

## SHORT PAPER

# Cell specific markers for eosinophils and neutrophils in sputum and bronchoalveolar lavage fluid of patients with respiratory conditions and healthy subjects

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**Background:** Highly specific protein markers for eosinophils and neutrophils could be a valuable diagnostic aid in various respiratory disorders. The cell specificity of monoclonal antibodies against eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), human neutrophil lipocalin (HNL), and myeloperoxidase (MPO) was investigated using immunocytochemical techniques.

**Methods:** Induced sputum and bronchoalveolar lavage fluid samples from 14 patients with respiratory conditions and four healthy individuals were studied. Antigens were detected at their intracellular sites in cells with well preserved structures using optimal techniques for fixation, permeabilisation, and immunolabelling.

**Results:** Anti-EPO antibodies reacted only with eosinophils, and anti-HNL antibodies only with neutrophils. Anti-ECP antibodies reacted with both eosinophils and neutrophils and anti-MPO antibodies with neutrophils and monocytes. Cells not stained by monoclonal anti-EPO and anti-HNL antibodies included lymphocytes, monocytes, macrophages, squamous epithelial cells, and ciliated epithelial cells.

**Conclusions:** EPO, a unique component of eosinophils, and HNL, a unique component of neutrophils, are useful markers for the identification of eosinophils and neutrophils, respectively, in sputum and bronchoalveolar lavage fluid.

In inflamed airways increased numbers of inflammatory cells such as eosinophils and neutrophils are found in the bronchial mucosa, bronchoalveolar lavage (BAL) fluid, sputum and, in more severe cases, in peripheral blood. There is a need for eosinophil and neutrophil specific markers to identify accurately the type of granulocyte present in the airways. The aim of this study was to investigate whether eosinophil peroxidase (EPO) and human neutrophil lipocalin (HNL) could serve as more cell specific markers for eosinophils and neutrophils than the established eosinophil cationic protein (ECP)<sup>1</sup> and myeloperoxidase (MPO). Sputum and BAL fluid cells were immunolabelled with antibodies directed to two eosinophil derived (EPO, ECP) and two neutrophil derived (HNL, MPO) intracellular markers and their cell specificity was studied.

## METHODS

Sputum samples were obtained from 14 subjects (four healthy individuals, two patients with acute respiratory infections, six with asthma, one with chronic obstructive pulmonary disease (COPD), and one with prolonged cough). The mean age of the patients (three men) was 49.4 years and of the healthy subjects (one man) was 35.5 years. BAL fluid samples were obtained from five patients (one man) of mean age 49.4 years (two with asthma, one with COPD, and two with chronic cough of unknown cause). Asthma and COPD were diagnosed according to ATS guidelines.<sup>2</sup>

