

Fluorocytometric analysis of induced sputum cells in an asthmatic population

J. Domínguez Ortega, F. León^{+#}, JC Martínez Alonso, A. Alonso Llamazares*, E. Roldán⁺, T. Robledo*, M. Mesa*, A. Bootello⁺, C. Martínez-Cócer^a*

Allergy Unit. Hospital Virgen de la Concha. Zamora, Spain
Lab. of Clinical Investigation/NIAID/NIH. Bethesda, USA,

* Department of Allergy. H. Clínico San Carlos

⁺ Department of Immunology. H. Ramón y Cajal. Madrid. Spain

Abstract

Background: Bronchial mucosal inflammation is the major pathogenic process in asthma. In the latest years, induced sputum (IS) examination has become an important non-invasive method of assessing airway inflammation. Flow cytometry has been recently applied to the study of IS though it is not exempt of methodological difficulties. The aim of the present study was to further study if the fluorocytometric analysis of IS could represent a reliable tool to assess the presence of bronchial activated lymphocytes in stable mild asthmatic patients.

Methods: Induced sputa from controls and asthmatic patients were processed in isotonic 3mM dithiothreitol (DTT), a mucolytic agent required for cell dispersion. The individualized cells were then stained with monoclonal antibodies for three-colour flow-cytometric analysis. Total IgE and ECP were measured in serum and in the sputum fluid phase

Results: The cellularity of asthmatic sputa is enriched in eosinophils (mean, 26.63%) with respect to controls, but not in lymphocytes. However, lymphocytes from asthmatics show increased surface expression of activation markers (CD25 in T cells, CD23 in B cells). Surprisingly, no differences were observed in the detected levels of CD54 on IS lymphocytes and eosinophils between asthmatics and non-asthmatics. Furthermore, there was a significantly higher concentration of ECP and total IgE in the sputum from the asthmatic group.

Conclusion: Fluorocytometric analysis of induced sputum is a reliable non-invasive method for the study of bronchial immune cells. It could provide complementary information on activated cells in the bronchial mucosa even in non-smokers, mild and stable asthmatics and it is reasonable to speculate that it will be useful in monitoring the effect of the treatment in these patients.

Key words: Induced sputum, Asthma, ECP, Adhesion molecules, CD23, CD25, Lymphocytes, Eosinophils

Introduction

Asthma is a chronic inflammatory disease characterized by inflammatory cell infiltration of the bronchial mucosa by eosinophils, mast cells and activated lymphocytes. T-lymphocytes play a central role in this inflammatory process by means of several functional pathways and eosinophils would be involved as effector cells. This inflammation of the airways appears to be the primary event leading to the reversible airways obstruction and bronchial hyperresponsiveness [1].

Induced sputum (IS) by inhalation of hypertonic saline is currently used as a reliable tool to evaluate airway inflammation in airways diseases. Since its first description [2], it has been regarded as a highly reproducible method without significant adverse effects [3,4]. IS offers a more accurate assessment of bronchial inflammation than other non-invasive markers such as blood eosinophil count or serum eosinophil cationic protein (ECP) determination [5] while rendering similar results to those obtained with more invasive procedures such as the bronchoalveolar lavage [6].

To increase the information provided by IS, flow

cytometry has been recently applied to the analysis of its cellular subtypes and their activation state. However, flow cytometry of IS faces methodological difficulties. On the one hand, dithiothreitol (DTT), a mucolytic agent used to achieve cell dispersion in the sample, could alter the detection of some cellular markers [7]. On the other hand, the distinction between eosinophils and neutrophils still remains technically difficult [8].

Based on these considerations, the aim of the present study was to further evaluate whether the fluorocytometric analysis of IS could represent a reliable tool to assess the presence of bronchial activated immune lymphocytes in stable mild asthmatic patients.

