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REPORT OF WORKING GROUP 4

Analysis of fluid-phase mediators

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Sputum cellular indices are valid, reliable and responsive to change [1–7]. Increasingly, numerous inflammatory mediators are being measured in the fluid phase of sputum; these include cytokines, chemokines, granulocyte proteins, markers of vascular leakage, eicosanoids, proteases and others. Many of these mediators are included in table 1, which gives details of methods used by investigators to process sputum and measure mediator levels. The table also provides the median/mean levels measured in studied subject groups to give an indication of the expected levels of these mediators in sputum. However, the reproducibility, precision and validity of many of these measurements in sputum have not been investigated and, therefore, their utility as a research and clinical tool remains uncertain and requires confirmation.

The following issues are important in the analysis of mediators: 1) choice of methods for measuring fluid-phase mediators; 2) points to consider when planning an immunoassay; and 3) evaluation of the measurement of a soluble mediator in sputum, *i.e.* validation of the measurement method.

Methods for measuring fluid-phase mediators

The three main types of method used for the measurement of sputum fluid-phase mediators are bioassays, enzyme assays and immunoassays.

Bioassays

Bioassays rely on the retention of biological activity and the ability to exert a measurable effect, such as proliferation of cells, bone marrow colony formation or chemotaxis [3, 11, 58]. Sputum processing with mucolytics such as dithiothreitol (DTT) or dithioerythritol, which are strong reducing agents, may decrease the biological activity of cytokines, many of which rely on disulphide bonds to provide a stable structure for bioactivity. Bioassays also have the disadvantage of being inconvenient, time-consuming and lacking in specificity [59]. In addition, the presence of commonly occurring endogenous cytokine inhibitors, although allowing an estimate of net activity, may result in significant underestimation of total cytokine levels [58].

Enzyme assays

Many of the mediators of the inflammatory response are enzymes, released from cellular sites of synthesis into an environment replete with enzyme inhibitors. Again, an estimate of net activity is important when evaluating their potential contribution to tissue responses. The proteases neutrophil elastase [20, 22, 60, 61], cathepsin G [20, 61] and cathepsin B [22] have been assayed in sputum using specific chromogenic substrates, from which proteases release a coloured product that can be quantified spectrophotometrically. Net protease activity is determined using purified enzyme standards. Active forms of matrix metalloproteinases in sputum are identified by means of substrate gel zymography, and net activity can be quantified using radiolabelled substrates [62]. Other proteases, such as chymase and tryptase, are less robust. Their activity rapidly diminishes on freezing/thawing sputum samples, and immunoassays are the best means of detecting them (see below).

The activity of enzymes involved in oxidant/antioxidant balance, myeloperoxidase (MPO) [60, 61, 63] and catalase [63], similarly can be measured in sputum using spectrophotometry. The presence of sulphated glycoconjugates and deoxyribonucleic acid (DNA) in sputum in severe asthma [64] may inhibit MPO activity [63], and immunoassays carried out in parallel may also be required. However, studies in which sputum was spiked with MPO have

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Table 1. – Measurement of mediators levels in sputum

Mediator	Sputum processing	Recovery %	Effect of DTT on gradient of standard curve[#]	Mediator concentration[¶]	Subjects	Assay	[Ref.]
Eosinophil proteins							
ECP	1	ND	Decreased	288–1040 ng·mL^{-1}	N, A, SB	RIA, Pharmacia	[2]
	2	ND	ND	49–107 ng·mL^{-1}	A	RIA, Pharmacia	[3]
	3	>85	Not used	0–1800 ng·mL^{-1}	A, CF, P	RIA, Pharmacia	[8]
	4	100^{+}	Decreased 30–50%	12.8 ng·mL^{-1}	A, N	FEIA, Pharmacia	[9]
	2	ND	ND	730–910 ng·mL^{-1}	A, N	RIA, Pharmacia	[10]
	4	100	ND	6–26 ng·mL^{-1}	A, N	FEIA, Pharmacia	[11]
	Selected plus 10 vol 0.1% DTT for 30 min; 700×*g* for 10 min at 4°C	98.6^{+}	No effect	51–4605 ng·mL^{-1}	N, A, COPD, B	RIA, Pharmacia	[12]
	3 (centrifuged repeatedly)	ND	ND	1200 ng·mL^{-1}	CF	RIA, Pharmacia	[13]
EPX/EDN	3	ND	Not used	100–4700 ng·mL^{-1}	A, CF, P	RIA, Pharmacia	[8]
	1	ND	ND	270–1510 ng·mL^{-1}	N, A, SB	RIA	[2]
EPO	5	ND	No effect	40–178 ng·mL^{-1}	A, COPD	FEIA	[14]
	1	ND	ND	4.5–28.1 ng·mL^{-1}	N	FEIA	[15]
MBP	1	ND	ND	304–1176 ng·mL^{-1}	A, N, SB	IMRA	[2]
Neutrophil proteins							
HNE	5	ND	No effect	4.2–5.2 µg·mL^{-1}	COPD	EA (*N*-MSN)	[16]
	2	ND	ND	0–20 µg·mL^{-1}[§] (free) 0–52 µg·mL^{-1}[§] (total)	A, N, COPD	EA (*N*-MSN) ELISA	[17]
	Unselected plus 1 vol PBS; 25000×*g* for 20 min	ND	Not used	2.6–5.9 µM	CF	EA (*N*-MSN)	[18]
	2	ND	No effect	16 µM	CF	EA (*N*-MSN)	[19]
	6	ND	Not used	36.5–384.5 µg·mL^{-1}[§]	B	EA (*N*-MSN)	[20]
	7	ND	Not used	0–2.1 µM	COPD, B	EA (*N*-MSN)	[21]
Cathepsin G	6	ND	Not used	0.2–54.9 µg·mL^{-1}[§]	B	EA (*N*-SN)	[20]
Cathepsin B	7	ND	Not used	65.8 U·mL^{-1}	B	EA (*N*-MESA)	[22]
MPO	5	ND	No effect	0.6–8.1 ng·mL^{-1}	A, COPD	RIA, Pharmacia	[23]
	3 (centrifuged repeatedly)	ND	ND	110 ng·mL^{-1}	CF	RIA, Pharmacia	[13]
	Selected plus 9 vol 0.1% DTT in shaking waterbath, at 37°C	ND	ND	1–220 ng·mL^{-1}	A (children)	ELISA, R&D Systems	[24]
	4	78	ND	188–382 ng·mL^{-1}	A, N	RIA, Pharmacia	[11]
	1	ND	ND	1.9 ng·mL^{-1}	A	RIA, Pharmacia	[25]
	7	>85[f] 29–70	Not used Not used	3–15.7 U·mL^{-1}[§] 391–4557 ng·mL^{-1}	COPD, B	EA (*o*-DH_2Cl) ELISA, R&D Systems	[21]
HNL	5	ND	ND	1.7–10.5 mg·mL^{-1}	A, COPD	FEIA	[23]
	1	ND	ND	7.1–12.4 mg·mL^{-1}	N	FEIA	[15]
	5	ND	ND	3.7–5.1 mg·mL^{-1}	A	FEIA	[14]