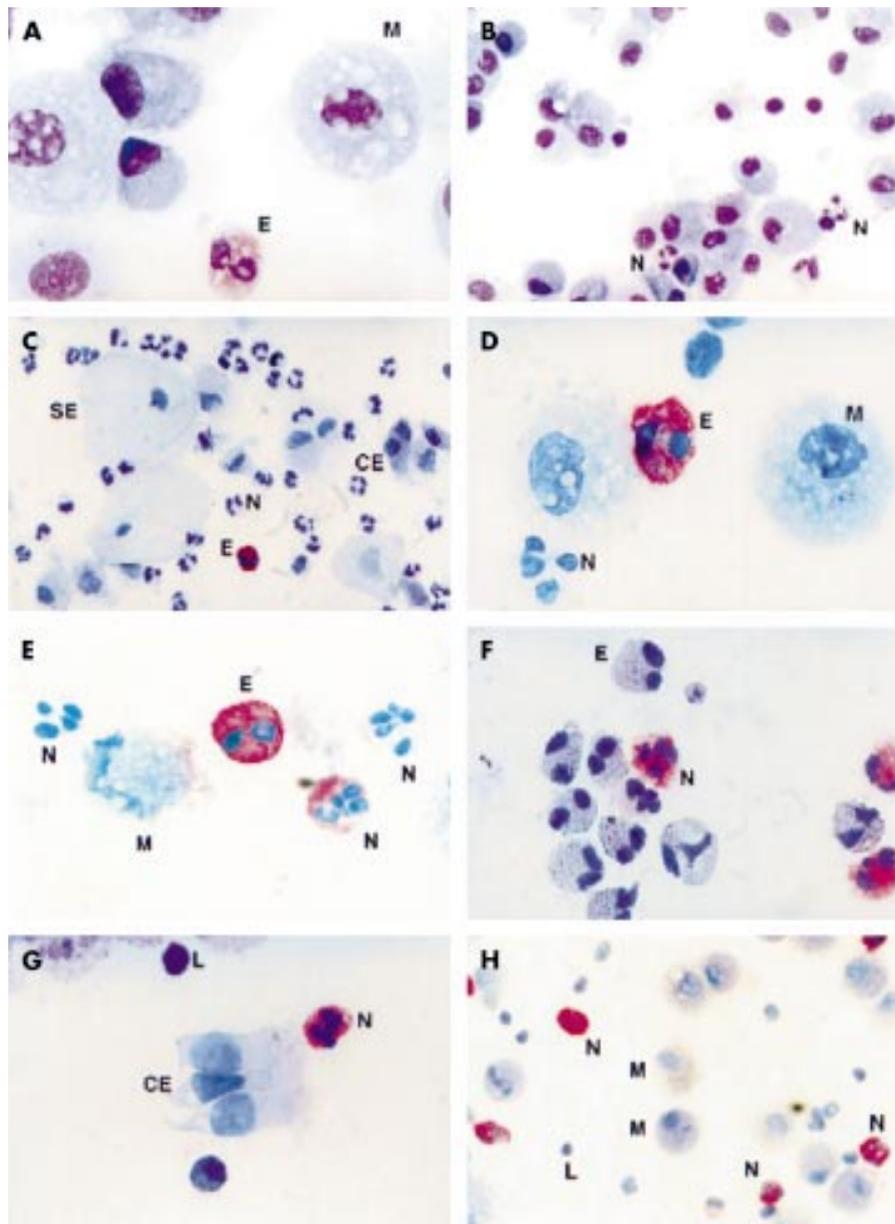
Sputum was induced by inhalation of 5 ml 3% NaCl solution from an ultrasonic nebuliser (Omron U1, Omron, Germany). Peak expiratory flow was measured before and after induction to ensure the safety of the procedure. No pre-medication was undertaken. The sputum was processed by the method described by Pizzichini *et al.*<sup>3</sup> Briefly, all sputum macroscopically free of salivary contamination was selected and treated with 6.5 mmol/l dithiothreitol (Sputolysin, CalbioChem, LaJolla, USA) and phosphate buffered saline (PBS).

The suspension was filtered using a 53 µm mesh nylon filter (Nybolt PA-53/35, Seidengaze, Germany) and centrifuged at 800g for 10 minutes in order to separate sputum cells.

Bronchoscopy and BAL were carried out according to a standardised protocol.<sup>4</sup> Cells were immediately separated from BAL fluid by centrifugation at 800g for 10 minutes at room temperature.

Cell pellets were resuspended in PBS and viability was determined using the trypan blue exclusion test. It was always greater than 90%. Suspensions of unfixed sputum and BAL cells were centrifuged onto Vectabond treated (Vectabond Reagent, Vector Laboratories Inc, CA, USA) microscope slides at 450 rpm for 4 and 6 minutes respectively. Slides were air dried for at least 30 minutes. One slide was used for differential staining using the May-Gr unwald-Giemsa (MGG) method. The remaining slides were wrapped in Parafilm foil, stored at –20°C, and used for the immunocytochemical experiments.

Frozen sputum and BAL cell samples on slides were thawed and immediately fixed and permeabilised for 40 minutes using undiluted Ortho PermeaFix (OPF; Ortho Diagnostic Systems Inc, UK). After permeabilisation, cell samples were incubated for 15 minutes in PBS with 0.2% bovine serum albumin (BSA) before addition of a primary antibody. The primary antibodies were (1) six anti-HNL antibodies (clones 697, 699, 736, 764, 765 and 766), (2) two anti-EPO antibodies (clones 672 and 673), (3) two anti-MPO antibodies (clones 720 and 724), and (4) two anti-ECP antibodies (clones EG1 and EG2). All were monoclonal antibodies and were obtained from Pharmacia AB Diagnostics (Uppsala, Sweden). The HNL antibodies were used as a cocktail at a final concentration of 0.02 mg/ml to increase the staining effect. All other antibodies were added individually at a final concentration of 0.01 mg/ml. When staining intensities and non-specific background staining were investigated, lower (EPO 0.001 mg/ml) and higher (EPO 0.5 mg/ml) concentrations than those mentioned above were used.



