

The air pollutant sodium sulfite enhances mite crude extract-stimulated detachment of A549 airway epithelium cells

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Background and Purpose: It has been previously reported that the pollutant sodium sulfite (Na_2SO_3) can activate airway epithelial cells; however, there is as yet no evidence of any direct relationship between house dust mite allergen exposure and Na_2SO_3 with regards to the pathogenesis of airway allergy. This study investigated the effect of sulfite on mite-stimulated human airway epithelial cells.

Methods: The A549 human lung epithelial cell line was used as an in vitro model. Cells were treated with 10 $\mu\text{g}/\text{mL}$ mite crude extract for 8 h and/or Na_2SO_3 (0, 10, 100, 500, 1000 and 5000 μM) for 16 h, and cell adhesion and dissociation on a cell culture plastic surface were quantitated with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay. Changes in cell adhesion were also analyzed by monitoring the expression of the cell surface of adhesion molecules integrin alpha2 (CD49b) and integrin alpha6 (CD49f) using flow cytometry.

Results: A549 cells treated with either mite crude extract only or Na_2SO_3 only did not show a significant increase in dissociation from the cell culture plastic surface. However, when cells were pretreated with mite extract for 8 h, followed by 16-h incubation with various concentrations of Na_2SO_3 , cell dissociation was enhanced in a dose-dependent manner. A dose-dependent decrease of CD49b and CD49f expression was also seen in cells treated with Na_2SO_3 only and in mite-pretreated cells. Mite treatment decreased CD49b expression, and a cumulative effect was seen in cells further treated with Na_2SO_3 .

Conclusion: Significant dissociation of airway epithelial cells with Na_2SO_3 stimulation only occurred in cells pretreated with mite extract. Mite pretreatment enhanced Na_2SO_3 -induced CD49f down-regulation; Na_2SO_3 and/or mite extract down-regulated CD49b expression of A549 cells. These findings indicate that a synergistic effect of mite extract and sulfite can severely disrupt the airway bronchial epithelial barrier.

Key words: Antigens, *Dermatophagoides*; Epithelial cells; Integrin alpha2; Integrin alpha6; Mites; Sulfites

Introduction

The proteolytic activity of the house dust mite allergen can activate cells and cause damage to the human airway epithelium. Clinical evidence suggests that air pollution can also be a key factor in triggering an asthma attack; specifically, the air pollutant sulfite may play a role in the exacerbation of asthma. Several components in traffic pollution have been identified as factors that can enhance airway sensitivity to allergens.

The association between daily air pollution levels and the number of daily hospital admissions for respiratory diseases suggests that air pollution may increase the incidence of airway inflammation, leading to an increase in the incidence of respiratory illness [1-5].

Previous studies have revealed that particular components of air pollution inappropriately activate a variety of airway cells, leading to cell inflammation and thereby causing respiratory difficulties [6]. Among these pollutants, sulfur-containing compounds, such as sulfur dioxide, can be converted into sulfite upon contact with the fluids that coat the lining of the airway. Sulfite derived from fossil fuels has been shown to be a key stimulator of airway epithelial cells and can cause

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bronchoconstriction [7-10]. Sulfite has also been found to activate human neutrophils and directly induce production of reactive oxygen species such as hydrogen peroxide and superoxide directly in neutrophils [1,2]. In addition, sodium sulfite (Na_2SO_3) stimulation can also increase neutrophil adhesion to human airway epithelial cells [3]. Therefore, it is likely that asthmatic patients living in areas of high air pollution levels or in an environment with a higher sulfite content will have a greater number of asthma attacks or incidence of respiratory illness.

Interestingly, studies have shown that exposure to house dust mite allergens may also lead to increased airway inflammation, as these allergens possess a peptidase activity, which is a significant functional modulator of the airway epithelium. It has been suggested that the major protease in the house dust mite allergen *Dermatophagoides pteronyssinus* (Der p) 1 acts enzymatically when inhaled [4,5] and so increases airway epithelial permeability [6].

We investigated whether or not there is a relationship between environmental exposure to house dust mite allergens and the pollutant sulfite and, if so, whether this affects the pathogenesis of airway allergy. This is an important problem to address, because human epithelial cells frequently come into contact with both dust mite allergens and air pollutants. It is suggested here that exposure to sulfite augments house dust mite-induced airway epithelial cell damage, and this possibility was investigated by studying the behavior of a human airway epithelial cell line, A549, which was pretreated with crude mite extract and then exposed to varying concentrations of Na_2SO_3 . Two outcomes were monitored: first, the amount of cell dissociation that occurred in response to these substances; and second, whether cell adhesion was affected in the A549 cells, as assessed by the expression of integrin α 2 (CD49b) and integrin α 6 (CD49f) cell surface adhesion molecules.

