Eur Respir J 2008; 31: 311–319
DOI: 10.1183/09031936.00003707

Elevated expression of adenosine A_1 receptor in bronchial biopsy specimens from asthmatic subjects

R.A. Brown*, G.W. Clarke#, C.L. Ledbetter#, M.J. Hurle¶, J.C. Denyer¶, D.E. Simcock#, J.E. Coote¶, T.J. Savage¶, R.D. Murdoch¶, C.P. Page*, D. Spina* and B.J. O'Connor#

ABSTRACT: Asthmatics, unlike healthy subjects, experience bronchoconstriction in response to inhaled adenosine, and extracellular adenosine concentrations are elevated in the bronchoalveolar lavage fluid and exhaled breath condensate of asthmatic subjects. However, little is known about the location and expression of adenosine receptors in asthmatic airways. The aim of the present study was to investigate the distribution of adenosine A_1 receptors in bronchial biopsy specimens from mildly asthmatic steroid-naïve subjects and then compare the degree of expression with that of healthy subjects.

Biopsy sections were immunostained using an adenosine A_1 receptor antibody, the selectivity of which was validated in specific experiments. Image analysis was then performed in order to determine differences in immunostaining intensity.

Immunostaining of biopsy sections from the asthmatic subjects revealed strong expression of the A_1 receptor, located predominantly in the bronchial epithelium and bronchial smooth muscle. In comparison, very weak immunostaining was observed in biopsy specimens obtained from healthy subjects. Image analysis revealed that the intensity of positive staining of the asthmatic bronchial epithelium and smooth muscle regions was significantly greater than that observed for the healthy epithelium and smooth muscle.

In conclusion, the sensitivity of asthmatics to inhaled adenosine coupled with increased adenosine A_1 receptor expression implies that these receptors play a role in the pathophysiology of this disease.

KEYWORDS: Adenosine, adenosine A_1 receptor, adenosine A_1 receptor expression, asthma

Adenosine is a purine nucleoside that is expressed in all cells of the body and involved in a wide range of physiological processes. The effects of adenosine are mediated predominantly through specific cell surface receptors, of which four subtypes (A_1, A_{2A}, A_{2B} and A_3) have been described. It is now well recognised that extracellular levels of adenosine markedly increase under metabolically stressful conditions, such as hypoxia and inflammation, and, although an acutely elevated level of extracellular adenosine is considered to mediate anti-inflammatory and protective effects, chronic accumulation has been associated with pathological consequences [1].

In asthmatic subjects, it has been demonstrated that adenosine levels in bronchoalveolar lavage fluid and exhaled breath condensate are significantly higher than those occurring in healthy subjects [2, 3], and current evidence strongly suggests that they may contribute to the pathophysiology of asthma. For example, it has been recognised since the mid-1980s that inhalation of adenosine 5′-monophosphate (AMP; 5′-nucleotidase rapidly hydrolyses AMP to adenosine in the lung) in asthmatic but not healthy subjects results in dose-related bronchoconstriction [4]. This is considered to be mediated predominantly, but not exclusively, by mast cell degranulation *via* A_{2B} receptor activation (reviewed in [5]). Furthermore, inhalation of AMP has been shown to increase airway eosinophilia [6].

A role for adenosine in asthma is further supported by the observation that plasma

This article has supplementary material accessible from www.erj.ersjournals.com

AFFILIATIONS
*Sackler Institute of Pulmonary Pharmacology, Division of Pharmaceutical Sciences, #Dept of Asthma and Allergy, Division of Medicine, King's College London, London, and ¶GlaxoSmithKline, Stevenage, UK.

CORRESPONDENCE
C.P. Page
King's College London
Sackler Institute of Pulmonary Pharmacology
5th floor
Hodgkin Building
Guy's Campus
London
SE1 1UL
UK
Fax: 44 2078486097
E-mail: clive.page@kcl.ac.uk

Received:
January 11 2007
Accepted after revision:
September 27 2007

STATEMENT OF INTEREST
Statements of interest for M.J. Hurle, J.C. Denyer, D.E. Simcock, J.E. Coote, T.J. Savage, R.D. Murdoch, C.P. Page and D. Spina and the study itself can be found at www.erj.ersjournals.com/misc/statements.shtml

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

adenosine levels increase rapidly following allergen challenge in asthmatic subjects [7], suggesting a possible involvement of endogenous adenosine in the early-phase airway response to allergen. This possibility was supported by the blockade of allergen-induced bronchoconstriction through the use of an antisense oligonucleotide directed against the A_1 receptor [8] and, in 2005, of a selective adenosine A_1 receptor antagonist [9] in allergic rabbits. Furthermore, it has been demonstrated that the concentrations of adenosine are increased in both plasma and exhaled breath condensate during exercise-induced bronchoconstriction in subjects with asthma [10, 11]. Finally, it has also been shown that isolated human asthmatic bronchial tissue contracts in response to adenosine *via* an A_1-receptor-dependent mechanism [12].

In order to further investigate the role of endogenous adenosine in asthma, it is crucial that current knowledge concerning the distribution and specific functions of adenosine receptors in asthmatic airways is extended. To date, binding studies in healthy peripheral lung tissue have suggested that A_2 receptor subtypes are more abundant than the A_1 and A_3 receptor subtypes [13], and expression of the A_3 receptor was observed to be upregulated in asthmatic lung tissue, where it was located predominantly to eosinophils [14]. Therefore, the aim of the present study was to extend these observations. Although the ultimate intention is to profile the expression of all four adenosine receptor subtypes and identify the subtype-specific cellular functions, it was hypothesised that characterisation of the distribution and expression of the A_1 receptor would particularly further understanding of the role of adenosine in asthma given the observations detailed above. Thus the distribution of the adenosine A_1 receptor in bronchial biopsy specimens from asthmatic subjects was investigated and then compared to the distribution and level of expression of biopsy specimens obtained from healthy subjects in order to provide further insight into the role of adenosine in asthma.

METHODS

Further details of all methods and procedures can be found in the supplementary material.

