

REVIEW

Indirect bronchial hyper-responsiveness: the coming of age of a specific group of bronchial challenges

J. Van Schoor, R. Pauwels and G. Joos

Department of Respiratory Diseases, Ghent University Hospital, Ghent, Belgium

Introduction

Bronchial hyper-responsiveness (BHR) can be defined as an increase in the ease and the degree of airflow limitation in response to bronchoconstrictor stimuli *in vivo*. Although BHR is not entirely specific for asthma, it is one of its major pathophysiological features [1, 2].

Airway narrowing in asthma is the ultimate result of an interaction between multiple mechanisms, not necessarily and uniquely related to airway inflammation. BHR and non-invasive markers of airway inflammation, such as sputum eosinophilia or exhaled nitric oxide (eNO), are only loosely related to each other. This implies that no inferences about BHR can be made from measurements of airway inflammation and vice versa. It is questionable whether good correlations between the outcome of indirect challenges and markers of inflammation are important and/or desirable: bronchial challenge tests provide integrated information about multiple pathophysiological pathways within the airways, information that is not picked up by the non-invasive surrogate markers of airway inflammation, which provide rather specific information on selected inflammatory pathways [3–5].

The phenomenon of BHR is sometimes referred to as 'non-specific', i.e. that most patients with active asthma will react to these stimuli. The stimuli, used to reveal it, however, act by highly specific mechanisms. As multiple pathophysiological pathways are involved, it is therefore no surprise that the results of the different challenge tests are only weakly correlated and not mutually interchangeable.

Bronchoconstrictor stimuli may be classified into 'direct' and 'indirect', according to the main mechanism through which they induce airflow limitation (Table 1). Combinations of mechanisms are possible. Direct stimuli induce airflow limitation through direct action on the effector cells, involved in the airflow limitation. These comprise in the first place airway smooth muscle cells, but bronchial vascular endothelial cells and/or mucus-producing cells may also be involved. Indirect stimuli, on the other hand, act on intermediary cells such as inflammatory cells, bronchial epithelial cells and/or neuronal cells; the pro-inflammatory mediators and/or neurotransmitters, liberated by these cells will then interact with the effector cells to cause airflow limitation (Fig. 1) [6, 7].

The role of epithelial cells as active players in mediating airflow limitation following indirect stimuli is being increasingly recognized; more specifically, human bronchial epithelial cells have been shown to carry adenosine A₁ [8], adenosine A_{2B} [9], tachykinin (TK) NK₁ [10], and bradykinin B₂ receptors [11].

The increased clinical and research interest in indirect challenges is being reflected by the generation of a consensus document on 'indirect airway challenges' by an *ad hoc* European Respiratory Society (ERS) taskforce. This taskforce proposed the following practical working definition of an indirect challenge: 'Indirect challenges act by causing the release of endogenous mediators that cause the airway smooth muscle to contract, with or without effect in inducing microvascular leakage. Because the responses to these challenges are modified or even completely inhibited by inhaled steroids, the airway response to these challenges may be a closer reflection of active airway inflammation' [12].

This review will focus on the recent evolutions and the emerging new concepts concerning the clinical and research applications of indirect stimuli in asthma.

The pharmacology of indirect challenges

Mechanisms – a general overview

Over the last decades, a considerable number of papers has been published on the underlying mechanisms of airflow limitation, induced by indirect challenges. As a general rule, it can be stated that the airflow limitation, caused by an indirect challenge can be prevented by pre-treatment by inhalation of a cromone (cromoglycate, nedocromil), furosemide or heparin, whereas this is not the case for direct challenges [7, 12–14]. Individual mediators such as histamine, leukotrienes, prostaglandins, acetylcholine and TKs are implicated in a varying degree in the mechanisms of the different indirect challenges [7, 12]. An in-depth review on the subject has been published in 2000 [7]. Since then, new data have continued to emerge in the literature, progressively completing our knowledge on the mechanisms and mediators involved (Tables 2 and 3) [13–22].

Mechanisms – what has been learnt from studies on mannitol-induced bronchoconstriction

In the past years, a novel osmotic bronchial challenge using mannitol dry powder has been developed as an alternative for the wet aerosol of hypertonic saline [23]. From a practical

Correspondence: Guy Joos, Department of Respiratory Diseases, Ghent University Hospital, De Pintelaan 185, B-9000 Ghent, Belgium.

E-mail: guy.joos@ugent.be

Table 1. Classification of bronchial challenge tests according to their main mechanism

Direct stimuli	Indirect stimuli
Pharmacological stimuli	Pharmacological stimuli
Cholinergic agonists	Adenosine (AMP)
Acetylcholine	Tachykinins (SP, NKA)
Methacholine	Bradykinin
Carbachol	Metabisulphite/SO ₂
Histamine	Propranolol
Prostaglandin D ₂ /F _{2α}	
Leukotriene C ₄ /D ₄ /E ₄	
	Physical and physicochemical stimuli
	Exercise
	Isocapnic hyperventilation with (cold) dry air
	Osmotic stimuli
	Hypertonic aerosols (e.g. HS)
	Hypotonic aerosols (e.g. UNDW)
	Hypertonic mannitol dry powder

AMP, adenosine 5'-monophosphate; SP, substance P; NKA, neurokinin A; HS, hypertonic saline; UNDW, ultrasonically nebulized distilled water; SO₂, sulphur dioxide.

point of view, it does not require a nebulizer and can be performed outside the laboratory setting using a simple hand held inhaler device, the mannitol being contained in gelatin capsules. *In vitro*, the human lung mast cell has been shown to release histamine in response to a hyperosmolar stimulus; at the same osmolarity, mannitol was found to be more potent than sodium chloride [24]. Recently, it has also been shown that hyperosmolar stimulation with mannitol resulted in leukotriene C₄ release from human peripheral blood eosino-

phils *in vitro* [25]. The presence of mast cell activation is further suggested by the observation that mannitol-induced bronchoconstriction is associated with increased urinary levels of the prostaglandin D₂ (PGD₂) metabolite and mast cell marker 9 α ,11 β -PGF₂, and of leukotriene E₄ (LTE₄) [26]. Another interesting supportive finding was the quite close correlation, in asthmatics, between the BHR to mannitol and to adenosine 5'-monophosphate (AMP), a stimulus which is thought to act largely through mast cell-dependent mechanisms [27]. Pre-treatment with specific mediator antagonists suggests a different role for histamine and leukotrienes; it has been shown that pre-treatment with the H₁ antagonist fexofenadine (two 180 mg doses taken over 14 h) decreased the airway sensitivity in asthmatic patients, while the cysLT₁ antagonist montelukast (three 10 mg doses taken over 36 h) shortened the recovery time [28]. Similar results were described in a second study in asthmatics, in which the effects of 10 mg montelukast, alone or in combination with 5 mg of the H₁ antagonist desloratidin, were studied on mannitol and AMP challenge; interestingly, the effects observed on both challenges appeared again quite similar [29]. As is the case with all indirect challenges, pre-treatment with 8 mg of inhaled nedocromil sodium was found to significantly inhibit the responsiveness to inhaled mannitol in asthmatic subjects [30]. Finally, a pilot study on 18 asthmatics suggested that 6–9 weeks of inhaled corticosteroids (ICS) (budesonide (BUD) 800–2400 μ g/day) reduces sensitivity and reactivity to mannitol [31] (Table 3).

Receptors

In addition, the development of specific antagonists for adenosine and TK receptors is currently opening new research directions. These molecules will not only allow to determine which receptors are involved in the adenosine and TK-induced airflow limitation, but will ultimately permit to conclude, by performing appropriate clinical trials, whether or not adenosine and TKs are asthma mediators of clinical relevance.

The adenosine receptor pharmacology in human airways is being increasingly studied *in vitro*; adenosine exerts its effects on human cells through interaction with specific adenosine (P1) receptors, of which four subtypes (A₁, A_{2A}, A_{2B}, and A₃) have been described [32]. Different animal models, among which knock-out mice for specific adenosine receptors [32] or mice, deficient for adenosine deaminase, a purine catabolic enzyme that converts adenosine to inosine [33], have provided insights into the physiology and pathophysiology of the different adenosine receptors. However, there are important interspecies differences and these results cannot be extrapolated as such to the human situation. Although the relative importance of the various adenosine receptor subtypes in asthma remains to be further elucidated, it appears that stimulation of adenosine A_{2B} receptors on the surface of human lung mast cells is the main trigger for adenosine-induced airflow limitation [34, 35]; in addition, its relevance has recently been further illustrated by the finding that adenosine increases cytokine release by human bronchial muscle cells *in vitro* via an A_{2B}-mediated mechanism [36]. The results of a rabbit study using an aerosolized antisense oligodeoxynucleotide to reduce the number of A₁ receptors

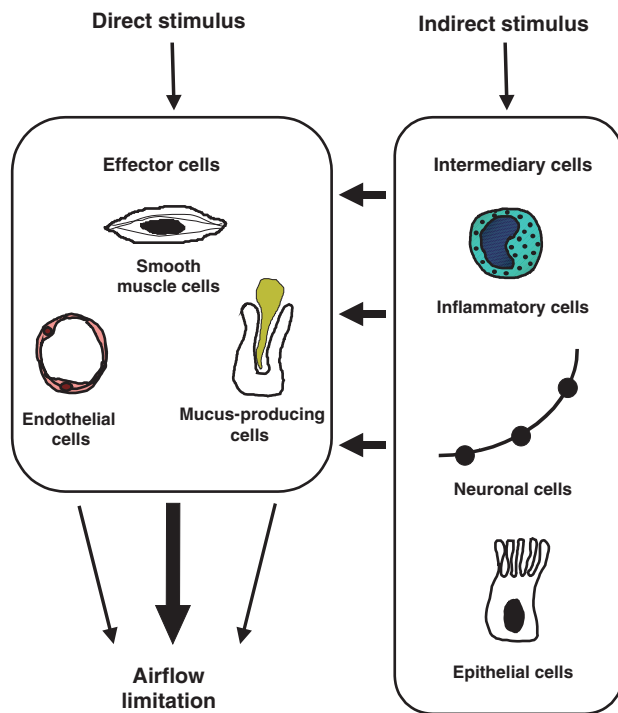
**Fig. 1.** Mechanisms via which direct and indirect stimuli induce airflow limitation.

