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

T Metso, P Venge, T Haahkela, C G B Peterson, L Sevés

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 and of the healthy subjects 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 individuals, two patients with acute respiratory infections, six with asthma, one with chronic obstructive pulmonary disease (COPD), and one with prolonged cough).

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.

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.
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 reference (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. 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.

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 May-Gruber’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. Similar observations have been reported by Grigg et al. 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 immunoaassays for EPO and HNL in various body fluids including BAL fluid and sputum would be useful in clinical studies.

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**Authors’ affiliations**

T Metso, T Hahtela, Skin and Allergy Hospital, Division of Allergy, Helsinki University Central Hospital, Helsinki, Finland

P Venge, L Sevéus, Department of Medical Sciences, University of Uppsala, University Hospital, Uppsala, Sweden

C G B Peterson, Pharmacia AB Diagnostics, Uppsala, Sweden

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