IgE-MEDIATED RELEASE OF LEUKOTRIENE C_4, CHONDROITIN SULFATE E PROTEOGLYCAN, β-HEXOSAMINIDASE, AND HISTAMINE FROM CULTURED BONE MARROW-DERIVED MOUSE MAST CELLS[*]

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Mouse bone marrow cells differentiate in vitro into a relatively homogeneous population of mast cells when cultured in the presence of conditioned medium obtained from concanavalin A-stimulated splenocytes (1–3). These bone marrow-derived mast cells are similar to mouse and rat serosal mast cells in terms of the histologic staining of their intracellular granules (1–3); the presence of $1-5 \times 10^5$ IgE Fc cell surface receptors (1, 4); and their overall ultrastructural morphology, which includes prominent granules, an oval nucleus, and extensive microvilli (3, 5, 6). There are, however, a number of criteria that distinguish cultured bone marrow-derived mast cells from mouse and rat serosal mast cells. Morphologically, the granules of the bone marrow-derived mast cells possess less electron-dense material (3, 5, 6) and only about one-tenth as much histamine as mouse and rat serosal mast cells (1, 5–8). The rate of proteoglycan synthesis in the bone marrow-derived mast cell, as assessed by $[^{35}S]$sulfate incorporation, is two- to sevenfold higher than that of serosal mast cells. In addition, >90% of the proteoglycan synthesized possesses covalently bound chondroitin sulfate E glycosaminoglycans rather than heparin glycosaminoglycans (9, 10). Upon stimulation with the calcium ionophore A23187, mouse bone marrow-derived mast cells release an average of 90 ng of 5(*S*), 6(*R*)-5-hydroxy-6-*S*-glutathionyl-7,9-*trans*,11,14-*cis*-eicosatetraenoic acid (leukotriene C_4, LTC_4)[1]/10^6 cells and only 5.7 ng

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[1] *Abbreviations used in this paper:* ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi-diS$_E$, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-6-di-*O*-sulfo-D-galactose; ΔDi-OS, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; DNP-BSA, dinitrophenyl-bovine serum albumin; LTC_4, LTD_4, LTE_4, leukotrienes C_4, D_4, and E_4; PGD_2, prostaglandin D_2; RIA, radioimmunoassay; RP-HPLC, reverse-phase high-performance liquid chromatography.

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of prostaglandin D_2 (PGD_2)/10^6 cells (11). In contrast, under comparable conditions, rat serosal mast cells release an average of 50 ng of $PGD_2/10^6$ cells and <0.7 ng of $LTC_4/10^6$ cells (11, 12). Because the cultured bone marrow-derived mast cells synthesize a different proteoglycan and metabolize arachidonic acid to a different predominant product, it is considered likely that they represent a subclass of mast cells (9, 11) rather than a different stage of development caused by the culture conditions for their differentiation.

Activation of the IgE-bearing bone marrow-derived mast cells with anti-IgE results in the accumulation of the intracellular granules in channels that extend to the cell surface (5) and in the exocytosis of the granular contents, resulting in a net percent release of histamine. Specific antigen-induced release of chondroitin sulfate E proteoglycan and LTC_4, as well as the conventional secretory granule markers β-hexosaminidase and histamine from cultured mast cells sensitized with monoclonal IgE, now establish that all of these molecules are derived from bone marrow-differentiated mast cells.

Materials and Methods

Culture of Mouse Bone Marrow-derived Mast Cells. Bone marrow cells, obtained from femurs of 2-mo-old male BALB/c mice (The Jackson Laboratory, Bar Harbor, ME), were cultured for 14 d at 37°C and at a starting density of 0.1×10^6 cells/ml in 50% RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 U/ml streptomycin, and 50 μM 2-mercaptoethanol (Grand Island Biological Co., Grand Island, NY), pH 7.2 (enriched medium), and 50% conditioned medium (1). Conditioned medium was obtained from the co-culture of C57BL/6J and C3H mouse spleen cells (1×10^6/ml) for 2 d in enriched medium containing 2 μg/ml concanavalin A (Sigma Chemical Co., St. Louis, MO). After being in culture for 14 d in a humidified atmosphere containing 5% CO_2, the cells were stained with toluidine blue at pH 3.5. Approximately 98% of the cells were identified as mast cells by the presence of metachromatic granules. Less than 3% of the cells in these bone marrow-differentiated mast cell cultures ingested opsonized zymosan particles when incubated for 1 h at 37°C in Tyrode's buffer at a zymosan concentration of 1,600 μg/10^6 cells and assessed after staining with Giemsa by light microscopy.