Table 2. Inhibitory effects of drugs on the airflow limitation, induced by indirectly acting pharmacological stimuli (modified and updated from [7])

	Adenosine	TKs	Bradykinin	MBS/SO ₂	Propranolol
H ₁ -antagonists	+++	–	+	–	+
Leukotriene modifiers	++	+ [17,18]	ND	+	–
COX-I	+	+	+	+	ND
Anti-cholinergics	+	+	++	–/+++*	+++
TK antagonists	– [16]	+	–/+	ND	ND
NOS inhibitors	?	ND	+	–	ND
ICS	++	+ [19]	+ [20]	–/+	–

*Wide inter-individual variations.

MBS, metabisulphite; SO₂, sulphur dioxide; COX-I, cyclooxygenase inhibitor; TK, tachykinins; NOS, nitric oxide synthase; ICS, inhaled corticosteroids; ND, no data available; ?, inconclusive data; +, inhibitory effect; –, no inhibitory effect.

suggested a role of these receptors in asthma [37], but the relevance to human asthma has yet to be proven. In contrast to A_{2B} receptors, A_{2A} activation results in suppression of histamine and tryptase release from human mast cells *in vitro* [38] and was found to induce broad anti-inflammatory activity in a rat model of allergic airways inflammation [39]. Adenosine A₃ receptor protein, on the other hand, could not be demonstrated in human lung mast cells, but is abundantly expressed by human airway eosinophils [40]. Activation of the A₃ receptor was found to inhibit human eosinophil chemotaxis [40, 41], as well as human eosinophil degranulation and superoxide anion release [42]. The development of potent and specific agonists and antagonists for the different adenosine receptors for human use will allow definitive conclusions as to the role of these receptors in clinical asthma. Based on the limited human data currently available, blocking A₁ or A_{2B} receptors by specific antagonists, or activating the A_{2A} or A₃ receptors by specific agonists could potentially become novel therapeutic strategies for asthma.

As potent and specific TK receptor antagonists for use in man are currently available, still new ones being in development, increasingly more data are becoming available on the involvement of the different TK receptors in human asthma [43]. The initial observation that the TK NK₂ receptor antagonist SR 48968 (saredutant) was able to partially inhibit NKA-induced airflow limitation [44], was subsequently

confirmed by a similar trial with another NK₂ antagonist, MEN 11420 (nepadutant) [45]. The finding that the dual NK₁/NK₂ antagonist DNK333A had a major blocking effect on NKA challenge suggests that TK NK₁ receptors are relatively more involved than the NK₂ subtype [46]; dual NK₁/NK₂ antagonists therefore appear to be more efficacious than single receptor antagonists. Recently, the triple TK receptor antagonist CS-003 was equally found to offer a significant protection against NKA challenge [47].

Further research in appropriately designed clinical trials may potentially result in the emergence of novel classes of anti-asthma medications, blocking the effects of adenosine or TKs, respectively.

Different informations yielded by indirect vs. direct challenges

We have now come to a detailed understanding of the mechanisms and mediators of many direct and indirect stimuli. It has become clear that these mechanisms are heterogeneous, which explains the poor correlation between individual challenges, direct as well as indirect ones; this also means that the results of the different challenge tests probably reflect different phenomena in the airways. This suggests that different informations, of potential clinical relevance, may be obtained through performing these different challenges.

Airway inflammation and direct/indirect stimuli

Currently, there is an important research interest into the relationship between the different airway challenges and airway inflammation in asthma, as reflected by non-invasive surrogate markers of inflammation, the effects of natural allergen exposition or avoidance, or therapeutic interventions with anti-inflammatory treatments (essentially ICS).

The main conclusion of these studies is that the results of direct and indirect challenges are providing us with different and complementary information. The statistical method of factor analysis allows the many parameters that characterize the disease to be reduced to a few independent factors, each factor grouping associated parameters. A study on 99 stable asthmatics using factor analysis concluded that baseline BHR (measured by histamine challenge), airway function and airway inflammation are to be considered separate dimensions in the description of chronic asthma and thus supports the utility of routine measurement of all these dimensions [48]. A subsequent study, which performed factor analysis on 66 stable asthmatics, assessed whether the relationship between BHR (measured by methacholine challenge), non-invasive markers of airway inflammation, and baseline lung function depended on the duration of the disease; the results of the analysis suggested that with a shorter duration of the disease, BHR is associated with airway inflammation, whereas with a longer duration it is associated with impaired lung function, suggesting that in long-standing asthma ongoing alterations become the primary determinant of functional characteristics [49]. To the best of our knowledge, no data on factor analysis for indirect challenges has been published to date. Indirect challenges are said to be better reflecting ongoing inflammation than direct challenges; this

Table 3. Inhibitory effects of drugs on the airflow limitation, induced by indirectly acting physical and physicochemical stimuli (modified and updated from [7])

	Exercise	Hypertonic saline	Distilled water	Isocapnic hyperventilation	Mannitol
H ₁ -antagonists	+	+	+	+	+ [28]
Leukotriene modifiers	+++	ND	++	++	+ [28]
COX-I	++	+	++	–	ND
Anti-cholinergics	+++*	+++*	+++*	+++*	ND
TK antagonists	+	–	ND	ND	ND
ICS	++	++	++	++	++ [31]

*Wide inter-individual variations.

COX-I, cyclooxygenase inhibitor; TK, tachykinins; ICS, inhaled corticosteroids; ND: no data available; +, inhibitory effect; –, no inhibitory effect.

hypothesis is based on data which are obtained from studies, in which indirect and direct challenges have been compared head to head [12]. This has been most extensively studied in the case of adenosine challenge [50, 51]. Allergen avoidance at high altitude led to a significant diminution of eosinophils and ECP in blood; this was accompanied by a significant decrease in BHR to AMP (2.1 doubling concentrations), BHR to methacholine remaining unchanged [52]. BHR to AMP is more closely associated with airway inflammation (especially sputum eosinophils) than is BHR to methacholine [53, 54]. It had been noted before that BHR to AMP improves to a larger extent to a short course of ICS (SC-ICS) than BHR to methacholine [7, 50, 51]. Formal proof that these more marked improvements are indeed associated with a more marked reduction in airway inflammation (again most clearly with sputum eosinophils) has recently been published; these changes were noted after 2 weeks of treatment [55]. The correlations between PC₂₀ AMP and individual surrogate markers of inflammation (albeit stronger than for PC₂₀ methacholine), however, remain rather weak. This is no surprise, as bronchial challenge tests are reflecting the global, integrated airway response, which involves many cell types and their mediators. As AMP-induced bronchoconstriction is thought to mainly involve activated mast cell-related pathways, it can be hypothesized that a stronger association might be found between PC₂₀ AMP and tryptase or other markers of mast cell activation in sputum or blood. It has been shown that sputum tryptase levels significantly decrease following a 2 weeks course of 400 µg ciclesonide daily [56]. It has recently been shown that there exists a mast cell infiltration of the smooth muscle layer in asthmatic airways, causing a mast cell myositis. In addition, the number of mast cells in the smooth muscle was found to be inversely correlated with the degree of BHR to methacholine, suggesting that this microlocalization of mast cells may be a key factor in the development of BHR in asthma [57]. It is tempting to speculate that the correlation between mast cell numbers and BHR to AMP, as mast cell marker, would be even more marked.