Subjects

All of the asthmatic volunteers provided a clinical history consistent with intermittent reversible airflow obstruction and atopy to common aeroallergens. Thus all of the asthmatic subjects (nonsmokers) were characterised on the basis of a previous history of wheeze, a forced expiratory volume in one second (FEV_1) of >70% of the predicted value, atopy as defined by a positive cutaneous response to intradermal allergen, and airways hyperresponsiveness to both methacholine and AMP. In addition, all asthmatic subjects demonstrated an increase in baseline FEV_1 of ⩾15–20% in response to inhaled salbutamol (data not shown). Of the 16 mildly asthmatic subjects screened for the present study, 12 went on to complete bronchial provocation challenge with AMP and bronchoscopy (four subjects withdrew their consent to undergo bronchoscopy). Each subject exhibited infrequent symptoms, which were controlled using occasional inhaled short-acting β_2-agonists alone. No subject was taking any regular anti-asthma therapy, and none had taken inhaled corticosteroids for ⩾3 months prior to entry. None of the subjects had had an exacerbation of asthma or respiratory infection during the preceding 6 weeks, and all subjects were nonsmokers. Patient demographics are summarised in table 1. For comparison, biopsy specimens from eight healthy age-matched nonsmoking subjects obtained under the same conditions in a separate but recent previous study were also analysed. The demographics of these subjects are also described in table 1. Written informed consent was obtained from each subject, and the study was approved by the Ethics Committee of King's College Hospital (London, UK).

Study design

Visit 1 procedures for all subjects included medical history taking and physical examination, an asthma characterisation questionnaire, intradermal skin-prick tests, spirometry and methacholine challenge to determine the provocative concentration of agent causing a 20% fall in FEV_1 (PC_{20}). After ~1 week, asthmatic subjects returned for visit 2, when they underwent AMP challenge in order to determine the PC_{20} to AMP. All subjects refrained from using rescue medication and caffeinated beverages for ⩾8 h before each visit. After a period of 3–4 weeks, all subjects returned for visit 3, during which bronchoscopy was performed.

Immunohistochemistry

Using the methods described below to validate the adenosine receptor subtype specificity of an antibody, it was found that the specificity of commercially available antibodies against all adenosine receptor subtypes was unsatisfactory; hence an antibody directed against the adenosine A_1 receptor was created specifically for the present study (the cost involved in obtaining a custom-made antibody was also a contributory factor in the decision not to concurrently profile the expression of the other adenosine receptor subtypes). Thus the custom-made affinity-purified A_1 receptor polyclonal antibody was raised in the rabbit against a synthetic peptide corresponding to amino acids 309–326 (CQPAPPIDEDLPEERPDD) of the carboxy terminus of the human A_1 receptor (Cambridge Research Biochemicals, Billingham, UK).

Brain cortex and cardiac tissue sections from three subjects were co-analysed as a positive control, since it is well

TABLE 1 Patient demographics

	Asthmatic	Healthy
Subjects n	16	8
Males/females n	11/5	4/4
Age yrs	26.6 (20–36)	25.6 (20–37)
Baseline FEV_1 % pred	92.7 (74–120)	106.8 (89–127)
Height cm	175.2 (160–186)	179.0 (159–188)
Weight kg	74.0 (56–95)	79.2 (52–106)
AMP PC_{20} mg·mL^{-1}	28.1 (0.88–136)	NA
Methacholine PC_{20} mg·mL^{-1}	1.13 (0.29–2.88)	>16

Data are presented as geometric mean (range), unless stated otherwise. FEV_1: forced expiratory volume in one second; % pred: % predicted; AMP: adenosine 5′-monophophate; PC_{20}: provocative concentration of agent causing a 20% fall in FEV_1; NA: not available.

established that these tissues highly express A_1 receptors. Cadaveric brain cortex and cardiac tissue samples were obtained from the Institute of Psychiatry Brain Bank (London, UK) and Peterborough Tissue Bank (Peterborough, UK), respectively, following GlaxoSmithKline (Stevenage, UK) and UK (Human Tissue Authority, London, UK) guidelines for the acquisition and use of human tissues, including ethical approval and the use of appropriate consent forms.

Frozen biopsy specimens of brain cortex, cardiac tissue and bronchial tissue were sectioned at a thickness of 6 μm. Sections were placed on Vectabond™-coated slides (Vector Laboratories, Peterborough, UK), and the brain cortex and cardiac tissue sections fixed in ice-cold 4% weight (w)/volume (v) paraformaldehyde for 5 min (bronchial biopsy sections were fixed with 4% w/v paraformaldehyde prior to cryopreservation). Sections were then immunostained for the A_1 receptor using an Optimax automatic immunostaining machine (Biogenex, San Ramon, CA, USA). Positive staining was detected with diaminobenzidine in addition to counterstaining with Mayer's haematoxylin.

Image analysis

Fully automated densitometry of A_1 receptor expression was developed using the Zeisss Vision KS400 system (Carl Zeiss, Göttingen, Germany). Stain intensity was described using arbitrary units (AU). All image analysis and measurements were performed blind by one observer.

Antibody validation with adenosine-receptor-transfected cells

Further antibody validation experiments were performed through flow cytometric analysis of A_1 receptor antibody binding to each adenosine receptor subtype expressed on CHO-K1 cells (which lack any known subtype of adenosine receptor) transfected with recombinant human A_1, A_{2A}, A_{2B} or A_3 receptor. Comparative expression levels of the adenosine receptor subtypes in each cell line were first investigated using real-time RT-PCR analysis. Data are expressed as gene copy number per 50 ng of complementary DNA. For flow cytometric analysis, cell lines were analysed, following antibody labelling, by appropriate gating for immunofluorescence using a FACSCalibur flow cytometer (Beckton Dickinson, Oxford, UK) after excitation at 488 nm. At least 3,000 events were collected and mean fluorescence intensities recorded.

Statistics

Data were analysed using an unpaired t-test, and, unless otherwise stated, are presented as mean±SEM.

RESULTS

Subjects

Patient demographics are described in table 1. All asthmatic subjects who completed the present study were hyperresponsive to AMP and methacholine (table 1). The PC_{20} to methacholine in the eight nonatopic healthy subjects were all >16 mg·mL^{-1}. All asthmatic subjects exhibited a positive skin response to at least one allergen, whereas none of the healthy subjects responded to any of the allergens tested.

Immunohistochemistry

Positive immunostaining appeared brown against a blue background as a result of the Mayer's haematoxylin counterstain. Immunostaining of bronchial biopsy specimens obtained from asthmatic subjects with the A_1 receptor antibody consistently revealed strong positive staining on the bronchial epithelium and moderate-to-strong positive staining on the bronchial smooth muscle, these distinctive regions were identified morphologically (fig. 1). The immunostaining appeared to be distributed uniformly throughout the whole of these regions, rather than localised to specific areas or structures within these regions. By comparison, immunostaining of bronchial biopsy specimens obtained from healthy subjects with the A_1 receptor antibody was strikingly less intense, with weak positive staining of the epithelium and very weak or sometimes undetectable positive staining of the smooth muscle (fig. 2). In all asthmatic and healthy bronchial biopsy sections, a negligible level of nonspecific staining was consistently observed with the isotype control antibody (data not shown).