Antigen-induced, IgE-mediated Release of Preformed Mediators and of Sulfidopeptide Leukotrienes. Bone marrow-derived mast cells (1×10^6), in 0.2 ml of Tyrode's buffer containing 0.32 mM Ca^{2+}, 0.2 mM Mg^{2+}, and 0.5% gelatin (modified Tyrode's buffer), were sensitized by incubation for 1 h at 37°C with 0.1–10 μg of mouse monoclonal IgE directed against dinitrophenyl-bovine serum albumin (DNP-BSA) (13). Sensitized cells were washed with 2 ml of modified Tyrode's buffer, sedimented at 400 *g* at room temperature, and suspended in 0.5 ml of prewarmed (37°C) modified Tyrode's buffer containing 0.8-500 ng of DNP-BSA (18 mol DNP/mol BSA). Reactions were terminated by the addition of EDTA to give a final concentration of 2 mM and by sedimentation at 400 *g* for 5 min at room temperature. The supernatants were collected, and the cell pellets were suspended in 1 ml of 10 mM Tris-HCl, 1 M NaCl, pH 7.4, and sonicated at 4°C with a Branson sonifier (Branson Sonic Power Co., Danbury, CT; setting 3, 50% pulse cycle, 10 pulses). All experiments were carried out in duplicate and included sensitized cells that were not incubated with antigen. Both the supernatants and the disrupted cell pellets were assayed for their content of various mediators, as described below. In certain experiments, the cell pellets were washed with 0.5 ml of 10 mM Tris-HCl and 1.5 M NaCl, pH 7.4, before sonication in order to solubilize any granule-bound enzymes and proteoglycans that were still cell associated (7).

Cell viability was assessed by exclusion of Trypan blue and by the measurement of the release of cytosol lactate dehydrogenase (7). Histamine was measured by radioenzymatic assay (14) with ^{3}H-labeled histamine, methyl ^{14}C-labeled *S*-adenosyl-L-methionine (New England Nuclear, Boston, MA) and rat kidney histamine methyltransferase. β-Hexosaminidase was assayed by hydrolysis of *p*-nitrophenyl-β-D-2-acetamido-2-deoxyglucopyranoside (Sigma Chemical Co.); 1 U of enzyme cleaves 1 μmol of substrate/h at 37°C (15, 16).

For quantitation of chondroitin sulfate E proteoglycan, bone marrow-derived mast cells (1 × 10^7) were preincubated for 4 h at 37°C in 10 ml of enriched medium containing 50–100 μCi [^{35}S]sulfate/ml (New England Nuclear Corp). Radiolabeled mast cells were sedimented at 400 *g* for 5 min at room temperature and washed with enriched medium. The cells were then sensitized with monoclonal IgE and challenged with DNP-BSA under the same experimental conditions as for unlabeled cells. After separation of the supernatant, intracellular ^{35}S-labeled proteoglycans were liberated at 4°C by the addition of 0.1 ml of a solution containing 1% Zwittergent 3–12 detergent (Calbiochem-Behring Corp., La Jolla, CA), 0.1 M 6-aminohexanoic acid, 0.1 M sodium EDTA, 5 mM benzamidine HCl, 1 mM sodium iodoacetamide, and 0.1 M sodium acetate, pH 6.0, followed 30–60 s later by the addition of 1 ml of 4 M guanidine hydrochloride containing the same protease inhibitors (9). The ^{35}S-labeled macromolecules released and those remaining cell associated were both quantitated by measurement of radioactivity with a Searle Beta Counter (model 6880; Searle Analytic, Des Plaines, IL).

The net percentages of release of histamine, β-hexosaminidase, and ^{35}S-labeled chondroitin sulfate E proteoglycan were calculated by the following formula: net percent release = $[S - S_{\text{control}}]/[(S + P) - S_{\text{control}}] \times 100$, where S is the mediator content of supernatant of stimulated cells, P is the mediator content of pellet of stimulated cells, and S_{control} is the mediator content of supernatant of unstimulated cells.

Generation of the sulfidopeptide leukotrienes was quantitated with radioimmunoassay (RIA). The supernatants were diluted to 100 μl with 10 mM Tris-HCl, 0.15 M NaCl, and 0.1% gelatin, pH 7.3 (Isogel buffer); mixed with 50 μl of buffer containing 5,000–7,000 cpm of ^{3}H-labeled LTC_4 (New England Nuclear) and 100 μl of class-specific rabbit immune plasma (17); and incubated for 1 h at 37°C. ^{3}H-labeled LTC_4 bound to the rabbit antibodies was precipitated by an overnight incubation at 4°C with 200 μl of goat anti-rabbit IgG. The precipitates were pelleted by centrifugation, solubilized in 0.1 N NaOH, and counted. Synthetic LTC_4 was detectable on the linear portion of a net radioligand binding inhibition curve over a dose range from 0.1 to 1.0 ng (17). Because unstimulated cells did not generate immunoreactive leukotrienes, the antigen-induced release of leukotrienes is expressed as nanograms of product released into the supernatant per 10^6 cells.