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Table 1. – Continued

Mediator	Sputum processing	Recovery %	Effect of DTT on gradient of standard curve[#]	Mediator concentration[¶]	Subjects	Assay	[Ref.]
Mast cell products							
Histamine	2	100[f]	ND	7–10 ng·mL^{-1}	A, N	RIA, AMAC, Inc.	[26]
	4	100[+]	No effect (but lower levels with DTE)	0.95 ng·mL^{-1}	A, N	RIA, Serotec, Inc.	[9]
Tryptase	1	ND	ND	12–20.8 U·L^{-1}	A, N, SB	IMRA, Pharmacia	[2]
	4	ND	ND	0–7.2 ng·mL^{-1}	A, N	RIA, Pharmacia	[11]
	2	100[f]	ND	1.3–6.8 U·L^{-1}	A, N, SB	RIA, Pharmacia	[26]
	4	100[+]	No effect	8.8 ng·mL^{-1}	A, N	RIA, Pharmacia	[9]
	2	ND	ND	1–6.1 ng·mL^{-1}[§] (detectable in 18%)	A, N	FEIA, Pharmacia	[27]
Plasma exudate markers							
Albumin	1	ND	No effect	288–704 μg·mL^{-1}	N, A, SB	ELISA	[2]
	2	ND	ND	52–140 μg·mL^{-1}	A	Nephelometry	[3]
	2	ND	ND	9.9–32.7 μg·mL^{-1}	A	Turbidimetry	[28]
	8	SSP 99[f]	Decreased, restored by NEM	SSP 29.3–3300 ng·mL^{-1}	A, COPD	Immunoturbidimetry, Roche Diagnostics	[29]
Fibrinogen	1	ND	ND	0.44–2.08 μg·mL^{-1}	N, A, SB	ELISA	[2]
	2	ND	ND	0.17–1.29 μg·mL^{-1}	A	ELISA	[3]
	1	ND	ND	2.7–250 μg·mL^{-1}	A, COPD	ELISA	[30]
	2	100[f]	ND	11.9–44.2 μg·mL^{-1}	A, N	ELISA	[26]
α_2-M	8	106[f] SSP 93 SGP (after NEM)	Not used Decreased, restored by NEM	SSP 900–1100 ng·mL^{-1} SGP 1900–2100 ng·mL^{-1}	A, COPD	ELISA	[29]
	7	ND	Not used	0.16–0.21 μM	COPD	Radial immunodiffusion	[31]
	Frozen, ultrasonicated 15 min, and centrifuged at 32000×*g* for 15 min	ND	Not used	1.66–18.38 μg·mL^{-1}	N	RIA	[32]
Cytokines							
GM-CSF	3	>85	Not used	0–91.8 pg·mL^{-1}	A, CF, P	ELISA, R&D Systems	[8]
	4	68[f]	ND	Detectable <5%	A, N	ELISA, R&D Systems	[11]
	9	77	Not used	Detectable <1%	A, N	ELISA Amersham Biosciences	[33]
	5	ND	ND	1.3–1.5 ng·mL^{-1}	A	ELISA	[14]
IL-1β	Selected plus 7 vol 0.05% DTT	ND	ND	4.0–9.0 ng·mL^{-1}	A	ELISA, R&D Systems	[34]
	10	ND	Not used	0.269–12.22 ng·mL^{-1}	COPD, CF, N	ELISA, R&D Systems	[35]

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Table 1. – Continued

Mediator	Sputum processing	Recovery %	Effect of DTT on gradient of standard curve[#]	Mediator concentration[¶]	Subjects	Assay	[Ref.]
	9	80.9	Not used	0–0.40 ng·mL^{-1}	A, N	ELISA, Amersham Biosciences	[33]
	Unselected, frozen, thawed and centrifuged at 100000×*g* for 30 min at 4°C	ND	Not used	8.21–17.96 ng·mL^{-1}	B	ELISA, R&D Systems	[36]
IL-2	9	77.8	Not used	40–70 pg·mL^{-1}	A, N	ELISA, Amersham Biosciences	[33]
IL-3	3	>85	Not used	0–39 pg·mL^{-1}	A, CF, P	ELISA, R&D Systems	[8]
IL-5	1	ND	No effect	9.4–46 pg·mL^{-1} (detectable 26.5%)	N, A, SB	ELISA, R&D Systems	[2]
	3	>85	Not used	0–0.17 ng·mL^{-1}	A, CF, P	ELISA, R&D Systems	[8]
	1	ND	ND	0–0.16 ng·mL^{-1}	A	ELISA, R&D Systems	[25]
	5	ND	ND	0.11–0.16 ng·mL^{-1} (detectable <30%)	A, COPD	ELISA, R&D Systems	[14]
	Selected plus 9 vol 0.1% DTT in shaking waterbath at 37°C	ND	ND	Detectable <5%	A (children)	ELISA, R&D Systems	[24]
	4	80[f]	ND	Detectable <5%	A, N	ELISA, R&D Systems	[11]
	1	ND	ND	Detectable 0%	A, N	ELISA, R&D Systems	[30]
	1	ND	ND	44–67 pg·mL^{-1} (detectable 62.5%)	A	ELISA, R&D Systems	[37]
	Unselected plus 3 vol PBS centrifuged at 40000×*g* for 30 min at 4°C	ND	Not used	1.18 ng·mL^{-1}	A	ELISA, GIF	[38]
	5	ND	No effect	2.1–3.6 ng·mL^{-1}	A	ELISA	[14]
IL-6	2	ND	ND	79–187 ng·mL^{-1}	A	ELISA	[28]
	2	ND	ND	27–45 pg·mL^{-1}	N	ELISA, R&D Systems	[39]
	1	ND	ND	64–120 pg·mL^{-1}	COPD	ELISA, R&D Systems	[40]
	10	ND	Not used	26–620 pg·mL^{-1}	COPD, CF, N	ELISA, R&D Systems	[35]
	9	80	Not used	50–310 pg·mL^{-1}	A, N	ELISA, Amersham Biosciences	[33]
IL-10	1	ND	ND	0–68 pg·mL^{-1}	A, N, COPD	ELISA, R&D Systems	[41]
	10	ND	Not used	24–177 pg·mL^{-1}	COPD, CF, N	ELISA, R&D Systems	[35]
TNF-α	5	ND	ND	1.02–1.73 ng·mL^{-1}	A	ELISA	[14]
	7	52–60[+]	Not used	0–7.5 nM[§]	N, A, COPD	ELISA	[42]
	3	67–75[f]	Not used	0.10–0.15 ng·mL^{-1}	CF	ELISA, R&D Systems	[43]
	Unselected, frozen, thawed, and centrifuged at 100,000 *g*, for 30 min at 4°C	ND	Not used	44–120 pg·mL^{-1}	B	ELISA, R&D Systems	[36]

Table 1 continued on next page.

