

## Immunological findings in blood and bronchoalveolar lavage fluid in chronic bronchitis patients with recurrent infectious exacerbations

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**ABSTRACT:** Bronchial infections are common in smokers and seem to be related to the presence of chronic bronchitis (CB). Why only some smokers develop repeated bronchial infections is not known. The aim of this study was to screen for immunological changes associated with disease in patients with CB and recurrent infectious exacerbations compared to asymptomatic smokers.

Sixteen smokers with stable CB and recurrent infectious exacerbations, and 18 asymptomatic smokers, all without any immunomodulating treatment, underwent bronchoscopy and bronchoalveolar lavage (BAL). Smoking history and current smoking status were comparable. Serum levels of immunoglobulin (Ig)A, IgM, IgG and IgG subclasses were measured. Blood and BAL lymphocyte phenotypes and proliferative responses of peripheral blood mononuclear cells (PBMCs) to various stimulators were analysed. Unstimulated and tetanus toxoid-stimulated production of cytokines in PBMC cultures was measured. Natural killer (NK-) cell activity was analysed.

A significantly ( $p < 0.05$ ) lower level of IgG<sub>3</sub> was found in the CB group, and a significantly ( $p < 0.01$ ) higher proliferative response of PBMCs was found in the CB group after stimulation with diphtheria toxoid. Detectable levels of interleukin (IL)-6, tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon- $\gamma$ , but not of IL-2, IL-4 or transforming growth factor- $\beta$ 2, were found in supernatants from cultured cells in both study groups. Stimulated TNF- $\alpha$  production was significantly ( $p < 0.05$ ) higher in the CB group. NK-cell activity did not differ significantly between the study groups. There were no major differences between the groups in lymphocyte subpopulations in blood or BAL.

In conclusion, no major alterations in the analysed indices of cell-mediated and humoral immunity were found in patients with chronic bronchitis prone to recurrent infectious exacerbations when compared with asymptomatic smoking controls.

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Tobacco smoking induces profound immunological and inflammatory changes, both in the airways and systemically. Smoking impairs host defences and increases susceptibility to infection [1, 2].

The literature on the effects of smoking on the immune system is extensive and has been reviewed by several authors [2–4]. Evidence of a considerable influence on both humoral and cell-mediated immunity has been presented. Decreased serum immunoglobulin (Ig) levels, mainly IgG, have been reported in smokers [5, 6]. Data concerning immediate skin reactivity to common allergens [7, 8] as well as specific antibody responses to inhaled antigens [9] and vaccinations [10], indicate depressed immune responses in smokers. There are several reports of decreased activity of natural killer (NK-) cells, both from peripheral blood [11, 12] and the lung [13]. Results from functional studies of circulating T-lymphocytes are conflicting, with reports of increased [14], unchanged [15] and decreased [16, 17] responses to mitogenic stimulation *in vitro*. Alterations in

immunoregulatory T-lymphocyte subsets with a decreased fraction of helper/inducer and an increased fraction of cytotoxic/suppressor cells have been reported, both locally in the lung [18], and in the peripheral circulation [19, 20].

Bronchial infections are common in smokers [2], but why only some smokers develop problems with repeated bronchial infections is largely unknown. Hypothetically, smokers prone to bronchial infections might differ immunologically from smokers without this disposition. To our knowledge, there are no studies addressing this issue in which smoking habits of study and control groups have been taken fully into account.

Since the disposition for bronchial infections in smokers seems to be related to the presence of chronic bronchitis (CB) [21], a state of chronic mucus hypersecretion [22], and appear in the form of recurrent infectious exacerbations, patients with CB prone to infectious exacerbations were chosen for the study group. We decided to analyse most of the immunological parameters known or thought

to be influenced by smoking, to investigate whether patients with CB and recurrent infections differed from asymptomatic smokers with comparable smoking habits.

## Materials and methods

### Design

The study was a cross-sectional, parallel group study investigating different indices of airway and systemic immunity in patients with CB and recurrent infectious exacerbations compared with asymptomatic smokers. The subjects were recruited from patient files at the Department of Pulmonary Medicine and by advertisement in a daily newspaper. A medical examination, a lung function test, and blood sampling for haematology and clinical chemistry took place at one visit. Approximately 2 weeks later, venous blood for immunological analyses was drawn and fiberoptic bronchoscopy with bronchoalveolar lavage (BAL) performed. The study was performed between May 1993 and June 1994.

The study was approved by the Ethics Committee at the University of Göteborg. The subjects gave their consent after both written and oral information.