Sections obtained from only 11 asthmatic subjects and seven healthy subjects were deemed of suitable quality for image analysis, since sections from one subject in each study group lacked sufficient epithelial and smooth muscle area for the analysis. Quantification of the staining intensity of the epithelium revealed that the level of positive staining of the asthmatic epithelium was significantly greater than that observed with the healthy epithelium (175.2 ± 5.3 *versus* 78.9 ± 30.9 AU; $p<0.01$). Similarly, the intensity of immunostaining of the asthmatic bronchial smooth muscle was significantly greater than that observed with the healthy bronchial smooth muscle (132.3 ± 9.9 *versus* 58.1 ± 24.4 AU; $p<0.01$; fig. 3).

Submucosal glands were identified on sections from two asthmatic bronchial biopsy specimens and one healthy biopsy specimen. Again, A_1 receptor expression appeared to be greater in the asthmatic biopsy specimens (fig. 4a) compared with the healthy biopsy specimen (fig. 4b).

Both the brain cortex and cardiac tissue samples from all three subjects immunostained very strongly for the A_1 receptor (fig. 5a and c, respectively), with no detectable staining observed with the isotype control antibody (fig. 5b and d). The small degree of brown colouration observed in the cardiac tissue (fig. 5d) was considered to be due to the presence of lipofuscin pigments.

A_1 receptor antibody validation

In order to provide additional support for the observations resulting from the immunohistochemical analysis described above, antibody validation experiments were also performed in order to analyse the affinity and selectivity of the A_1 receptor antibody for the A_1 receptor.

Quantitative RT-PCR analysis of the CHO-K1 cell lines transfected with each of the four adenosine receptor subtypes revealed that each of the cell lines expressed very high transcript levels of the transfected receptor, and that the gene expression levels of each receptor were generally of a similar magnitude (fig. 6), although slightly lower in the A_3 cell line. Subsequent flow cytometric analysis of the CHO-K1 cell lines using the A_1 receptor antibody showed a significant increase in

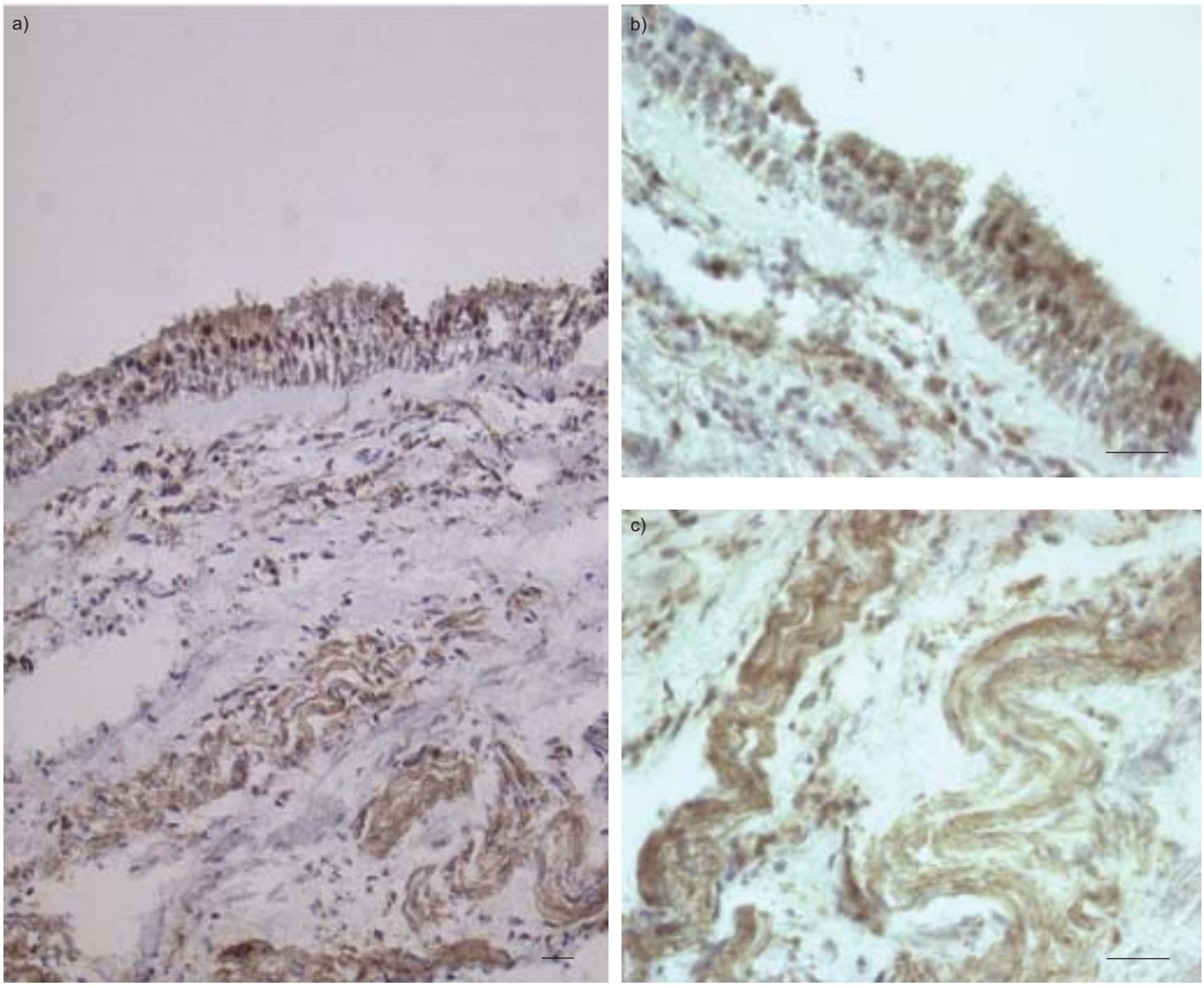

FIGURE 1. Representative photomicrographs showing positive immunostaining of asthmatic bronchial biopsy sections with an A_1 receptor antibody (a), with high expression of the A_1 receptor on the epithelium (b) and smooth muscle (c), shown at higher magnification. Positive immunostaining appears brown against a blue background as a result of the Mayer's haematoxylin counterstain. Scale bars=25 μm.

mean fluorescence intensity compared with wild-type CHO-K1 cells in the A_1 cell line alone (478 ± 84 *versus* 35 ± 8 AU; $p<0.01$). There were no significant differences between any of the other cell lines and the wild-type cells (fig. 6).