The effects of inhaled corticosteroids on indirect stimuli

The response of AMP challenge to SC-ICS has been studied in great detail, although some important questions remain to be answered. The AMP challenge responsiveness appears to react very rapidly to treatment with ICS, as a significant attenuation (1.9 doubling doses) has been demonstrated within 2 h of a single inhaled dose as low as 100 µg of fluticasone propionate (FP) in steroid-naïve asthmatics [58]; this acute effect is not specific to FP [59]. A more recent study from the same group has shown that the acute ICS effect on AMP is seen as early as 1 h, reaching its maximal at 4 h after single inhalation [60]. Other authors have specifically studied the duration of the protective effect, and have found that a single inhaled dose of 1000 µg FP protects against AMP challenge up to 26 h after dosing [61]. The mechanism, underlying this phenomenon, is not well established; it has been proposed that it involves an effect on airway mucosal blood flow and airway microvascular permeability [62]. In addition, a dose-dependent effect of 2 weeks of inhaled ciclesonide (at three different doses) on BHR to AMP has been reported; the observed reduction in percentage of

sputum eosinophils, however, was not found to be dose dependent [63]. Detailed longitudinal assessment of changes in BHR to methacholine and AMP, as well as in sputum cell counts was carried out before, during and up to 3 weeks after withdrawal of a 6-week therapy with inhaled BUD, 800 µg daily. When compared with the other markers, BHR to AMP detected inflammatory changes already after only 1 week of BUD treatment, continued to improve progressively over the treatment period, and returned to near-baseline levels as early as the first week of treatment discontinuation [64]. This suggests that ICS exert their effects on AMP through both acute (vascular) and chronic mechanisms (depletion of airway mast cells) [62]. Changes in BHR to methacholine and sputum eosinophil counts, on the other hand, were observed only by the fourth week of treatment; BHR to methacholine returned to baseline values at 3 weeks after treatment discontinuation, at which moment sputum eosinophil counts were still significantly lowered. In addition, the ICS provided a significantly greater reduction in BHR to AMP than to methacholine [64], a finding consistent with the other data in the literature [50, 51]. BHR to AMP thus appears to be characterized by an exquisite sensitivity to the effects of ICS, which suggests that it may be one of the most accurate means to monitor ICS requirements in asthma and to establish the appropriate dose needed to control airway inflammation. These promising data, obtained in a small population of highly selected asthmatics, now need to be extended in clinical trials involving greater numbers of patients for longer periods of time. It would be interesting to know after which duration of ICS treatment the changes in BHR to AMP reach a plateau maximal value, if any. In this context, it is interesting to note the findings of a 6-month trial, during which the effects of inhaled fluticasone (200 vs. 500 µg/day) on exercise-induced bronchoconstriction (EIB) in asthmatic children were studied. Three weeks after starting active treatment, EIB was significantly reduced and these improvements were sustained throughout the 24 weeks of treatment, the protective effect of the two doses of FP not differing significantly [65].

It is now a well-established fact that BHR to adenosine is better correlated with current non-invasive surrogate markers of airway inflammation than BHR to direct stimuli, at baseline as well as in reaction to SC-ICS (up to 6 weeks). Studies, comparing the effects of SC-ICS on BHR to indirect and direct stimuli are summarized in Table 4 [19, 20, 55, 58, 64–77]. ICS induced an attenuation of BHR to the majority of indirect stimuli, although to different degrees, illustrating differences in underlying mechanisms. Although currently studied in much less detail than for AMP, the protective effect of SC-ICS does also appear to be more marked for some other indirect challenges, in comparison with direct stimuli. Based on the currently available literature data, this appears to be most convincing for NKA [19] and bradykinin [20]. The results of the three studies on bradykinin appear inconsistent on first glance. In the first study, failure to detect a greater effect on bradykinin than on histamine challenge may have been because of a variety of reasons: a treatment duration of 3 weeks may have been too short, specific airways conductance was used instead of forced expiratory volume in 1 s (FEV₁) to measure airways responses, bradykinin responsiveness was assessed immediately following histamine challenge, and bradykinin was prepared in solution contain-

Table 4. Studies comparing the relative effects of short courses of ICS on the BHR against direct and indirect stimuli

Author	Treatment			Relative effect (direct vs. indirect stimuli)
	Compound	Dose ($\mu\text{g/day}$)	Duration	
O'Connor et al. [66]	BUD	1600	2 weeks	AMP > methacholine
Doull et al. [67]	BDP	400	12 weeks	AMP > methacholine (no effect)
Weersink et al. [68]	FP	500	6 weeks	AMP > methacholine
van den Berge et al. [55]	FP	500	2 weeks	AMP > methacholine
	FP	2000	2 weeks	AMP > methacholine
Prosperini et al. [64]	BUD	800	6 weeks	AMP > methacholine
Ketchell et al. [58]	FP	1000	Single dose	AMP > histamine (no effect) at 2 h post-dose
Van Schoor et al. [19]	FP	1000	2 weeks	NKA > methacholine
Fuller et al. [69]	BUD	1200	3 weeks	Bradykinin $\approx$ histamine
Doull et al. [67]	BDP	400	12 weeks	No effects on bradykinin or methacholine
Reynolds et al. [20]	FP	440	16 weeks	Bradykinin > methacholine
Wiebicke et al. [70]	BDP	2000	3 weeks	SO ₂ (no effect) < methacholine $\approx$ histamine
O'Connor et al. [66]	BUD	1600	2 weeks	MBS $\approx$ methacholine
Kraan et al. [71]	BUD	400	4 weeks	Propranolol (no effect) < histamine
Vathenen et al. [72]	BUD	1600	6 weeks	Exercise $\approx$ histamine
Hofstra et al. [65]	FP	200	6 weeks	Exercise > methacholine
	FP	500	6 weeks	Exercise $\approx$ methacholine
Wiebicke et al. [70]	BDP	2000	3 weeks	IHV (room air) ? Methacholine $\approx$ histamine
Vathenen et al. [72]	BUD	1600	6 weeks	IHV (room air) $\approx$ histamine
Claussen and Sill [73]	BUD	800	4 weeks	IHV (cold air) ? carbachol
Nielsen and Bisgaard [74]	BUD	800	8 weeks	IHV (cold air) > methacholine
du Toit et al. [75]	BUD	1000	12 weeks	HS $\approx$ histamine
Groot et al. [76]	BDP	800	8 weeks	UDNW $\approx$ histamine
Bootsma et al. [77]	BDP	1500	6 weeks	UDNW $\approx$ histamine
	FP	750	6 weeks	UDNW $\approx$ histamine

?, no statistical comparison performed; ICS, inhaled corticosteroids; BHR, bronchial hyper-responsiveness; BUD, budesonide; BDP, beclomethasone dipropionate; FP, fluticasone propionate; AMP, adenosine 5'-monophosphate; NKA, neurokinin A; SO₂, sulphur dioxide; MBS, metabisulphite; IHV, isocapnic hyperventilation with (cold) dry air; HS, hypertonic saline; UNDW, ultrasonically nebulized distilled water.

ing ethanol [69]. In the second study, the failure to find any significant effect of ICS on either methacholine or bradykinin may have been because of the fact that subjects were selected on the basis of recurrent wheezing; the majority showed no BHR to any of both stimuli at baseline and was arbitrarily assigned the maximal theoretical dose of spasmogen. The observation that, even with these limitations, it was possible to detect a significant effect on AMP challenge, is an illustration of the greater sensitivity of the latter [67]. The third study, however, yields convincing evidence [20]. Somewhat in analogy to AMP, a better correlation between bradykinin challenge and eosinophil count in bronchoalveolar lavage and bronchial biopsies [78] or in sputum [79] has been demonstrated, in comparison with methacholine challenge. Moreover, allergen-induced BHR to bradykinin is more pronounced than that to methacholine [80].

In contrast to direct challenges, indirect challenges depend on the activity of intermediary cells and intercellular pathways in order to induce airway narrowing. In analogy to the discussion on the effect of ICS on eNO [81], it is important to bear in mind that it cannot be excluded that the exquisite sensitivity of some indirect challenges (c.q. AMP) to steroid intervention is caused by direct blockade of some of these cellular pathways that are required for their bronchoconstrictive effects. Such effects are then to be considered as 'functional antagonism' that is not related to the desired 'disease modifying effects'; if this were the case, indirect challenges would not be very useful as outcome parameter of

steroid intervention. Appropriately designed clinical studies of sufficient duration are necessary to address this outstanding issue.

The contention that indirect stimuli in general show a faster and more pronounced reaction to SC-ICS than direct stimuli, however, would be an incorrect generalization. More in particular, effects on metabisulphite/SO₂ [66, 70] and UNDW [76, 77] challenge show a comparable response, while propranolol does not appear to react at all to SC-ICS [71, 82]. For the remaining types of indirect challenges (i.e. exercise, IHV, and HS) results are equivocal and inconclusive, and further work is needed to further explore the behaviour of these stimuli. The recently introduced stimulus with mannitol dry powder has yet to be put to the test and can therefore not be classified for the moment.

In the current state of the knowledge, two different profiles of indirect stimuli seem to emerge and it would appear that a further subclassification of indirect stimuli is warranted, based on their clinical correlation with ongoing inflammation. We propose to coin the term 'Type 1' or 'adenosine-type' indirect stimuli for those challenges which show a better correlation with ongoing inflammation than direct challenges; this is reflected by a more rapid and profound attenuation of BHR in response to a therapeutic test with SC-ICS. We propose the term 'Type 2' or 'metabisulphite/SO₂-type' indirect stimuli, on the other hand, for those challenges, displaying similar characteristics as compared with direct challenges, in response to the therapeutic test (or no response

at all as for propranolol) (Table 5). One explanation for this heterogeneity might be that 'Type 1' challenges rely predominantly on real 'indirect mechanisms' (with inflammatory cells and/or epithelial cells as principal intermediary cells), while the underlying mechanisms of 'Type 2' challenges also involve a significant (or even predominant) direct component.

It should be stressed that there is no consensus in the literature on how a SC-ICS should be performed (choice of compound, its posology and the duration of administration) in the context of exploring the mechanisms of airways challenges. Differences in the schemes used may account for a part of the equivocal results. Standardization is needed to allow better comparisons; this applies as well for the comparisons of indirect with direct challenges as for the comparisons of indirect challenges among themselves. Standardization of the majority of indirect stimuli has only fairly recently been agreed upon [12]; this will allow valid and relevant comparisons between future studies. Another issue to be tackled is to define quantitative criteria in order to study differences in response between challenges, e.g. to ICS intervention. This will allow a fully objective and stringent way of classifying stimuli, compared with the rather semi-quantitative interpretation of the data we currently have at our disposition. Such studies will ultimately allow to determine which is the most optimal challenge for studying integrated and physiological information on ongoing inflammation, and by which parameter (surrogate markers of inflammation, therapeutic test with ICS, etc.) this should be explored.