Methods

Mite crude extract

An extract of the mite Der p purchased from Allergon AB (Ängelholm, Sweden) was prepared by homogenizing lyophilized mites in phosphate-buffered saline (PBS) using an ultrasonic homogenizer. After centrifugation at 10,000 g for 30 min, the supernatant was filtered through a 0.2- μm filter. The crude extract was quantified using a Bio-Rad protein assay, and aliquots were stored at -70°C .

Cell culture

The human airway epithelial cell line A549 (American Type Culture Collection [ATCC], Rockville, MD, USA), derived from carcinoma cells of type II pneumocytes, was cultured in RPMI-1640 medium (Cambrex Corp., East Rutherford, NJ, USA). The culture medium contained 10% fetal bovine serum (ATCC), 100 U/mL penicillin (Invitrogen, Carlsbad, CA, USA) and 100 $\mu\text{g}/\text{mL}$ streptomycin (Invitrogen). One-mL aliquots of A549 cells (1×10^5 cells) were seeded out into 12-well plates (BD Biosciences, San Jose, CA, USA) and the cells were allowed to settle and adhere for 16 h. They were then incubated with 10 $\mu\text{g}/\text{mL}$ mite crude extract for 8 h, followed by the addition of Na_2SO_3 (Sigma, St. Louis, MO, USA) of various concentrations (0, 10, 100, 500, 1000 and 5000 μM) and then incubated for an additional 16 h. As an experimental control, A549 cells were also cultured in the presence of different concentrations of Na_2SO_3 but without mite pretreatment. After incubation, cell adhesion and the expression of cell surface adhesion molecules were analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Serva, Germany) and flow cytometry.

Measurement of cell dissociation and adhesion

The measurement of cell dissociation and adhesion in this study was based on the ability of mitochondrial enzymes in living cells to chemically reduce the yellow MTT to a purple formazan dye [7,8]. After incubation, the detached cells from the culture medium and the cells that remained attached to the culture plates were collected. After centrifugation (450 g for 5 min), cell numbers were quantified with the MTT assay by re-suspending each cell pellet in 100 μL of 0.2 mg MTT/mL. Cells were incubated at 37°C for 4 h before spinning down and re-suspending in 2 mL dimethyl sulfoxide (Tedia, Fairfield, OH, USA), and the absorbance at 450 nm was immediately recorded with an enzyme-linked immunosorbent assay reader (Thermo Labsystems, Vantaa, Finland).

Expression of intercellular adhesion molecule-1, CD49b and CD49f

For flow cytometry analysis, A549 cells were washed twice with PBS then detached from the culture dish by incubating with 0.25% trypsin for 10 min at 37°C . Expression of the surface molecules CD49b, CD49f and intercellular adhesion molecule-1 (ICAM-1; CD54) was assessed by immunostaining. Washing and immunolabeling was carried out in Hanks' Balanced Salt

Solution (Invitrogen) containing 1% bovine serum albumin and 0.1% sodium azide at 4°C. The A549 cells were incubated for 60 min with 10 µL of 100 µg/mL fluorescein isothiocyanate-conjugated monoclonal antibodies to CD49b, CD49f and ICAM-1 (BD Biosciences) or an isotype-matched control antibody. After washing off unbound antibodies, the cells were analyzed using a fluorescence-activated cell sorter (FACS; FACSCalibur™ [BD Biosciences]). For each sample, 10,000 counts were collected. The mean values of fluorescence intensity were used for comparison.

Statistical analysis

Each of the experiments described above was performed independently. Each experiment was performed in quadruplicate each time, and at least 3 experiments were performed. Cell dissociation data were expressed as mean ± standard error of the mean. All flow cytometry data were expressed as mean ± standard deviation fluorescence values. Statistical significance was assessed using the non-paired two-group *t* test.

Results

Effect of Na₂SO₃ concentration on A549 cell viability

MTT assay only measures living cells, and thus the potentially damaging effect of sulfite was assessed at a wide range of Na₂SO₃ concentrations. The results show that there was no decrease in cell viability with sulfite treatment up to 5000 µM (Table 1). However, at higher sulfite concentrations, 20,000 and 10,000 µM, there was a significant decrease in cell viability. Therefore, in this study, the maximum Na₂SO₃ concentration used was 5000 µM.