Table 1.–Continued

Mediator	Sputum processing	Recovery %	Effect of DTT on gradient of standard curve[#]	Mediator concentration[¶]	Subjects	Assay	[Ref.]
TGF-β_1	Unselected plus 3 vol PBS centrifuged at 40000×*g* for 30 min at 4°C. Acid activation	ND	Not used	21.7 ng·mL^{-1}	A	ELISA, R&D Systems	[38]
ET-1	Selected, centrifuged at 13000 rpm, for 15 min. HPLC, on C18 column	88	Decreased, therefore not used	11–16 pg·mL^{-1}	N, A	RIA, Nichols Institute Diagnostics	[44]
IFN-γ	1	ND	ND	Detectable 0%	A, N	ELISA, R&D Systems	[30]
	9	75.3	Not used	30–70 pg·mL^{-1} (detectable 23%)	A, N	ELISA, Amersham Biosciences	[33]
Chemokines							
IL-8	3	>85	Not used	1.27–4.95 ng·mL^{-1}	A, P, CF	ELISA, Bender MedSystsems	[8]
	2	ND	ND	2.1–2.7 ng·mL^{-1}	A	ELISA	[3]
	5	ND	No effect	2.1–3.5 nM	A	RIA	[23]
	7	>90[f]	Not used	0–3.5 nM[§]	A, N, COPD	RIA	[42]
	3	98[f]	Not used	30–49 ng·mL^{-1}	CF	ELISA, R&D Systems	[43]
	4	21[f]	ND	41–87 pg·mL^{-1} (free; detectable 45%)	A, N	ELISA	[11]
	4	38.3 (free)$^{+}$; 100 (total)$^{+}$	No effect	0 ng·mL^{-1} (free)	A, N	ELISA	[9]
	1	ND	ND	2.88–16 ng·mL^{-1}	A, N	ELISA, R&D Systems	[30]
	5	ND	Decreased	6.9–10.9 ng·mL^{-1} (total)	COPD	ELISA, Amersham Biosciences	[16]
RANTES	3	>85	Not used	15.6–151.5 ng·mL^{-1}	A, P, CF	ELISA, R&D Systems	[8]
	4	82[f]	ND	Detectable <5%	A, N	ELISA, R&D Systems	[11]
	9	74.6	Not used	40–251 pg·mL^{-1}	A, N	ELISA, Amersham Biosciences	[33]
Eotaxin	10	ND	Not used	14–530 pg·mL^{-1}	A, N	ELISA	[45]
	1 (on ice)	ND	ND	0.31 ng·mL^{-1} (detectable 86%)	A	ELISA, R&D Systems	[46]
MCP-1	2	ND	ND	1.6–3.9 μg·mL^{-1}	A	ELISA, R&D Systems	[28]
	3 (centrifuged 1000×*g* for 10 min)	ND	Not used	0–25 mg·mL^{-1}[§]	A	ELISA	[47]
MIP-1α	3 (centrifuged 1000×*g* for 10 min)	ND	Not used	0–25 mg·mL^{-1}[§]	A	ELISA	[47]
Soluble cytokine receptors							
IL-1RA	10	ND	Not used	31–94 ng·mL^{-1}	COPD, CF, N	ELISA, R&D Systems	[35]
sTNFRII	10	ND	Not used	0.44–4.9 ng·mL^{-1}	COPD, CF, N	ELISA, R&D Systems	[35]
sICAM-1	3	35–50[f]	Not used	0.63–1.9 ng·mL^{-1}	CF	ELISA, British Biotech	[43]
	2 (1 mM DTT)	ND	ND	1.1–11.9 ng·mL^{-1}	A, N	ELISA, Boehringer Mannheim	[48]
	4	86[f]	ND	12–52 ng·mL^{-1}	A, N	ELISA, R&D Systems	[11]

Table 1 continued on next page.

Table 1. – Continued

Mediator	Sputum processing	Recovery %	Effect of DTT on gradient of standard curve[#]	Mediator concentration[¶]	Subjects	Assay	[Ref.]
Proteases/inhibitors							
MMP-1	5	ND	Decreased	19.1–20.5 ng·mL^{-1}	COPD	ELISA, Amersham Biosciences	[16]
MMP-9	2	ND	ND	23–80.5 ng·mL^{-1}	A, N, COPD	ELISA, Amersham Biosciences; zymography	[49]
	5	ND	Decreased	13.2–13.7 ng·mL^{-1}	COPD	ELISA, Amersham Biosciences	[16]
	3	ND	Not used	500–3000 ng·mL^{-1}	A	ELISA, Amersham Biosciences	[50]
TIMP-1	2	ND	ND	60–475 ng·mL^{-1}	A, N, COPD	ELISA, Amersham Biosciences	[50]
	5	ND	Decreased	14.7–15.1 ng·mL^{-1}	COPD	ELISA, Amersham Biosciences	[16]
	3	ND	Not used	200–550 ng·mL^{-1}	A	ELISA, Amersham Biosciences; zymography	[50]
SLPI	5	ND	Decreased	5.3–5.7 μg·mL^{-1}	COPD	ELISA, Amersham Biosciences	[16]
	7	>85[f]	Not used	Not given	COPD, B	ELISA, R&D Systems	[21]
	7	ND	Not used	1.5–1.8 μM	COPD	ELISA	[31]
α_1-AT	2	ND	ND	0–78 μg·mL^{-1}[§]	A, N, COPD	Nephelometry	[17]
	7	ND	Not used	2.4–2.6 μM	COPD	Radial immunodiffusion	[31]
Eicosanoids							
LTB_4	12 (with HPLC)	27.8[f] (radiolabelled, all stages assessed)	Not used	44.3 nM	CF	RIA	[51]
	7	>85[f]	Not used	Not given	COPD, B	ELISA, Amersham Biosciences	[21]
	Unselected, frozen, centrifuged at 100000×*g* for 30 min at 4°C	ND	Not used	1.62–3.17 ng·mL^{-1}	B	ELISA, R&D Systems	[36]
LTC_4, LTD_4, LTE_4	12 (with HPLC)	19–27.2[+] (radiolabelled, all stages assessed)	Not used	74.4 nM	CF	RIA	[51]
	1 (on ice); C18 column	80–85[f] (radiolabelled)	ND	6.4–13 ng·mL^{-1}	A, N	ELISA, Cayman Chemicals	[52]
	1 (on ice); C18 column	80–85[f] (radiolabelled)	ND	3.45–11.95 ng·mL^{-1}	A	ELISA, Cayman Chemicals	[53]
	12	ND	Not used	1.0–5.7 nM	B, COPD, CF	RIA	[54]
	Ethanol, C18 column	ND	Not used	2.31 ng·mL^{-1}	A, N	ELISA, Cayman Chemicals	[55]
PGD_2	1 (on ice)	ND	ND	0.15–0.4 ng·mL^{-1}	A, N	MS	[52]
	1 (on ice)	ND	ND	0.36–0.51 ng·mL^{-1}	A	MS	[53]

Table 1 continued on next page.

