

QUANTITATION OF HISTAMINE, TRYPTASE, AND CHYMASE IN DISPERSED HUMAN T AND TC MAST CELLS¹

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Levels of histamine, chymase, and tryptase were assessed in preparations of dispersed human TC (tryptase⁺, chymase⁺) mast cells obtained from foreskin and of dispersed human T (tryptase⁺, chymase⁻) mast cells obtained from lung. Consistent with previous immunohistochemical results, extracts of T mast cells, the predominant mast cell type in lung (93% T and 7% TC mast cells), were deficient in human chymase (<0.3 μ g and 0.04 U/10⁶ mast cells) but not tryptase (10.8 μ g and 0.3 U/10⁶ mast cells) by corresponding immunologic and enzymatic (suc-L-ala-ala-pro-phe-p-nitroanilide in the presence of aprotinin and tosyl-L-gly-pro-lys-p-nitroanilide in the presence of soybean trypsin inhibitor, respectively) assays. The minor presence of chymase activity in lung could be accounted for by the minor presence of lung TC mast cells. Extracts of TC mast cells, the predominant mast cell type (1% T and 99% TC mast cells) in foreskin, contained both proteases. However, TC mast cells from adult foreskin contained eightfold to 10-fold higher levels of chymase (4.5 μ g and 1.01 U/10⁶ mast cells) and twofold to threefold higher levels of tryptase (11.5 μ g and 0.27 U/10⁶ mast cells) than did TC mast cells from newborn foreskin (<0.6 μ g and 0.09 U of chymase and 35 μ g and 0.62 U of tryptase/10⁶ mast cells). In contrast, histamine levels were not significantly different in adult foreskin TC (1.9 μ g/10⁶ mast cells), newborn foreskin TC (1.6 μ g/10⁶ mast cells), and adult lung T (1.5 μ g/10⁶ mast cells) mast cells. The relative ratio of each mediator in newborn foreskin mast cells to that in adult foreskin mast cells is highest for histamine, followed by tryptase and then chymase. Tryptase from TC and T mast cells had identical subunit compositions by Western blot analysis and similar apparent specific activities. This study extends the previously reported immunohistochemical distinction between human T and TC mast cells in tissue sections by direct quantitation of chymase and tryptase in dispersed preparations of T and TC mast cells.

In human mast cells, two serine proteinases have been

identified, which are referred to as tryptase (1-3) and chymase (4, 5). Immunohistochemical techniques performed by using antibody preparations against each proteinase have indicated the presence of two types of human mast cells, termed T and TC (6). T mast cells stain positively with anti-tryptase but not anti-chymase antibodies and are the predominant type in lung (particularly in alveoli) and intestinal mucosa. TC mast cells stain positively with antibodies against both neutral proteases and are the predominant type in skin and intestinal submucosa. In normal nasal mucosa and bronchial/bronchiolar regions a mixture of the two mast cell types are found.

The current study compares the content of histamine, tryptase, and chymase in dispersed preparations of T and TC mast cells. Mast cells from lung were used as a source of predominantly T mast cells, and those from foreskins of newborn and adult subjects were used as a source of TC mast cells. Specific immunoassays for tryptase (7) and chymase (5) were used. In addition, enzymatic assays with synthetic substrates were made highly preferential for tryptase by inclusion of soybean trypsin inhibitor (which does not inhibit tryptase) (2, 8) and for chymase by inclusion of aprotinin (which inhibits cathepsin G but not chymase) (4, 9).

MATERIALS AND METHODS

Reagents. Indoxylphosphate, nitroblue tetrazolium, collagenase, hyaluronidase, histamine, bovine serum albumin, acetonitrile, iodoplatinate, soybean trypsin inhibitor (SBTI),³ aprotinin, and toluidine blue (Sigma Chemical Co., St. Louis, MO); barbital, H₂O₂, and glycerol (Fisher Scientific Co., Fairlawn, NJ); Tween-20, acrylamide, bisacrylamide, SDS, nitrocellulose membranes, gelatin, and Tris (Bio-Rad Laboratories, Richmond, CA); Percoll (Pharmacia, Piscataway, NJ); 2(N-morpholino)ethanesulfonic acid (MES) and N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) (Calbiochem-Behring Corp., La Jolla, CA); ³H-S-adenosylmethionine and Blofluor LSC (New England Nuclear, Boston, MA); Whatman LK6D Linear-K-Silica TLC plates (Whatman Chemical Separation Inc., Clifton, NJ); tosyl-L-gly-pro-lys-p-nitroanilide (TGPL) (Boehringer Mannheim Biochemical Co., Indianapolis, IN); and suc-L-ala-ala-pro-phe-p-nitroanilide (SAAPP) (Vega Biotechnologies Inc., Tucson, AZ) were obtained as indicated.

