Online Data Supplement

Mast cell phenotype, location and activation in severe asthma. Data from the Severe Asthma Research Program

Silvana Balzar, Merritt L. Fajt, Suzy A.A. Comhair, Serpil C. Erzurum, Eugene Bleecker, William W. Busse, Mario Castro, Benjamin Gaston, Elliot Israel, Lawrence B. Schwartz, Douglas Curran-Everett, Charity G. Moore and Sally E. Wenzel

METHODS

Questionnaires

Information collected through questionnaires included general demographics and medical and smoking history. Subjects also graded the frequency of respiratory symptoms over the past 3 months (wheezing, chest tightness, shortness of breath, nighttime symptoms) on 1 (never) - 6 (daily occurrence) scale. For this study, the scale was compressed to 3 levels: never or rarely (1), weekly but less than daily (2) and daily or several times/day (3). They also reported need for 3 or more steroid bursts, asthma-related urgent care visits or hospitalizations, all in the last 12 months. Any of those events were considered a severe exacerbation of asthma.

Pulmonary function and atopy testing

Pulmonary function testing was performed according to ATS guidelines and as previously described (1). The parameters measured included pre-bronchodilator FEV1 and FVC (as % predicted) and FEV/FVC. Maximal (or best) FEV1 (% predicted) was measured following increasing number of puffs of albuterol (to a maximum of 8 puffs). The percent improvement from the pre-bronchodilator FEV1 was the maximal FEV1 (in liters)-prebronchodilator FEV1 (in liters) divided by the prebronchodilator FEV1 (in liters) x 100%. Methacholine provocation concentration (PC_{20}) was determined in all subjects with an FEV1>55% predicted. Atopy was assessed by skin prick testing to 14 common aeroallergens. Subjects were considered to be atopic if there was $\geq$1 positive skin reaction (wheal>the saline control). Serum IgE was measured as well.

Bronchoscopy, bronchoalveolar lavage (BAL) and sample processing

BAL cells and fluid were separated by centrifugation at 400g. BAL fluid from the combined first two syringes was aliquoted and stored at -80ºC for later measurements of tryptase and PGD2 levels. BAL cell counts were performed as previously described and differentials performed on cytospin slides using Protocol Hema 3 set of stains (Fisher Scientific) and counting 300 cells (2).

Immunostaining and tissue morphometric analysis

All tissue samples were immunostained at the Cleveland Clinic site using an automated system. Five-micron tissue sections were deparaffinized, rehydrated and immunostained using automated system Ventana-320-ES (Ventana Medical Systems, Tucson, AZ). Following antigen retrieval (proteinase K treatment for tryptase epitopes and heating in citrate buffer pH 6 for chymase epitopes), and appropriate blocking steps, primary antibodies against tryptase (Promega, G3361) and chymase (Abcam, clone CC1) were applied. Positive staining was visualized with biotin-avidin-peroxidase detection kit (Ventana) and 3-amino-9-ethylcarbazole (AEC) chromogen. Sections were counterstained with Mayer's hematoxylin and overlaid with Crystal-mount (Electron Microscopy Sciences, Hatfield, PA). Positively stained cells were counted in the area between the subepithelial basement membrane and smooth muscle and within an intact epithelial layer, at 400X magnification (3-6). Analyzed tissue areas were measured using Image-Pro software (MediaCybernetics) and cell counts reported per mm^2 of submucosa or, for epithelial cell counts, per 10 mm length of subepithelial basement membrane.