Table 1. – Continued

Mediator	Sputum processing	Recovery %	Effect of DTT on gradient of standard curve[#]	Mediator concentration[¶]	Subjects	Assay	[Ref.]
PGE_2	1 (on ice)	ND	ND	1.2–1.6 ng·mL^{-1}	A, N	MS	[52]
	1 (on ice)	ND	ND	1.09–1.11 ng·mL^{-1}	A	MS	[53]
	12	ND	Not used	19.3–45.7 nM	B, COPD, CF	RIA	[54]
TxB_2	1 (on ice)	ND	ND	0.7–1.3 ng·mL^{-1}	A, N	MS	[52]
	12	ND	Not used	6.3–10.5 nM	B, COPD, CF	RIA	[54]
	Ethanol, C18 column	ND	Not used	0.88 ng·mL^{-1}	A, N	ELISA, Cayman Chemicals	[55]
$PGF_2\alpha$	1 (on ice)	ND	ND	0.3–0.7 ng·mL^{-1}	A, N	MS	[52]
	12	ND	Not used	4.5–10.4 nM	B, COPD, CF	RIA	[54]
Miscellaneous							
NO products	Selected plus 7 vol 0.05% DTT	ND	ND	330–400 μM	A	Greiss reaction	[34]
	Unselected plus 4 vol 0.005% DTT, centrifuged at 13000×*g* for 30 min	ND	ND	387–774 μM	N, CF	Greiss reaction	[56]
Lactoferrin	2	ND	ND	35.2–118.9 μg·mL^{-1}	A, N	ELISA	[57]
MLG	2	ND	ND	562–2574 μg·mL^{-1}	A, N	ELISA	[57]
DNA	2	ND	ND	3.6–7.1 μg·mL^{-1}	A, N	Microfluorimetry, Calbiochem-Behring	[57]
Total IgA	4	ND	ND	10.5–18.4 μg·mL^{-1}	A, N	ELISA	[11]
SC	4	ND	ND	3.2–4.1 μg·mL^{-1}	A, N	ELISA	[11]

Sputum was processed as follows: 1) selected sputum, 4 volumes (vol) 0.1% (6.5 mM) dithiothreitol (DTT) and 4 vol phosphate-buffered saline (PBS) centrifuged at 500×g for 10 min; 2) unselected sputum and 1 vol 0.1% DTT in shaking water-bath at 37°C for 15 min, centrifuged at 2,000 revolutions per minute (rpm) for 5 min; 3) unselected sputum and 1–4 vol PBS centrifuged at 2000×g for 20–30 min at 4°C; 4) unselected sputum and 1 vol 10 mM dithioerythritol (DTE) centrifuged at 400×g for 10 min at 4°C; 5) unselected sputum and 2 mL 1% DTT centrifuged at 300×g for 10 min; 6) unselected sputum and 3 vol PBS centrifuged at 8,800×g for 20 min; 7) unselected sputum centrifuged at 50,000–60,000×g for 60–90 min at 4°C; 8) thawed frozen spontaneous sputum centrifuged at 50,000×g for 90 min at 4°C (sputum sol phase (SSP)); rest mixed with 1 vol 10 mM DTT and 150 U DNAse type IV (Sigma, St Louis, MO, USA) for 60 min at 4°C and then centrifuged at 50,000×g for 90 min at 4°C (sputum gel phase (SGP)); 9) unselected sputum and 10 vol PBS glass homogenised at 4°C and then centrifuged at 3,000 rpm for 15 min at 4°C; 10) selected sputum and 1 vol PBS centrifuged at 8,800×g for 20 min; and 11) unselected sputum collected into ethanol on ice and centrifuged at 12,000×g for 20 min, evaporated to dryness and separated on C18 column; The locations of the companies from which commercial assays were obtained are as follows: Pharmacia: Uppsala, Sweden; R&D Systems: Minneapolis, MN, USA;; AMAC, Inc.: Westbrook, ME, USA; Serotec, Inc.: Raleigh, NC, USA; Roche Diagnostics: Grenzacherstrasse, Basle, Switzerland; Amersham Biosciences: Little Chalfont, UK; GIF: Munster, Germany; Nichols Institute Diagnostics: San Clemente, CA, USA; Bender Medsystems: Vienna, Austria; British Biotech: Oxford, UK; Boehringer Mannheim: Mannheim, Germany; Cayman Chemicals: Ann Arbor, MI, USA; and Calbiochem-Behring: La Jolla, CA, USA; ECP: eosinophil cationic protein; EPX/EDN: eosinophil protein X or eosinophil-derived neurotoxin; EPO: eosinophil peroxidase; MBP: major basic protein; HNE: human neutrophil elastase; MPO: myeloperoxidase; HNL: human neutrophil lipocalin; α_2-M; α_2-macroglobulin; GM-CSF: granulocyte-macrophage colony-stimulating factor; IL: interleukin; TNF: tumour necrosis factor; TGF-β_1: transforming growth factor-β_1; ET-1: endothelin-1; IFN-γ: interferon gamma; RANTES: regulated on activation, normal T-cell expressed and secreted; MCP-1: monocyte chemotactic protein-1; MIP-1α: macrophage inflammatory protein-1α; IL-1RA: IL-1 receptor antagonist; sTNFRII: type II soluble TNF receptor; sICAM-1: soluble intercellular adhesion molecule-1; MMP: matrix metalloproteinase; TIMP: tissue inhibitor of metalloproteinase; SLPI: secretory leucocyte protease inhibitor; α_1-AT: α_1-antitrypsin; LT: leukotriene; PG: prostaglandin; TxB_2: thromboxane B_2; MLG: mucin-like glycoprotein; DNA: deoxyribonucleic acid; Ig; immunoglobulin; SC: secretory component of IgA; ND: not determined; N: normal control; A: asthmatic; SB: smokers' bronchitis; RIA: radioimmunoassay; CF: cystic fibrosis; P: pneumonia; FEIA: fluorimetric enzyme immunoassay; COPD: chronic obstructive pulmonary disease; B: bronchiectasis; IMRA: immunoradiometric assay; EA: enzyme assay (specified substrate); *N*-MSN: *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide; ELISA: enzyme-linked immunosorbent assay; *N*-SN: *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide; *N*-MESA: 2-(*N*-morpholino)ethanesulphonic acid; *o*-DH_2Cl: *o*-dianisidine dihydrochloride; NEM: *N*-ethylmaleimide; HPLC: high-performance liquid chromatography (reverse phase); MS: mass spectroscopy. [#]: ND indicates that the effect of DTT on the standard curve was not investigated despite DTT being used to process the sputum; not used indicates that DTT was not used to process the sputum. [¶]: data are presented as range of mean/median values; [+]: sputum spiked preprocess (no label indicates that it is not known when the sputum was spiked); [§]: range (not mean range); [*f*]: sputum supernatant spiked.

yielded better recovery in the enzyme assay than the immunoassay [21].