DISCUSSION

Although the expression and functions of adenosine receptors have been studied in individual cell types, usually obtained from healthy subjects, very little information is available concerning the distribution and relative expression of the four adenosine receptor subtypes in healthy or asthmatic airways. The aim of the present study, therefore, was to investigate the distribution of A_1 receptors in bronchial biopsy specimens obtained from mildly asthmatic subjects and then to compare the level of expression with that in biopsy specimens taken from healthy subjects. The present data reveal that there is a low level of A_1 receptor expression in healthy bronchial tissue, which appears to be predominantly located to the bronchial epithelium, whereas A_1 receptor expression is markedly elevated in asthmatic bronchial tissue, particularly in the bronchial epithelium and bronchial smooth muscle. Although the precise location of this receptor was not determined, it is possible that it is expressed on ciliated epithelial cells [15], goblet cells [16], sensory nerves [17] and smooth muscle cells [18]. Future studies using double labelling and confocal microscopy may discern the cell types involved.

In an attempt to quantify the increased expression in asthmatic tissue, image analysis was performed on the epithelial and smooth muscle areas of one tissue section from each biopsy specimen. Image analysis thus revealed that the mean intensity of the staining in these regions of asthmatic tissue was significantly greater than that observed with healthy tissue, which, therefore, confirmed the higher A_1 receptor expression.

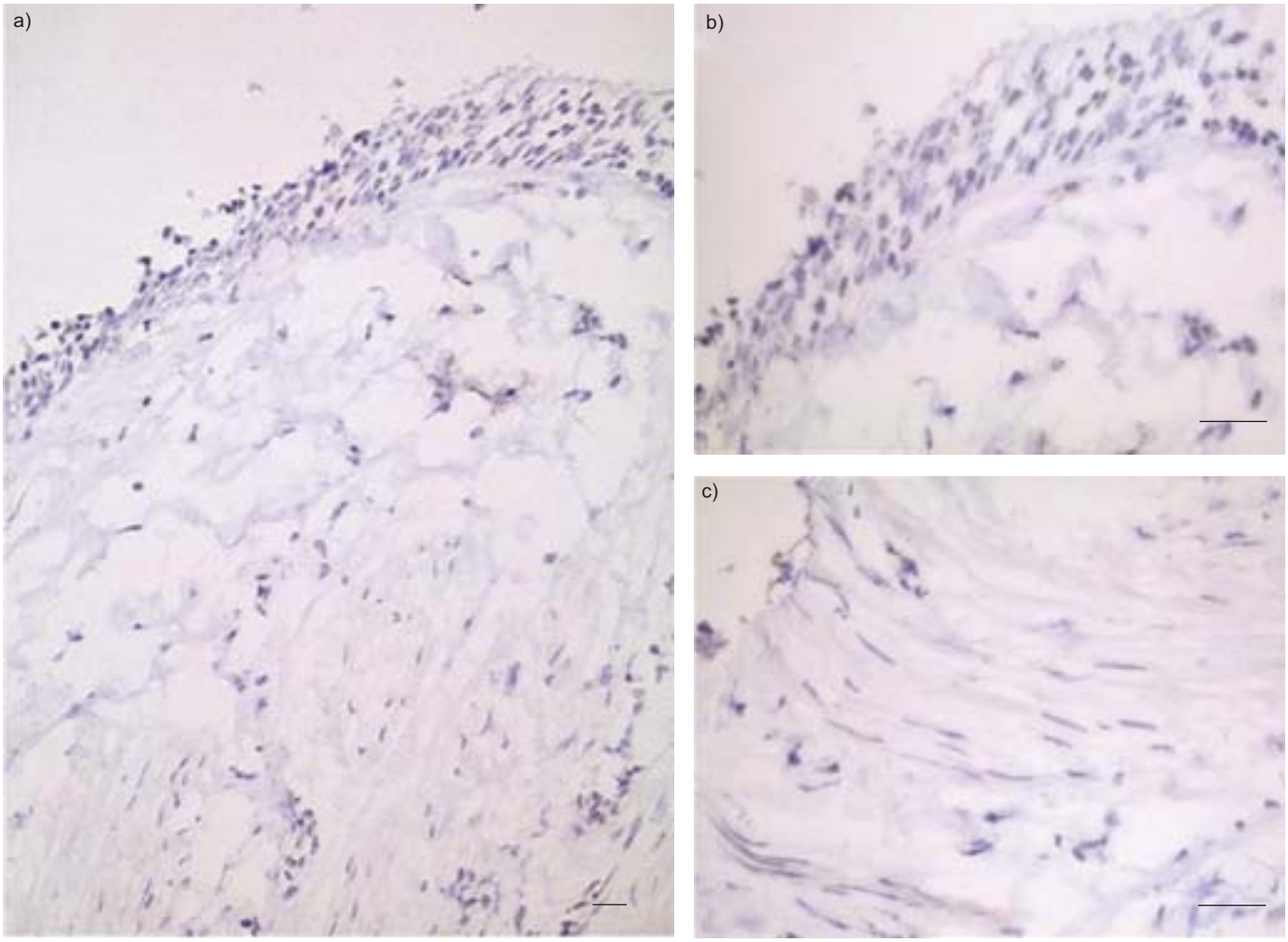

FIGURE 2. Representative photomicrographs showing positive immunostaining of healthy bronchial biopsy sections with an A_1 receptor antibody (a), with weak expression of the A_1 receptor on the epithelium (b) and virtually no positive immunostaining of smooth muscle (c), shown at higher magnification. Positive immunostaining appears brown against a blue background as a result of the Mayer's haematoxylin counterstain; scale bars=25 μm.