Tissues and cells. Surgical specimens of human lung were mechanically minced and digested with proteases to disperse cells as described (10). Approximately 0.25 to 1.0 $\times$ 10⁶ mast cells of 2 to 15% purity were obtained per gram of lung. Neutrophils accounted for less than 20% of dispersed nucleated cells as described (11). Foreskins were obtained at circumcisions of normal newborn infants (less than 4 days old) and of adults (at least 21 yr old) with phimosis or penile lesions where the foreskin appeared to be unin-

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³ Abbreviations used in this paper: MES, 2(N-morpholino)ethanesulfonic acid; TGPL, tosyl-L-gly-pro-lys-p-nitroanilide; SAAPP, Suc-L-ala-ala-pro-phe-p-nitroanilide; and TBS, 0.02 M Tris, pH 7.4, 0.5 M NaCl; SBTI, soybean trypsin inhibitor.

volved; cells were dispersed by using a modification of the technique described by Holgate et al. (12). Samples were minced, were washed with Hanks' balanced salt solution without calcium (HBSS⁻), and were incubated with 1.5 mg collagenase and 0.5 mg hyaluronidase/ml at 37°C for 60 min with gentle agitation. About 2 to 5 ml of this solution per gram of tissue were used. Dispersed cells were separated from the tissue by washing them through a No. 80 mesh stainless steel sieve; they were then washed in HBSS⁻. The remaining tissue was digested three more times, and dispersed cells were collected, washed, combined, and filtered through a porous plastic filter (Vyon F, 0.75 mm, Porvair Ltd., Norfolk), and subjected to centrifugation onto a 70% (v/v) Percoll cushion. The latter separates erythrocytes and residual debris from nucleated cells. Approximately 0.2 to 0.5 × 10⁶ mast cells of 3 to 8% purity were typically obtained per gram of tissue. Neutrophils were rarely (<2%) observed. A portion of each cell preparation was used to make cytospin preparations; the remaining cells were pelleted and stored at -70°C until used.

Preformed mediators were solubilized from mast cells by sonication of thawed cell preparations (1 to 2 × 10⁶ mast cells/ml of 0.01 M MES, pH 6.5, 1 M NaCl) at 4°C with a Branson sonifier and microtip at power 3 to 4, 50% pulse cycle, 20 pulses times three. Solubilized material was obtained from the supernatant after centrifugation of the sonicate at 40,000 × G for 45 min at 4°C. The pellet was resuspended in the above buffer, was sonicated, and was centrifuged two more times in order to extract all of the preformed mediators of interest. Selected pellets were then sonicated and extracted in 0.01 M MES, pH 6.5, 2 M NaCl in order to ensure that each protease had been solubilized completely, which was the case in each instance.

Mediator assays. Histamine was measured in cell extracts by modification of a previously described radioenzyme technique (13). Histamine methyl transferase was extracted from rat kidneys, was subjected to ammonium sulfate precipitation and dialysis against PBS as described (14), and was adjusted to 5 mg protein/ml before use. ³H-S-adenosylmethionine (12 µCi in 22 µl, 50 to 85 Ci/mmol) was mixed with 1 ml of histamine methyltransferase immediately before use. Of this enzyme mixture, 25 µl were added to 25 µl of sample (diluted appropriately in 0.05 M Tris, pH 7.4, 0.15 M NaCl, 0.1% sodium azide, 0.1% [w/v] bovine serum albumin) or standard (histamine, 0.1 to 1.0 ng/ml of the sample buffer) and 25 µl of distilled/deionized H₂O. Reactions were performed in duplicate at 4°C for 90 min. Ice-cold acetonitrile/water (80:20) (75 µl) containing 0.2 mg of 1-methylhistamine/ml was then added to stop the reaction and precipitate protein, which was removed by microcentrifugation at 10,000 × G for 15 min at 4°C. The supernatant (100 µl) was subjected to thin-layer chromatography for 40 to 60 min in freshly prepared acetone/aqueous ammonia (92:8), was dried, and was sprayed with iodoplatinate to detect methyl histamine. The silica with each stained band was transferred to a scintillation vial, was bleached with 0.03 ml of 30% H₂O₂ added to 0.22 ml 1 N HCl for 60 min at 22°C, and was mixed with 5 ml of Biofluor LSC cocktail. Histamine levels were then calculated based on the dpm determined in each vial.