Cell adhesion

A549 cells pretreated with 10 µg/mL house mite extract for 8 h showed no significant change in the amount of cell dissociation or adhesion ($p=0.307$ and $p=0.590$, respectively) as compared to the experimental control of culture medium alone (Fig. 1A and 1B).

With Na₂SO₃ treatment alone, there was no significant decrease in cell adhesion at concentrations up to 1 mM (Fig. 1B). At a concentration of 5000 µM, there was a reduction of 28% in cell adhesion ($p<0.01$); however, total cell numbers (dissociated + adhered; Fig. 1A and 1B) were similar in both the Na₂SO₃-only and mite + Na₂SO₃ groups.

Table 1. Cell survival under various sodium sulfite (Na₂SO₃) concentrations

Na ₂ SO ₃ concentration	Cell numbers (% of control) [mean ± SD] ^a
0 (control)	100±1.0
100 µM	92.4±1.5
1000 µM	90.3±4.3
5000 µM	101.8±0.6
10,000 µM	60.4±6.2
20,000 µM	32.8±3.3

Abbreviation: SD = standard deviation

^aCells were cultured in media with or without Na₂SO₃ for 24 h. Cell number was measured as the sum of the cells suspended in the culture medium and those adherent on the cell culture surface by MTT assay.

Cell dissociation in mite-pretreated cells

As described above, cell dissociation was unaffected when A549 cells were incubated with 10 µg/mL mite extract or treated with Na₂SO₃. A test was then performed to ascertain whether the addition of sulfite would induce cell dissociation in A549 cells that had been pretreated with house mite extract. It was shown that this combination of treatment caused a dramatic increase in cell detachment (Fig. 1A), this increase in cell detachment directly correlating with Na₂SO₃ concentration. Similarly, there was an augmented decrease in cell adherence of mite-pretreated cells incubated with Na₂SO₃ (Fig. 1B). In mite-pretreated cells, the addition of Na₂SO₃ induced 15-20% cell detachment when sulfite concentration was higher than 100 µM.

Expression of CD49b and CD49f

Flow cytometry analysis showed that CD49b expression in mite-treated cells was reduced by 26.5% (± 2.05; $p<0.01$), while there was no significant difference ($p=0.471$) in CD49f expression (Fig. 2A and 2B). Down-regulation of both CD49b and CD49f was seen with Na₂SO₃ treatment alone at concentrations higher than 100 µM (CD49b) and 500 µM (CD49f) [$p<0.05$ compared with the medium-only control]. The dose-dependent relationship is seen in Fig. 2A and 2B, and the accumulative effect on CD49b expression can be seen in Fig. 2A ($p<0.01$ compared with the Na₂SO₃ group). In order to clarify whether the change of CD49b and CD49f expression is a general effect of Na₂SO₃ on cell surface molecules, the expression of ICAM-1 in the cells was also examined. No change in the levels of ICAM-1 expression was seen in cells cultured with either mite extract or Na₂SO₃ alone. Interestingly, mite-cells pretreated with mite 10 µg/mL, followed by Na₂SO₃ 1000 or 5000 µM