The effects of ICS on direct stimuli

The BHR to some indirect stimuli thus appears to react faster and to a greater extent to an SC-ICS than the BHR to the typical direct stimuli methacholine or histamine. The BHR to the latter does not react as quickly and markedly, and thus seems less appropriate to monitor ongoing airway inflammation. BHR to direct stimuli, however, provides us with equally interesting information, which is different and complementary to data, obtained with indirect stimuli. It had already been noted by several authors that although BHR to direct stimuli already shows improvement over the first few weeks [83, 84], it continues to improve with prolonged ICS therapy for at least 1 year [85–88]. Very interesting conceptual observations were made in a 6-month trial, where the effects of two doses of FP (100 or 250 µg b.i.d.) on the BHR of the direct stimulus methacholine were compared with those to the indirect stimulus exercise. The improvements in BHR to exercise were evident after 3 weeks of treatment; these

improvements did not differ between the two doses and were sustained throughout the rest of the treatment period. The BHR to methacholine, however, improved significantly during the first 6 weeks and steadily increased with time at both doses; the difference between doses approached significance after 6 months. From this study, it would appear that the protection afforded by ICS against methacholine challenge is time and dose dependent, whereas the protection against exercise challenge is not [65].

The improvement of direct BHR over time under prolonged ICS treatment is thought to be the result of their effects on two different phenomena. The early improvement (detectable after 2–8 weeks of ICS) appears to be correlated with the reduction in parameters of airway inflammation [55], while the subsequent slowly progressive additional improvement (over and beyond 1 year) is thought to be the reflection of effects on chronic phenomena in the airways, relating to airway remodelling [89–91]. This interesting hypothesis is awaiting further confirmation through specifically designed long-term clinical trials. The acute effects of ICS on BHR are better reflected by indirect stimuli, such as AMP, as discussed above. Whether AMP or other indirect stimuli show a similar response to prolonged ICS remains to be established in appropriately designed clinical trials. It would be interesting to investigate whether (and to what extent) the improvement in BHR to the direct stimulus would catch up in time with the one to the indirect stimulus. In this context, it is interesting to note that thickening of the subepithelial layer, as component of airway remodelling, was found to correlate with responsiveness to methacholine, but not to UNDW in one study [92]. The obtained improvements in direct BHR appear to level off to a plateau value after about 1–2 years of fixed-dose ICS treatment, following which the improvement is maintained, without showing further improvement (at least not at the studied dose of 800 µg BDP/day), as suggested by a 5-year ICS intervention study [93]. This would suggest that the airway processes, underlying this factor of persistent BHR would require higher doses of ICS to resolve, or are completely steroid resistant. The finding that prolonged (9 months) ICS treatment did not cause a significant improvement in the BHR of adolescents with long-term asthma remission suggests that the mechanism underlying BHR in this clinical setting may be different from that in symptomatic asthma [94].

Recent findings indicate that using direct BHR as a parameter might be of practical clinical relevance in the optimization of the long-term maintenance treatment of asthma. Commonly used clinical indices of asthma severity, such as symptoms, β_2 -agonist use, FEV₁ and peak flow variability, were shown not to be significantly correlated with inflammatory cell counts in the lamina propria of the airways in non-ICS-treated [95] as well as in ICS-treated asthmatics [96]. In addition, infiltration with inflammatory cells in the lamina propria seems to persist despite regular treatment with ICS; the number of infiltrating leucocytes appears to be correlated with direct BHR, but not with symptoms or lung function [96]. In many patients whose disease is considered clinically controlled, it appears that BHR and airways inflammation persist [96–98]. According to international guidelines, the level and adjustment of anti-inflammatory treatment is based solely on symptoms and

Table 5. Proposed subclassification of indirect stimuli based on their correlation with ongoing inflammation, compared with that of direct stimuli

Type 1 (AMP type)	Type 2 (MBS/SO ₂ type)	Currently not classified
AMP	MBS/SO ₂	Exercise
NKA	Propranolol	IHV
Bradykinin	UNDW	Hypertonic saline
		Mannitol dry powder

AMP, adenosine 5'-monophosphate; NKA, neurokinin A; MBS, metabisulphite; SO₂, sulphur dioxide; UNDW, ultrasonically nebulized distilled water; IHV, isocapnic hyperventilation with (cold) dry air.

lung function [1], and, in the light of the above, one can question whether such an approach really leads to the optimal long-term outcome of asthma. Researchers from the Leiden group have postulated that, in addition to the current guidelines, asthma treatment should also be directed towards reducing BHR [96]. They have elegantly shown in a subsequent 2-year prospective parallel-group trial (the AMPUL study) that a treatment strategy with stepwise adjustments of ICS doses, aimed at reducing BHR on top of optimizing symptoms and lung function, indeed leads to more effective asthma control (significantly greater improvements in FEV₁, significantly 1.8-fold decrease in mild exacerbation rate) and greater improvement of chronic airways remodelling (significantly greater reduction in subepithelial reticular layer thickness), when compared with a 'reference' strategy, based on current treatment guidelines [89]. It would be very interesting to know what the outcome of a similar approach would be, if an indirect Type 1 challenge, especially AMP itself, were used, given its stronger correlation with inflammatory processes. The 'fil rouge' of this story is the poor correlation between BHR, airways inflammation and abnormal lung function, whereby different and complementary information about the characteristics of the disease can be obtained along each of these three axes. Moreover, their interrelationship may depend on the duration of the disease. This was illustrated by a recent study, which showed that BHR was associated with airway inflammation with a shorter duration of the disease (≤ 16 years), whereas with a longer duration (> 16 years) it is associated with impaired baseline lung function, suggesting that in chronic asthma ongoing processes of airway remodelling lead to a dissociation with markers of inflammation, and structural alterations become the primary determinant of BHR [49].

In summary, evidence indicating that measurements of BHR are not only of pathophysiological interest, but also of clinical importance, is continuously increasing. Information obtained from bronchial challenge tests provides additional and complementary information about different aspects of asthma, ranging from remodelling over diagnosis to guidance in optimization of anti-inflammatory treatment. In addition, the information, obtained by direct and indirect challenges is different, providing us with increasingly more refined information, the applications of which are currently only partially grasped. This intriguing research area is in full expansion and will undoubtedly provide us with very interesting new insights in asthma, chronic obstructive pulmonary disease (COPD) and other illnesses.

Specific uses of individual indirect challenges

We now have at our disposition a broad and heterogeneous array of stimuli, which can be used for bronchial challenge testing in asthmatic patients. How we should best measure BHR is as yet uncertain. The choice of stimulus depends at least in part on the reason for performing the challenge and on the type of information one wants to obtain.

In general, BHR to direct stimuli is extremely sensitive for clinically current asthma, and a negative test permits to exclude with reasonable certainty the diagnosis of asthma in

symptomatic patients. These tests, however, lack specificity both in differentiating asthma from normal and asthma from COPD; moreover, they perform less well in the epidemiological setting. Parameters of direct BHR appear to be better correlated with the long-term structural alterations of remodelling than with indices of inflammation. By contrast, tests of indirect BHR are both more specific (but less sensitive) for asthma and correlate better with the airway inflammation of the disease. Bronchial responsiveness to indirect stimuli may be preferred to confirm a diagnosis of asthma, for monitoring the disease activity and the anti-inflammatory effects of anti-asthma therapy in the short and medium term, while direct stimuli may be preferred to exclude asthma and to optimize anti-inflammatory treatment in the long term [12, 99]. Both types of challenges thus appear to have their value in the assessment of the different pathophysiological components of asthma.

Pharmacological stimuli

Adenosine occupies a unique place in the group of indirect stimuli, as it is the challenge which, to date, is the best reflection of Th2-like-mediated inflammation, dominated by activated mast cells and eosinophils [100]. This means that BHR to AMP can also be present in other conditions, characterized by mast cell priming, such as allergic rhinitis (AR) and a subgroup of COPD patients. Adenosine challenge may be a useful tool in making the differential diagnosis of asthma and COPD, in cases where the diagnosis is uncertain. This is especially the case in non-smokers, as smokers with COPD may show AMP responsiveness as well [50, 51].

The role of the mast cell in COPD is currently poorly defined. It has been shown that the number of epithelial mast cells in the bronchioli of smokers with COPD is increased [101]. Interestingly, a subset of COPD patients has been described, characterized by sputum eosinophilia and mast cell activation; the hypothesis has been raised that responders to steroids have both eosinophil- and mast cell-driven disease, regardless of whether they have asthma or COPD [102]. Extending this hypothesis, we would expect such patients to display BHR to AMP challenge. If this were the case, this would imply the existence of a distinct pathological COPD phenotype; a specific study, however, should be performed to confirm or to refute this hypothesis.

Concerning the association of BHR to AMP with upper airway allergic inflammation, an increasing dose-response slope along the symptom axis asymptomatic controls – AR/conjunctivitis – allergic asthma has been demonstrated, which was in addition stronger than for methacholine [54].

