Study design

This was a cross sectional study with two visits within 3 weeks. In visit 1, all subjects completed clinical questionnaires, provided informed consent, underwent a physical examination and had allergy skin tests. At this time, subjects also performed a methacholine inhalation test, had a urine test for pregnancy (where applicable) and subjects in the CA group were provided with salmeterol and salbutamol. At visit 2, spirometry was performed including bronchodilator reversibility, sputum and blood samples were taken for multiple analyses (see below). Remaining salmeterol and salbutamol were collected from subjects in the CA group.

Inhaled corticosteroid medication was discontinued in the CA group 2 weeks prior to subject testing and sample collection. At the time inhaled steroid was discontinued, 2 puffs bid salmeterol was provided. Forty eight hours before samples were taken, salmeterol was stopped and substituted with salbutamol 2 inhalations qid. Salbutamol was stopped at least 6 hours before blood samples were taken. Laboratory testing was performed simultaneously on three (matched) subjects, one from each group. Laboratory personnel performing the tests were not informed of the groups to which each subject belonged.

Procedures

Routine physical examination, questionnaire and clinical tests were performed according to standard protocols. Spirometry included measurement of FVC, FEV_1, FEV_1/VC and methacholine challenge tests. Blood counts included a manual eosinophil count.

Sputum cell induction and examination

Sputum was induced with an aerosol of inhaled hypertonic saline by a modification of the method of Pin *et al.* [32] after pretreatment with inhaled Salbutamol 200 μg. The modification consisted of inhaling the hypertonic saline in concentrations of 3, 4 and 5% each for 7 minutes. Sputum examination followed the method of Pizzichini *et al.* [33]. Sputum mediator assessments were performed using routine protocols. The concentration of eosinophil cationic protein (ECP) was determined using a sensitive radioimmunoassay (Kabi-Pharmacia Diagnostics AB, Uppsala, Sweden). Fibrinogen was measured using an "in house" ELISA assay which employed rabbit anti-fibrinogen antibody (Dako# A080). Cytokines were assayed by ELISA (IL-5 using Biotrak kit, Amersham, UK and TNF-α by "in house" ELISA).

Peripheral blood cytokine analysis

Peripheral blood mononuclear cells (PBMC) were isolated from the blood of each subject. Matched triplets which included one subject from each group were sampled and assayed in parallel. Short term cultures of PBMC were established for 24 or 72 hours in the presence or absence of a series of stimulants. These included anti-CD3 antibody 1 μg/ml (OKT3, Cedarlane , Burlington, Ont), killed *Staphylococcus aureus* Cowan strain 1 bacteria (SAC) 0.07% w/v (Sigma, St Louis, Mo). *E-coli* derived LPS (Sigma) 5 μg/ml, Tetanus toxoid (0.1 μg/ml) and a combination of phorbol ester PDBu (Sigma) (10^{-6} M) and Calcium ionophore A23187 (Sigma) (10^{-7} M). The concentrations and timing of these stimuli were selected on the basis of pilot experiments.

The cytokine content of cell free supernatants, was assessed using ELISA kits with the exception of TNF-α which was assessed using a well validated "in house" ELISA. The cytokine kits employed were: IL-4- Biotrak RPN 2753, IL-5- Biotrak RPN2761, IL-6 Biotrak RPN 2754, IL-10 Biotrak RPN 2755, IL-12- Biotrak RPN 2765, GM-CSF Biotrak RPN 2757. All of these kits were obtained from Amersham (Toronto, ON, Canada).

Flow cytometric protocols and analysis

Panels of antibodies were employed in 2 and 3 colour analysis protocols to identify and calculate frequencies of T-cell subsets, B cells, monocytes and subsets of these cells expressing important functional markers. A complete listing of B and T lymphocyte, and monocyte phenotypes analysed by flow cytometry are listed in Figure 1. All staining was done in whole blood with subsequent fixation and wash. Flourescent labelled antibodies against CD3, CD4, CD8, CD14, HLADR, CD45RO CD45RA, CD40 and CD23 were purchased from Becton Dickinson Canada (Mississuaga., Ontario). Antibody to CD40 ligand

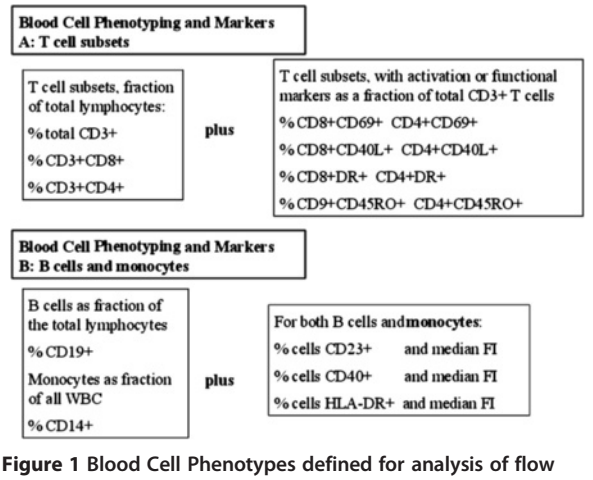

Figure 1 Blood Cell Phenotypes defined for analysis of flow cytometric data. Major lymphocyte subsets are reported as % of total lymphocytes. Activated or functional lymphocyte subsets are reported as % of each lymphocyte type. Expression of functional B or monocyte surface markers are reported as median fluorescence intensity.