For quantitation of PGD_2, supernatant diluted to 100 μl in Isogel buffer was mixed with 50 μl of buffer containing 6,000 cpm of ^{3}H-labeled PGD_2 (New England Nuclear) and 100 μl of rabbit anti-PGD_2 immune plasma for 1 h at 37°C. Normal rabbit plasma (100 μl) and goat anti-rabbit IgG antiserum (200 μl) were successively added, and samples were precipitated overnight at 4°C and centrifuged. The pellets were resolubilized in 0.1 N NaOH and counted. Synthetic PGD_2 was detectable on the linear portion of a net radioligand binding inhibition curve over a dose range from 0.1 to 2.0 ng (17). Antigen-induced release of PGD_2 is expressed as nanograms of product released into the supernatant per 10^6 cells; release from unstimulated cells was not measurable.

Characterization of Released Chemical Mediators. The radiolabeled proteoglycans released into the medium and those remaining associated with the cells were characterized by gel filtration chromatography on Sepharose CL-4B and, after purification by cesium chloride density gradient sedimentation, by the disaccharide content of their bound glycosaminoglycans (9). The Sepharose CL-4B column (0.6 × 120 cm) was equilibrated and eluted with a 4-M guanidine HCl solution containing 50 μg/ml of pig mucosa heparin glycosaminoglycan (Sigma Chemical Co.), 0.1 M sodium sulfate, and 0.1 M Tris-HCl, pH 7.5, at a flow rate of 1.5 ml/h. Samples of each 0.5-ml fraction were analyzed for radioactivity. Rat mast cell ^{35}S-labeled proteoglycan, rat mast cell ^{35}S-labeled glycosaminoglycan, pig dermatan sulfate, and [^{35}S]-sulfate were used as reference standards (9).

The ^{35}S-labeled proteoglycans that were purified by cesium chloride density gradient centrifugation (9) were incubated for 1 h with 0.2 U of chondroitinase ABC in 100 μl Tris-HCl buffer (50 mM Tris-HCl, 50 mM NaCl, 35 mM sodium acetate, pH 8.0) containing 0.05% BSA (Sigma Chemical Co.) and 100 μg of both chondroitin sulfate A and chondroitin sulfate C (Miles Laboratories, Inc., Elkhart, IN) carriers. The reaction products were characterized by their mobility relative to disulfated and monosulfated disaccharide standards during ascending thin-layer chromatography on precoated cellulose acetate plates (EM Laboratory, Inc., Elmsford, NY) (9, 18). The standards were: 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose (ΔDi-4S); 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose (ΔDi-6S); 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-6-di-*O*-sulfo-D-galactose (ΔDi-diS$_E$); 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose (ΔDi-diS$_D$); and 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-OS). Carrier disaccharides were visualized under ultraviolet light. [^{35}S]Sulfate-labeled digestion products were localized by autoradiography with XR-5 x-ray film (Eastman Kodak Co., Rochester, NY) and quantitated by β-scintillation counting after elution with 1 ml of 0.5 M HCl for 2 h at 55°C.

The bioassay of sulfidopeptide leukotrienes was performed on guinea pig ileum strips; a contraction amplitude of one SRS-A unit was defined as equal to that elicited by 5 ng/ml of histamine (19). Before their introduction onto reverse-phase high-performance liquid chromatography (RP-HPLC), the supernatants were mixed with 4 vol of ethanol for 30 min at 4°C, centrifuged at 1,500 *g* for 10 min at 4°C to remove precipitated proteins, evaporated to dryness, and redissolved in methanol:water (1:1). The leukotrienes were resolved by RP-HPLC with an isocratic solvent of methanol:water:acetic acid (65:34.9:0.1, pH 5.6) at a flow rate of 1 ml/min (17). 1-ml samples were collected for 45 min with on-line monitoring of absorbance at 280 nm. Fractions were evaporated to dryness and redissolved in 0.5 ml Isogel buffer for measurement of biological activity and immunoreactivity. Synthetic LTC$_4$, LTD$_4$, and LTE$_4$ (17) standards eluted with retention times of 15.4 ± 0.5, 22.3 ± 0.7, and 28.2 ± 0.6 min (mean ± SEM, n = 3), respectively, with >85% recovery for each compound (11, 20).