### Subjects

Eighteen asymptomatic control subjects were studied. All were current smokers for more than 10 yrs consuming at least 10 cigarettes·day<sup>-1</sup> without fulfilling the American Thoracic Society (ATS)-defined criteria of CB [22]. All had normal lung function defined as forced expiratory volume in one second (FEV<sub>1</sub>) >80% of predicted. In addition, 16 patients with symptoms of CB as defined by ATS [22], *i.e.* chronic or recurrent productive cough on most days for a minimum of 3 months in the year during the past 2 yrs, were studied. Co-existing chronic airway obstruction defined as FEV<sub>1</sub> <80% pred was allowed. All patients were current smokers for more than 10 yrs, consuming at least 10 cigarettes·day<sup>-1</sup>. In addition, they all had a history of two or more infectious exacerbations during the past 12 months as defined by BOMAN *et al.* [23]. The total number of exacerbations during the past 2 yrs was recorded.

No subject was allowed to use N-acetylcysteine (NAC) or antihistamines or to undergo vaccination or other immunomodulating treatment within 4 weeks prior to the first investigation. No glucocorticosteroid treatment (oestrogen included), whether oral, for inhalation, dermal or nasal, or other immunosuppressant treatment was allowed within 3 months prior to the first investigation. All subjects were to be free of symptoms of infectious respiratory disease 4 weeks prior to the investigation. All subjects were to be 35–65 yrs of age. Subject demographic and clinical data are presented in table 1.

Criteria for exclusion were: baseline FEV<sub>1</sub> <45% pred; a postbronchodilator increase in FEV<sub>1</sub> >15% pred abnormal chest radiograph; bronchial hypersecretion caused by other known active pulmonary diseases such as sarcoidosis or cystic fibrosis; known immunodeficiency;  $\alpha$ 1-antitrypsin deficiency; or a history of asthma. Patients with

Table 1. – Demographic and clinical data

|  | Asymptomatic smokers | CB with exacerbations | p-value |
|--|----------------------|-----------------------|---------|
| Subjects n                                   | 18                   | 16                    |         |
| Age yrs                                      | 45 (35–58)           | 53 (38–63)            | 0.007   |
| FEV <sub>1</sub> % pred                      | 93 (81–114)          | 88 (62–124)           | NS      |
| Pack-years                                   | 28 (15–57)           | 29 (16–44)            | NS      |
| Current smoking cigarettes·day <sup>-1</sup> | 20 (10–30)           | 20 (10–30)            | NS      |
| Gender Male/Female                           | 7/11                 | 4/12                  | NS      |
| Duration of CB yrs                           | 0                    | 8 (4–26)              |         |
| Exacerbations in 2 yrs                       | 0                    | 7 (4–12)              |         |

Values are presented as median with range in parenthesis. CB: chronic bronchitis; NS: nonsignificant.

known atopy were also excluded, as were patients with concurrent severe diseases of any kind as judged by the investigators.

Ventilatory lung function (FEV<sub>1</sub> % pred) was measured with a Vitalograph Alpha (Vitalograph Ltd., Buckingham, UK) in a standardized manner according to the directions of the European Coal and Steel Community [24].

### Clinical chemistry and haematology

Five millilitres of venous blood was drawn at the first visit for analysis of haemoglobin, platelet count, leucocytes (total and differential count) and C-reactive protein. The Sahlgrenska University Hospital laboratory's instructions for handling of samples and its reference ranges for normality were used.

### Fiberoptic bronchoscopy

Immediately preceding bronchoscopy, 90 mL of venous blood for immunological analyses was drawn.

Premedication was given as oral diazepam (5–10 mg) together with 0.5–1 mL morphine-scopolamine (10 mg·mL<sup>-1</sup> + 0.4 mg·mL<sup>-1</sup>) *i.m.* 45 min before bronchoscopy. Local anaesthesia of the oropharynx was given as 1% tetracaine sprayed by a deVilbiss aerosol device (de Vilbiss Ltd., Heston, UK) (~5 mL), followed by additional (4–5 mL) instillation of tetracaine into the larynx. All bronchoscopies were performed transorally by one of two experienced bronchoscopists, and with the subject in the supine position.

### Bronchoalveolar lavage

With the bronchoscope wedged in a middle lobe bronchus, BAL was performed by instilling 2 × 100 mL sterile phosphate-buffered saline (PBS) at 37°C, and immediately aspirating the fluid after each instillation. The aspirated BAL fluid was pooled in a siliconized glass container and immediately transported on ice to the Department of Clinical Immunology for analysis.

Recovery was measured, and the fluid filtered through a nylon web with a pore size of 100  $\mu$ m for retention of mucus and cell debris. The lavage fluid was then centrifuged at 250 × g for 10 min at +4°C. The supernatant was

separated, and the cell-pellet was resuspended in PBS. The total number of cells in BAL fluid from the first eight asymptomatic smokers and the first four CB patients was determined with a haematological cell counter (Sysmex S300, TOA Medical Electronics Co Ltd, Kobe, Japan). We noted, however, that the values from these determinations did not correspond with determinations using a haemocytometer, probably due to debris in the BAL samples. The remainder of the determinations, 10 from the asymptomatic smoker (AS) and 12 from the CB group, were therefore performed using a haemocytometer. Cell viability was estimated by means of trypan blue exclusion. Calculation of cell differentials was done on cytocentrifuged preparations (Cytospin 2; Shandon Southern Products Ltd., Runcorn, UK) stained with May-Grünwald-Giemsa and after counting of 1,000 cells.