In summary, the specificity of AMP challenge for asthma, together with its high repeatability, could be useful for epidemiological purposes. AMP challenge testing will also become increasingly used for pharmacological testing, once specific adenosine receptor antagonists will become available for use in man. Such investigations will allow to determine whether adenosine is a clinically important mediator of asthma, as well as to identify which adenosine receptor subtypes play a main role in asthma. Adenosine is inexpensive, and the challenge is technically easy to perform; its future widespread use is therefore very probable. There are, however, two remaining issues to be resolved. Firstly, the challenge is not

yet internationally acknowledged, but the ERS task force document [12] has paved the way for pharmaceutical grade AMP to be registered and the scientific community should now take action, otherwise it will become increasingly difficult to use AMP (and other substances) in research and clinical practice. Secondly, the limited solubility of AMP is preventing higher concentrations to be used, thereby preventing full dose-response curves to be constructed.

TKs are of potential importance as mediators of asthma. Performing bronchial challenges with TKs is mainly of pathophysiological importance, to elucidate the actions of the different TKs and to study the role of the airway TK receptors. Challenge tests with substance P and NKA are currently employed to evaluate newly developed TK receptor antagonists. The high cost of these peptides, however, will probably limit their use to fundamental and clinical research purposes [43].

The other pharmacological stimuli have been much less studied to date. To date specific clinical uses have not been identified and these challenges remain essentially of pathophysiological interest in the laboratory setting. The high cost of bradykinin may be a limiting factor for its use, while the use and value of metabisulphite/MBS challenge is hampered by the absence of well-documented reproducibility studies. The limited data for propranolol support higher specificity and lower sensitivity compared with histamine or methacholine. Propranolol inhalation tests were negative in the majority of subjects with chronic airflow limitation, supporting better specificity of propranolol for asthma [12].

Physical and physicochemical stimuli

This group of challenges mimics much better the stimuli which asthmatic patients are likely to encounter in their everyday life. In this respect, exercise challenge is considered to be the most physiological of all stimuli used for challenge testing.

Several international guidelines have described the indications for exercise testing, and these include: to make a diagnosis of EIB in asthmatic patients with a history of breathlessness during or after exertion, to evaluate the ability to perform demanding or lifesaving work (e.g. military, police, or fire fighting work) in persons with a history suggesting asthma, to determine the effectiveness and optimal dosing of medications prescribed to prevent EIB, and to evaluate the effects of anti-inflammatory treatment [103, 104].

Isocapnic hyperventilation with (cold) dry air mimics exercise and has the same clinical significance. In contrast to exercise challenge, dose-response curves can be constructed [7, 12, 103].

Hypertonic saline challenge is easy to perform and allows construction of a dose-response curve. The principal indications for this challenge are to identify BHR consistent with active asthma and to evaluate bronchial responsiveness that will respond to treatment with anti-inflammatory drugs. A HS challenge is an alternative to exercise or hyperventilation to identify patients with EIB [12]. It can also be used in the assessment of patients with a history of asthma who wish to self-contained underwater breathing apparatus (SCUBA) dive [105, 106]. Hypertonic saline challenge has gained widespread use to induce sputum, and this procedure has

become an important research tool for assessing acute and chronic airway inflammation in a non-invasive fashion. The mechanisms by which sputum is induced are not known, but different mechanisms are thought to be involved, such as increased vascular permeability, stimulation of mucus production, humidification of airway mucus, stimulation of mucociliary clearance and of the cough reflex [107]. It is therefore a unique feature of HS challenge that it permits to document BHR at the same time as collecting sputum. Recently, HS challenge has found a novel application in the non-invasive assessment of microvascular leakage in the monitoring of airway inflammation, via the model of 'dual induction' (first leakage, then sputum). In this model, pharmacological agents, which induce leakage (e.g. TKs), are inhaled in a first step; this is then followed by sputum induction through a HS challenge, allowing quantification of macromolecules which serve as markers of leakage [108].

Another hypertonic challenge is the recently developed inhalation of hypertonic mannitol dry powder, the results of which show a good correlation with the other physical challenges exercise, HS, and IHV. A major advantage of this procedure is its technical simplicity as there is no need for an energy source, nor for sophisticated machinery to aerosolize the molecule; the system is portable, which could make it very useful for epidemiological purposes. The technique also allows sputum induction, but this application needs more study, as experience is limited to date [109].

There are no specific uses of UNDW challenge, which fundamentally differentiate it from the others described above [12].

The use of bronchial challenges in the evaluation of athletes and self-contained underwater breathing apparatus divers

A specific topic of interest, which is currently generating a great deal of attention, is the diagnosis of asthma and EIB in elite athletes. Since 1984, an ever increasing number of athletes, reaching 5.5% of participants of the 2000 Summer Olympic Games in Sydney, notified the International Olympic Committee Medical Commission (IOC-MC) of the use of inhaled β_2 -agonists for the treatment of putative asthma. Such a high reported prevalence of asthma might reflect a clinical asthma syndrome unique to the elite athlete group. The incidence of notification varies between sports, varying between 20% for triathlon and less than 2.2% for gymnastics, wrestling, boxing and soccer at the 2000 Sydney Summer Olympics. The IOC-MC, however, raised concerns over the accuracy of the diagnosis. Effective September 1, 2001, athletes who require the use of inhaled β_2 -agonists to treat asthma/EIB at the Olympic Games, are required to submit to the IOC-MC objective evidence to justify the need for and to obtain approval for use of this medication; in practice, this procedure has become effective from the 2002 Winter Olympic Games on [110]. More in particular, positive bronchial challenge test results for exercise, IHV or methacholine may be accepted for those athletes, who experience airway obstruction only on exercise [111, 112]. Recent findings, however, suggest that methacholine challenge has but a low sensitivity for identifying BHR to IHV, and that it should not be used as an alternative challenge for EIB in

elite athletes [113]. Isocapnic hyperventilation therefore appears to be an appropriate challenge in this specific group; specific information about the test and the interpretation of its results have recently been published [114]. Very recently, it has been shown that mannitol dry powder challenge is both very sensitive and specific for identifying responses to IHV, and, as such, could be used as alternative to the latter to identify EIB [115].

Another specific clinical situation in which bronchial challenge tests play a role is the evaluation of asthmatic patients who want to engage in SCUBA diving. Although the risk, incurred by asthmatic patients when they SCUBA dive is still much a matter of debate, BHR, when present, may implicate an increased risk. While diving, unconditioned, cold dry air is directly administered to the lower airways by breathing through the mouth. In addition, human deep-sea diving requires the use of low density gas mixtures to reduce the work of breathing. Helium gas is commonly used for this purpose, but has a specific heat and thermal conductivity which are both high compared with nitrogen. Breathing of helium–oxygen mixtures has been shown to enhance the cold-induced increase in lung resistance [116]. In addition, while diving, the diver is performing physical exercise, which may lead to hyperventilation. Several bronchial challenge tests, such as HS, IHV and exercise, have been proposed in the assessment of BHR in this setting, but there is no general consensus on which challenge to use [105, 106].

Conclusions

The knowledge relating to indirect challenges has greatly increased from the 1980s on. The concept of the fundamental pathophysiological distinction between indirect and direct challenges is now firmly established. The mechanisms through which the different indirect challenges induce airflow limitation have been studied in great detail, and further evolutions in this research area are to be expected, as novel-specific receptor antagonists (e.g. for adenosine or TKs), synthesis inhibitors and/or enzyme inhibitors will become available in the future. In the meantime, still new challenges (such as mannitol dry powder) [109] or new approaches (such as dual induction) [108] are being developed. Moreover, the characteristics of bronchial challenges are currently being studied in greater detail in other diseases than asthma, such as COPD and AR; this may lead to new applications in the future.

It has become evident that the mechanisms by which the different indirect stimuli act are clearly heterogeneous, and that their classification will have to be further refined, beyond the simple classification based on the nature of the stimulus. One further distinction that has become evident, is the degree to which an indirect stimulus is reflecting the ongoing inflammatory process in the airways; the behaviour of the different stimuli is clearly different in this respect. We propose to subdivide indirect challenges into two subgroups, based on the degree by which they reflect ongoing airway inflammation, revealed indirectly by a therapeutic test with ICS. 'Type 1' or 'adenosine-type' indirect challenges are characterized by a significantly greater correlation with surrogate markers of inflammation and a significantly larger response to a therapeutic test with SC-ICS, in comparison with a direct

challenge. 'Type 2' or 'metabisulphite-type' indirect challenges, on the other hand, do not behave significantly different from direct stimuli on the parameters mentioned above.

The area of indirect BHR is in full evolution, and is only beginning to unveil its secrets; much more work, however, remains to be done. An important conceptual point that needs addressing is whether the exquisite sensitivity of some indirect challenges to steroid intervention is related to their desired disease modifying effects, or merely because of functional antagonism, as this would determine in a fundamental way the value of these challenges as outcome parameter. The ERS Task Force on 'indirect airway challenges' has identified the remaining gaps in our knowledge, and proposed areas for future research for the following topics: mechanisms and receptors involved, assessment of the value of the different challenges for diagnostic, monitoring, and epidemiological purposes, and technical issues, such as standardization and reproducibility [12]. On a more fundamental level, testing of specific and potent adenosine or TK receptor antagonists is eagerly awaited, because this will allow further pharmacological dissection of mechanisms of airflow limitation, which may in turn potentially lead to novel ways of treating asthma. On a more clinical level, there currently exists a great need to perform long-term trials of at least 1 year, comparing indirect stimuli (such as AMP) with direct ones (such as methacholine) in order to be able to determine their relative value in monitoring treatment in the long run. The AMPUL study by Sont et al. [89] with methacholine challenge and the study by Green et al. [117] using sputum eosinophils are examples of approaches, to which the monitoring value of indirect challenges will have to be weighted. In addition, as combination inhaler treatment with ICS+LABA is becoming increasingly commonplace, there is also a need to study the long-term effects of such treatment on both direct and indirect challenges. The future holds great promise, and it appears that where we are going will indeed be quite far away from where we have been [118].