Immunoreactive tryptase was measured by a specific sandwich enzyme-linked immunosorbent assay (ELISA) (7). Extracts of lung and foreskin preparations showed no interference with the measurement of tryptase by the immunoassay. Enzymatically active enzyme was measured spectrophotometrically at 22°C by cleavage of 0.1 mM TGPL (15) in the presence of 50 µg SBTI per 0.6 ml assay to inhibit other neutral serine proteases that cleave trypsin-like substrates. Specificity of this assay for mast cell tryptase was shown by selective removal of >90% of the TGPL activity from adult lung and skin extracts by immunoprecipitation with goat IgG anti-tryptase (7) and Pansorbin (Calbiochem-Behring, La Jolla, CA) and removal of <10% by nonimmune goat IgG and Pansorbin. The percentage of SBTI-resistant vs total TGPL activity was 95 ± 4 (±SD, n = 8) for extracts from dispersed lung cells, 102 ± 9 (±SD, n = 4) for extracts from dispersed adult foreskin cells and 96 ± 14 (±SD, n = 5) for extracts from dispersed newborn foreskin cells.

Immunoreactive chymase was determined by radioimmunoassay (5). Enzymatically active enzyme was quantified by cleavage of 0.1 mM SAAPP (molar extinction coefficient = 8800) in 0.05 M HEPES, pH 7.6, 4 M NaCl containing 15 µM aprotinin at 22°C. The level of aprotinin in the assay was enough to inhibit >95% of the chymotryptic activity (predominantly cathepsin G) from 10⁶ neutrophil equivalents, more than 20-fold higher than the neutrophil equivalents in any of the mast cell extracts that were used. The percentage of aprotinin-resistant vs total SAAPP activity was 66 ± 14 (±SD, n = 4) for extracts from dispersed lung cells and 99 ± 2 (±SD, n = 3) for the three foreskin extracts in which this percentage was determined. For both enzymes, 1 U cleaves 1 µmol of substrate per minute.

Histochemistry. Metachromatic staining was performed by application of 0.5% (w/v) toluidine blue in 0.5 M HCl to Carnoy's fluid-

fixed specimens for 25 min. Slides were then rinsed with deionized water and were mounted in 90% glycerol/PBS. Single and double-indirect immunoperoxidase staining of mast cells was performed as described (6, 16). The mouse monoclonal IgG1, κ anti-tryptase (termed H4) (3) and rabbit polyclonal IgG anti-chymase were used; controls stained with a mouse myeloma IgG1, κ or unimmunized rabbit IgG were negative. T and TC mast cells were delineated as described (6).

Electrophoretic techniques. Western blots were performed after SDS-denatured samples (nonreduced) were subjected to electrophoresis in 12% (w/v) polyacrylamide slab gels (0.75 mm thick) as described (3). Nitrocellulose membranes containing the material transferred from the polyacrylamide gel were incubated with 3% (w/v) gelatin in 0.02 M Tris, pH 7.4, 0.5 M NaCl (TBS) for two 30-min periods in order to block nonspecific reactivity. Mouse anti-tryptase (3) was then applied at 50 µg/ml in TBS containing 0.5% (v/v) Tween-20, and the preparations were incubated overnight at room temperature. The nitrocellulose was then washed with 3% gelatin in TBS and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (1:3000, Boehringer Mannheim, Indianapolis, IN) in TBS containing 1% gelatin for 2 h at room temperature. After the second antibody step, membranes were washed sequentially with TBS containing 0.5% Tween-20 and Veronal buffer (0.188 g/100 ml sodium barbital, 0.248 g/100 ml barbital, and 4.38 g/100 ml NaCl in water) and developed with an alkaline phosphatase substrate solution consisting of nitroblue tetrazolium (0.1 mg/ml), indoxyl phosphate (50 µg/ml), and 4 mM MgCl₂ in Veronal buffer. Reactions were stopped by rinsing in tap water.