#### Assay of serum immunoglobulins

The content of IgG, IgM and IgA as well as of the IgG subclasses in serum was assessed by radial immunodiffusion, using class specific polyclonal rabbit antihuman IgG, IgM and IgA (Dakopatts a/s, Glostrup, Denmark) and monoclonal subclass specific antibodies to IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub> and IgG<sub>4</sub> (Oxoid Unipath Ltd, Hampshire, UK). The concentration of the Ig classes and subclasses was expressed in g·L<sup>-1</sup>, by comparison with a standard. The class reference ranges of the Department of Clinical Immunology (IgG: 7.6–22.1, IgM: 0.5–3.4 and IgA: 0.2–2.8 g·L<sup>-1</sup>) and the IgG subclass reference ranges published by OXELIUS [25] (IgG<sub>1</sub>: 4.22–12.92, IgG<sub>2</sub>: 1.17–7.47, IgG<sub>3</sub>: 0.41–1.29 and IgG<sub>4</sub>: <2.91 g·L<sup>-1</sup>) were used.

#### Flow cytometry analysis of lymphocytes in blood and BAL

In order to investigate the distribution in blood and BAL of major lymphocyte subsets (T-, B- and NK-cells, memory and unprimed T-cells, helper/inducer and cytotoxic/suppressor T-cells) and their expression of activation markers and cell adhesion molecules, subpopulations of lymphocytes were determined by flow cytometry. The samples were stained with combinations of murine monoclonal antibodies, directly conjugated with fluorochromes (fluorescein isothiocyanate, phycoerythrin, R-phycoerythrin-Cy5 or Tri-color). Whole blood (with ethylenediamine tetra-acetic acid (EDTA) as anticoagulant and in a volume corresponding to about 5×10<sup>5</sup> cells-sample<sup>-1</sup>) and BAL cells (1×10<sup>5</sup> cells-sample<sup>-1</sup>) were incubated at 4°C for 15 min with antibodies in the concentrations recommended by the manufacturer. The different monoclonal antibodies used are listed in table 2.

Cell analysis was done on a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA), calibrated with CALIBRITE® beads (Becton-Dickinson) and Auto-COMP® software (Becton-Dickinson). A lymphocyte gate was set manually according to the location in the forward scatter *versus* side scatter diagram. Negative isotype controls (Dakopatts a/s) were used to set quadrant markers which delineated positive fluorescent staining from non-antigen specific staining. Dot plots and quadrant statistics from three-colour analysis were generated by Lysis II software (Becton-Dickinson). The absolute number of blood lymphocytes was determined using a haematological cell counter (Sysmex-K1000; TOA Medical Electronics Co). In the majority of the BAL samples, it

Table 2. – Murine monoclonal antibodies against cell surface antigens used for staining of blood and bronchoalveolar lavage cells in flow cytometry

| Surface antigen | Cell population/antigen characteristic  | Fluorochrome               | Manufacturer                 |
|-----------------|---|----------------------------|------------------------------|
| CD3             | T-lymphocyte                            | Fluorescein isothiocyanate | Dakopatts a/s, *             |
| CD3             | T-lymphocyte                            | R-phycoerythrin-Cy5        | Dakopatts a/s*               |
| CD5             | T-lymphocyte                            | Phycoerythrin              | Dakopatts a/s*               |
| TCRαβ           | T-cell antigen receptor αβ              | Fluorescein isothiocyanate | Becton-Dickinson+            |
| TCRγδ           | T-cell antigen receptor γδ              | Fluorescein isothiocyanate | Becton-Dickinson+            |
| 45RA            | Unprimed cell                           | Fluorescein isothiocyanate | Dakopatts a/s*               |
| 45R0            | Memory cell                             | Phycoerythrin              | Dakopatts a/s*               |
| 45RB            | Subset of T-cells, B-cells              | Fluorescein isothiocyanate | Dakopatts a/s*               |
| CD4             | Helper/inducer cell                     | Phycoerythrin              | Dakopatts a/s*               |
| CD4             | Helper/inducer cell                     | R-phycoerythrin-Cy5        | Dakopatts a/s*               |
| CD8             | Cytotoxic/suppressor cell               | Phycoerythrin              | Dakopatts a/s*               |
| CD8             | Cytotoxic/suppressor cell               | R-phycoerythrin-Cy5        | Dakopatts a/s*               |
| HLA-DR          | Activated cell, transplantation antigen | Fluorescein isothiocyanate | Dakopatts a/s*               |
| HLA-DR          | Activated cell, transplantation antigen | Phycoerythrin              | Becton-Dickinson+            |
| CD69            | Activated cell, early activation marker | Phycoerythrin              | Becton-Dickinson+            |
| CD25            | Activated cell, IL-2 receptor           | Fluorescein isothiocyanate | Dakopatts a/s*               |
| CD2             | Adhesion molecule                       | Phycoerythrin              | Dakopatts a/s*               |
| CD11b           | Adhesion molecule                       | Phycoerythrin              | Dakopatts a/s*               |
| CD11c           | Adhesion molecule                       | Fluorescein isothiocyanate | Dakopatts a/s*               |
| CC18            | Adhesion molecule                       | Fluorescein isothiocyanate | Dakopatts a/s*               |
| CD54            | Adhesion molecule                       | Phycoerythrin              | Serotec <sup>#</sup>         |
| CD56            | NK-cells, subset of T-cells             | Phycoerythrin              | Becton-Dickinson             |
| CD56            | NK-cells, subset of T-cells             | Tri-color                  | Caltag Lab. Inc <sup>‡</sup> |
| CD19            | B-cells                                 | R-phycoerythrin-Cy5        | Dakopatts*                   |