Immunoassays

Immunoassays are the method of choice for measuring sputum fluid-phase mediators because of their convenience, reproducibility and specificity. Their sensitivity is improving, and now approaches that of bioassays. A full description of the theoretical basis of immunoassay is beyond the scope of this report; however, the most widely used are competitive radioimmunoassays (RIAs), using radiolabelled antigen, and immunometric assays such as enzyme-linked immunosorbent assays (ELISAs), which use enzyme-labelled antibodies.

Immunoassays are indirect methods whereby the concentration of analyte is measured with respect to a parameter related to its concentration, using either counts per minute (radioactivity) or the optical density of the product of the antibody-linked enzyme at a certain wavelength [65]. In competitive assays, the concentration of mediator present in the sample is inversely proportional to the radioactivity (RIA), whereas, in immunometric assays, the mediator concentration is proportional to the optical density of the product formed by the conjugated enzyme (ELISA). The concentration of the mediator is calculated with reference to a standard curve constructed by serial dilution of a standard mediator preparation. Since the standard is often a recombinant protein, its structure and degree of glycosylation and amidation depend on how it is produced [58]. Therefore, the antibodies used often have different affinities for the standard as opposed to the endogenous mediator. Standardisation between different assays for valid comparisons is, therefore, problematic [66]. In response to this, the World Health Organization Consultative Group on Cytokines is assigning International Standards or Reference Reagents [67–69] for each known cytokine, which are intended to be used to calibrate secondary and working standards. This will eventually allow valid interassay comparisons. Similar standards are also required for immunoassays of other soluble mediators [70].

Points to consider when planning an immunoassay

Immunoassay is the commonest means of measuring mediator levels. Therefore, its use is considered here in detail, although some of the following points could be applied to bioassays and enzyme assays. It is imperative that factors affecting the validity of the assay be recognised and controlled for, including those specific to: 1) the assay itself; 2) the soluble mediator being measured; and 3) the unique nature of sputum and the method used to process it.

Factors specific to the assay

The ease of production of monoclonal antibodies as well as recombinant antigens has permitted the development of numerous commercial and laboratory-specific ("in-house") immunoassays, particularly ELISAs. The immunoassay operator should be trained in the application of immunoassays and be able to troubleshoot [71]. Variability between production lots should be minimal, and the operator should be satisfied that the laboratory or company supplying the kits is reliable. Information on the validity, sensitivity, specificity, cross-reactivity, predictive value and precision of each assay should be available [21, 71], but additional quality control and optimisation often need to be performed by the operator. The sensitivity of the assay refers to the lowest concentration that can be reliably detected above the background, and is, therefore, its detection limit. It is usually expressed as the amount of analyte detectable per millilitre. However, some manufacturers convert this figure to the amount of analyte per well, which is a much smaller figure, and it is necessary to be aware of this. The lowest standard suggested by the manufacturer is usually the detection limit of the assay, although operators can test this for themselves. The stability of reagents such as the standard used in the assay should be ensured by storage at the correct temperature. If necessary, the standard should be stored frozen in aliquots so that it is not continually subjected to freezing and thawing. Internal laboratory controls should be used routinely in each assay so as to determine intra- and interassay variability in the hands of the user.

Factors specific to the soluble mediator

Cytokines are often bound to various molecules present in biological fluids (such as soluble receptors, α_2-macroglobulin and autoantibodies), and these are usually present in excess [72–78]. A cytokine may not be recognised by the capture antibody of the assay if the relevant epitope is hidden when the cytokine is bound within a complex. Polyclonal capture antibodies recognise several epitopes and, therefore, may be more effective in detecting complexed cytokines. Competitive assays using monoclonal antibodies are likely to be more accurate in the presence of complexed cytokine but are not widely available. Chemokines, such as interleukin (IL)-8, are small highly basic proteins, with an affinity for acidic negatively charged molecules, such as heparin, that are present in sputum samples [79]. Heparin inhibits the chemotactic function of soluble IL-8 *in vitro* [80], although binding does not necessarily imply an effect on function. Using gel filtration chromatographic analysis of IL-8 in sputum samples, very high-molecular-weight forms [81] that no longer bind to heparin are found, and these include IL-8 bound to α_2-macroglobulin [80, 81], immunoglobulin A [82], DNA [83] and actin [83]. These high-molecular-weight molecules not only potentially mask detection of IL-8 in immunoassays [83] but also affect the function of this chemokine. DNA is present in relatively high concentrations in sputum from patients with severe asthma [64], chronic obstructive pulmonary disease [85] and cystic fibrosis [64, 85]; this may inhibit the function of IL-8 in

sputum [85]. These factors should be recognised when interpreting results.

Factors specific to the unique nature of sputum and the method used to induce and process it

The unique nature of sputum. Many of the assays used to measure soluble mediator levels in sputum have been developed for serum or culture supernatants. However, in being a mixture of mucus, cellular degradation products, DNA, substances secreted by airway cells (including proteases, soluble receptors, autoantibodies, and binding and carrier proteins), and inflammatory and epithelial cells, sputum cannot be assumed to be equivalent to either of these. Sputum, like all biological fluids, may produce matrix effects in assays by alteration of pH or ionic strength. Spontaneously expectorated sputum from patients with infective bronchitis, bronchiectasis or cystic fibrosis is even more complex due to high levels of viscid DNA and actin released from necrotic inflammatory cells in addition to high concentrations of proteases [57]. As mentioned in the *Factors specific to the soluble mediator* section, α_2-macroglobulin, which is present in sputum, binds certain cytokines (platelet-derived growth factor, IL-2, IL-1β, IL-6, tumour necrosis factor-α (TNF-α), interferon gamma and IL-8), and may interfere with the recognition of cytokine epitopes by the assay [86].

The method used to obtain sputum. Induced *versus* spontaneous sputum. Fibrinogen levels have been found to be increased in spontaneous expectorated samples compared to induced sputum [87]. There is a trend towards increased eosinophil cationic protein (ECP) levels in spontaneously expectorated sputum.