Results

Exocytosis of Granule Contents and Generation of Leukotrienes. The bone marrow-derived mast cells, sensitized with monoclonal mouse IgE over a dose range of 0.1–10 µg/10^6 cells and then challenged for 10 min with 20 ng of DNP-BSA, released β-hexosaminidase, histamine, and sulfidopeptide leukotrienes in a dose-dependent relationship to the sensitizing concentration of IgE (Fig. 1). The plateau for the net percent release of β-hexosaminidase and histamine and that for the release of immunoreactive sulfidopeptide leukotrienes occurred at the same sensitizing dose, 5 µg of IgE/10^6 cells. In two consecutive experiments in which cells sensitized with 10 µg IgE were washed three times in Tyrode's buffer and then challenged with optimal antigen dose, the washing step increased antigen-induced release of leukotrienes by an average of 29 ± 5% (mean ± SEM).

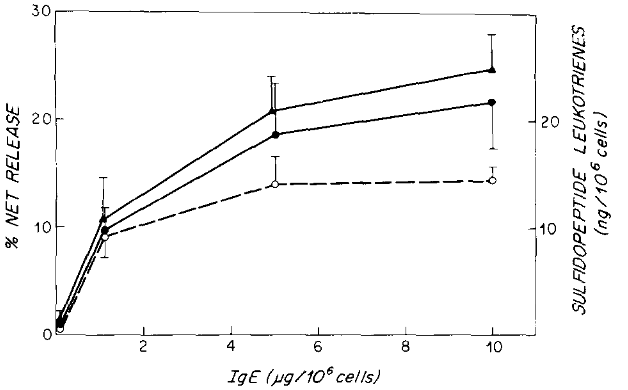

FIG. 1. Net percent antigen-induced release of β-hexosaminidase (○) and of histamine (●), and net release of sulfidopeptide leukotrienes (▲) from bone marrow-derived mast cells sensitized with incremental concentrations of mouse monoclonal anti-DNP IgE. Results are expressed as mean ± SEM of three experiments. Unstimulated cells released 10 ± 2% of β-hexosaminidase and 10 ± 3% of histamines, while failing to generate detectable leukotrienes.

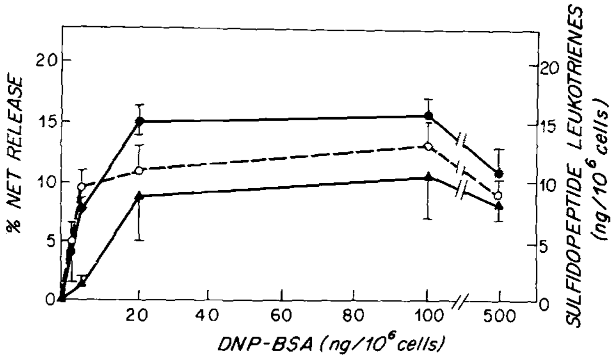

FIG. 2. DNP-BSA antigen dose-dependent net percent release of β-hexosaminidase (●) and [^{35}S]-proteoglycans (○), and release of sulfidopeptide leukotrienes (▲) from sensitized bone marrow-derived mast cells. Separate samples were radiolabeled for 4 h and washed before being assessed for antigen-induced release of ^{35}S-labeled proteoglycans. Results are expressed as the mean ± SEM of three experiments for β-hexosaminidase and leukotrienes and of six experiments for ^{35}S-labeled proteoglycans. Unstimulated cells released 9 ± 2% of β-hexosaminidase and 10 ± 3% of [^{35}S]-proteoglycans.

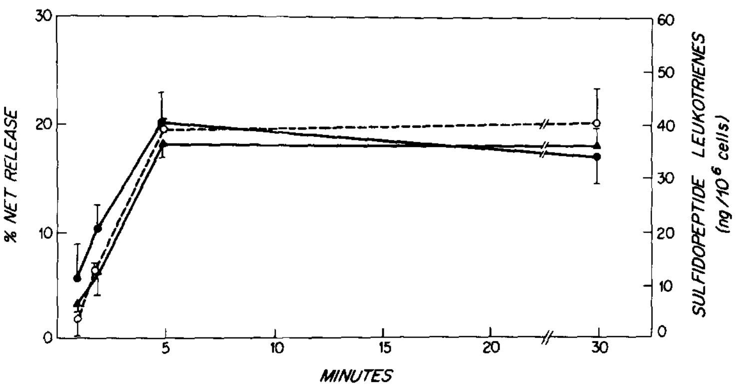

FIG. 3. Kinetics of antigen-induced net percent release of β-hexosaminidase (●) and ^{35}S-labeled proteoglycans (○), and release of sulfidopeptide leukotrienes (▲) from sensitized bone marrow-derived mast cells. The release of β-hexosaminidase and of [^{35}S]proteoglycans from unstimulated cells was 10 and 9%, respectively, at 5 min and did not increase with longer incubations.