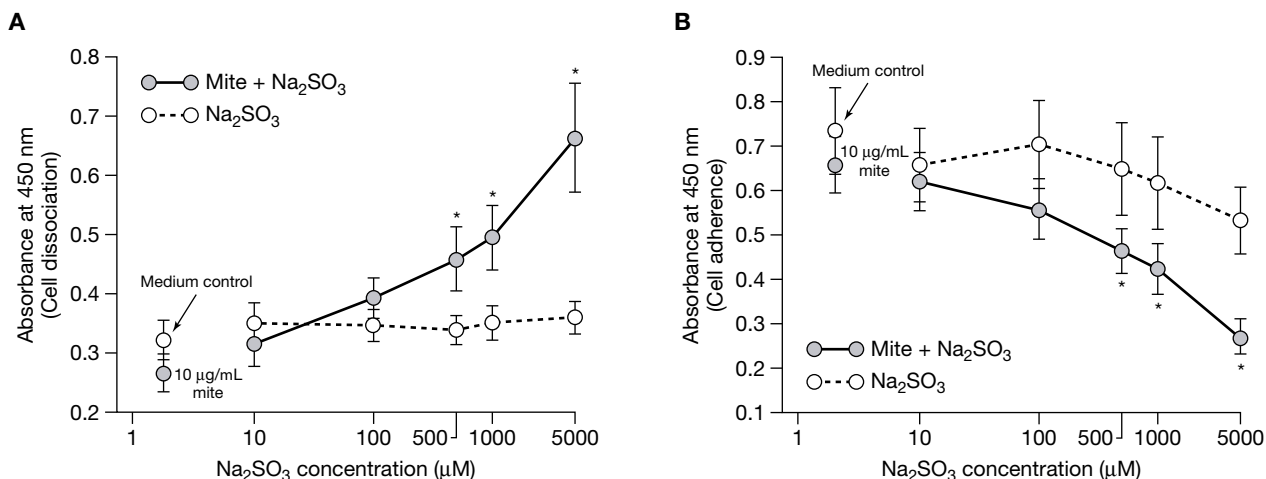


Fig. 1. Effect of mite/sodium sulfite (Na_2SO_3) treatment on dissociation (A) and adherence (B) in airway epithelial cells. A549 cells were incubated with or without 10 $\mu\text{g}/\text{mL}$ mite crude extract for 8 h, after which various concentrations of Na_2SO_3 (0, 10, 100, 500, 1000 and 5000 μM) were added for 16 h. Cell dissociation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Each value is mean \pm standard error of the mean. The axis of sulfite concentration is expressed on a log scale. * $p < 0.01$.

induced a 50% increase in ICAM-1 immunoreactivity, as detected by ICAM-1 antibody (Fig. 3).

Discussion

The present study has demonstrated that exposure to the environmental pollutant Na_2SO_3 enhances cell detachment of A549 cells pretreated with house dust mite extract in a dose-dependent manner. It is known that house dust mite proteins such as Der p are the major causative allergen of asthma [9]; Der p can induce a significant increase in bronchial

hyperresponsiveness in mild-to-moderate asthmatics at concentrations as low as 0.64 $\mu\text{g}/\text{g}$ of bedroom dust [10]. Proteinases present in crude mite extracts can disrupt intercellular adhesion, increase epithelial permeability and initiate epithelial cell death [11].

The increased prevalence of asthma in areas of high air pollution indicates that high levels of air pollution may contribute to an asthma attack. In this study, a possible mechanism by which the airway epithelial damage that occurs in asthma is enhanced by the presence of the air pollutant sulfite has been investigated.

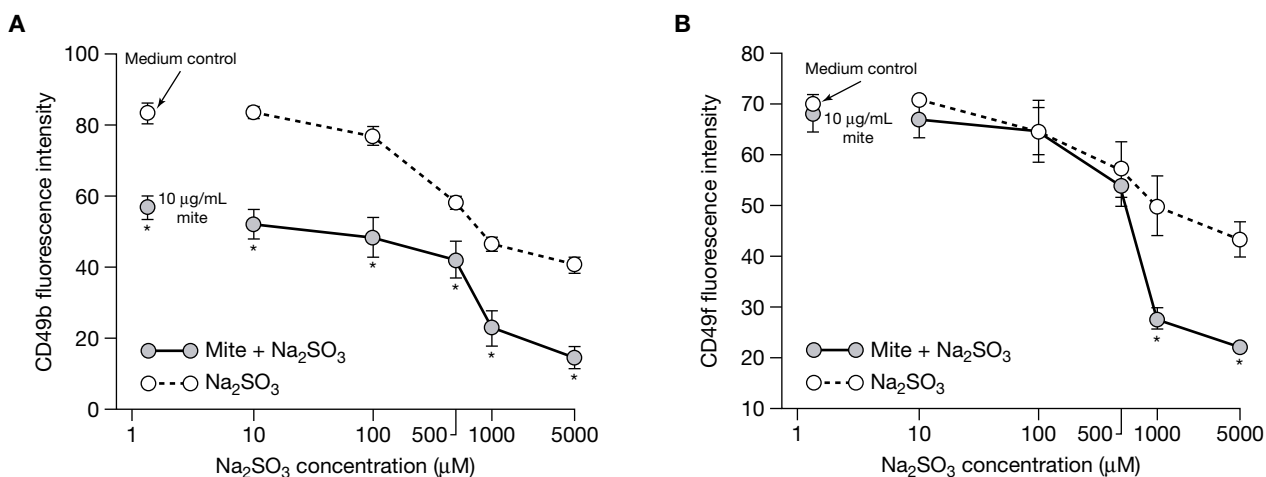


Fig. 2. Flow cytometry analysis of CD49 expression on A549 cells. Cells were incubated for 8 h with or without 10 $\mu\text{g}/\text{mL}$ mite extract, after which various concentrations of sodium sulfite (Na_2SO_3) were added for 16 h. Cell suspension was prepared with trypsin and stained with integrin alpha2 (CD49b) or integrin alpha6 (CD49f) antibodies. Flow cytometry results show mean values of fluorescence intensity. (A) CD49b; (B) CD49f. Each value is mean \pm standard deviation. The axis of sulfite concentration is expressed on a log scale. * $p < 0.01$.