Factors that affect detection in induced sputum. β_2-Agonist pretreatment. Histamine levels are slightly reduced after salbutamol pretreatment (as might be expected given the mast cell-stabilising properties of salbutamol), but ECP levels are unaffected [88].

Concentration of saline solution. One study looked at the effect of isotonic or hypertonic saline induction on ECP and histamine levels and found no effect [89].

Duration of inhalation. Since it has been shown that the composition of sputum changes as induction time increases (the proportion of granulocytes is reduced and that of macrophages and lymphocytes increased), it would be expected that concentrations of mediators might also change. During a 20-min induction, 4-min portions of sputum were examined and ECP and mucin-like glycoprotein levels found to be decreased, with surfactant protein A levels increasing during induction [90]. This suggests that, if the study of proximal bronchial secretions is the objective, it is best to analyse samples collected early on in the induction. Similar findings were obtained in another study, in which the induction lasted 30 min; the concentration of ECP decreased in sputum collected sequentially every 10 min [91].

Methacholine challenge. One study found no effect of methacholine on ECP or albumin level when challenge was performed 1 h before sputum induction [92]. Another study found that methacholine challenge results in a small, but significant, increase in measured α_2-macroglobulin levels [32].

Repeated induction. One study showed that ECP concentration increases along with neutrophil number [93] when sputum induction is repeated after 24 h. A 30-min period between inductions was found to have an effect on IL-8 measurements [94].

Method used for sputum processing. Delay before processing. This has not been systematically investigated, but recommendations are that sputum be processed as soon as possible and kept at 4°C in the meantime.

Selected sputum *versus* entire expectorate. As stated in the article entitled "Methods of sputum processing for cell counts, immunocytochemistry and *in situ* hybridisation" [95], two main methods are used for the processing of sputum: selection of viscid portions, in an attempt to minimise contamination by saliva, and processing of the entire expectorate, which contains variable amounts of saliva. Salivary contamination is often reduced by spitting out saliva separately during induction. Saliva generally contains much lower levels of ECP, tryptase, mucin-like glycoprotein, lactoferrin, DNA, elastase, α_1-antitrypsin, fibrinogen and albumin than unselected sputum [17, 26, 57], but histamine and endothelin-1 levels have been found to be higher in saliva than in unselected sputum [26, 44]. ECP levels have been found to be higher in selected portions of induced sputum than in the entire expectorate [96, 97]. The repeatability (in repeated sputum samples from the same subject) of measurements of albumin, fibrinogen, IL-8 and ECP has been found to be good in unselected/entire expectorates from asthmatics [3]. The repeatability in selected sputum of measurement of ECP, major basic protein, albumin and fibrinogen has been also good (intraclass correlation coefficient (ICC) of >0.8) but not as good for IL-5 (ICC 0.69) and tryptase (ICC 0.6) [2]. Further studies, comparing the repeatability of mediator measurement in samples processed by both methods, need to be conducted.

Dispersal method. Because of its viscid nature, sputum requires dispersal in order to permit extraction of mediators into the fluid phase. Furthermore, solubilisation of mucus enables more complete removal of cells and debris which, if left in the supernatant, continue to release mediators. Inadequate dispersal results from suspending the sputum in saline and agitating and rocking the sputum [9], which results in lower cell counts and ECP [98], IL-8 [83] and MPO (J. Shute, unpublished data) levels. This suggests binding of

these mediators to negatively charged mucins that are sensitive to sulphydryl reducing reagents such as DTT. Although DTT achieves good dispersal of most sputa, it may reduce disulphide bonds present in several mediators [99], such as IL-1, -7, -10 and -12, chemokines (monocyte chemotactic proteins and eotaxin), granulocyte-macrophage colony-stimulating factor, transforming growth factor-β (TGF-β), the interferon family, TNF-α, vascular cell adhesion molecule-1, the selectin family and α_2-macroglobulin [100–104]. Theoretically, DTT may also interfere with the disulphide bonds present in the capture antibodies of immunoassays, disrupting them and resulting in decreased assay sensitivity. Dithioerythritol is the optical isomer of DTT and exhibits similar actions.

In experiments using ultrasonication to disperse sputum followed by addition of DTT (final concentration 5 mM) to aliquots of sputum, DTT has been found to have no effect on ECP or eosinophil protein X (EPX) level but reduces recovery of eosinophil peroxidase and MPO [105]. In spiking studies, DTT, when added for sputum processing, does not interfere with measurement of ECP, EPX, IL-8, tryptase or immunoglobulin A [2, 9]. However, fresh DTT added to the ECP standard interferes with the radioimmunoassay, suggesting that the activity of the DTT is reduced after it has dispersed the sputum and been frozen and thawed. In spiking experiments, DTT has not been shown to affect measurement of IL-5 [2, 106, 107]. Good reproducibility (ICC 0.93) of TNF-α concentrations over a 2-week period has been found for samples dispersed with DTT. Conversely, the ICC is only 0.69 when sputum from the same subjects is ultracentrifuged only (V. Keatings, unpublished data). The levels detected in the ultracentrifuged samples were ~10% of those processed with DTT.

Other types of chemical dispersal using enzymes [108] have not yet been fully evaluated, and the effect of enzymes on soluble mediator levels is unknown.

Physical methods of dispersal using ultracentrifugation [8], glass homogenisation [33] or ultrasonication [105] may be used. Ultracentrifugation, with separation into a sol and gel phase, may exclude mediators that remain bound to the gel phase [109], and mediators may "stick" to glass beads. These methods have the disadvantage of cell disruption with release of intracellular mediators; samples processed in this manner are not suitable for estimation of cellular indices.

Temperature during processing. The processing temperature varies from 4°C (on ice) to room temperature and 37°C. Its effects on mediators have not yet been fully investigated, but it does not appear to affect ECP, EPX, eosinophil peroxidase or MPO levels [9, 105].

Osmolarity. Increasing osmolarity of processing fluids has been shown to increase ECP levels in sputum supernatant [110].

Loss of mediators to nonspecific binding sites on filters and tubes. The dispersed cell suspension is commonly filtered to remove clumps of mucus and debris, and a proportion of the soluble mediators may be lost to nonspecific binding to the filter or the sides of tubes. This has been found to be in the order of 15% overall for IL-5 when selected sputum is spiked with radiolabelled IL-5 [111]. High dilution of the sample during processing may increase this loss. In addition, if there is poor dispersal, this may be aggravated by filtration, with the mediator remaining bound to nonfiltered mucus. Filters and tubes should be made from material that does not stick to protein, polypropylene tubes being superior in this respect to polycarbonate or glass ones. For highly cationic proteins such as ECP, it is advisable to dilute the sample before storage and assessment with a buffer containing the cationic detergent cetyltrimethylammonium bromide (CTAB; 0.2%) (see appendix).