References

- 1 Global Initiative for Asthma, Revised 2002. National Institutes of Health. National Heart, Lung, and Blood Institute. NIH publication 02-3659A, July 2002. www.ginasthma.com
- 2 Pauwels R. Bronchial hyperresponsiveness. In: Kay AB, ed. Allergy and allergic diseases. Oxford: Blackwell Science Ltd, 1997; 682–91.
- 3 Sterk PJ. Airway hyperresponsiveness: using bronchial challenge tests in research and management of asthma. *J Aerosol Med* 2002; 15:123–9.
- 4 Joos GF. Do measures of bronchial responsiveness add information in diagnosis and monitoring of patients with asthma? *Eur Respir J* 2001; 18:439–41.
- 5 Brusasco V, Crimi E, Pellegrino R. Airway hyperresponsiveness in asthma: not just a matter of airway inflammation. *Thorax* 1998; 53:992–8.
- 6 Pauwels R, Joos G, Van Der Straeten M. Bronchial hyperresponsiveness is not bronchial hyperresponsiveness is not bronchial asthma. *Clin Allergy* 1988; 18:317–21.
- 7 Van Schoor J, Joos GF, Pauwels RA. Indirect bronchial hyperresponsiveness in asthma: mechanisms, pharmacology and implications for clinical research. *Eur Respir J* 2000; 16:514–33.
- 8 McCoy DE, Schieberr EM, Karlson KH, Spielman WS, Stanton BA. Identification and function of A₁ adenosine receptors in

- normal and cystic fibrosis human airway epithelial cells. *Am J Physiol* 1995; 268:C1520–7.
- 9 Clancy JP, Ruiz FE, Sorscher EJ. Adenosine and its nucleotides activate wild-type and R117H CFTR through an A_{2B} receptor-coupled pathway. *Am J Physiol* 1999; 276:C361–9.
 - 10 Bai TR, Zhou D, Weir T et al. Substance P (NK₁)- and neurokinin A (NK₂)-receptor gene expression in inflammatory airway diseases. *Am J Physiol* 1995; 269:L309–17.
 - 11 Lung CC, Jagels MA, Daffern PJ, Tan EM, Zuraw BL. Induction of human B₂ bradykinin receptor mRNA and membrane receptors by IFN γ . *Immunopharmacology* 1998; 39:243–53.
 - 12 Joos GF, O'Connor B, ERS Task Force. Indirect airway challenges. *Eur Respir J* 2003; 21:1050–68.
 - 13 Tutluoglu B, Gurbuz N, Atis S, Abanozlu S, Ibis R, Kanik A. Effects of heparin on hypertonic potassium chloride-induced bronchoconstriction. *Ann Pharmacother* 2001; 35:1161–5.
 - 14 Tranfa CME, Vatrella A, Parrella R, Pelaia G, Bariffi F, Marsico SA. Effect of inhaled heparin on water-induced bronchoconstriction in allergic asthmatics. *Eur J Clin Pharmacol* 2001; 57:5–9.
 - 15 Rorke S, Jennison S, Jeffs JA, Sampson AP, Arshad H, Holgate ST. Role of cysteinyl leukotrienes in adenosine 5'-monophosphate induced bronchoconstriction in asthma. *Thorax* 2002; 57:323–7.
 - 16 Kraan J, Vink-Klooster H, Postma DS. The NK₂ receptor antagonist SR 48968C does not improve adenosine hyperresponsiveness and airway obstruction in allergic asthma. *Clin Exp Allergy* 2001; 31:274–8.
 - 17 Joos G, Van De Velde V, Schelfhout V, Pauwels R. The leukotriene receptor antagonist zafirlukast inhibits neurokinin A-induced bronchoconstriction in patients with asthma. *Am J Respir Crit Care Med* 2001; 163:A418.
 - 18 Crimi N, Pagano C, Palermo F et al. Inhibitory effect of a leukotriene receptor antagonist (montelukast) on neurokinin A-induced bronchoconstriction. *J Allergy Clin Immunol* 2003; 111:833–9.
 - 19 Van Schoor J, Joos GF, Pauwels RA. Effect of inhaled fluticasone on bronchial responsiveness to neurokinin A in asthma. *Eur Respir J* 2002; 19:997–1002.
 - 20 Reynolds CJ, Togiias A, Proud D. Airways hyper-responsiveness to bradykinin and methacholine: effects of inhaled fluticasone. *Clin Exp Allergy* 2002; 32:1174–9.
 - 21 Carratù P, Morelli N, Freire AX et al. Effect of zafirlukast on methacholine and ultrasonically nebulized distilled water challenge in patients with mild asthma. *Respiration* 2003; 70:249–53.
 - 22 Richter K, Jörres RA, Magnussen H. Efficacy and duration of action of the antileukotriene zafirlukast on cold air-induced bronchoconstriction. *Eur Respir J* 2000; 15:693–9.
 - 23 Anderson SD, Brannan J, Spring J et al. A new method for bronchial-provocation testing in asthmatic subjects using a dry powder of mannitol. *Am J Respir Crit Care Med* 1997; 156:758–65.
 - 24 Eggleston PA, Kagey-Sobotka A, Lichtenstein LM. A comparison of the osmotic activation of basophils and human lung mast cells. *Am Rev Respir Dis* 1987; 135:1043–8.
 - 25 Moloney ED, Griffin S, Burke CM, Poulter LW, O'Sullivan S. Release of inflammatory mediators from eosinophils following a hyperosmolar stimulus. *Respir Med* 2003; 97:928–32.
 - 26 Brannan JD, Gulliksson M, Anderson SD, Chew N, Kumlin M. Evidence of mast cell activation and leukotriene release after mannitol inhalation. *Eur Respir J* 2003; 22:491–6.
 - 27 Currie GP, Haggart K, Brannan JD, Lee DKC, Anderson SD, Lipworth BJ. Relationship between airway hyperresponsiveness to mannitol and adenosine monophosphate. *Allergy* 2003; 58:762–6.
 - 28 Brannan JD, Anderson SD, Gomes K, King GG, Chan HK, Seale JP. Fexofenadine decreases sensitivity to and montelukast improves recovery from inhaled mannitol. *Am J Respir Crit Care Med* 2001; 163:1420–5.
 - 29 Currie GP, Haggart K, Lee DKC et al. Effects of mediator antagonism on mannitol and adenosine monophosphate challenges. *Clin Exp Allergy* 2003; 33:783–8.
 - 30 Brannan JD, Anderson SD, Freed R, Leuppi JD, Koskela H, Chan HK. Nedocromil sodium inhibits responsiveness to inhaled mannitol in asthmatic subjects. *Am J Respir Crit Care Med* 2000; 161:2096–9.
 - 31 Brannan JD, Koskela H, Anderson SD, Chan HK. Budesonide reduces sensitivity and reactivity to inhaled mannitol in asthmatic subjects. *Respirology* 2002; 7:37–44.
 - 32 Fredholm BB, Ijzerman AP, Jacobson KA, Klotz KN, Linden J. International union of pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev* 2001; 53:527–52.
 - 33 Young HWJ, Molina JG, Dimina D et al. A₃ adenosine receptor signaling contributes to airway inflammation and mucus production in adenosine deaminase-deficient mice. *J Immunol* 2004; 173:1380–9.
 - 34 Feoktistov I, Garland EM, Goldstein AE et al. Inhibition of human mast cell activation with the novel selective adenosine A_{2B} receptor antagonist 3-isobutyl-8-pyrrolidinoxanthine (IPDX). *Biochem Pharmacol* 2001; 62:1163–73.
 - 35 Ryzhov S, Goldstein AE, Matafonov A, Zeng D, Biaggioni I, Feoktistov I. Adenosine-activated mast cells induce IgE synthesis by B lymphocytes: an A_{2B}-mediated process involving Th2 cytokines IL-4 and IL-13 with implications for asthma. *J Immunol* 2004; 172:7726–33.
 - 36 Zhong H, Belardinelli L, Maa T, Feoktistov I, Biaggioni I, Zeng D. A_{2B} adenosine receptors increase cytokine release by bronchial smooth muscle cells. *Am J Respir Cell Mol Biol* 2004; 30:118–25.
 - 37 Nyce JW, Metzger WJ. DNA antisense therapy for asthma in an animal model. *Nature* 1997; 385:721–5.
 - 38 Suzuki H, Takei M, Nakahata T, Fukamachi H. Inhibitory effect of adenosine on degranulation of human cultured mast cells upon cross-linking with Fc ϵ RI. *Biochem Biophys Res Commun* 1998; 242:697–702.
 - 39 Fozard JR, Ellis KM, Villela Dantas MF, Tigani B, Mazzoni L. Effects of CGS 21680, a selective adenosine A_{2A} receptor agonist, on allergic airways inflammation in the rat. *Eur J Pharmacol* 2002; 438:183–8.
 - 40 Walker BAM, Jacobson MA, Knight DA et al. Adenosine A₃ receptor expression and function in eosinophils. *Am J Respir Cell Mol Biol* 1997; 16:531–7.
 - 41 Knight D, Zheng X, Rocchini C, Jacobson M, Bai T, Walker B. Adenosine A₃ receptor stimulation inhibits migration of human eosinophils. *J Leukoc Biol* 1997; 62:465–8.
 - 42 Ezeamuzie CI, Philips E. Adenosine A₃ receptors on human eosinophils mediate inhibition of degranulation and superoxide anion release. *Br J Pharmacol* 1999; 127:188–94.
 - 43 Joos GF, Pauwels RA. Pro-inflammatory effects of substance P: new perspectives for the treatment of airway diseases? *Trends Pharmacol Sci* 2000; 21:131–3.
 - 44 Van Schoor J, Joos GF, Chasson BL, Brouard RJ, Pauwels RA. The effect of the NK₂ tachykinin receptor antagonist SR 48968 (saredutant) on neurokinin A-induced bronchoconstriction in asthmatics. *Eur Respir J* 1998; 12:17–23.
 - 45 Joos G, Schelfhout V, Van De Velde V, Maggi C, Pauwels R. The effect of the tachykinin NK₂ receptor antagonist MEN11420 (nepadutant) on neurokinin A-induced bronchoconstriction in patients with asthma. *Am J Respir Crit Care Med* 2001; 163:A628.
 - 46 Joos GF, Vincken W, Louis R et al. Dual tachykinin NK₁/NK₂ antagonist DNK333 inhibits neurokinin A-induced bronchoconstriction in asthma patients. *Eur Respir J* 2004; 23:76–81.
 - 47 Schelfhout V, Louis R, Lenz W, Pauwels R, Joos G. The triple neurokinin receptor antagonist CS-003 inhibits neurokinin A (NKA)-induced bronchoconstriction in patients with asthma. *Eur Respir J* 2003; 22 (Suppl. 45):415s.
 - 48 Rosi E, Ronchi MC, Grazzini M, Duranti R, Scano G. Sputum analysis, bronchial hyperresponsiveness, and airway function in