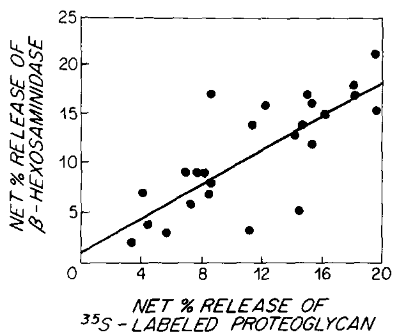

FIG. 4. Correlation of the net percent release of ^{35}S-labeled proteoglycan and β-hexosaminidase.

Because bone marrow-derived mast cells were to be routinely sensitized with 10 μg of IgE, the dose of DNP-BSA was varied from 0.8 to 500 ng/10^6 cells to determine the optimal dose for mediator release (Fig. 2). The net percent release of β-hexosaminidase and the release of immunoreactive sulfidopeptide leukotrienes reached their plateau values with 20 ng of DNP-BSA. In separate experiments, the net percent release of ^{35}S-labeled proteoglycan reached a plateau at the same antigen concentration.

The kinetics of the release of β-hexosaminidase, immunoreactive sulfidopeptide leukotrienes, and ^{35}S-labeled proteoglycan were examined with three duplicate sets of 1×10^6 cells sensitized with 10 μg of IgE and challenged with 20 ng DNP-BSA for 1–30 min (Fig. 3). The net percent release of β-hexosaminidase and ^{35}S-labeled proteoglycans and the release of sulfidopeptide leukotrienes had similar time courses, which reached plateaus 5 min after challenge. Washing radiolabeled mast cells with 1.5 M NaCl in 10 mM Tris HCl after challenge did not increase the net percent release of ^{35}S-labeled proteoglycan.

In two experiments with bone marrow-derived mast cells sensitized with 10 μg IgE/10^6 cells and challenged with 20 ng DNP-BSA for 5 min at 37°C, the average release of PGD_2 was <0.5 ng as compared with 26 ng of sulfidopeptide leukotrienes. Increasing the time period of antigen challenge did not result in further PGD_2 release.

When the data for the net percent release of histamine and β-hexosaminidase, obtained from cells sensitized with incremental doses of IgE (Fig. 1), were compared by linear regression analysis (21, 22), a correlation coefficient (r) of 0.91 ($n = 10$, $P < 0.01$) was obtained. A similar comparative analysis, examining the relationship of the net percent release of β-hexosaminidase and ^{35}S-labeled proteoglycan (data from Figs. 2 and 3) yielded $r = 0.77$ ($n = 27$, $P < 0.001$), with the line intersecting the y axis at a point not statistically different from zero (Fig. 4) ($t = 0.75$, $P > 0.05$).

Identification of Released ^{35}S-labeled Proteoglycan and Sulfidopeptide Leukotrienes. Two sets of duplicate samples of 1×10^6 ^{35}S-labeled bone marrow-derived mast cells were sensitized with 10 μg of IgE. The optimal concentration of antigen was added to one set while the other set remained in buffer alone. Both sets were then incubated at 37°C for 10 min and the cells sedimented by centrifugation. 100-μl samples of the supernatants from the stimulated cells, the extracts from the challenged cell pellets, and the extracts from the unchallenged cell pellets were chromatographed sequentially on Sepharose CL-4B. The ^{35}S-labeled proteoglycans from the unchallenged cells (Fig. 5 A), from the challenged cells (Fig. 5 B), and from the supernatant of the challenged cells (Fig. 5 C) each filtered with an apparent 150,000–250,000 mol wt, which indicates that the hydrodynamic size of the proteoglycan was not altered during the release reaction.

The ^{35}S-labeled proteoglycan from the cell extracts of unchallenged and challenged cells and from the supernatants of challenged cells were each purified by cesium chloride density-gradient centrifugation and digested with chondroitinase ABC. Ascending thin-layer chromatography revealed ultraviolet-absorbing products migrating at the positions of ΔDi-4S and ΔDi-6S, which is consistent with the reported mobility of the digestion products of the two carrier chondroitin sulfates. As assessed by autoradiography, digestion of the ^{35}S-labeled proteoglycans from the unstimulated bone marrow-derived mast cells, from the stimulated cells, and from the supernatants of the stimulated cells yielded two products. These radiolabeled products migrated in the positions of ΔDi-4S and of an oversulfated disaccharide previously shown to