Methods

Subjects

Sputum was induced in June, 2001 from 15 patients with seasonal bronchial asthma, aged 19-43, and 13 healthy non-atopic subjects. Asthma was defined as a clinical history of cough, dyspnea, chest tightness or intermittent wheezing during the grass pollen season in Madrid (May and June), with documented reversible airflow limitation (an improvement in forced expiratory volume in one second (FEV1) of >12% after inhaled salbutamol) and a FEV1 > 70% predicted during the study. Atopy was indicated by a positive skin-prick test (>3mm wheal) to grass pollen extract (ALK-Abelló, Madrid, Spain) or by increased serum concentrations (>0.35 Ku/L) of specific IgE for grass pollen as measured by CAP System (Pharmacia-Upjohn, Uppsala, Sweden). The patients' medication consisted merely of inhaled cromoglycate or budesonide (less than 800 mg per day) to control their asthmatic disease. For 15 days before the moment of sputum induction, they were allowed to use exclusively short acting bronchodilators if required, and oral antihistamines (loratadine, cetirizine or ebastine) if they presented with rhinitis or conjunctivitis.

The healthy control subjects had no history of lower respiratory affliction. Skin prick tests to a range of common airborne allergen extracts including grass pollen, were all negative. A positive control (histamine acid phosphate 10 mg/ml) and a negative one (saline solution) were used in all the subjects. Specific IgE for grass pollen was lower than 0.35 Ku/L in all cases. They all had a FEV1 >80% predicted with no significant changes after Salbutamol (200mg) and no airway responsiveness after the inhalation of >16 mg/ml of methacholine. For every subject, exclusion criteria included any history of smoking in the past four years and an upper respiratory tract infection in the previous 8 weeks.

All subjects signed a written informed consent for participation. The protocol was approved by the Hospital Research Committee.

Sputum induction and processing

The sputum was induced and processed according to Pizzichini's method with some modifications [4], by inhalation of 3% hypertonic saline nebulized for 15 min. after premedication with 400 µg of inhaled salbutamol. Saline solutions were nebulized by an ultrasonic nebulizer Ultra Ned 2000 (De Vilbiss), with output set at 1.5 ml/min. The subjects wore a nose clip and quietly inhaled aerosol for up to four 5-min periods. After each inhalation, the subjects rinsed their mouths with water to minimize saliva contamination.

Samples were collected in a plastic sterile container, kept on ice, and processed within 2 hours of expectoration. To avoid as far as possible the presence of salivary contamination in the sputum sample, selected portions from the whole expectorate were taken using an inverted microscope. Osmolarity was corrected by addition of phosphate buffer saline (PBS) solution, four times the volume of the sputum. DTT 0.1 M (Sigma Co. St Louis, USA) was added to reach a 3 mM final concentration. After agitating in a vortex mixer for 15 seconds, the samples were placed on a shaking plate for 30 min., at room temperature. The collected cell suspensions were then filtered through a 30 µm filter (Miltenyi Biotec S.L, Madrid., Spain) and spun at 1400 r.p.m. for 7 min. The pelleted cells were resuspended in PBS and total cell counts were performed in a Neubauer haemocytometer. Cell viability was determined using the trypan blue exclusion method. The supernatants were stored at -40°C for later analysis.

Measurement of IgE and ECP

Total IgE and ECP were measured in serum and in the sputum fluid phase with fluoroimmunoassay (UniCAP System), following the manufacturer's instructions. For ECP concentration in sputum, the results were adjusted for the dilution factor and the concentration expressed in µg L⁻¹. The detection threshold was 2 µg/L.

Table 1. Monoclonal antibodies used in the study

Specificity-Fluorochrome	Clone	Isotype
Irrelevant (-Pe, -FITC and PerCP) (isotypic controls)	X-40	IgG ₁
CD3-FITC	UCHT1	IgG ₁
CD8-PE	T8	IgG ₁
CD23-PE	M-L233	IgG ₁
CD25-PE	2A3	IgG ₁
CD45-PerCP	2D1	IgG ₁
CD19-FITC	4G7	IgG ₁
CD-54-PE	6.5B5	IgG ₁

Table 2. Summary of the various inflammatory parameters determined in the sputum samples. The asterisk indicates statistical significance ($p < 0.05$)