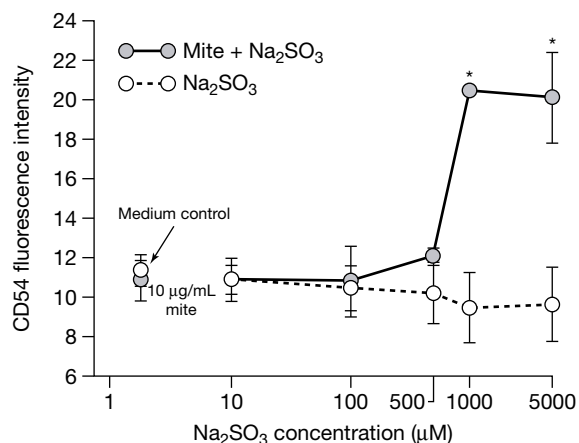


Fig. 3. Effect of sodium sulfite (Na_2SO_3) on CD54 expression in A549 cells. Cells were stained with CD54 antibody. Flow cytometry results are shown as mean fluorescence intensity. Each value is mean \pm standard deviation. Sulfite concentration is expressed on a log scale. * $p < 0.01$.

Pelletier and coworkers [12] showed that 1 mM of Na_2SO_3 does not induce apoptosis in A549 cells; this study similarly found that no significant cell death occurred in cells treated with Na_2SO_3 at concentrations up to 5000 μM . In these results, quantification of cell detachment using the MMT assay showed that the pathway leading to cell damage was triggered in mite-pretreated airway epithelial cells, and that later exposure to sulfite completed this process in these sensitized cells.

There is evidence that adhesion molecules play an important role in the mechanism of airway inflammation. The beta (β)1 integrin dimers are known ligands for matrix proteins. VLA-6 (also called $\alpha 6\beta 1$ or CD49f/CD29) interacts exclusively with laminin [13], while VLA-2 (also called $\alpha 2\beta 1$ or CD49b/CD29) serves not only as a receptor for laminin and collagen [13], but also plays a role in cell-cell adhesion [14]. The finding of this study, that sulfite exposure down-regulates CD49b and CD49f expression in a dose-dependent manner, suggests that the increase in cell detachment detected in mite-pretreated cells is associated with the integrins VLA-2 and VLA-6. Although cells in this study were not cultured on collagen- or laminin-coated surfaces, studies of several airway epithelial cell lines have shown that these cells are able to secrete both of these matrix proteins [15,16].

Our results show that Na_2SO_3 does not down-regulate the expression of ICAM-1 as seen in CD49b or CD49f, which reveals that the damaging effect of Na_2SO_3 occurs only on CD49b and CD49f, which are relevant to cell-substratum binding. CD54 is mainly

expressed on the apical surface of airway epithelial cells [17,18], and is a receptor for leukocyte function-associated antigen 1; it is likely that the increase in CD54 correlates with the accumulation of granulocytes and lymphocytes accompanying the inflammation. The increase in CD54 expression could also play an important role in binding some airborne pollutants, such as viruses and pollen, in the airways, and may further influence the binding of epithelial cells to neutrophils or other cell types at the sites of epithelial damage. In this study, the increased expression of CD54 revealed that there is no generalized decrease in protein expression under mite and sulfite stimulation. Physiopathologically, the increased ICAM-1 expression could enhance the adhesion of polymorphonuclear cells on airway epithelial cells and initiate epithelial damage. On the other hand, the expression of CD49b and CD49f suppressed by Na_2SO_3 can further augment cell dissociation.

In this study, an alveolar epithelial cell line, A549, was used to reveal a synergistic effect on cell dissociation from culture plates caused by sulfite and house dust mites. A possible mechanism is proposed, by demonstrating the dose-dependent decrease in expression of CD49b/CD49f on cell surfaces. These data emphasize the importance of sulfite toxicity, which is proposed as being able to augment damage to human airways, especially in individuals with an existing allergy to house dust mites. The mechanism by which dual mite/sulfite exposure leads to airway inflammation is worthy of further investigation.

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