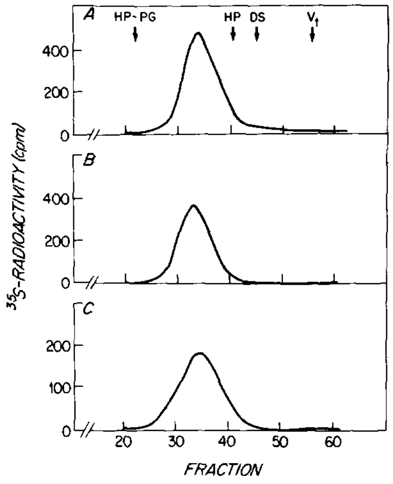

FIG. 5. Sepharose CL-4B gel filtration, under dissociative conditions, of cell-associated ^{35}S-labeled proteoglycan in unchallenged cells (A) and challenged cells (B) and of [^{35}S]proteoglycan released into the supernatant after antigen challenge (C). HP-PG, ^{35}S-labeled rat heparin proteoglycan, 750,000 mol wt; HP, ^{35}S-labeled rat heparin glycosaminoglycan, 100,000 mol wt; DS, pig dermatan sulfate, 40,000 mol wt. Unlabeled markers were identified by determination of their uronic acid content.

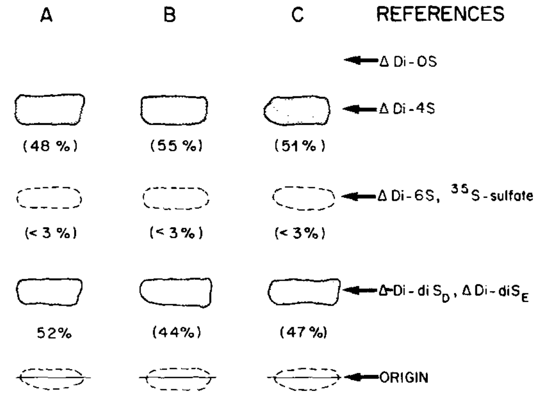

FIG. 6. Ascending thin-layer chromatography of chondroitinase ABC-treated ^{35}S-labeled proteoglycans from (A) unstimulated, sensitized bone marrow-derived mast cells, (B) antigen-challenged, sensitized, bone marrow-derived mast cells, and (C) supernatant from antigen-challenged, sensitized, bone marrow-derived mast cells. The digestion products from the carrier glycosaminoglycans and the disaccharide standards were visualized by ultraviolet absorption, whereas the ^{35}S-labeled digestion products were detected by autoradiography. The radioactivity eluted from the chromatograms is indicated in parentheses in terms of percentage of distribution; recoveries were >95%.

contain *N*-acetyl-galactosamine 4,6-disulfate, ΔDi-diS$_E$ (Fig. 6). Quantitation of the radiolabeled digestion products revealed two-thirds of the radioactivity to be associated with the disulfated disaccharide, which indicates that the disulfated and monosulfated *N*-acetyl-galactosamines were equally present in the undigested glycosaminoglycan, as previously observed by analysis of both radiolabeled and unlabeled chondroitin sulfate E from the resting mouse bone marrow-derived mast cells (9). Furthermore, the highly sulfated proteoglycan present in these mast cell granules did not undergo a change in sulfation of its bound glycosaminoglycans upon exocytosis.

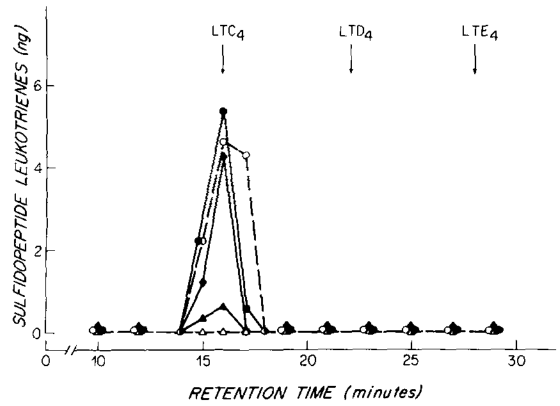

FIG. 7. RP-HPLC elution pattern of immunoreactive leukotrienes obtained from 1 × 10^6 sensitized bone marrow-derived mast cells subjected to antigen challenge for 0 (△), 1 (▲), 2 (◆), 5 (○), and 30 (●) min at 37°C.

To characterize the sulfidopeptide leukotriene products obtained at various times after antigen challenge, five duplicate sets of sensitized bone marrow-derived mast cells were challenged; the supernatants were harvested at 0, 1, 2, 5, and 30 min and resolved by RP-HPLC. The recoveries after RP-HPLC were 73 and 76% of the total immunoreactive material applied from sensitized cells challenged 5 and 30 min, respectively, with antigen. In each experiment, >95% of the immunoreactive sulfidopeptide leukotrienes eluted at a retention time of 15–16 min (Fig. 7), the same as that of authentic LTC_4, which indicates that further processing to LTD_4 and LTE_4 did not occur within this time period after antigen challenge. In two separate experiments involving duplicate sets of 1 × 10^6 cells sensitized with IgE and challenged with the optimal concentration of antigen for 10 min at 37°C, the immunoreactive leukotrienes, which again eluted at 15–16 min, had an average spasmogenic activity of 1.4 U/pmol in assays in which synthetic LTC_4 had a specific activity of 1.3 U/pmol.