Quantitative real-time polymerase chain reaction

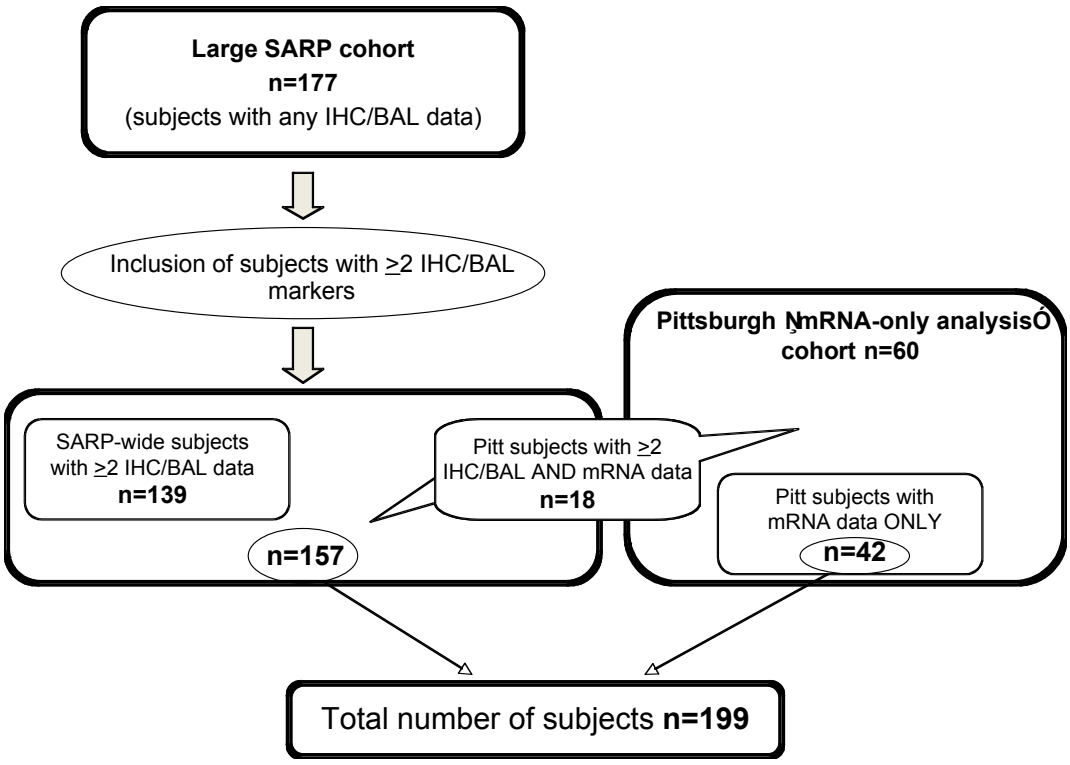
Tissue and epithelial cell expression of tryptase, chymase and carboxypeptidase 3 (CPA3) mRNA was determined by real-time quantitative PCR as described previously (7). The primers and probes were purchased from Applied Biosystems (Foster City, CA, USA). Real-time PCR was performed on the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA) in the core facilities of the University of Pittsburgh. The mRNA levels for each of the mast cell markers were determined by indexing to GAPDH using the formula $1/2^{\Delta Ctx1000}$. Analysis was performed on samples only if the GAPDH threshold was <28 cycles for epithelial cells and ≤32 cycles for tissue.

Final selection of study population

For reasons of the diverse interests of the SARP sites, not all subjects in the initial bronchoscopy dataset/large SARP cohort had measurements available for all four of the mast cell markers (IHC tryptase and chymase, BAL fluid tryptase and PGD2) analyzed. An *a priori* decision was made to include only subjects with data for at least 2 of the 4 mast cell markers. From the initial 177 subjects, 142 subjects had IHC tryptase. Forty-five subjects in that group lacked paired IHC chymase due to: 1) unavailable additional tissue sections (n=24), 2) unsatisfactory quality of staining for chymase (n=15) or 3) loss/damage of sections during staining procedure (n=6), leaving 97 subjects with paired IHC tryptase/chymase data. Thirty-three subjects with IHC tryptase who were missing chymase data had at least one BAL parameter measured, totaling 130 subjects

with IHC tryptase data, while 12 were eliminated as only IHC tryptase was available. An additional 27 subjects were included as they had both BAL tryptase and PGD2 measured (but no IHC data). The final study population included 157 subjects (Table 1A). Of these 157 subjects in Table 1A, 18 were from the PITT center. In addition to IHC and BAL data, these 18 subjects had epithelial brushings and biopsy tissue available for mRNA analysis and are thus also included in the PITT-only mRNA cohort (n=60; Figure E1). Intact epithelium to evaluate epithelial mast cells was present in 96 of the 130 tryptase-stained and 74 of the 97 chymase-stained tissue samples. The five groups were not statistically different in fractions of missing data for submucosal tyrptase or chymase (overall p=0.11 and 0.06, respectively), or for epithelial tryptase or chymase (overall p=0.11 and 0.69, respectively).

Figure E1.



RESULTS

Table E1. Association of mast cell markers (MC_{TC}/MC_{Tot} and BAL fluid PGD2) with frequency of symptoms in subjects with asthma. Presented are median (25^{th}-75^{th} percentile) values for mast cell markers in each of the symptom frequency groups (Kruskal-Wallis tests).

Symptom Mast cell marker	**Frequency of asthma symptoms**			**Overall p-value**
	Group 1 (Never/rarely)	**Group 2** (weekly, but <daily)	**Group 3** (daily, or several times/day)	
Chest tightness				
MC_{TC}/MC_{Tot}	0.32 (0.22-0.44)	0.37(0.16-1.1)	0.77 (0.39-1.1)	0.03
BAL fluid PGD2 (pg /ml)	3 (2-6)	4(2-6.75)	6 (4-15.5)	0.005
Wheezing				
MC_{TC}/MC_{Tot}	0.26 (0.11-0.34)	0.54 (0.28-1)	0.62 (0.31-1.4)	0.002
BAL fluid PGD2 (pg /ml)	3 (2-6)	4.5 (2-6)	6 (4-16.75)	0.003
Shortness of breath				
MC_{TC}/MC_{Tot}	0.25 (0.15-0.34)	0.41(0.25-1.1)	0.74 (0.31-1)	0.01
BAL fluid PGD2 (pg /ml)	3 (2-6)	4 (2-6)	7 (5-13)	<0.001
Nighttime symptoms				
MC_{TC}/MC_{Tot}	0.30 (0.1-0.43)	0.45(0.22-1.1)	0.92 (0.38-2.2)	0.001
BAL fluid PGD2 (pg /ml)	4 (2-5.75)	5 (2-7)	7 (4-18.5)	0.009

Table E2. Strength of relationships (Spearman's correlations) between mast cell markers measured in different airway compartments (r_s and p-value). **A**) by immunostaining/IHC of tissue, **B**) by mRNA levels in tissue samples vs. epithelial brushings.