Centrifugation. High centrifugal forces may result in activation of cells and release of mediators; a centrifugal force of $400\times g$ is recommended.

Storage time and temperature. Supernatants should be stored in airtight tubes at -70°C. ECP has been shown to be stable for 72 h at 4°C or 6 h at 25°C [105], and is most stable if 0.4% CTAB is added (see appendix). In addition, repeated freeze/thaw cycles should be avoided. Therefore, if several mediators are to be measured, the supernatant should be frozen in aliquots.

Use of reagents to maximise levels of specific mediators. For some mediators, special treatment has been shown to result in better recovery. For example, TGF-β is present in latent form and requires acid activation for quantification of total TGF-β. The addition of CTAB to sputum samples during processing extracts intracellular proteins, *e.g.* ECP and MPO, and provides an estimate of the total content of the sample (see appendix) [112, 113]. Storage of supernatant for later measurement of ECP and MPO is optimised by addition of 0.4% CTAB to the supernatant. (The addition of CTAB is not recommended if other mediators are to be measured.) Processing sputum after addition of protease inhibitors has been shown to improve measurement of IL-5 (see appendix) [114].

Evaluation of the measurement of a soluble mediator in sputum

Bearing in mind the factors considered above, the discussion now focuses on the evaluation of the measurement of a specific soluble mediator in sputum and the questions that should be asked (fig. 1).

Is the measurement in sputum valid?

The validity of a measurement is best established by comparison to a gold standard, but there is none available for most mediators. Therefore, validity

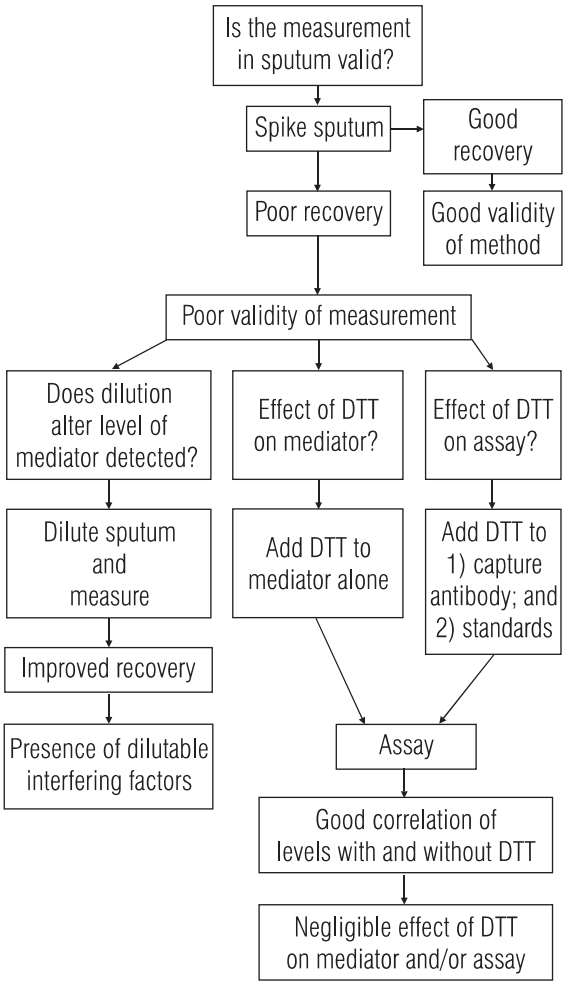

Fig. 1.–Evaluation of the measurement of a soluble mediator in sputum. DTT: dithiothreitol.

should be examined in a series of "spiking" experiments [107]. This involves adding a known amount of mediator to unprocessed sputum, processing the sputum as usual and then measuring recovery by immunoassay. It is important that the commercial source of the mediator used for spiking be the same as that used as the standard in the immunoassay. Unspiked sputum is simultaneously processed and assayed so that percentage recovery can be calculated. Any factors operating to reduce the amount of spike detected also reduce the recovery of endogenous mediator, masking to some extent the low recovery of the spike. However, the masking effect is usually slight, and poor recovery should be easily detected. The amount of mediator added to sputum should be such that the final concentration in supernatant, if most of it is recovered, lies on the straight part of the standard curve, in the middle/upper range of the limit of detection of the assay. A useful control experiment consists of spiking phosphate-buffered saline (PBS) containing 1% (weight/volume) bovine serum albumin (BSA) when the sputum is spiked. This is then frozen at the same time as the sputum supernatant, thawed and assayed simultaneously with it. This controls for errors due to pipetting technique and for any effect of freezing and thawing. The sputum should be spiked over a range of concentrations. It is not known whether recovery differs between individuals due to differences in factors in sputum which interfere with detection. Therefore, sputum from a range of subjects with different clinical conditions and degrees of severity should be used in spiking experiments. Dilution studies should be performed as a complement to or in combination with spiking. If the mediator is present in high concentration, the supernatant can be directly diluted and the mediator assayed. If not, exogenous mediator can be added to the supernatant after dilution and then assayed. The dilution buffer composition should be the same as for the standard. The concentration of mediator measured should not change significantly after correcting for dilution. If the measurement increases with dilution, this suggests the presence of interfering substances, such as soluble receptors of the mediator. In addition, parallel-line studies may be performed, whereby mediator is added to supernatant which is then diluted. The mediator concentration can then be plotted against absorbance, and the gradient line should be parallel to that of the standard curve if no interfering factors are present.

If there is good recovery (>80%) of spike, it can be accepted that the assay is reasonably valid, and, if endogenous mediator is not detected, it can be assumed that there is insufficient mediator present to be detected. If recovery is poor, the next step will depend on whether the mediator is easily measured or not.

What to do if validity is poor

If the mediator level is readily measured, at least in some clinical settings, and is able to provide useful information, it may be appropriate to continue using the assay, accepting that a certain percentage of mediator is being lost. However, it cannot be assumed that the same proportion is lost in all subjects and at all mediator concentrations, unless this is established by spiking.

If the mediator is not readily detectable, it cannot be assumed that the level of mediator is below the detection limit of the assay. The following steps need to be taken in order to ascertain the cause of poor recovery and attempt to minimise loss.

What is the effect of dithiothreitol? The effect of DTT on the mediator itself and on the immunoassay antibodies should be investigated. A solution of mediator of known concentration (in PBS containing 1% BSA) is "processed" with DTT (as for sputum) and stored as usual. It is later thawed and assayed, allowing the effect of DTT on the mediator to be examined (without sputum present). DTT may have an effect on the immunoassay itself; thus DTT is added to the assay standard (to the same final concentration as in sputum) and compared to the standard diluted in the usual way with assay buffer. In addition, if an ELISA is used, the effect on the

capture antibody can be checked by adding DTT to wells coated with capture antibody and incubating for an additional 15–30 min before washing [107]. If DTT does not affect measurement of mediator standard, other causes of poor recovery/loss should be considered.