- asthma: results of a factor analysis. *J Allergy Clin Immunol* 1999; 103:232–7.
- 49 Grönke L, Kannies F, Holz O, Jörres RA, Magnussen H. The relationship between airway hyper-responsiveness, markers of inflammation and lung function depends on the duration of the asthmatic disease. *Clin Exp Allergy* 2002; 32:57–63.
 - 50 Polosa R, Rorke S, Holgate ST. Evolving concepts on the value of adenosine hyperresponsiveness in asthma and chronic obstructive pulmonary disease. *Thorax* 2002; 57:649–54.
 - 51 van den Berge M, Kerstjens HAM, Postma DS. Provocation with adenosine 5'-monophosphate as a marker of inflammation in asthma, allergic rhinitis and chronic obstructive pulmonary disease. *Clin Exp Allergy* 2002; 32:824–30.
 - 52 van Velzen E, van den Bos JW, Benckhuijsen JAW, van Essel T, de Bruijn R, Aalbers R. Effect of allergen avoidance at high altitude on direct and indirect bronchial hyperresponsiveness and markers of inflammation in children with allergic asthma. *Thorax* 1996; 51:582–4.
 - 53 van den Berge M, Meijer RJ, Kerstjens HAM et al. PC₂₀ adenosine 5'-monophosphate is more closely associated with airway inflammation in asthma than PC₂₀ methacholine. *Am J Respir Crit Care Med* 2001; 163:1546–50.
 - 54 De Meer G, Heederik D, Postma DS. Bronchial responsiveness to adenosine 5'-monophosphate (AMP) and methacholine differ in their relationship with airway allergy and baseline FEV₁. *Am J Respir Crit Care Med* 2002; 165:327–31.
 - 55 van den Berge M, Kerstjens HAM, Meijer RJ et al. Corticosteroid-induced improvement in the PC₂₀ of adenosine monophosphate is more closely associated with reduction in airway inflammation than improvement in the PC₂₀ of methacholine. *Am J Respir Crit Care Med* 2001; 164:1127–32.
 - 56 Kannies F, Richter K, Böhme S, Jörres RA, Magnussen H. Effect of inhaled ciclesonide on airway responsiveness to inhaled AMP, the composition of induced sputum and exhaled nitric oxide in patients with mild asthma. *Pulm Pharmacol Ther* 2001; 14:141–7.
 - 57 Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. *N Engl J Med* 2002; 346:1699–705.
 - 58 Ketchell RI, Jensen MW, Lumley P, Wright AM, Allenby MI, O'Connor BJ. Rapid effect of inhaled fluticasone propionate on airway responsiveness to adenosine 5'-monophosphate in mild asthma. *J Allergy Clin Immunol* 2002; 110:603–6.
 - 59 Ketchell RI, Jensen MW, Clarke GW, Allenby MI, O'Connor BJ. Rapid effect of single dose inhaled glucocorticosteroid on airway responsiveness to AMP in asthma is not molecule-dependent. *Am J Respir Crit Care Med* 2003; 167:A770.
 - 60 Ketchell RI, Clarke GW, Jensen MW, Vos TP, Allenby MI, O'Connor BJ. Time course evaluation of the rapid effect of a single dose of inhaled fluticasone propionate on airway responsiveness to AMP in asthma. *Am J Respir Crit Care Med* 2003; 167:A770.
 - 61 Luijk B, Kempersford RD, Wright AM, Zanen P, Lammers JWJ. Duration of effect of single-dose inhaled fluticasone propionate on AMP-induced bronchoconstriction. *Eur Respir J* 2004; 23:559–64.
 - 62 Prosperini G, Spicuzza L, Polosa R. Rapid effect of inhaled fluticasone on airway responsiveness to AMP: research implications (letter to the editor+reply). *J Allergy Clin Immunol* 2003; 111:1416–7.
 - 63 Taylor DA, Jensen MW, Kanabar V et al. A dose-dependent effect of the novel inhaled corticosteroid ciclesonide on airway responsiveness to adenosine 5'-monophosphate in asthmatic patients. *Am J Respir Crit Care Med* 1999; 160:237–43.
 - 64 Prosperini G, Rajakulasingam K, Cacciola RR et al. Changes in sputum counts and airway hyperresponsiveness after budesonide: monitoring anti-inflammatory response on the basis of surrogate markers of airway inflammation. *J Allergy Clin Immunol* 2002; 110:855–61.
 - 65 Hofstra WB, Neijens HJ, Duiverman EJ et al. Dose-responses over time to inhaled fluticasone propionate treatment of exercise- and methacholine-induced bronchoconstriction in children with asthma. *Pediatr Pulmonol* 2000; 29:415–23.
 - 66 O'Connor BJ, Ridge SM, Barnes PJ, Fuller RW. Greater effect of inhaled budesonide on adenosine 5'-monophosphate-induced than on sodium-metabisulphite-induced bronchoconstriction in asthma. *Am Rev Respir Dis* 1992; 146:560–4.
 - 67 Doull IJM, Sandall D, Smith S, Schreiber J, Freezer NJ, Holgate ST. Differential inhibitory effect of regular inhaled corticosteroid on airway responsiveness to adenosine 5' monophosphate, methacholine, and bradykinin in symptomatic children with recurrent wheeze. *Pediatr Pulmonol* 1997; 23:404–11.
 - 68 Weersink EJM, Douma RR, Postma DS, Koëter GH. Fluticasone propionate, salmeterol xinafoate, and their combination in the treatment of nocturnal asthma. *Am J Respir Crit Care Med* 1997; 155:1241–6.
 - 69 Fuller RW, Choudry NB, Eriksson G. Action of budesonide on asthmatic bronchial hyperresponsiveness. Effects on directly and indirectly acting bronchoconstrictors. *Chest* 1991; 100: 670–4.
 - 70 Wiebicke W, Jörres R, Magnussen H. Comparison of the effects of inhaled corticosteroids on the airway response to histamine, methacholine, hyperventilation, and sulfur dioxide in subjects with asthma. *J Allergy Clin Immunol* 1990; 86:915–23.
 - 71 Kraan J, Koëter GH, van der Mark TW, Sluiter GH, de Vries K. Changes in bronchial hyperreactivity induced by 4 weeks of treatment with antiasthmatic drugs in patients with allergic asthma: a comparison between budesonide and terbutaline. *J Allergy Clin Immunol* 1985; 76:628–36.
 - 72 Vathenen AS, Knox AJ, Wisniewski A, Tattersfield AE. Effect of inhaled budesonide on bronchial reactivity to histamine, exercise, and eucapnic dry air hyperventilation in patients with asthma. *Thorax* 1991; 46:811–6.
 - 73 Claussen M, Sill V. Influence on non-specific bronchial hyper-reactivity to cold air hyperventilation and carbamol by nedocromil and budesonide. *Pneumologie* 1993; 47:209–14.
 - 74 Nielsen KG, Bisgaard H. The effect of inhaled budesonide on symptoms, lung function, and cold air and methacholine responsiveness in 2-to 5-year-old asthmatic children. *Am J Respir Crit Care Med* 2000; 162:1500–6.
 - 75 du Toit JI, Anderson SD, Jenkins CR, Woolcock AJ, Rodwell LT. Airway responsiveness in asthma: bronchial challenge with histamine and 4.5% sodium chloride before and after budesonide. *Allergy Asthma Proc* 1997; 18:7–14.
 - 76 Groot CAR, Lammers JWJ, Molema J, Festen J, van Herwaarden CLA. Effect of inhaled beclomethasone and nedocromil sodium on bronchial hyperresponsiveness to histamine and distilled water. *Eur Respir J* 1992; 5:1075–82.
 - 77 Bootsma GP, Dekhuijzen PNR, Festen J, Mulder PGH, van Herwaarden CLA. Comparison of fluticasone propionate and beclomethasone dipropionate on direct and indirect measurements of bronchial hyperresponsiveness in patients with stable asthma. *Thorax* 1995; 50:1044–50.
 - 78 Roisman GL, Lacroinque JG, Desmazes-Dufeu N, Carré C, Le Cae A, Dusser DJ. Airway responsiveness to bradykinin is related to eosinophilic inflammation in asthma. *Am J Respir Crit Care Med* 1996; 153:381–90.
 - 79 Polosa R, Louis R, Cacciola R, Prosperini G, Crimi N, Djukanovic R. Sputum eosinophilia is more closely associated with airway responsiveness to bradykinin than methacholine in asthma. *Eur Respir J* 1998; 12:551–6.
 - 80 Berman AR, Togias AG, Skloot G, Proud D. Allergen-induced hyperresponsiveness to bradykinin is more pronounced than that to methacholine. *J Appl Physiol* 1995; 78:1844–52.

