the total $^{35}$S-labeled disaccharides were oversulfated, the remainder being 15 and 68% ADi-6S and ADi-4S, respectively. In addition, the disulfated disaccharide containing N-acetylgalactosamine-4,6-disulfate was not detected in $^{35}$S-labeled glycosaminoglycans synthesized by radio-labeled rat basophils, rat peritoneal mast cells, rat chondrocytes, or mouse peritoneal mast cells under these in vitro conditions.

**DISCUSSION**

Mouse bone marrow cultures differentiate, in the presence of conditioned medium derived from mouse spleen cells in the
process of mitosis, into a relatively homogeneous population of mast cells defined by their ultrastructure characteristics (2, 3, 7), metachromatic staining granules (1–3), and cell surface IgE receptors (1). These criteria do not fully exclude the possibility that these cells are basophils, and cells differentiated from guinea pig bone marrow under comparable experimental conditions have been defined as basophils by their indented nuclei observed by light microscopy (33). The finding that the mouse bone marrow-derived mast cell contains a novel oversulfated chondroitin sulfate proteoglycan in which approximately one-half of the N-acetylgalactosamines are sulfated in both the 4 and 6 positions distinguishes this cell from conventional mast cells or basophils. The proteoglycan, defined after radiolabeling or by direct chemical analysis of rat peritoneal mast cells (11, 16), dispersed concentrated human lung mast cells (12), or in human skin mastocytes (13), is heparin proteoglycan, whereas the 35S-labeled glycosaminoglycans present in enriched populations of normal guinea pig basophils (14) and in rat leukemic (15) and human leukemic (34) basophils are predominantly chondroitin sulfates A, B, and C.

The mouse bone marrow-derived mast cell incorporated [35S]sulfate during a 4-h period in conditioned medium at a rate 2–12-fold higher than that of rat leukemic basophils, and normal mouse and rat peritoneal mast cells (Table 1), but was lower than that of Swarm rat chondrosarcoma chondrocytes, which continuously secrete a chondroitin sulfate proteoglycan into the culture medium. The doubling time, based on [3H]thymidine incorporation and visual enumeration, is less than 34 h for the bone marrow-derived mast cell, and the distribution of the 35S-labeled proteoglycan to an intracellular compartment rather than to the culture medium is presumably related to the differentiation of granule-deficient cells to granule-rich mast cells. The proteoglycan content of approximately 1.9 μg/10^6 cells based on chemical determination for uronic acid is in the range observed for human mast cells (12) and rat basophils (15), but is substantially less than that of the rat peritoneal mast cells (35). The oversulfated 35S-labeled proteoglycan of the mouse bone marrow-derived mast cell is smaller in size than mouse (Fig. 2A) and rat peritoneal mast cell heparin proteoglycan (16), and has a lesser buoyant density than mouse mast cell proteoglycan (Fig. 1) or chondrosarcoma chondroitin sulfate-rich proteoglycan (19). The lesser buoyant density reflects a large amount of protein core relative to the amount of covalently bound glycosaminoglycans, as is the case in pig ovarian follicular fluid proteoglycan (36), human skin fibroblast proteoglycan (37), and rhesus monkey cornea keratan sulfate proteoglycan (38). The [3H]serine-labeled proteoglycan core isolated after chondroitinase ABC treatment filtered with M_w 26,000, which represents a minimum figure due to possible proteolytic degradation during isolation of the proteoglycan molecule and its core peptide. As the 8-eliminated 35S-labeled glycosaminoglycans from the bone marrow mast cell proteoglycan have an apparent M_w 25,000 (Fig. 2B), the polydispersed proteoglycan of average M_w 200,000 (Fig. 2A) has a maximum of seven side chains attached to its protein core.

Because the 35S-labeled glycosaminoglycan was electrophoretically indistinguishable from squid cartilage chondroitin sulfate E (Fig. 3) and was resistant to nitrous acid degradation (Fig. 4), further studies were directed toward determining whether the disaccharides in the mast cell 35S-labeled glycosaminoglycan were identical in sulfation with those of squid glycosaminoglycan. Chondroitinase ABC digestion of the 35S-labeled glycosaminoglycan yielded two cleavage products which co-migrated on descending paper chromatography with the monosulfated and disulfated disaccharides obtained simultaneously from squid glycosaminoglycan (Fig. 5). The disulfated disaccharide from shark chondroitin sulfate (ΔDi-diS), which has one of its sulfate residues on the uronic acid, had a distinctly slower mobility in this chromatographic system than ΔDi-diSe (28, 29), which has both sulfates on the hexosamine. High voltage electrophoresis of the same chondroitinase digest also demonstrated two products which co-electrophoresed with those from squid chondroitin sulfate E (Fig. 6). The specificity of the two sulfates for sulfate residues on C-4 and C-6 of the N-acetylgalactosamine moiety of chondroitin sulfate (39) confirms the structure of the monosulfated and oversulfated disaccharides resolved on high voltage electrophoresis as ΔDi-4S and ΔDi-diSe, in agreement with their mobility on paper chromatography (Fig. 5).

As studied by Sephadex G-25 gel filtration (Fig. 7), the disulfated disaccharide and the monosulfated disaccharide were present in approximately equal concentrations and in a molar ratio similar to that of the squid hybrid chondroitin sulfate. The oversulfation of proteoglycan due to a substantial representation of N-acetylgalactosamine-4,6-disulfate in hybrid glycosaminoglycan chains is most unusual for mammalian cells and tissues. The recognition of chondroitin sulfate E localized intracellularly in bone marrow-derived mast cells by chemical as well as radiotracer techniques establishes this oversulfated chondroitin sulfate as the characteristic glycosaminoglycan of this subclass of mast cells. Pharmacologic concentrations of N^6,O^2-dibutyryl cyclic AMP have recently been shown to increase synthesis of N-acetylgalactosamine-4,6-disulfate by fetal calf articular cartilage in vitro (40), implying a regulatory role of cyclic AMP-dependent protein kinase in formation of the oversulfated product. The detection of N-acetylgalactosamine-4,6-disulfate as a minor component of chondroitin sulfates isolated from rat urine (41), beef liver (42), and human cartilage (43) indicates that the synthesis of a proteoglycan containing this oversulfated chondroitin sulfate is not limited to in vitro culture. The equal proportion of ΔDi-diSe residues relative to ΔDi-4S in the bone marrow mast cell hybrid glycosaminoglycan indicates that the oversulfated residues must reside within the chain rather than only at the nonreducing terminal position of the chondroitin sulfate chain as found in cultured chick embryo cartilage chondroitin sulfate (44).

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