	Healthy subjects	Asthmatics
Serum ECP ($\mu\text{g/L}$)	2.53 (1-15)	9.05 (2-19)*
Serum total IgE (Ku/L)	38.2 (12.6 - 99.2)	261 (45-1304)*
Sputum ECP ($\mu\text{g/L}$)	16.9 (1- 61)	68 (55-189)*
Sputum total IgE (Ku/L)	1 (0 -15)	25.6 (1-146)*
CD3 ⁺ -CD25 ⁺ lymphocytes	12.23 (4.7 - 27.6)	39.64 (13.04-68)*
CD19 ⁺ -CD23 ⁺ lymphocytes	6.88 (1.2 - 16.6)	16.2 (2-57)*
CD54 ⁺ eosinophils	4.7 (1- 48)	2 (0 - 23)

Flow cytometry

For each parameter analyzed, 100.000 cells were incubated with a combination of 3 fluorochrome-conjugated monoclonal antibodies (mAb) (Becton Dickinson Immunocytometry Systems, BDIS, San Jose, CA, USA) for 30 min. at 4°C. The mAb used are listed in Table 1. After washing the cells in PBS, a FACScan flow cytometer (BDIS) was used for cell acquisition and Cell Quest Software (BDIS) was used for the expression of different markers. The analysis windows for lymphocytes and eosinophils were established on the basis of their granularity/complexity (side-scatter, SSC) and their level of expression of CD45 (Figure 1), CD8⁺, CD23⁺, CD25⁺, and ICAM-1⁺ cells were shown as percentages of the cells in the lymphocyte gate. All markers were compared with the background of conjugated isotypic-control mAb.

Statistical analysis

Results are expressed as mean (SEM) or median (range) and compared between asthmatics and healthy subjects using the unpaired t test or the non-parametric Mann-Whitney test, as appropriate. Statistical significance was assumed at a first kind error of $p < 0.05$. All analysis were carried out by means of SPSS statistical package program (Release 5.0.1:SPSS Inc., Cary, NC,USA).

Results

Eosinophil enrichment of asthmatic sputa

All subjects produced an adequate sputum specimen. There were no significant differences in terms of sputum volume between asthmatics and non-asthmatics. In both groups, a total cell count of 1.800.000 \pm 200.000 cells/ml was obtained in all the samples. There was no significant squamous cell contamination in any of them and the mean cellular viability was 65% of total sputum cell. All subjects tolerated the procedure without any salbutamol requirement after the induction.

The mean eosinophil counts expressed as percentage of total cell counts, were significantly higher in the asthmatics compared with control subjects (26.63 \pm 11.47% and 5.45 \pm 2.81% respectively, $p < 0.01$). By contrast, there was no difference between asthmatics and healthy subjects in the number of lymphocytes in the sputum samples (2.51 \pm 1.37 % and 2.75 \pm 1.48% respectively).

Increased concentration of ECP and IgE

Sputum from the asthmatic group, in comparison with the sputum of healthy subjects, had a significantly higher concentration of ECP ($p < 0.01$) and total IgE ($p < 0.05$) (Table 2). Similarly, we could observe a statistically significant difference in serum levels of ECP and total IgE ($p < 0.05$ and $p < 0.01$ respectively) between asthmatics and non-asthmatics (Table 2).

Increased expression of lymphocyte activation markers in asthmatic sputa

The profiles of the inflammatory markers of the sputum samples are summarised in Table 2. The relative amount of CD23⁺ B-cells and CD25⁺ T-lymphocytes were significantly higher in the asthmatic group ($p < 0.05$ and $p < 0.001$ respectively)(Table 2). No differences were observed in the detected levels of CD54 (ICAM-1) in the surface of either lymphocytes or eosinophils from asthmatics and non-asthmatics' sputa (Table 2), though some asthmatics showed high levels of these two markers.