- 81 van Rensen ELJ, Straathof KCM, Veselic-Charvat MA, Zwinderman AH, Bel EH, Sterk PJ. Effect of inhaled steroids on airway hyperresponsiveness, sputum eosinophils, and exhaled nitric oxide levels in patients with asthma. *Thorax* 1999; 54:403–8.
- 82 Carpentiere G, Castello F, Marino S. Effect of beclomethasone dipropionate on the bronchial responsiveness to propranolol in asthmatics. *Chest* 1990; 98:263–5.
- 83 van Grunsven PM, van Schayck CP, Molema J, Akkermans RP, van Weel C. Effect of inhaled corticosteroids on bronchial responsiveness in patients with "corticosteroid naive" mild asthma: a meta-analysis. *Thorax* 1999; 54:316–22.
- 84 Sovijärvi ARA, Haahtela T, Ekroos HJ et al. Sustained reduction in bronchial hyperresponsiveness with inhaled fluticasone propionate within three days in mild asthma: time course after onset and cessation of treatment. *Thorax* 2003; 58:500–4.
- 85 Kerrebijn KF, van Essen-Zandvliet EEM, Neijens HJ. Effect of long-term treatment with inhaled corticosteroids and beta-agonists on the bronchial responsiveness in children with asthma. *J Allergy Clin Immunol* 1987; 79:653–9.
- 86 Juniper EF, Kline PA, Vanzieleghe MA, Ramsdale EH, O'Byrne PM, Hargreave FE. Effect of long-term treatment with an inhaled corticosteroid (budesonide) on airway hyperresponsiveness and clinical asthma in nonsteroid-dependent asthmatics. *Am Rev Respir Dis* 1990; 142:832–6.
- 87 Kerstjens HAM, Brand PLP, Dutch chronic non-specific lung disease study group et al. A comparison of bronchodilator therapy with or without inhaled corticosteroid therapy for obstructive airways disease. *N Engl J Med* 1992; 327:1413–9.
- 88 van Essen-Zandvliet EE, Hughes MD, Dutch chronic non-specific lung disease study group et al. Effects of 22 months of treatment with inhaled corticosteroids and/or beta₂-agonists on lung function, airway responsiveness, and symptoms in children with asthma. *Am Rev Respir Dis* 1992; 146:547–54.
- 89 Sont JK, Willems LNA, AMPUL study group. Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. *Am J Respir Crit Care Med* 1999; 159:1043–51.
- 90 Ward C, Pais M, Bish R et al. Airway inflammation, basement membrane thickening and bronchial hyperresponsiveness in asthma. *Thorax* 2002; 57:309–16.
- 91 Kips JC, Pauwels RA. Airway wall remodeling: does it occur and what does it mean? *Clin Exp Allergy* 1999; 29:1457–66.
- 92 Chetta A, Foresi A, Del Donno M et al. Bronchial responsiveness to distilled water and methacholine and its relationship to inflammation and remodeling of the airways in asthma. *Am J Respir Crit Care Med* 1996; 153:910–7.
- 93 Douma WR, Kerstjens HAM, Dutch chronic nonspecific lung disease study group. Initial improvements in lung function and bronchial hyperresponsiveness are maintained during 5 years of treatment with inhaled beclomethasone dipropionate and terbutaline. *Chest* 2002; 121:151–7.
- 94 Koh YY, Sun YH, Lim HS, Kim CK, Hong SJ. Effect of inhaled budesonide on bronchial hyperresponsiveness in adolescents with clinical remission of asthma. *Chest* 2001; 120:1140–6.
- 95 Djukanović R, Wilson JW, Britten KM et al. Quantitation of mast cells and eosinophils in the bronchial mucosa of symptomatic atopic asthmatics and healthy control subjects using immunohistochemistry. *Am Rev Respir Dis* 1990; 142:863–71.
- 96 Sont JK, van Krieken JHJM, Evertse CE, Hooijer R, Willems LNA, Sterk PJ. Relationship between the inflammatory infiltrate in bronchial biopsy specimens and clinical severity of asthma in patients treated with inhaled steroids. *Thorax* 1996; 51:496–502.
- 97 Boulet LP, Turcotte H, Brochu A. Persistence of airway obstruction and hyperresponsiveness in subjects with asthma remission. *Chest* 1994; 105:1024–31.
- 98 van Essen-Zandvliet EE, Hughes MD, Waalkens HJ, Duiverman EJ, Kerrebijn KF, the Dutch chronic non-specific lung disease study group. Remission of childhood asthma after long-term treatment with an inhaled corticosteroid: can it be achieved? *Eur Respir J* 1994; 7:63–8.
- 99 Cockcroft DW. How best to measure airway responsiveness. *Am J Respir Crit Care Med* 2001; 163:1514–5.
- 100 Holgate ST. Adenosine provocation: a new test for allergic type airway inflammation. *Am J Respir Crit Care Med* 2002; 165:317–9.
- 101 Grashoff WFH, Sont JK, Sterk PJ et al. Chronic obstructive pulmonary disease. Role of bronchiolar mast cells and macrophages. *Am J Pathol* 1997; 151:1785–90.
- 102 Louis RE, Cataldo D, Buckley MG et al. Evidence of mast-cell activation in a subset of patients with eosinophilic chronic obstructive pulmonary disease. *Eur Respir J* 2002; 20:325–31.
- 103 Sterk PJ, Fabbri LM, Quanjer PhH et al. Airway responsiveness. Standardized challenge testing with pharmacological, physical and sensitizing stimuli in adults. Report Working Party Standardization of Lung Function Tests. European Community for Steel and Coal. Official statement of the European Respiratory Society. *Eur Respir J* 1993; 6 (Suppl. 16):53–83.
- 104 American Thoracic Society. Guidelines for methacholine and exercise challenge testing – 1999. *Am J Respir Crit Care Med* 2000; 161:309–29.
- 105 Anderson SD, Brannan J, Trevillion L, Young IH. Lung function and bronchial provocation tests for intending divers with a history of asthma. *SPUMS J* 1995; 25:233–48.
- 106 British Thoracic Society Guidelines on Respiratory Aspects of Fitness for Diving. British Thoracic Society Fitness to Dive Group, a Subgroup of the British Thoracic Society Standards of Care Committee. *Thorax* 2003; 58:3–13.
- 107 Djukanović R, Sterk PJ, Fahy JV, Hargreave FE, ERS Task Force Standardised methodology of sputum induction and processing. *Eur Respir J* 2002; 20 (Suppl. 37):1s–55s.
- 108 Van Rensen ELJ, Hiemstra PS, Rabe KF, Sterk PJ. Assessment of microvascular leakage via sputum induction. The role of substance P and neurokinin A in patients with asthma. *Am J Respir Crit Care Med* 2002; 165:1275–9.
- 109 Leuppi JD, Brannan JD, Anderson SD. Bronchial provocation tests: the rationale for using inhaled mannitol as a test for airway hyperresponsiveness. *Swiss Med Wkly* 2002; 132:151–8.
- 110 McKenzie DC, Stewart IB, Fitch KD. The asthmatic athlete, inhaled beta agonists, and performance. *Clin J Sport Med* 2002; 12:225–8.
- 111 Weiler JM. Why must Olympic athletes prove that they have asthma to be permitted to take inhaled beta₂-agonists? *J Allergy Clin Immunol* 2003; 111:36–7.
- 112 International Olympic Medical Commission., Beta₂-adrenoceptor agonists and the Olympic Winter Games in Salt Lake City. www.olympic.org
- 113 Holzer K, Anderson SD, Douglass J. Exercise in elite summer athletes: challenges for diagnosis. *J Allergy Clin Immunol* 2002; 110:374–80.
- 114 Anderson SD, Argyros GJ, Magnussen H, Holzer K. Provocation by eucapnic voluntary hyperpnea to identify exercise induced bronchoconstriction. *Br J Sports Med* 2001; 35:344–7.
- 115 Holzer K, Anderson SD, Chan HK, Douglass J. Mannitol as a challenge test to identify exercise-induced bronchoconstriction in elite athletes. *Am J Respir Crit Care Med* 2003; 167:534–7.
- 116 Jammes Y, Burnet H, Cosson P, Lucciano M. Bronchomotor response to cold air or helium-oxygen at normal and high ambient pressures. *Undersea Biomed Res* 1988; 15:179–92.
- 117 Green RH, Brightling CE, McKenna S et al. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *Lancet* 2002; 360:1715–21.
- 118 Anderson SD, Brannan JD, Chan HK. Use of aerosols for bronchial provocation testing in the laboratory: where we have been and where we are going. *J Aerosol Med* 2002; 15:313–24.