The A_1 receptor antibody used in the present study was raised against a C-terminal portion of the A_1 receptor, the aim being to limit recognition of the other adenosine receptor subtypes. In order to validate the antibody, experiments were performed to provide evidence that this A_1 receptor antibody was able to selectively bind A_1 receptors. Flow cytometric analysis of CHO-K1 cells transfected with recombinant human adenosine receptors showed a significant increase in fluorescence only for the cell line expressing A_1 receptors compared with wild-type cells following incubation with the A_1 receptor antibody. The caveat to this is the assumption that there is similar adenosine receptor protein expression in each cell line. Although a formal comparison of protein expression was not attempted in the present study, analysis of mRNA transcript levels revealed similar levels of gene expression in each cell line used in the present experiments, and, although the A_3 receptor transcript level was slightly lower than that of the other subtypes, it was still of a magnitude expected to yield a very high degree of receptor protein expression. Further confirmation that the present antibody recognises and binds to the A_1 receptor was demonstrated by a high level of positive immunostaining on the brain cortex and cardiac tissue samples, tissues recognised to exhibit high expression of the A_1 receptor. Therefore, having validated the selectivity of the A_1 receptor antibody, it was conclusively demonstrated, for the first time, that the A_1 receptor is markedly upregulated in bronchial tissue from subjects with asthma who are responsive to AMP challenge. That this difference was a consistent observation was confirmed with image analysis. Given the limitations of immunohistochemistry and image analysis, it would be expected that the image analysis data are certainly not proportionally representative of total A_1 receptor number but simply an indication that there is a difference in the pattern of A_1 receptor expression between the mildly asthmatic and healthy phenotypes.

The present observations support a number of studies investigating adenosine receptor expression in animal models of allergic lung inflammation, which also identified an increase in airway A_1 receptor expression compared with healthy naïve animals [8, 19–22]. In a rabbit model of allergic lung inflammation, it was demonstrated that the A_1 receptor was

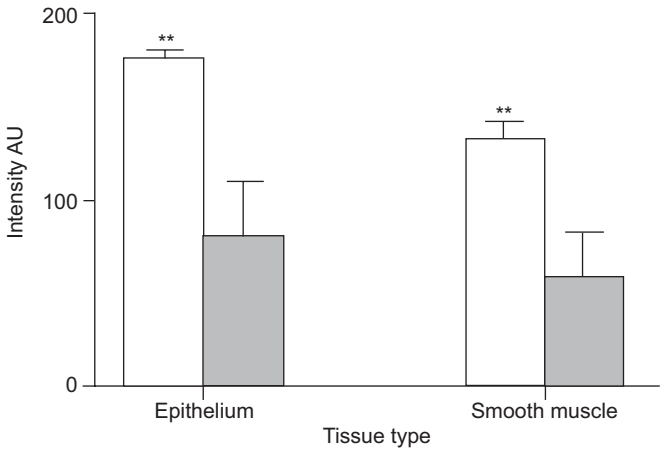

FIGURE 3. Image analysis of asthmatic (□) and healthy (■) bronchial biopsy sections immunostained with an A_1 receptor antibody. The intensity of positive staining specifically on the epithelium and smooth muscle areas of bronchial biopsy sections from 11 asthmatic subjects and seven healthy subjects (one section per subject) was quantified using Zeiss Vision KS400 software, from four different views of both epithelium and smooth muscle at a magnification of 1,000×. Data are presented as mean±SEM. **: $p<0.01$ *versus* healthy subjects.

upregulated on airway smooth muscle, and a functional consequence of this increase was that it mediated bronchoconstriction following inhalation of adenosine [8, 23]. Given the present observations, it could, therefore, be speculated that an increase in A_1 receptor numbers on bronchial smooth muscle in atopic mildly asthmatic subjects may contribute to the bronchoconstrictor response to AMP observed in these subjects. In support of this possibility, an investigation into the spasmogenic effect of adenosine on isolated human bronchial strips showed a variable but small overall contractile response in tissue from healthy subjects, the magnitude of which was significantly increased in tissue from asthmatic subjects [12]. The adenosine-induced contraction in isolated tissue from asthmatic subjects was significantly inhibited by the A_1 receptor antagonist 2-thio-[(1,3-dipropyl)-8-cyclopentyl]-xanthine (DPCPX). The same study also showed that the response could be blocked by pre-incubation with a histamine H_1 receptor antagonist and leukotriene synthesis inhibitor, suggesting that the response was mast cell-mediated. However, a subsequent investigation demonstrated that isolated human bronchial tissue has a high degree of intrinsic tone, and that histamine and leukotrienes constitute a major part of this basal tone [24]. Thus it is difficult to ascertain to what degree the inhibition of adenosine-induced contractile responses by the H_1 receptor antagonist and leukotriene synthesis inhibitor in the former study can be attributed to the inhibition of mast cell products released by adenosine, or simply to a reduction in basal tone. Hence further studies investigating the effects of selective adenosine receptor agonists and antagonists on isolated asthmatic bronchial tissue are required in order to provide further insight into the A_1-mediated contraction of bronchial smooth muscle obtained from subjects with asthma.

On a cellular level, however, an investigation into adenosine receptor signalling pathways in isolated airway smooth muscle cells from healthy donors identified A_{2B} receptors as the predominant adenosine receptor subtype [25]. The A_1 receptor is the subtype showing the greatest affinity for adenosine (reviewed in [26]), and so it could be speculated that adenosine preferentially activates the upregulated A_1 receptor on asthmatic