Discussion

IS has been shown to constitute a powerful tool to study the inflammatory process in bronchial asthma. The present study not only confirms the effectiveness of IS to assess the presence of inflammation but also demonstrates the feasibility of finely analysing specific IS cell markers at a single cell level by flow cytometry. In 1996, Kidney et al. reported the first fluorocytometric

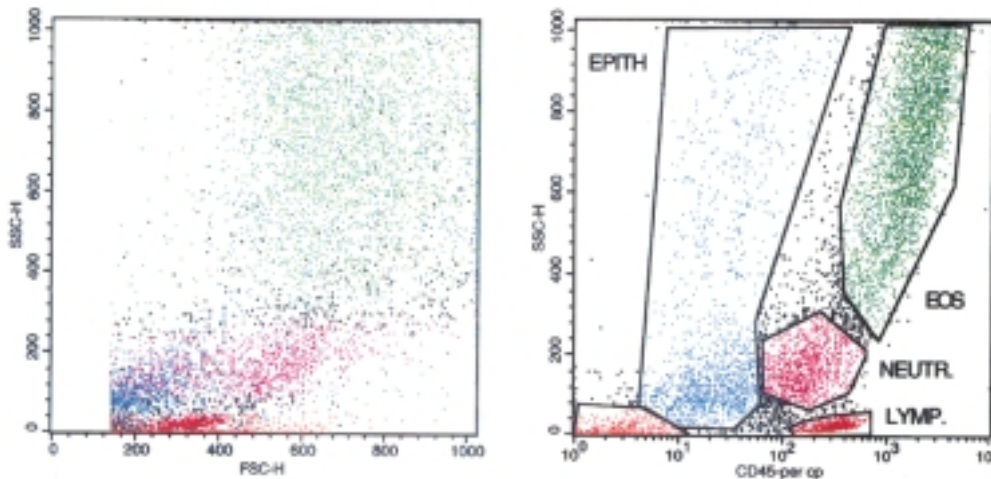


Figure 1. The first dot-plot (FSC/SSC) is ungated and shows the debris and complex cellularity of the IS sample. However, the use of CD45 grants a fine cell discrimination between eosinophils (upper gate) and lymphocytes (lower gate) in this type of studies.

examination of lymphocytes in sputum from asthmatics [9]. The results of that study have to be interpreted, however, under the consideration that non asthmatic smokers were selected as the control group and cigarette smoke could alter any inflammatory parameter, since it has the capacity to damage the bronchi in a number of ways (direct toxicity to the epithelium, recruitment of inflammatory cells, oxidative damage and increased epithelial permeability [10]. In our study, an adequate sputum specimen was obtained in all the subjects to conclude that a healthy non smoker subject is able to generate enough sample to obtain an appropriate number of cells for the fluorocytometric analysis. Moreover, rinsing the mouth with water before each expectoration and properly selecting the sample are useful ways to avoid the presence of squamous cells indicating salivary contamination [11], without significant alteration of viable cell counts to be studied.

In this study, asthmatics showed a significantly higher mean eosinophil count in IS than that observed in the non-asthmatics. One of the hallmarks of allergic inflammation is the infiltration of eosinophilic granulocytes into the affected tissues. Eosinophils are clearly participants responding to lymphocytes and cytokines, stimulating lymphocyte function, as antigen-presenting cells or sources of lymphocyte-active cytokines [12], and directly damaging the respiratory epithelium of the upper and lower respiratory tract [13]. In agreement with this sequence of events, we found increased sputum levels of ECP (a good marker of eosinophil activation in serum and sputum from asthmatics) [14], which means that a significant percentage of the eosinophils were likely activated. On the other hand, there were no significant differences in the sputum lymphocyte counts between the two groups of subjects. Our findings are in agreement with earlier published results [9,15], although Louis et al [16] observed a significant increase in CD4+ T cells in sputum from asthmatics by flow cytometry. To explain this controversy, two factors should be considered. Processing the sputum for flow cytometry could alter

lymphocytes viability, although it has been previously reported that DTT have no direct effect on inflammatory cells [7,17]. On the other hand, transepithelial migration of inflammatory cells from the airway tissue into the lumen is a highly specific process, requiring multiple pathways of cellular adherence and activation [18]. Considering this idea, induced sputum would only reflect an estimation of intraluminal lymphocyte inflammation in asthma [6].

Although the percentages of CD3+ and CD19+ lymphocytes did not differ between atopic asthmatic patients and control subjects, an increased expression of low-affinity IgE receptor (CD23) and Interleukine-2 receptor α chain (CD25) were observed in sputum lymphocytes from asthmatics. On human B cells, membrane CD23 is closely involved with cell activation, antigen presentation and IgE synthesis [19] and is high in atopic individuals, rising with flares of disease [20]. Furthermore, increased number of peripheral blood CD25+ T-cells has been described in atopic asthmatics as compared with controls [21]. We have obviated the effect of smoking over the CD25 detection involving a non-smoker population [9]. These observations suggest an activation of sputum lymphocytes in atopic asthmatics during the pollen season, without clinical implications.