**Figure 1** (A) Cytocentrifuged sputum cells and (B) BAL fluid cells after MGG staining. (C) A strongly EPO positive eosinophil among negative neutrophils, macrophages, and epithelial cells in sputum from a healthy subject. (D) At high magnification bright red granules are visible in an eosinophil in the BAL fluid from a patient with asthma and immunostained with anti-EPO antibodies while macrophages, neutrophils, and lymphocytes remain unstained. (E) A brightly EG2 positive eosinophil and an EG2 positive neutrophil in sputum from a patient with asthma. (F) Three strongly and one faintly HNL positive neutrophils and nine HNL negative eosinophils in sputum from a patient with COPD. (G) One HNL positive neutrophil, two negative lymphocytes, and three negative ciliated cells of bronchial epithelium in sputum from a patient with asthma. (H) Four strongly MPO positive neutrophils in sputum from a patient with chronic cough. E=eosinophil, N=neutrophil, M=macrophage, L=lymphocyte, CE=ciliated epithelial cell, SE=squamous epithelial cell.

Incubation with a primary antibody was performed at room temperature in a humid chamber for 30 minutes and terminated by washing with PBS containing 0.2% BSA. In negative controls a primary antibody was omitted. The antigen-antibody complex was visualised using a commercial alkaline phosphatase-antialkaline phosphatase (APAAP) kit (K670, Dako, Glostrup, Denmark) and a Fast Red substrate, in accordance with manufacturer's instructions. Samples were counterstained with Mayer's haematoxylin (Merck, Darmstadt, Germany) for 6 minutes and examined microscopically. Fuji 200 negative film was used in photography.

## RESULTS

Identification of APAAP stained cells was based on cell morphology using a corresponding MGG stained slide as ref-

erence (fig 1A, B). Eosinophils (stained with anti-EPO or EG1/EG2 antibodies) and neutrophils (stained with anti-HNL or anti-MPO antibodies) appeared bright red on the slides (fig 1C-H). All other cells remained blue (counterstained with haematoxylin) and recognisable.

Eosinophils remained unstained when anti-HNL was used, and neutrophils remained unstained when anti-EPO was used. Granules in eosinophils and neutrophils were visible and stained very bright red, indicating that antibodies had penetrated cell and granule membranes. Cells stained by neither anti-EPO nor anti-HNL included lymphocytes, squamous epithelial cells, and ciliated epithelial cells. Macrophages positive for anti-EPO and anti-HNL were occasionally seen.

When antibodies against ECP (EG2 and EG1) were used, eosinophils were always positive. However, neutrophils were

frequently positive in sputum and BAL fluid samples (fig 1E). When anti-MPO antibodies were used as a primary antibody, all cells except neutrophils remained unstained (fig 1H). In one patient with asthma, monocytes and neutrophils were positive for MPO antibodies in the sputum and BAL fluid samples. Monocytes were seldom found in sputum or BAL fluid samples. No background reaction was seen in any cell in negative control samples.

## DISCUSSION

Immunohistological localisation of eosinophil and neutrophil granule proteins has been used widely to study the pathophysiology of various respiratory diseases. Thus, antibodies to ECP (clones EG1 and EG2) have been used to localise eosinophils and to claim eosinophil activation. The latter has been questioned by Jahnsen *et al.*<sup>3</sup> Like eosinophils, neutrophils are cytotoxic and therefore damage tissues. Most neutrophil derived markers are not specific to neutrophils—for example, MPO is also expressed by monocytes.<sup>6</sup>

Jahnsen *et al.* have emphasised the importance of fixation in relation to intensities and specificities when labelling granular proteins with antibodies. In our studies we have found that the organic solvents often used as fixatives in immunocytochemical analysis are unsuitable for detection of intracellular proteins of low molecular weight (Metso *et al.*, unpublished). In addition, if intracellular antigens are to be identified using labelled antibodies, as is the case in our study, the cell membranes need to be permeable and the intracellular antigens need to be fixed in situ. Best results in induced sputum and BAL cells were obtained by using Ortho Permeafix which allows fixation and permeabilisation in one step.

We did not use any double staining of the cells to identify eosinophils and neutrophils. Since there is no consensus on the best method for fixation, recognition of immunolabelled cells was based on the morphology of the cells. After fixation, permeabilisation, immunolabelling, and staining with Mayer's haematoxylin, different cell types could be identified with the same certainty as with conventional MGG staining.

Macrophages positive for all antibodies studied were occasionally observed among sputum and BAL fluid cells. This was not unexpected, as in vitro studies have shown that apoptotic eosinophils are recognised and phagocytosed by macrophages.<sup>8</sup> Similar observations have been reported by Grigg *et al.*<sup>9</sup> who observed free and ingested apoptotic neutrophils in BAL fluid samples.

It is important to identify the type of granulocyte which predominates in various respiratory disorders, and to stage eosinophilic and neutrophilic inflammation. EPO seems to detect eosinophils specifically and may be more useful than the other eosinophil marker, ECP. HNL is a constituent unique to neutrophils. Rapid and sensitive immunoassays for EPO and HNL in various body fluids including BAL fluid and sputum would be useful in clinical studies.

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