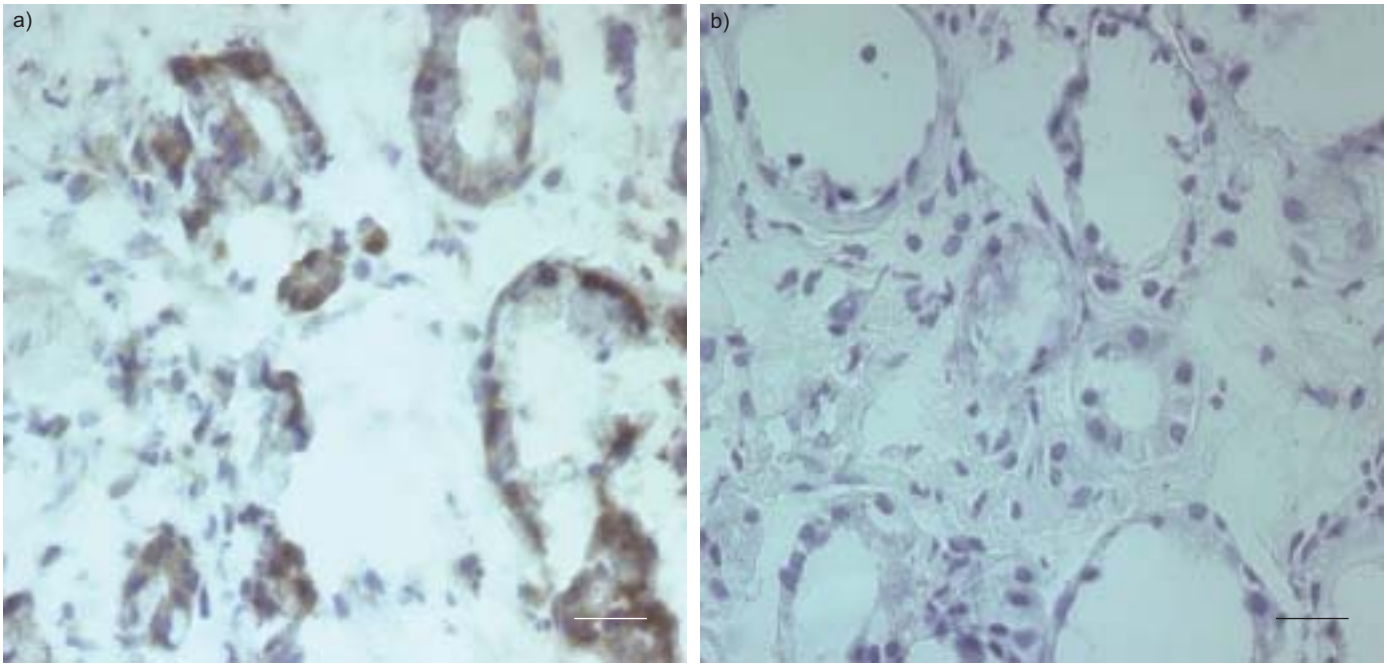

FIGURE 4. Representative photomicrographs showing immunostaining of a) asthmatic, and b) healthy bronchial biopsy sections with an A_1 receptor antibody. Positive immunostaining appears brown against a blue background as a result of the Mayer's haematoxylin counterstain. There was high expression of the A_1 receptor on asthmatic submucosal glands, but no apparent positive immunostaining of healthy submucosal glands. Scale bars=25 μm.

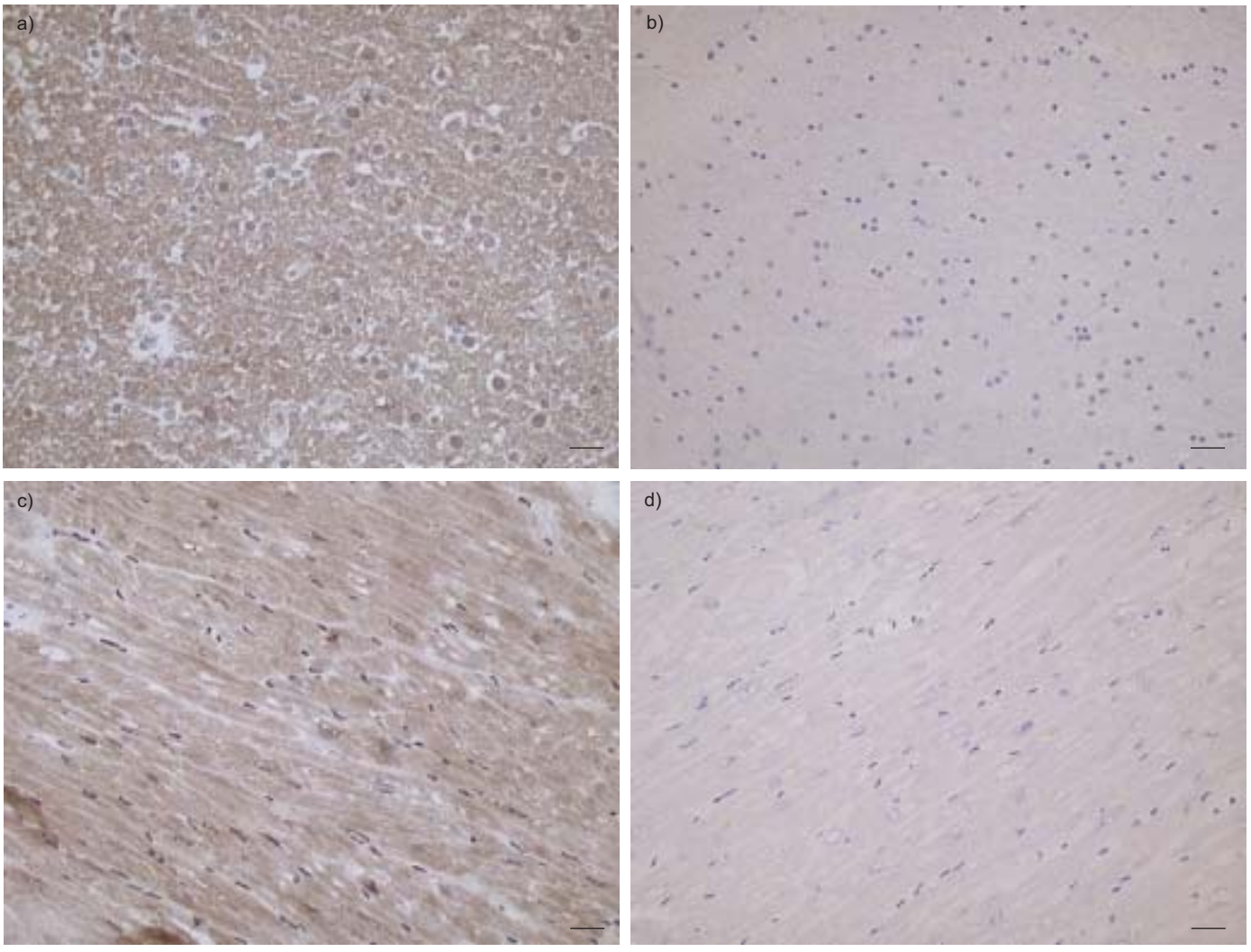

FIGURE 5. Photomicrographs showing immunostaining of a, b) brain cortex, and c, d) cardiac tissue with the A_1 antibody (a, c) and an isotype control antibody (b, d). Positive immunostaining appears brown against a blue background as a result of the Mayer's haematoxylin counterstain. There was positive staining of brain cortex and cardiac tissue with the A_1 antibody, but negligible staining of sections from the same samples using the isotype control. Scale bars=25 μm.

bronchial smooth muscle cells. Since the A_1 receptor is an inhibitory-G-protein-coupled receptor, its stimulation would result in a decrease in cyclic AMP level, leading to smooth muscle contraction. In support of this, it has recently been demonstrated that activation of the A_1 receptor on healthy human airway smooth muscle cells with a selective A_1 receptor agonist induces calcium mobilisation *in vitro* [18]. Thus, given that H_1 receptor antagonists do not completely block AMP-induced bronchoconstriction in asthmatic subjects and that co-administration of inhibitors of other mast cell products are not additive (reviewed in [4]), it is likely that other mechanisms beside mast cell degranulation are involved in the response to AMP. Further studies are thus required in order to investigate these other mechanisms, including the potential direct spasmogenic effects of adenosine in asthmatic subjects.