RESULTS

Dispersed human lung mast cells were predominantly of the T phenotype, whereas dispersed adult and newborn foreskin mast cells were almost exclusively of the TC phenotype as summarized in Table I. Cytochrome preparations of mast cells from each tissue are shown in Figure 1 after staining in parallel with toluidine blue (A-C), mouse anti-tryptase (D-F), and rabbit anti-chymase (G-I). Mast cells incubated with mouse IgG1 and nonimmune rabbit IgG as controls were negative (not shown). Mast cells from the newborn foreskin stained less intensely with toluidine blue than mast cells from adult foreskin and lung. The mast cells from newborn foreskin also stained less intensely with anti-chymase than mast cells from adult foreskin and the TC subpopulation of lung mast cells. The intensity of mast cell staining with anti-tryptase was not appreciably different for the three cell preparations. Mast cells stained directly with toluidine blue decreased in apparent size as the staining time increased and therefore appeared smaller than mast cells subjected to the immunoperoxidase staining procedure. There was no difference in numbers of mast cells from each tissue detected by the immunoperoxidase technique

TABLE I
Mean levels (± SD) of histamine, tryptase, and chymase in T and TC mast cells^a

Tissue of Origin	Lung (n = 8)	Adult Foreskin (n = 4)	Newborn Foreskin (n = 5)
Histamine			
µg/10 ⁶ mast cells	1.5 ± 0.7 ^b	1.9 ± 0.8	1.6 ± 0.8
Tryptase			
U/10 ⁶ mast cells	0.30 ± 0.21	0.62 ± 0.16	0.27 ± 0.20
µg/10 ⁶ mast cells	10.8 ± 6.7	35.0 ± 12.3	11.5 ± 6.8
Chymase			
U/10 ⁶ mast cells	0.04 ± 0.02	1.01 ± 0.54	0.09 ± 0.05
µg/10 ⁶ mast cells	<0.3 ^{b,c}	4.5 ± 0.9 ^d	<0.6 ^c
%T/%TC mast cells	93/7 ^b	1/99	1/99

^a Mast cell % purities ± SD were 5.1 ± 2.7 for lung, 6.7 ± 2.5 for adult foreskin, and 3.2 ± 0.9 for newborn foreskin.

^b n = 4.

^c Chymase levels were below the limit of sensitivity (0.3 µg/ml) of the radioimmunoassay.

^d n = 3.