Asthmatic IS eosinophils have been shown to express increased levels of adhesion molecules of the integrin family [22]. Intercellular cell adhesion molecule (ICAM-1/CD54) is an adhesion molecule involved in leukocyte recruitment by its binding to lymphocyte function associated antigen-1 (LFA-1) [23] and plays a direct role in the inflammatory response [24]. Surprisingly, we found very little ICAM-1 expression with no significant differences between both analysed groups. This finding might have several explanations. On the one hand, it might reflect the mild disease of the subjects studied. On the other, we have to consider the use of oral antihistamines for the rhino-conjunctivitis symptoms. Seasonal allergic bronchial asthma is often associated with rhinitis and at least half of the patients who are being treated for asthma also have nasal or sinus

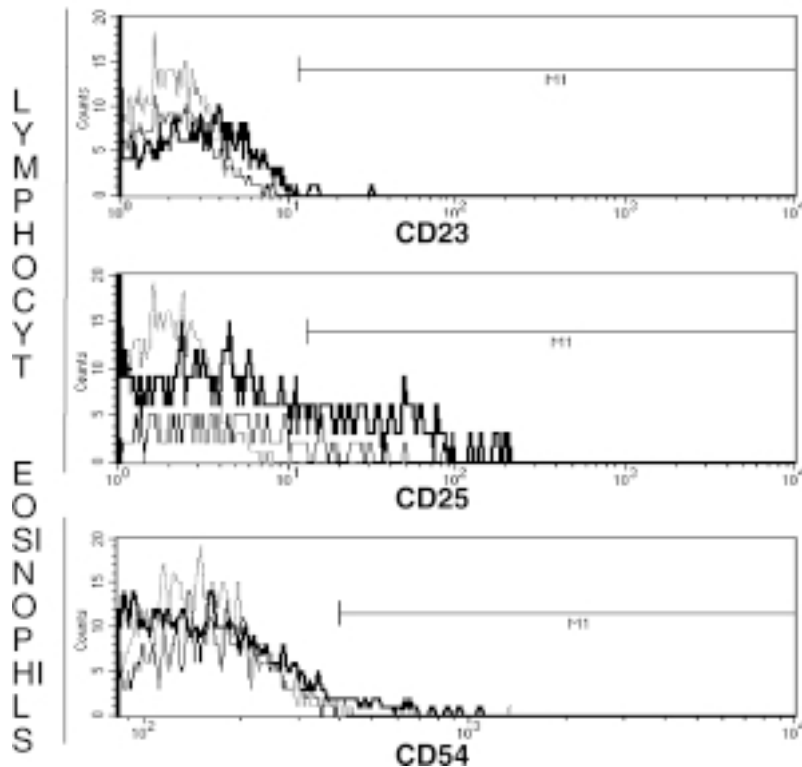


Figure 2. Expression of CD23, CD25 and CD54 on IS cells. Flow-cytometric overlay histograms showing the expression of C23 and CD25 on lymphocytes (top two histograms) and of CD54 on eosinophils (lower panel). The figure shows a representative control (solid thin line) and a representative asthmatic (solid bold line). The grey dotted line corresponds to an isotype control that serves to set the level of background fluorescence.

symptoms of some kind [25]. It has been reported that cetirizine reduces ICAM-1 levels after antigen challenge in vivo in allergic conjunctivitis[26] and the administration of azelastine to nose and eyes reduces ICAM-1 expression after allergen-specific challenge in pollinotic subjects[27]. Furthermore, in vitro studies, have shown that some antihistamines such as levocabastine or fexofenadine are capable of reducing ICAM-1 expression on cultured WK cell line [28]. In summary, it is possible that our data could reflect the effect of other common antihistamines modulating ICAM-1 expression. Finally, very late antigen-4 (VLA-4) could not be determined on eosinophils, since DTT significantly affects this parameter [29,30].