\*: Glostrup, Denmark; +: Mountain View, CA, USA; #: Oxford, UK; ‡: South San Francisco, CA, USA. CD: cluster of differentiation; TCR: T-cell receptor; HLA-DR: human leucocyte antigen DR gene cluster product; IL-2: interleukin 2; NK: natural killer.

was difficult to discriminate events representing lymphocytes from events due to debris in the forward scatter *versus* side scatter diagram. To compensate for this, all percentages of lymphocyte subpopulations from BAL and blood from each individual were divided by the sum of the percentages of CD3+, CD19+ and CD3-56+. This sum includes T-cells, B-cells and NK-cells and represents all major lymphocyte populations. These adjusted values were then used in all calculations. One BAL sample from a control subject contained mainly cell debris, probably due to accidental lysis of the lymphocytes in the sample while lysing contaminating erythrocytes in the preparation for flow cytometry. The result of the analysis of this sample was therefore discarded.

Results for each subpopulation were expressed in blood as percentage of lymphocytes and as number of cells  $\times 10^9 \cdot L^{-1}$ , and in BAL as percentage of lymphocytes.

#### *Blood lymphocyte stimulation and proliferation assay*

Mononuclear cells from blood collected in heparinized tubes were prepared by centrifugation on Ficoll-Hypaque (Lymphoprep; Nycomed Pharma a/s, Oslo, Norway) at  $827 \times g$  for 10 min at room temperature. A total of  $1 \times 10^5$  cells in a volume of 200  $\mu L$  was put in the wells of a microtiter plate with round-bottom wells (Nuclon; Nunc a/s, Roskilde, Denmark). Triplicates of each of the following stimulators were added: tetanus toxoid (final concentration: 10  $\mu g \cdot mL^{-1}$ ; SBL Vaccin Ltd., Stockholm, Sweden), staphylococcus enterotoxin A (SEA; final concentration: 5  $ng \cdot mL^{-1}$ ; Toxin Technology, Inc., Sarasota, FL, USA), purified protein derivative (PPD; 10  $\mu g \cdot mL^{-1}$ ; Statens Seruminstitut, Copenhagen, Denmark), phytohaemagglutinin (PHA; final concentration: 10  $\mu g \cdot mL^{-1}$ ; Wellcome Foundation Ltd., Beckenham, UK), diphtheria toxoid (100  $\mu g \cdot mL^{-1}$ ; SBL) and monoclonal anti-CD3 (125  $ng \cdot mL^{-1}$ ; Ortho Diagnostic Systems, Inc., Raritan, NJ, USA). A triplicate of wells without any stimulator was used as a negative control. The cells were then incubated in Iscoves's medium supplemented with  $5 \times 10^{-5}$  M 2-mercaptoethanol, 10% human AB-serum (Sera-Lab, Sussex, UK) and gentamicin (final concentration: 100  $U \cdot mL^{-1}$ ; Schering-Plough Int., Kenilworth, NJ, USA), in 5%  $CO_2$  at 37°C for 48 h. A sample of 150  $\mu L$  of culture supernatant was removed from each well and frozen at -70°C for later analysis of cytokine content. Then, 150  $\mu L$  of fresh medium and stimulator were added and the plate was incubated for another 24 h.  $^3H$ -thymidine (Amersham International plc, Amersham, UK) was then added and the plate was incubated over night. The cells were harvested (96-well cell harvester, INOTECH, Dottikon, Switzerland) and radioactivity measured as counts per minute (cpm) using a  $\beta$ -counter (Matrix<sub>96</sub>; Packard Instrument Co., Inc., Chicago, IL, USA). Results were presented as stimulatory index (SI) = cpm of stimulated cells/cpm of unstimulated cells.