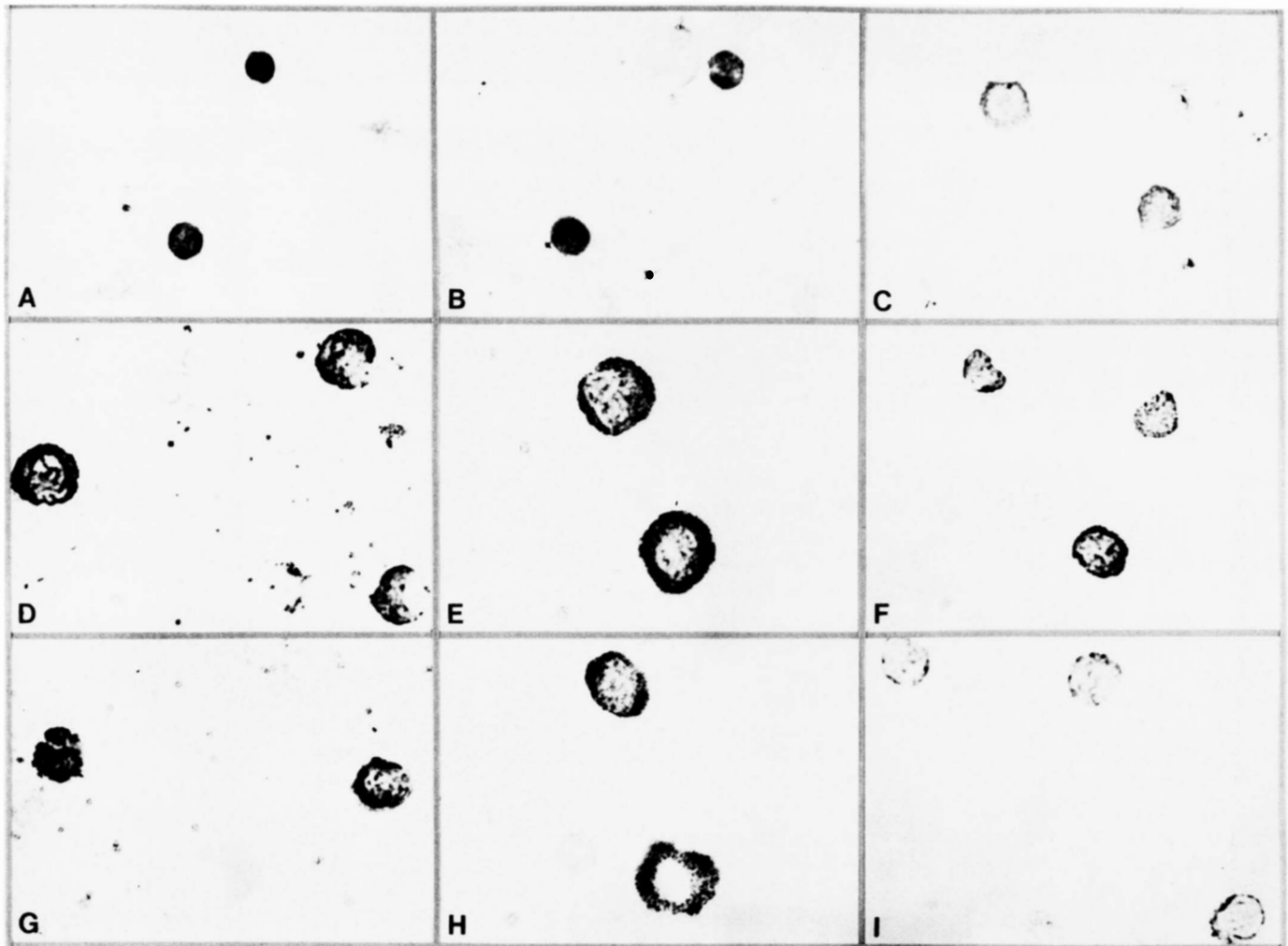


Figure 1. Staining of T and TC mast cells. Cytocentrifuge preparations of mast cells dispersed from lung (A, D, G), adult foreskin (B, E, H), and newborn foreskin (C, F, I) were stained in parallel with toluidine blue (A-C), mouse anti-tryptase (D-F), and rabbit anti-chymase (G-I) as described in *Materials and Methods*.

with anti-tryptase compared with toluidine blue; lung preparations, as expected, showed markedly fewer anti-chymase than anti-tryptase positive mast cells. These observations are consistent with previously reported immunohistochemical results with dispersed lung mast cells and, in addition, show that the predominant phenotype of the mast cells dispersed from adult and newborn foreskin is the TC type.

Preparations of human mast cells dispersed from lung, newborn foreskin, and adult foreskin were analyzed for mediator content as summarized in Table I. The extraction procedure yielded greater than 75% of each mediator in the first of two extractions and minimal additional amounts with a third extraction. There was a tendency for less of the total chymase to be extracted initially than for tryptase and histamine, but this was not statistically significant ($p > 0.05$). Although there were no significant differences between cellular levels of histamine among mast cells from the three types of tissue ($p > 0.4$), this was not the case for the two proteases.

Twofold to threefold greater amounts of immunoreactive tryptase were found in adult foreskin TC mast cells ($35 \mu\text{g}/10^6$ mast cells) than in adult lung ($10.8 \mu\text{g}/10^6$ mast cells) and newborn foreskin ($11.5 \mu\text{g}/10^6$ mast cells) mast cells ($p < 0.025$), mast cells from the latter two

tissue types having similar amounts of this enzyme. The calculated average specific activity ($\pm$ SD) of tryptase in lung cell extracts (28 ± 10 U/mg, $n = 8$) was not significantly different ($p > 0.2$) from that obtained in extracts from adult foreskin (18 ± 4 U/mg, $n = 3$) and newborn foreskin (23 ± 10 U/mg, $n = 5$) preparations. These specific activities were not different from the specific activity of 21 U/mg determined for the purified lung mast cell enzyme (15).