In conclusion, fluorocytometric analysis of induced sputum can be used successfully to examine inflammation cells in asthma, even in non-smokers, mild and stable asthmatics. It could provide complementary information on activated cells in the bronchial mucosa and it is reasonable to speculate that it will be useful in monitoring the effect of the treatment.

Acknowledgements:

We thank Luis Zayas, Maria Angeles Castillo and Francisca Casco for technical assistance. This study has been supported by a grant from the Allergy and Clinical Immunology Spanish Society Foundation. Francisco

Leon was recipient of a grant from the Spanish "Fondo de Investigación Sanitaria" (01/9417)

References

1. Louis R, Lau LCK, Bron AO, Roldaan AC, Radermecker M, Djukanovic R. The relationship between airways inflammation and asthma severity. *Am J Respir Crit Care* 2000; 161: 9-16.
2. Pin I, Gibson PG, Kolendowicz R, Girgis-Gabardo A, Denburg JA, Hargreave FE, Dolovich J. Use of induced sputum cell counts to investigate airway inflammation in asthma. *Thorax* 1992; 47: 25-29.
3. Pavord ID, Pizzichini MMM, Pizzichini E, Hargreave FE. The use of induced sputum to investigate airway inflammation. *Thorax* 1997;52:498-501.
4. Pizzichini E, Pizzichini MM, Efthimiadis A, Evans S, Morris MM, Squillace D, Gleich GJ, Dolovich J, Hargreave FE. Indices of airway inflammation in induced sputum: Reproducibility and validity of cell and fluid phase measurements. *Am J Respir Crit Care Med* 1996;154:308-317.
5. Pizzichini E, Pizzichini MM, Efthimiadis A, Dolovich J, Hargreave FE. Measuring airway inflammation in asthma: eosinophils and eosinophil cationic protein in induced sputum compared with peripheral blood. *J Allergy Clin Immunol* 1997; 99:539-544.
6. Grootendorst DC, Sont JK, Willems LNA, Kluin-Nelemans JC, Van Krieken JHJM, Veselic-Charvat M, Sterk PJ. Comparison of inflammatory cell counts in asthma: Induced sputum versus bronchoalveolar lavage and bronchial biopsies. *Clin Exp Allergy* 1997;27:769-779.