#### *Cytokine analyses*

Cytokines were analysed in supernatants from cells stimulated with tetanus toxoid. Commercially available kits were used according to the instructions of the manufacturer (R&D Systems Inc., Minneapolis, MN, USA) for

the determination of interleukin (IL)-4, tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and transforming growth factor- $\beta 2$  (TGF- $\beta 2$ ).

Interferon- $\gamma$  (IFN- $\gamma$ ) was determined with a sandwich enzyme-linked immunosorbent assay (ELISA) technique in which a polystyrene microtitre plate (Maxisorb, Nunc a/s) was coated with a monoclonal antibody against IFN- $\gamma$  (Chromogenix AB, Mölndal, Sweden) and incubated over night at 4°C. After washing and blocking with 1% bovine serum albumin (BSA) in PBS, the sample and standard (recombinant human IFN- $\gamma$ , Genzyme Corporation, Cambridge, MA, USA) was added and the plate was incubated for 2 h at 37°C. After washing, biotinylated anti-IFN- $\gamma$  (Chromogenix AB) was added and the plate was incubated over night at 4°C. After washing, alkaline phosphatase conjugated extravidin (Sigma Chemical Co, St Louis, MO, USA) was added and the plate was incubated for 1 h at room temperature. After washing, substrate for alkaline phosphatase extravidin (Sigma Chemical Co) dissolved in diethanolamine buffer was added and the absorbance at 405 nm was recorded using a photometer (Multiscan® BICHROMATIC, Labsystems Oy, Helsinki, Finland).

IL-2 was determined as in a previously described bioassay using the cytotoxic T-lymphocyte line (CTLL)-2 cell line which is dependent on IL-2 for its proliferation [26].

IL-6 was determined as in a previously described bioassay using the B9 subclone of the cell line B13.29 [27].

#### *Assay of blood NK-cell activity*

NK-cell activity was assayed as previously described [28]. NK-cell activity was expressed as the percentage of target cell lysis at an effector to target cell ratio of 5:1.

#### *Statistical analysis*

A StatView® 4.5 (Abacus Concepts, Berkeley, CA, USA) software package was used for the statistical analysis. Since most of the data did not show a normal distribution, data are presented as median and range unless otherwise stated. For comparisons between groups, a Wilcoxon rank sum test or a Fisher's exact test was performed for unpaired, and a Wilcoxon signed rank test for paired observations. All tests were two-tailed and p-values <0.05 were accepted as significant. A Spearman rank correlation coefficient was calculated to investigate correlations between clinical variables.

## **Results**

#### *Haematology, clinical chemistry and serum immunoglobulins*

There were no significant differences in haemoglobin level, platelet and total or differential leucocyte counts between the groups. No abnormal values of clinical significance were observed. All measurements of C-reactive protein were below the detection limit, *i.e.* <10  $mg \cdot L^{-1}$  (data not shown).

Table 3. – Serum immunoglobulins

|                  |                   | Asymptomatic<br>smokers<br>(n=18) | CB with<br>exacerbations<br>(n=16) | p-value |
|------------------|-------------------|-----------------------------------|------------------------------------|---------|
| IgG              | g·L <sup>-1</sup> | 11.2 (7.1–15.1)                   | 9.9 (6.4–14.0)                     | NS      |
| IgA              | g·L <sup>-1</sup> | 2.8 (1.7–4.2)                     | 2.4 (1.0–4.8)                      | NS      |
| IgM              | g·L <sup>-1</sup> | 1.9 (1.5–4.4)                     | 1.9 (0.8–4.7)                      | NS      |
| IgG <sub>1</sub> | g·L <sup>-1</sup> | 6.1 (2.9–7.8)                     | 5.7 (3.2–7.8)                      | NS      |
| IgG <sub>2</sub> | g·L <sup>-1</sup> | 2.3 (1.1–4.3)                     | 2.2 (0.5–4.0)                      | NS      |
| IgG <sub>3</sub> | g·L <sup>-1</sup> | 0.8 (0.1–1.2)                     | 0.5 (0.1–2.2)                      | 0.03    |
| IgG <sub>4</sub> | g·L <sup>-1</sup> | 0.2 (0.1–1.2)                     | 0.3 (0.1–0.6)                      | NS      |

Values are presented as median with range in parenthesis. Ig: immunoglobulin; CB: chronic bronchitis; NS: nonsignificant.