Discussion

Mouse bone marrow-derived mast cells respond to IgE-dependent activation with the release of the preformed mediators, histamine, β-hexosaminidase, and chondroitin sulfate E proteoglycan and the generation and release of LTC_4. The release of these mediators was related in a dose-dependent fashion to the concentration of monoclonal IgE used during the sensitization (Fig. 1) and to the concentration of the specific antigen used for activation (Fig. 2). The antigen-initiated response was not diminished by washing of the sensitized cells three times before antigen challenge. This indicated that the release of mediators involved the interaction of antigen with IgE fixed to the cell surface and not the phagocytosis of immune complexes formed in the fluid phase. The release of the preformed mediators, β-hexosaminidase and ^{35}S-labeled chondroitin sulfate E proteoglycan, and of LTC_4 followed similar kinetics beginning within 60 s after antigen challenge and reaching plateaus within 5 min at 37°C (Fig. 3). The common dose-response relationships observed for the sensitizing concentration of monoclonal IgE and for the quantity of antigen required for optimal release of preformed mediators and of LTC_4, as well as the superimposable time courses for their release, indicate the origin of these mediators from a common cell with IgE receptors.

As assessed by histochemical staining for esterase-positive cells, differential cell count after fixation and staining with Giemsa, and capacity to engage in a phagocytic response, <3% of the cells in the 14-d cultures of bone marrow-derived mast cells could be defined as macrophage-like cells (1). Previous experiments indicated that these bone marrow-derived mast cell cultures produce ~90 ng of $LTC_4/10^6$ cells after calcium ionophore A23187 activation over a 40-min time period, with <18 ng being released by 5 min (11). Whereas the ionophore-induced generation and release of oxidative products of arachidonic acid do not define the nature of the responding cell in the culture, the release of preformed mediators and LTC_4, after fixation of IgE and challenge of the cell with antigen, unequivocally indicates a bone marrow-derived mast cell origin for these molecules.

The release of chondroitin sulfate E proteoglycan was not accompanied by an apparent change in hydrodynamic size as assessed by gel filtration on Sepharose CL-4B (Fig. 5). That the released ^{35}S-labeled macromolecules were indeed the unique chondroitin sulfate E proteoglycan (9) was established by the digestion of their radiolabeled side chains with chondroitinase ABC and identification of the disaccharide products containing approximately equal amounts of *N*-acetyl-galactosamine 4,6-disulfate and *N*-acetyl-galactosamine 4-sulfate (Fig. 6). Linear regression analysis of the relationship of net percent release of histamine to that of β-hexosaminidase yielded a straight line which intersected the origin. Similarly, the statistical analysis of the relationship of net percent release of β-hexosaminidase to ^{35}S-labeled chondroitin sulfate E proteoglycan yielded a similar regression line (Fig. 4). These mediators, therefore, are localized in the secretory granules of the bone marrow-derived mast cells. Similar analysis of mediator release data has previously localized heparin proteoglycan, neutral proteases (chymase and carboxypeptidase B), and acid hydrolases (β-hexosaminidase and β-glucuronidase) to the secretory granules of rat peritoneal mast cells (7, 16). As the ratio of the net percent release of ^{35}S-labeled chondroitin sulfate E proteoglycan to β-hexosaminidase of 0.75 is substantially greater than the ratio of net percent release of heparin to β-hexosaminidase for rat peritoneal mast cells (0.24), it is apparent that the ^{35}S-labeled chondroitin sulfate E solubilizes from the released granule much more readily than does heparin. Indeed, even when immunologically activated rat peritoneal mast cells are washed with 1 M NaCl to solubilize the cell-associated proteoglycan and increase the release of heparin proteoglycan, the release ratio rises to only 0.51 (16). Thus, whereas the heparin proteoglycan of conventional mast cells forms an insoluble complex with the neutral proteases, which remains cell-associated even after the secretory granules have released soluble mediators (such as β-hexosaminidase and histamine), the chondroitin sulfate E proteoglycan of the bone marrow-derived mast cells is not substantially retarded in its diffusion into the microenvironment. The localization of ^{35}S-labeled chondroitin sulfate E proteoglycan to the secretory granules and the finding that >90% of the proteoglycan isolated from bone marrow-derived mast cells is chondroitin sulfate E (9) suggest that this proteoglycan is responsible for the characteristic metachromasia of the granule after staining with cationic dyes.