The functional significance of the increased expression of the A_1 receptor on the asthmatic bronchial epithelium remains to be established. However, a recent study demonstrated that activation of the A_1 receptor on tracheal epithelial cells resulted in an increase in mucin gene expression [16]. The present observation of an increase in A_1 receptor expression on the bronchial epithelium in asthmatic subjects thus further implicates adenosine in the promotion of mucus hyperscretion *via* activation of the A_1 receptor. Furthermore, submucosal glands were identified in tissue sections from three subjects. Two were from asthmatic subjects and showed high A_1 receptor expression; however, this was not apparent in sections from the healthy subject, but these are only preliminary observations and further investigations are required before definitive conclusions can be reached. However, adenosine has been shown to induce canine tracheal mucus secretion *in vivo* through an A_1-receptor-dependent mechanism [27], supporting the notion of adenosine as a secretagogue.

Interestingly, in 2006, it was demonstrated that subjects with chronic obstructive pulmonary disease, who also exhibit an increased extracellular level of adenosine in their airways,

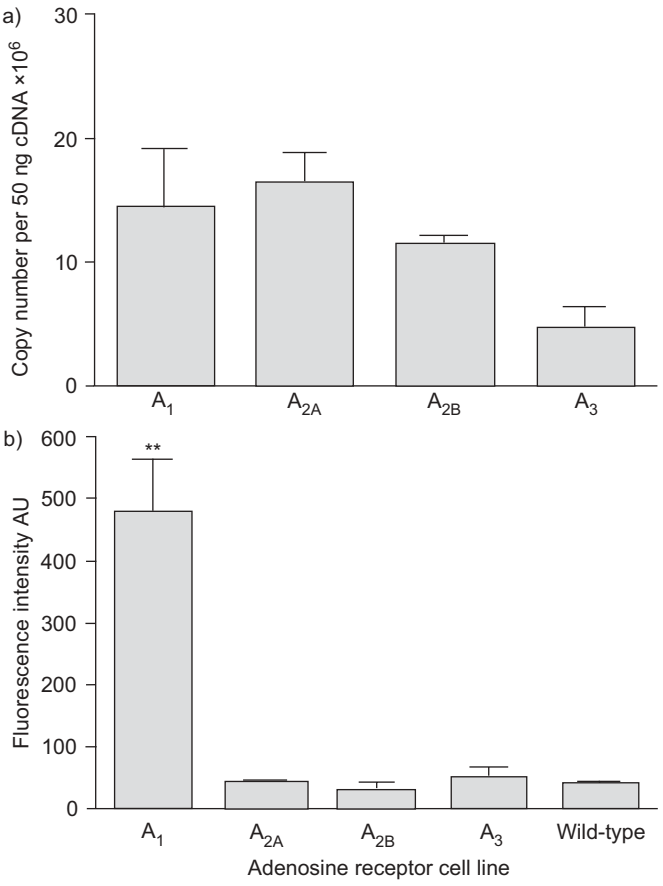

FIGURE 6. a) Quantitative real-time RT-PCR, and b) flow cytometric analysis of CHO-K1 cells transfected with recombinant human adenosine A_1, A_{2A}, A_{2B} or A_3 receptor. Expression of each transfected receptor was analysed using the appropriate primer pair and probe, described in the table in the supplementary material. Phycoerythrin-labelled cells were analysed by appropriate gating for immunofluorescence after excitation at 488 nm. At least 3,000 events were collected and mean fluorescence intensities recorded. Data are presented as mean±SEM and are from three separate experiments. An increase in fluorescence was observed only in the A_1-transfected cell line. AU: arbitrary unit. **: $p<0.01$ *versus* wild-type cells

show increased A_1, A_{2A} and A_3 receptor densities in the lung parenchyma compared with age-matched smoking controls [28]. However, in order to investigate the role of adenosine in asthma further, the aim of future studies will be to characterise the expression of the other adenosine receptor subtypes in asthmatic subjects, in addition to investigating the effects of glucocorticosteroid treatment on adenosine receptor expression, since, clinically, glucocorticosteroids decrease hyperresponsiveness to AMP in subjects with asthma [29].

The present evidence of increased adenosine A_1 receptor expression in asthmatic subjects reflects studies characterising adenosine receptor expression in mice partially lacking adenosine deaminase [30]. Such mice accumulate high levels of adenosine extracellularly and exhibit severe pulmonary inflammation. Some of the pathological features reported in this model, such as mucus hypersecretion, were consistent with those observed in asthma, but the pulmonary inflammatory cell profile differed considerably and consisted mainly of macrophages, with no increase in eosinophil numbers. However, transcript levels of the A_1, A_{2B} and A_3 receptors were found to be significantly increased, suggesting that an increase in extracellular adenosine promotes adenosine receptor signalling. The pulmonary inflammation in mice totally deficient in adenosine deaminase was even more severe, and the mice died aged 3 weeks due to respiratory distress [31]. Interestingly, concurrent knockout of the adenosine A_1 receptor was reported to exacerbate this inflammation further, suggesting a protective role of the A_1 receptor in this model [32]. However, eosinophil numbers in this model (<2% of total cells, compared with ~40% of total in a murine model of allergic inflammation [33]) were of a magnitude unlikely to result in alterations in airways responsiveness, and thus the relevance of these findings to asthma should be interpreted with caution.

In conclusion, therefore, it has been demonstrated, for the first time, that adenosine A_1 receptor expression is markedly upregulated in bronchial tissue obtained from subjects with asthma who are responsive to adenosine 5′-monophophate challenge, particularly on bronchial smooth muscle and the airway epithelium.

ACKNOWLEDGEMENTS

The authors would like to thank D. Hall and K. Saunders (GlaxoSmithKline, Stevenage, UK) for technical assistance with flow cytometry.