The results of the serum immunoglobulin analyses are presented in table 3. No subjects with IgA or IgM isotype deficiencies were detected. Eleven (32%) of the 34 subjects were deficient in total IgG or one or more IgG subclasses, eight out of 16 (50%) in the CB group and three out of 18 (17%) in the AS group. This difference was not statistically significant. Three subjects, two in the CB group and one in the AS group, had a deficiency in more than one IgG subclass resulting in a mild deficiency in total IgG. Four individuals, three in the CB group and one in the AS group had a minor IgG<sub>1</sub> deficiency. Only one, in the CB group, had a low IgG<sub>2</sub> value. The most common IgG subclass deficiency was IgG<sub>3</sub>, with more individuals with this deficiency in the CB group (seven out of 16, 44%) than in the AS group (two out of 18, 11%). This difference was not statistically significant. However, the level of IgG<sub>3</sub> differed significantly (p=0.03) between the groups, with a lower level in the CB group.

In view of the fact that IgG<sub>3</sub> differed significantly between the two groups, we investigated the possible correlation between IgG<sub>3</sub> and the number of exacerbations during the past 2 yrs, but found none.

#### Bronchoalveolar lavage

Volume recovery, cell viability, cell concentration, differential cell counts and albumin levels were determined and data are presented in table 4. Recovery was slightly lower in the CB group but the difference was not statis-

Table 4. – Recovery, cell viability and cell populations in bronchoalveolar lavage

|   | Asymptomatic<br>smokers<br>(n=18) | CB with<br>exacerbations<br>(n=16) | p-value |
|---|-----------------------------------|------------------------------------|---------|
| Volume recovered %  | 45 (23–58)                        | 40 (20–60)                         | NS      |
| Viability %   | 80 (59–94)                        | 70 (38–89)                         | 0.03    |
| Cell concentration*<br>10 <sup>4</sup> cells·mL <sup>-1</sup> | 37 (19–91)                        | 29 (18–54)                         | NS      |
| Albumin mg·L <sup>-1</sup>                                    | 60 (23–94)                        | 47 (24–96)                         | NS      |
| Macrophages %   | 95 (82–97)                        | 95 (87–99)                         | NS      |
| Lymphocytes %   | 3.5 (1–14)                        | 4 (1–7)                            | NS      |
| Neutrophils %   | 1 (0–5)                           | 1 (0–4)                            | NS      |
| Eosinophils %   | 1 (0–3)                           | 0 (0–3)                            | NS      |

Values are presented as median with range in parenthesis. \*: In the asymptomatic smokers group n=10, in the CB group n=12. CB: chronic bronchitis; NS: nonsignificant.

tically significant. Cell viability was significantly lower (70% versus 80%, p=0.03) in the CB group. Reliable values of cell concentrations were obtained in 12 and 10 subjects in the CB and AS groups, respectively (see Materials and methods). There were no significant differences between the groups in cell concentration or the fractions of macrophages, lymphocytes, eosinophils or neutrophils. Albumin levels in BAL fluid were comparable.

#### Flow cytometry of blood and BAL lymphocytes

The following lymphocyte subpopulations were characterized and the mean percentages (blood and BAL) and absolute numbers (blood) for each study group determined: T-, B- and NK-cells, T-cells with  $\alpha\beta$  or  $\gamma\delta$  antigen receptors, memory and unprimed T-cells, helper/inducer and cytotoxic/suppressor T-cells, T-cells expressing activation markers and cell adhesion molecules, NK-cells expressing human leucocyte antigen DR (HLA-DR) and cell adhesion molecules (table 2).

In blood the mean counts of the different subpopulations were similar in both groups and no significant differences between the groups could be detected in any subset (data not shown).

In the BAL samples the number of cells analysed varied between 10,000 and 50,000. For markers with a positive fraction of  $\geq 11\%$  the median number of positive cells was <50. Since this results in a minimal theoretical variation of 14%, comparisons between groups were only performed for markers with a positive fraction of >11%. These included T-cells, T-memory cells, T-cells with  $\alpha\beta$  antigen receptor, helper/inducer and cytotoxic/suppressor T-cells, T-cells expressing the activation markers DR or CD69 and the adhesion molecules CD2 or CD18. Of these, the fractions of CD3+45RB+ and CD4+5+ (helper/inducer cell) were significantly (p<0.05) lower in the CB group. For all other subpopulations, no significant differences between the groups in the mean percentages were found (data not shown).

#### Comparison of lymphocyte subpopulations in BAL and blood

To analyse possible differences in the distribution of major lymphocyte subpopulations in BAL compared to blood, the fractions in BAL and blood of 10 selected subpopulations were compared. Data from 33 complete pairs of observations were used for this comparison. Results are presented in table 5. The differences between BAL and blood were large and highly significant for most subpopulations, the only exception being IL-2 receptor positive T-cells (CD3+25+). The fractions of T-cells, T-memory cells, T-cytotoxic cells and two populations of activated T-cells were, on average, larger in BAL than in blood. The fractions of B-cells, unprimed or "naive" T-cells and T-helper cells were on average smaller in BAL than in blood. The T-helper/T-cytotoxic (TH/TC) ratio was significantly (p=0.0003) lower in BAL (geometric mean 1.1, coefficient of variation 0.8), than in blood (2.0, 0.3).