7. Loppow D, Böttcher M, Gercken G, Magnussen H, Jörres RA. Flow-cytometric analysis of the effect of dithiothreitol on leukocyte surface markers. *Eur Respir J* 2000; 16: 324-329.
8. Loppow D, Böttcher M, Jörres RA, Gercken G, Magnussen H. Flow cytometric detection of induced sputum eosinophils. *Eur Respir J* 1998;12:65s
9. Kidney JC, Wong AG, Efthimiadis A, Morris MM, Sears MR, Dolovich J, Hargreave FE. Elevated B cells in sputum of asthmatics. *Am J Respir Crit Care Med* 1996;153:540-4
10. Chalmers GW, MacLeod KJ, Thomson L, Little SA, McSharry C, Thomson NC. Smoking and airway inflammation in patients with mild asthma. *Chest* 2001;120:1917-1922.
11. Pizzichini E, Pizzichini A, Efthimiadis A, Hargreave J, Dolovich J. Measurement of inflammatory indices in induced sputum: effects of selection of sputum to minimize salivary contamination. *Eur Respir J* 1996; 9: 1174-1180.
12. Weller PF, Lim K, Wan HC, Dvorak AM, Wong DTW, Cruikshank WW, Kornfeld H, Center DM. Role of the eosinophil in allergic reactions. *Eur Respir J* 1996; 9 Suppl.22: 109-115.
13. Stoop AE, Van der Heijden HA, Biewenga J, Van der Baan S. Eosinophils in nasal polyps and nasal mucosa. An immunohistochemical study. *J Allergy Clin Immunol* 1993;91:616-622.
14. Venge P, Bystrom J, Carlson M, Hakansson L, Karawacjzyk M, Peterson C, Seveus L, Trulsson A. Eosinophilic cationic protein (ECP): molecular and biological properties and the use of ECP as a marker of eosinophil activation in disease. *Clin Exp Allergy* 1999;29:1172-1186
15. Synek M, Beasley R, Frew AJ, Goulding D, Holloway L, Lampe FC, Roche WR, Holgate ST. Cellular infiltration of the airways in asthma of varying severity. *Am J Respir Crit Care Med* 1996; 154:224-30.
16. Louis R, Shute J, Biagi S, Stanciu L, Marreli F, Tenor H, Hidi R, Djukanovic R. Cell infiltration, ICAM-1 expression and eosinophil chemotactic activity in asthmatic sputum. *Am J Respir Crit Care Med* 1997;155:466-472.
17. Louis R, Shute J, Goldring K, Perks B, Lau LCK, Radermecker M, Djukanovic R. The effect of processing on inflammatory markers in induced sputum. *Eur Respir J* 1999; 13: 660-667.
18. Hamid QA, Minshall EM. Molecular pathology of allergic disease: Lower airway disease. *J Allergy Clin Immunol* 2000;105:20-36.
19. Hakanson L, Heinrich C, Rak S, Venge P. Activation of B-lymphocytes during pollen season. Effect of immunotherapy. *Clin Exp Allergy* 1998;28:791-798.
20. Muller KM, Rocken M, Joel D, Bonnefoy JY, Saurat JH, Hauser C. Mononuclear cell-bound CD23 is elevated in both atopic dermatitis and psoriasis. *J Dermatol Sci* 1991;2:125-133.
21. Menz G, Ying S, Durham SR, Corrigan CJ, Robinson DS, Hamid Q, Pfister R, Humbert M, Kay AB. Molecular concepts of IgE-initiated inflammation in atopic and non-atopic asthma. *Allergy* 1998;53:15-21.
22. Thorne KJI, Richardson BA, Mazza G, Butterworth AEA. New method for measuring eosinophil activating factors, based on the increased expression of CR3 alpha (CD11b) on the surface of activated eosinophils. *J Immunol Meth* 1990;133:47-54.
23. Hansel TT, Braunstein JB, Walker C, Blaser K, Bruijnzeel PLB, Virchow JC, Virchow C. Sputum eosinophils from asthmatics express ICAM-1 and HLA-DR. *Clin Exp Immunol* 1991;86:271-277.
24. Schleimer RP, Bochner BS. The role of adhesion molecules in allergic inflammation and their suitability as targets of antiallergic therapy. *Clin Exp Allergy* 1998;28 (Suppl 3):15-23.
25. Ciprandi G, Pronzato C, Ricca V, Bagnasco M, Canonica GW. Evidence of intracellular adhesion molecule-1 expression on nasal epithelial cells in acute rhinoconjunctivitis caused by pollen exposure. *J Allergy Clin Immunol* 1994; 94:738-746..
26. Tosca M, Ciprandi G, Pasalacqua G, Canonica GW. Cetirizine reduces conjunctival non-specific hyperreactivity in children with mite allergy. *J Investig Allergol Clin Immunol* 1998;8:23-26.
27. Platts-Mills TAE, Pauwels RA, Holgate ST. Asthma. In: Holgate ST, Church MK, Lichtenstein LM. *Allergy* (2nd edn). London: Mosby;200:17-35.
28. Ciprandi G, Pasalacqua G, Canonica GW. Effects of H1 antihistamines on adhesion molecules: a possible rationale for long term treatment. *Clin Exp Allergy* 1999;29 (Suppl 3):49-53..
29. Quiu D, Tan WCh. Dithiothreitol has a dose-response effect on cell surface antigen expression. *J Allergy Clin Immunol* 1999;103:873-876
30. Dominguez Ortega J, Leon F, Alonso Llamazares A, Roldan E, Robledo T, Agustin P, Bootello A, Martinez-Cocera C. The effect of Dithiothreitol on VLA-4 in peripheral blood and induced sputum cells. *Immunol et Allergol* 2002;30:203-208

Dr. Javier Domínguez Ortega

Allergy Unit. Hospital Virgen de la Concha
Avenida de Requejo, 35
49022 Zamora. Spain
Tel.: +34 980548200
E-mail: jdort@mixmail.com