Is the mediator physically lost during processing? The mediator may be physically lost during processing by 1) binding to nonspecific sites on containers and filters, or 2) remaining bound to nonfiltered mucus particles. An effective means of investigating this is to spike the sputum with radiolabelled (*e.g.* with iodine-125) mediator and measure recovery of radioactivity. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of the radiolabelled mediator or analytical chromatography (alone, incubated with DTT and processed with sputum) may be performed to determine whether degradation of the mediator has occurred [111].

Has masking or alteration of epitopes occurred? If the mediator is not physically lost during processing, masking or alteration of its epitopes or those of the antibodies used in the assay may have occurred. If recovery of radiolabelled mediator is better than that detected by immunoassay, it can be assumed that the low measured levels in the latter are due to poor immunological recognition by the antibodies. Parallel-line analysis (dilution studies) can be used to examine whether or not the detecting antibody of the immunoassay is able to detect the endogenous mediator similarly to the standard [65]. The straight part of the standard curve is compared to that produced by assaying serially diluted native supernatant or supernatant with added exogenous mediator. If the lines are parallel, it suggests that the assay is responding to active components in the preparation similarly. If the lines are not parallel, the presence of a substance that interferes with recognition of mediator epitopes by the immunoassay and which is dilutable is suggested. This may be due to masking of epitopes by autoantibodies [78, 115], soluble receptors [72, 73, 116, 117] or other binding proteins such as albumin and α_2-macroglobulin [78, 86]. Specific assays are available for the soluble receptors of only certain cytokines, *e.g.* IL-2 and IL-4. Theoretically, use of polyclonal capture antibodies (more epitopes recognised) or competitive assays (which compete with the interfering substance for the epitope) would result in more sensitive measurement if interfering substances are suspected. The addition of a non-ionic detergent such as 2% Tween 20 may decrease nonspecific protein interactions.

Assuming that DTT is not interfering, sputum proteases may also be responsible for degrading the mediator and altering its epitopes or interfering with the antibodies in the assay. Proteases may attain high levels in sputum, proportional to the clinical severity of asthma [64]. There is evidence that they may be activated by DTT, which disrupts proteoglycan bonds and releases bound protease/antiprotease complexes [118]. STOCKLEY and BAYLEY [21] found that the addition of elastase to sputum resulted in decreased levels of secretory leucocyte protease inhibitor and MPO by immunoassay. The addition of protease inhibitors to sputum before processing has resulted in increased rates of detection and levels of IL-5, suggesting that proteases are interfering substances in some cases [114].

Full reporting of results

When reporting results, the methods used for validation should be provided. The procedure used to calculate the sensitivity of the assay should be stated, and estimates below the lowest (nonzero) standard concentration used are not valid. Reproducibility should be assessed and described in terms of intra- and interassay variation.

Before assays of soluble mediators can be used in a clinical situation, each laboratory needs to establish the normal range for that mediator as well as the levels expected in particular clinical settings.

Key points

1) The production, preparation and dispersal of sputum can all affect the levels of soluble mediators in sputum, and each mediator should be evaluated individually. 2) Different sources of antibodies used in immunological assays for soluble mediators can produce varying results. 3) If a soluble mediator is found to be below the level of detection of a specific assay, it cannot be assumed that the true level of that mediator is very low/zero unless spiking studies show good recovery of the mediator. If spiking studies show dramatic loss of added mediator, it should be assumed that it is unmeasurable using that assay. 4) The measurement of soluble mediators in sputum has been proven a useful investigative tool in airway inflammation as long as careful attention is paid to the use of appropriate laboratory methods and proper interpretation of results. 5) Where indicated, spiking experiments should be performed to validate results. 6) Sputum soluble mediator measurements reported in studies failing to follow a rigorous methodology should be interpreted with caution.

Outstanding questions

The following research questions still need to be addressed: 1) the differences in levels of mediators (other than ECP) between selected and unselected sputum; 2) the reproducibility of measurements of most of the mediators (apart from ECP, major basic protein, albumin, fibrinogen and IL-8); 3) the validity of measurements of most of the mediators in both selected and unselected sputum (apart from ECP, IL-5, histamine, total IL-8 and tryptase in selected sputum and albumin, fibrinogen, IL-8 and ECP in unselected sputum); 4) the effect of DTT on many mediators (apart from ECP, IL-5, histamine, total IL-8 and tryptase); 5) the effect of delay in processing

sputum; 6) the effect of temperature (room temperature *versus* 37°C *versus* 4°C) during processing (apart from ECP, IL-8, histamine and tryptase (room temperature *versus* 37°C)); 7) the effects of dilution, filtration and centrifugation of the sputum sample during processing; 8) the effect of soluble receptors, autoantibodies and other binding proteins in assays of most mediators (apart from IL-8); 9) the effect of proteases on most mediators; 10) the effect of duration of sputum induction on mediators (other than ECP and mucin-like glycoprotein) and the optimal period of induction; 11) the effect of the time between inductions; and 12) the proportion of free *versus* bound mediator detected in each assay.

Appendix: Notes on optimal measurement of specific mediators

Eosinophil cationic protein

If measurement of total (intracellular and released) ECP is required, the sputum sample should be processed with the addition of 0.4% (weight/volume) CTAB in PBS (0.05 M sodium phosphate, 0.9% sodium chloride, 0.05% sodium azide, 0.01 M disodium ethylenediamine tetraacetic acid (EDTA), 0.1% BSA; pH7.4±0.05) after the dispersal step. If released ECP alone is required, an equal volume of 0.4% CTAB should be added to the sputum supernatant before storing at -70°C. Further dilutions, if required, should be made with 0.2% (weight/volume) CTAB in PBS. All PBS and PBS-containing solutions should be filtered through a 0.22-μM mesh.

Myeloperoxidase

The same procedure as used for ECP should be followed. MPO is affected by freeze/thaw cycles and so the number of these should be minimised.

Interleukin-8

Processing of sputum with DTT yields higher concentrations of a number of basic proteins such as IL-8 in the soluble phase than in sputum treated with PBS; thus comparison can only be made between samples treated in the same way.

Interleukin-5

The addition of a combination of protease inhibitors to sputum before processing has been shown to increase the levels of IL-5 detected by enzyme immunoassay. The reagents used and their final concentrations are 4-(2-aminoethyl)-benzensulphonyl fluoride (2 mM), pepstatin A (1.4 μM), leupeptin (1.0 μM) and EDTA; 1.3 mM). The effect of protease inhibitors on other mediators has not been examined.

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