To investigate a possible difference between the asymptomatic smokers and the CB patients in the BAL to blood lymphocyte subset composition, we created BAL/blood

Table 5. – Comparison of lymphocyte subpopulations in BAL and blood

|                                     | Surface antigen | BAL (n=33) | Blood (n=33) | p-value |
|-------------------------------------|-----------------|------------|--------------|---------|
| <b>Cell population*</b>             |                 |            |              |         |
| T-lymphocyte                        | CD3+            | 92±5       | 80±7         | <0.0001 |
| B-lymphocyte                        | CD19+           | 3±4        | 13±7         | <0.0001 |
| NK-cell                             | CD3-56+         | 5±3        | 7±3          | <0.001  |
| <b>T-lymphocyte subpopulations+</b> |                 |            |              |         |
| Unprimed cell                       | CD3+45RA+       | 6±7        | 33±20        | <0.0001 |
| Memory cell                         | CD3+45R0+       | 93±12      | 61±13        | <0.0001 |
| Helper/inducer cell                 | CD3+4+          | 49±18      | 65±8         | <0.001  |
| Cytotoxic/suppressor cell           | CD3+8+          | 46±20      | 32±7         | <0.001  |
| Activated cell                      | CD3+DR+         | 23±15      | 13±9         | <0.01   |
| Activated cell                      | CD3+69+         | 48±23      | 3±8          | <0.0001 |
| Activated cell                      | CD3+25+         | 9±9        | 6±4          | NS      |

Values are presented as arithmetic mean±SD. \*: percentage of total lymphocytes; +: percentage of T-lymphocytes. BAL: bronchoalveolar lavage; NK: natural killer; NS: nonsignificant.

quotients of the same selected lymphocyte subpopulations and compared the two groups. Except for memory T-lymphocytes, which had a significantly lower BAL/blood quotient in the CB group, no significant differences were detected (data not shown).

*Lymphocyte proliferation*

The proliferative response of peripheral blood mononuclear cells (PBMCs) to stimulation with different antigens

and mitogens is shown in figure 1. There was a significantly higher (p=0.005) proliferative response in the CB group after stimulation with diphtheria toxoid. There was no significant difference between the groups for any of the other antigens or mitogens, and no tendency or pattern separating the two groups could be detected in the lymphoproliferative responses. Stimulation with the superantigen SEA and the mitogen PHA resulted in prominent responses. Responses to the three antigens tetanus toxoid, diphtheria toxoid and PPD were much lower and responses to the monoclonal CD3 antibody were intermediate.

*Cytokine production*

Data of IL-6, IFN-γ and TNF-α concentrations in supernatants from cultured PBMCs without and with stimulation with tetanus toxoid are presented in figures 2a–c. Some values were below the detection limit and three were above the upper limit. Values below the limit value are represented by half that value, and values above the upper limit by that value.

Stimulation with tetanus toxoid resulted in an increased concentration of TNF-α in both groups, with a significantly higher concentration in the CB group (p<0.05). TNF-α levels without stimulation were also higher in the CB group, but this difference was not significant. Tetanus toxoid stimulation did not noticeably increase concentrations of IL-6 and IFN-γ in any group, nor were there any significant differences between the groups for these cytokines.