A

		Submucosa (IHC)		**Epithelium (IHC)**	
		MC_{Tot}	MC_{TC}	MC_{Tot}	MC_{TC}
Submucosa (IHC)	MC_{Tot}		0.34 <0.001	0.49 <0.0001	0.12 0.32
	MC_{TC}	0.34 <0.001		0.27 0.025	0.30 0.009
Epithelium (IHC)	MC_{Tot}	0.49 <0.0001	0.27 0.025		0.21 0.11
	MC_{TC}	0.12 0.32	0.30 0.009	0.21 0.11	

Table E2.

B

		Tissue mRNA		Epithelial mRNA	
		Tryptase	CPA3	Tryptase	CPA3
Tissue mRNA	Tryptase		0.81 <0.0001	0.73 <0.0001	0.63 0.0002
	CPA3	0.81 <0.0001		0.56 0.001	0.57 0.001
Epithelial mRNA	Tryptase	0.73 <0.0001	0.56 0.001		0.74 <0.0001
	CPA3	0.63 0.0002	0.57 0.001	0.74 <0.0001	

A) MC_{Tot} = tryptase-positive (total) mast cells by immunostaining.

MC_{TC} = chymase-positive mast cells by immunostaining.

B) Tryptase and CPA3 (carboxypeptidase A3) mRNA represent MC_{Tot} and MC_{TC}, respectively.

Table E3. Differences between clinical centers in population structure and main parameters measured. Presented are median (25^{th}-75^{th} percentile) values for numerical variables. University of Virginia (contributed 1 subject) was not included in analysis. *Statistically significant differences across sites: African American p=0.0012 (Chi-square test); Severity p<0.0001 (Chi-square test); MC_{TC}/MC_{Tot} p=0.0037 (Kruskal-Wallis); BAL fluid tryptase p<0.0001 (Kruskal-Wallis); BAL fluid PGD2 p<0.0001 (Kruskal-Wallis).

	BWH		CLC		PITT		WFU		WIS		WSL	
	n		n		n		n		n		n	
	8		20		40		20		45		23	
African American*		4 (50%)		5 (25%)		6 (15%)		9 (45%)		4 (9%)		11 (48%)
ASTHMA SEVERITY*												
Normal		0		8 (40%)		15 (38%)		0		7 (16%)		4 (17%)
Mild/no ICS		0		3 (15%)		4 (10%)		5 (25%)		8 (18%)		2 (9%)
Mild/+ICS		6 (75%)		1 (5%)		1 (3%)		4 (20%)		17 (38%)		2 (9%)
Moderate		1 (13%)		2 (10%)		0		5 (25%)		3 (7%)		2 (9%)
Severe		1 (13%)		6 (30%)		20 (50%)		6 (30%)		10 (22%)		13 (57%)
MC_{Tot}/mm^2	8	133	17	215	28	107	18	107	36	163	23	78

		(44- 230)		(164-323)		(20-208)		(35-256)		(76-201)		(12-283)
MC_{TC}/mm^2	6	46 (9-76)	14	31 (3-84)	21	54 (28-80)	18	61 (14-119)	19	30 (0-72)	19	63 (31-136)
MC_{TC}/MC_{Tot}*	5	0.30 (0.29-0.34)	14	0.16 (0.05-0.33)	21	0.45 (0.27-1)	15	0.42 (0.26-1)	18	0.28 (0.03-0.54)	18	1.2 (0.30-3.8)
Epithelial MC_{Tot}/10mm	3	0	11	0 (0-40)	24	6.3 (0-56)	11	8.7 (0-48)	26	0 (0-24)	21	0 (0-39)
Epithelial MC_{TC}/10mm	5	0	9	0 (0-5.4)	17	0 (0, 0)	12	0 (0, 0)	15	0 (0, 0)	16	3 (0-19)
BAL fluid tryptase*	0		19	34 (10-137)	34	209 (90-546)	16	96 (51-329)	44	63 (29-113)	18	38 (10-83)
BAL fluid PGD2*	8	5 (3.5-9.5)	19	7 (5-11)	33	7 (5-13)	17	2 (2-6)	42	3 (2-5)	18	2 (2-5)

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