Although it has long been recognized that immediate-type hypersensitivity reactions, including IgE-dependent activation of dispersed human lung cells (23), generate

SRS-A, now known to be sulfidopeptide leukotrienes, the bone marrow-differentiated mouse mast cells are the first population of mast cells of >95% purity to consistently release large quantities of LTC_4 in response to IgE antigen-specific activation. The quantity of immunoreactive leukotrienes generated by bone marrow-derived mast cells under optimal conditions of IgE-dependent sensitization and antigen challenge in nine separate experiments averaged 23 ± 3 (mean ± SEM) $ng/10^6$ cells. The single immunoreactive sulfidopeptide leukotriene generated and released by antigen-dependent activation of IgE-sensitized bone marrow-derived mast cells was defined as LTC_4 by its retention time on RP-HPLC (Fig. 7) and by its biological activity on guinea pig ileum. The product was not converted to LTD_4 or LTE_4 within 30 min of its generation (Fig. 7). Previous studies demonstrated that bone marrow-derived mast cells also generated LTC_4 upon activation with calcium ionophore A23187 and this product was not metabolized to either LTD_4 or LTE_4 within 1 h of stimulation (11). Thus, the bone marrow-derived mast cells resemble zymosan-activated mouse peritoneal or pulmonary interstitial macrophages (24, 25) and ionophore-activated mouse mastocytoma (26) in not processing the LTC_4 that they generate. This finding contrasts with the ionophore-stimulated rat basophilic leukemia (RBL-1) cells, mouse pulmonary alveolar macrophages, and rat peritoneal mononuclear cells (25, 27, 28), which generate LTC_4 and then convert it to LTD_4 in a time-dependent fashion. Because γ-glutamyl transpeptidase is likely to be the enzyme responsible for this conversion (29), it is either absent or biologically unavailable in bone marrow-derived mast cells activated to release LTC_4 with either calcium ionophore or an IgE-dependent stimulus.

The finding that bone marrow-derived mast cells generate LTC_4 in approximately a 25:1 ratio to PGD_2 in response to either activation with the calcium ionophore (11) or perturbation of the IgE receptor contrasts with the finding that rat peritoneal mast cells respond to activation with a preferential generation of PGD_2 such that the ratio of LTC_4 to PGD_2 is <1:40. This difference in the oxidative metabolism of arachidonic acid in response to immunologic activation of the two mast cell types may have important biologic implications in view of recent findings of the differing pharmacologic actions of these two mediators on the vasculature of humans, guinea pigs, and hamsters (30–32) and the remarkable potency of aerosolized LTC_4, relative to histamine in compromising pulmonary function of normal humans (33).

Summary

Mouse bone marrow-derived mast cells differentiated in vitro and sensitized with monoclonal IgE respond to antigen-initiated activation with the release of histamine, β-hexosaminidase, chondroitin sulfate E proteoglycan, and leukotriene C_4 (LTC_4). The chondroitin sulfate E nature of the glycosaminoglycan side chain was established by demonstrating that the chondroitinase ABC disaccharide digestion products were composed of equal quantities of 4-sulfated and 4,6-disulfated *N*-acetyl-galactosamine. The single immunoreactive sulfidopeptide leukotriene, released and quantitated with a class-specific antibody, was identified as LTC_4 by its retention time on reverse-phase high-performance liquid chromatography and by its specific spasmogenic activity on the guinea pig ileum. The release of the preformed mediators, as well as of LTC_4, was related in a dose-response fashion to the concentration of monoclonal IgE used during the sensitization step and to the concentration of specific antigen used to initiate the

activation-secretion response. The optimal concentrations of IgE for sensitization and of antigen for challenge were the same for the release of preformed mediators and of LTC_4. In addition, the time courses of their release were superimposable, with a plateau at 5 min after antigen challenge. The release of three preformed mediators and of LTC_4 after fixation of IgE, washing of the sensitized cells, and antigen challenge unequivocally indicates a bone marrow-derived mast cell origin for these products. Linear regression analyses of the net percent release of β-hexosaminidase to histamine and of ^{35}S-chondroitin sulfate E to β-hexosaminidase yielded straight lines that intersected at the origin, which indicates that the three preformed mediators are localized in the secretory granules of the bone marrow-derived mast cells. The concomitant generation of 23 ng of $LTC_4/10^6$ sensitized bone marrow-derived mast cells represents the first example of IgE-dependent release of substantial amounts of LTC_4, a component of slow reacting substance of anaphylaxis, from a mast cell population of >95% purity. The IgE-dependent generation of LTC_4, rather than prostaglandin D_2, by the chondroitin sulfate E proteoglycan-containing bone marrow-derived mast cells contrasts with the predominant generation of prostaglandin D_2 by heparin proteoglycan-containing mast cells. These differences together support the existence of two phenotypically different mast cell subclasses.

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