The specific activity of chymase from adult foreskin TC mast cells was calculated to be 224 U/mg. Levels of the enzyme in extracts of newborn foreskin mast cells (99% TC) and lung mast cells (7% TC) were below the limit of sensitivity of the radioimmunoassay. Therefore, immunoreactive levels of chymase in mast cells from adult foreskin ($4.5 \mu\text{g}/10^6$ mast cells) were estimated to be at least sevenfold higher than in those from newborn foreskin ($<0.6 \mu\text{g}/10^6$ mast cells) and at least 15-fold higher than in those from lung ($<0.3 \mu\text{g}/10^6$ mast cells). By comparison, chymase-like enzymatic activity in mast cells from adult foreskin was 11-fold higher than in those from newborn foreskin and 25-fold higher than in those from lung ($p < 0.025$), consistent with the radioimmunoassay results. Assuming that the specific activity of chymase derived from each tissue is the same and that

essentially all chymase resides in the TC mast cell type, chymase levels in newborn foreskin should be $0.40 \mu\text{g}/10^6$ TC mast cells and those in lung should be about $2.6 \mu\text{g}/10^6$ TC mast cells.

The electrophoretic mobilities of the subunits of un-reduced, denatured tryptase were compared for enzyme purified from lung mast cells with that present in extracts of adult lung and foreskin mast cell preparations by Western blot analysis as shown in Figure 2. Tryptase from each tissue showed the same pattern with predominant bands at 31,000 m.w. and 33,000 m.w., as described previously for the purified lung mast cell tryptase in Coomassie Blue-stained polyacrylamide gels (1, 2, 15). This suggests that the tryptase in lung T mast cells is physicochemically identical to that in skin TC mast cells.

DISCUSSION

The levels of tryptase and chymase in dispersed preparations of predominantly T (lung) or TC (skin) mast cells extends the immunohistochemical data that phenotypically defines these two cell types (6) and is consistent with previous quantitative data for tryptase extracted from dispersed lung mast cells (1), and for chymase extracted from skin (4, 5) and from dispersed lung mast cells (9). These levels were determined not only by specific immunoassays, but also by enzymatic assays made relatively specific for chymase by inclusion of aprotinin to inhibit other chymotryptic enzymes such as cathepsin G and for tryptase by inclusion of SBTI. In the case of tryptase, specificity in the lung and skin extracts was indicated by the nearly complete removal of enzyme activity with goat IgG anti-tryptase.

TC mast cells from adult skin contained $4.5 \mu\text{g}$ of chymase (1.01 U of chymase activity) and $35.0 \mu\text{g}$ of tryptase (0.62 U of tryptase activity) per 10^6 mast cells. The weight ratio of tryptase to chymase in adult TC mast

cells was about 7.8:1, indicating the predominance of the tryptic enzyme in TC as well as T mast cells. In addition, the threefold increase in tryptase levels from adult TC vs T mast cells provides a further distinguishing feature between these mast cell types.

Mast cells from adult lung (93% T, 7% TC) contain $10.8 \mu\text{g}$ of tryptase (0.30 U of tryptase activity) and $<0.3 \mu\text{g}$ of chymase (0.04 U of chymase activity) per 10^6 mast cells. The level of chymase-like activity in lung mast cells can be completely accounted for by the fact that 7% of the lung mast cells were of the TC type. Thus, chymase is virtually absent from T mast cells. The apparent correlation between chymase enzymatic and immunoreactive activities in skin and lung extracts suggests the absence in T mast cells of a chymotryptic enzyme that is antigenically distinct from TC mast cell chymase.