REFERENCES

1 Hasko G, Cronstein BN. Adenosine: an endogenous regulator of innate immunity. *Trends Immunol* 2004; 25: 33–39.
2 Driver AG, Kukoly CA, Ali S, Mustafa SJ. Adenosine in bronchoalveolar lavage fluid in asthma. *Am Rev Respir Dis* 1993; 148: 91–97.
3 Huszar E, Vass G, Vizi E, *et al*. Adenosine in exhaled breath condensate in healthy volunteers and in patients with asthma. *Eur Respir J* 2002; 20: 1393–1398.
4 Meade CJ, Dumont I, Worrall L. Why do asthmatic subjects respond so strongly to inhaled adenosine? *Life Sci* 2001; 69: 1225–1240.
5 Forsythe P, Ennis M. Adenosine, mast cells and asthma. *Inflamm Res* 1999; 48: 301–307.
6 van den Berge M, Kerstjens HA, de Reus DM, Koeter GH, Kauffman HF, Postma DS. Provocation with adenosine 5′-monophosphate, but not methacholine, induces sputum eosinophilia. *Clin Exp Allergy* 2004; 34: 71–76.
7 Mann JS, Holgate ST, Renwick AG, Cushley MJ. Airway effects of purine nucleosides and nucleotides and release with bronchial provocation in asthma. *J Appl Physiol* 1986; 61: 1667–1676.
8 Nyce JW, Metzger WJ. DNA antisense therapy for asthma in an animal model. *Nature* 1997; 385: 721–725.
9 Obiefuna PC, Batra VK, Nadeem A, Borron P, Wilson CN, Mustafa SJ. A novel A_1 adenosine receptor antagonist (L-97-1) reduces allergic responses to house dust mite in an allergic rabbit model of asthma. *J Pharmacol Exp Ther* 2005; 315: 329–336.

10 Vizi E, Huszar E, Csoma Z, *et al.* Plasma adenosine concentration increases during exercise: a possible contributing factor in exercise-induced bronchoconstriction in asthma. *J Allergy Clin Immunol* 2002; 109: 446–448.
11 Csoma Z, Huszar E, Vizi E, *et al.* Adenosine level in exhaled breath increases during exercise-induced bronchoconstriction. *Eur Respir J* 2005; 25: 873–878.
12 Bjorck T, Gustafsson LE, Dahlen SE. Isolated bronchi from asthmatics are hyperresponsive to adenosine, which apparently acts indirectly by liberation of leukotrienes and histamine. *Am Rev Respir Dis* 1992; 145: 1087–1091.
13 Joad JP. Characterization of the human peripheral lung adenosine receptor. *Am J Respir Cell Mol Biol* 1990; 2: 193–198.
14 Walker BA, Jacobson MA, Knight DA, *et al.* Adenosine A_3 receptor expression and function in eosinophils. *Am J Respir Cell Mol Biol* 1997; 16: 531–537.
15 McCoy DE, Schwiebert EM, Karlson KH, Spielman WS, Stanton BA. Identification and function of A_1 adenosine receptors in normal and cystic fibrosis human airway epithelial cells. *Am J Physiol* 1995; 268: C1520–C1527.
16 McNamara N, Gallup M, Khong A, *et al.* Adenosine up-regulation of the mucin gene, *MUC2*, in asthma. *FASEB J* 2004; 18: 770–1772.
17 Chuaychoo B, Lee MG, Kollarik M, Pullmann R, Undem BJ. Evidence for both adenosine A_1 and A_{2A} receptors activating single vagal sensory C-fibres in guinea pig lungs. *J Physiol* 2006; 575: 481–490.
18 Ethier MF, Madison JM. Adenosine A_1 receptors mediate mobilization of calcium in human bronchial smooth muscle cells. *Am J Respir Cell Mol Biol* 2006; 35: 496–502.
19 Ali S, Mustafa SJ, Metzger WJ. Adenosine receptor-mediated bronchoconstriction and bronchial hyperresponsiveness in allergic rabbit model. *Am J Physiol* 1994; 266: L271–L277.
20 Ali S, Mustafa SJ, Metzger WJ. Adenosine-induced bronchoconstriction and contraction of airway smooth muscle from allergic rabbits with late-phase airway obstruction: evidence for an inducible adenosine A_1 receptor. *J Pharmacol Exp Ther* 1994; 268: 1328–1334.
21 el-Hashim A, D'Agostino B, Matera MG, Page C. Characterization of adenosine receptors involved in adenosine-induced bronchoconstriction in allergic rabbits. *Br J Pharmacol* 1996; 119: 1262–1268.
22 Pauwels RA, Joos GF. Characterization of the adenosine receptors in the airways. *Arch Int Pharmacodyn Ther* 1995; 329: 151–160.
23 Abebe W, Mustafa SJ. A_1 adenosine receptor-mediated $Ins(1,4,5)P_3$ generation in allergic rabbit airway smooth muscle. *Am J Physiol* 1998; 275: L990–L997.
24 Ellis JL, Undem BJ. Role of cysteinyl-leukotrienes and histamine in mediating intrinsic tone in isolated human bronchi. *Am J Respir Crit Care Med* 1994; 149: 118–122.
25 Mundell SJ, Olah ME, Panettieri RA, Benovic JL, Penn RB. Regulation of G protein-coupled receptor-adenylyl cyclase responsiveness in human airway smooth muscle by exogenous and autocrine adenosine. *Am J Respir Cell Mol Biol* 2001; 24: 155–163.
26 Marx D, Ezeamuzie CI, Nieber K, Szelenyi I. Therapy of bronchial asthma with adenosine receptor agonists and antagonists. *Drug News Perspect* 2001; 14: 89–100.
27 Johnson HG, McNee ML. Adenosine-induced secretion in the canine trachea: modification by methylxanthines and adenosine derivatives. *Br J Pharmacol* 1985; 86: 63–67.
28 Varani K, Caramori G, Vincenzi F, *et al.* Alteration of adenosine receptors in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2006; 173: 398–406.
29 Spicuzza L, Bonfiglio C, Polosa R. Research applications and implications of adenosine in diseased airways. *Trends Pharmacol Sci* 2003; 24: 409–413.
30 Chunn JL, Young HWJ, Banerjee SK, Colasurdo GN, Blackburn MR. Adenosine-dependent airway inflammation and hyperresponsiveness in partially adenosine deaminase-deficient mice. *J Immunol* 2001; 167: 4676–4685.
31 Blackburn MR, Volmer JB, Thrasher JL, *et al.* Metabolic consequences of adenosine deaminase deficiency in mice are associated with defects in alveogenesis, pulmonary inflammation, and airway obstruction. *J Exp Med* 2000; 192: 159–170.
32 Sun CX, Young HW, Molina JG, Volmer JB, Schnermann J, Blackburn MR. A protective role for the A_1 adenosine receptor in adenosine-dependent pulmonary injury. *J Clin Invest* 2005; 115: 35–43.
33 Riffo-Vasquez Y, Spina D, Page C, *et al.* Effect of *Mycobacterium tuberculosis* chaperonins on bronchial eosinophilia and hyperresponsiveness in a murine model of allergic inflammation. *Clin Exp Allergy* 2004; 34: 712–719.