The respective levels of tryptase and chymase in mast cells from newborn foreskin are 33% and $<13\%$ of those in mast cells from adult foreskin. Almost all of the mast cells from these tissues are stained with both mouse anti-tryptase and rabbit anti-chymase antibodies (Fig. 1), indicating that the TC mast cell is the predominant type. The lower mediator levels in the newborn mast cells may be indicative of a distinct fetal mast cell type. However, a more likely explanation is that these are TC mast cells that have not yet attained full maturity in terms of mediator content. In this case, the data suggest a delayed production of chymase relative to tryptase and of tryptase relative to histamine in the developing human TC mast cell. A somewhat similar delay in the appearance of rat mast cell chymase relative to histamine and proteoglycan has been observed in developing rat peritoneal cells (17). Whether or not such immaturity is also expressed in terms of the coupled activation-secretion response may need further clarification. Previous reports that mast cells dispersed from the foreskins of children (12) and those in tissue fragments of infant foreskin (18) release histamine by an IgE-dependent mechanism indicate a functional secretory response.

The identification of two types of human mast cells, each with a distinct tissue distribution, provides a situation comparable to that in rodents for consideration of mast cell differentiation. In rodents evidence indicates that different mast cell types originate from a common stem cell (19). How they further differentiate into mucosal and connective tissue mast cells is not certain. The current observations concerning histamine, tryptase, and chymase levels in mast cells from newborn and adult foreskins and adult lung are compatible with a sequential lineage where all mast cells pass through a T mast cell stage, as well as a divergent lineage where a common stem cell gives rise to one or the other type of mast cell. Recent observations indicate that in human subjects with either congenital or acquired disorders of T lymphocyte function a selective deficiency of the T mast cells occurs with normal amounts of TC mast cells (20). This indicates a role for the T lymphocyte in the maturation/proliferation of T but not TC mast cells (as is the case in rodents for the mucosal mast cell type) (21) and favors a divergent lineage between the two human mast cell types.

Tryptases from lung T mast cells and that in skin TC mast cells have been suggested to be distinct enzymes based upon analyses of the kinetics of cleavage of various synthetic substrates (22). This could have reflected the

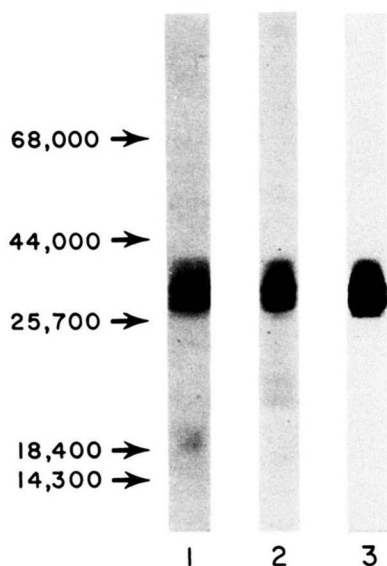


Figure 2. Comparison of unpurified extracts of lung (lane 1, $0.5 \mu\text{g}$ tryptase) and adult foreskin (lane 2, $0.5 \mu\text{g}$ tryptase) mast cell preparations with purified lung tryptase (lane 3, $0.6 \mu\text{g}$) by Western blot analysis. Amounts of tryptase were determined by immunoassay. Molecular weight standards include, from top to bottom, bovine serum albumin, ovalbumin, α -chymotrypsinogen, β -lactoglobulin, and lysozyme. Different photographic exposure times were used to optimize visualization of the darkly stained bands of purified tryptase along with the lighter stained bands of tryptase in the crude extracts.

predominance of the T mast cell subset in human lung versus the TC mast cell type in skin. However, different isolation procedures and salt concentrations under which assays were performed for the skin and lung enzymes, lack of data on the homogeneity of each preparation, and the absence of a stabilizing molecule, like heparin, during performance of the assays make tentative the conclusion that distinct tryptase enzymes reside in skin and lung mast cells. A close similarity between skin and lung tryptase has been suggested by the results of previous immunologic studies (6, 15, 23). The identical subunit structures shown by Western blot analysis (Fig. 2) and similar calculated specific activities together with the immunologic data strongly favor the concept that tryptase from each tissue and mast cell type is the same enzyme.

In summary, examination of dispersed preparations of predominantly T and TC mast cells showed a lack of chymase in T mast cells, thereby explaining prior immunohistochemical findings. Histamine levels were no different between these cell types. The tryptase in both cell types appeared identical. Lower amounts of proteases but not histamine in newborn vs adult TC mast cells suggest a developmental sequence for production of preformed mediators in this type of mast cell in the order of histamine, tryptase, and chymase.

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