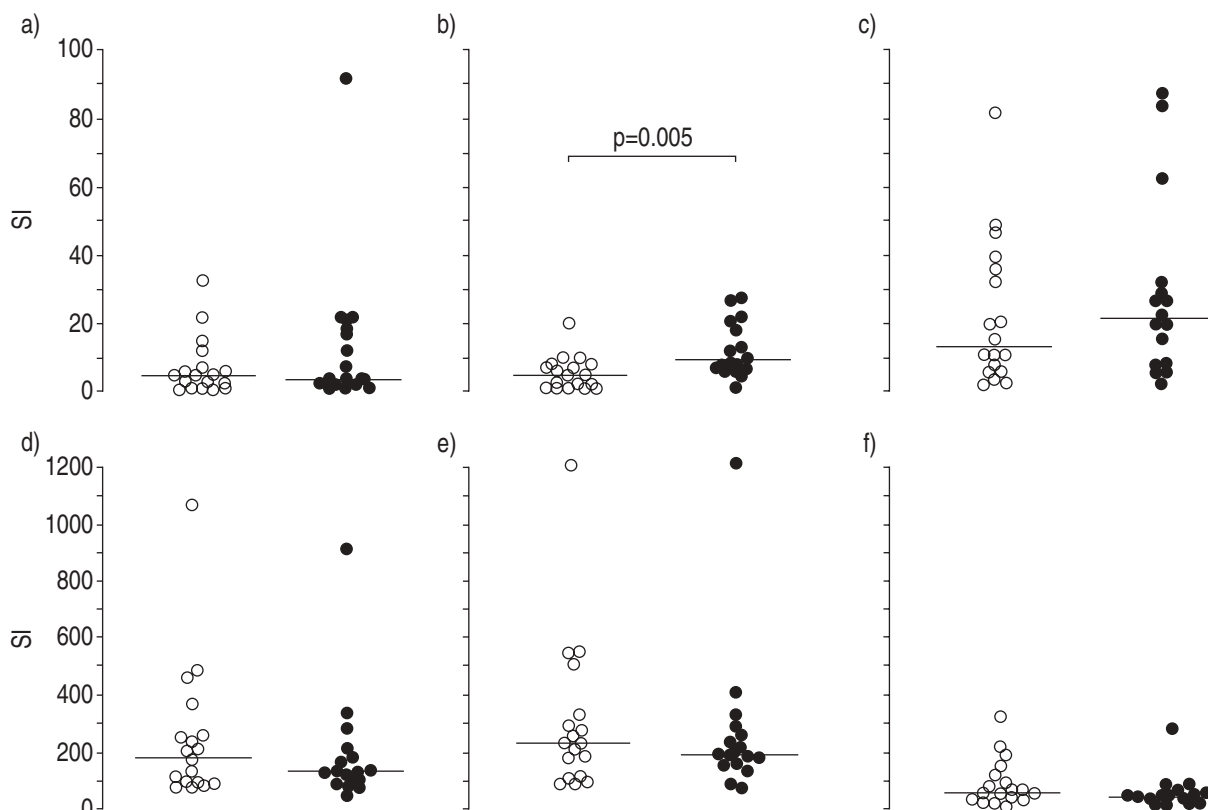


Fig. 1. – Proliferation of blood mononuclear cells from asymptomatic smokers (○) and chronic bronchitis patients (●) after stimulation with: a) tetanus toxoid; b) diphtheria toxoid; c) purified protein derivative; d) staphylococcus enterotoxin A; e) phytohaemagglutinin; and f) anti-CD3. Individual and median values of stimulatory index (SI) are shown.

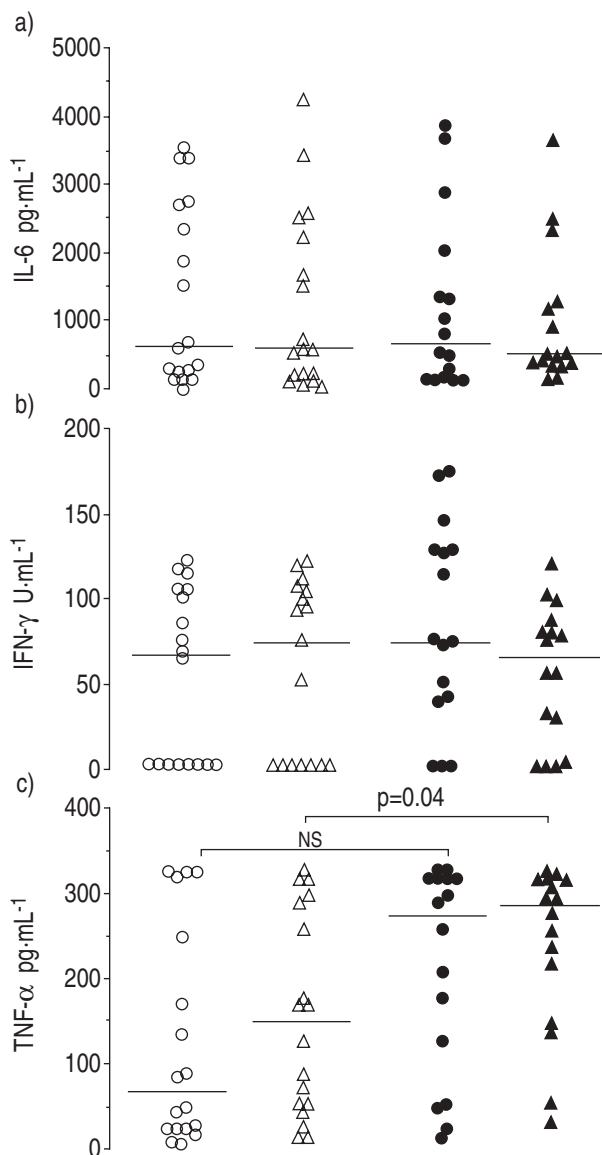


Fig. 2. – Concentrations of: a) interleukin-6 (IL-6), b) interferon- $\gamma$  (IFN- $\gamma$ ) and c) tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) in supernatants from blood mononuclear cell cultures without ( $\circ$ ,  $\bullet$ ) and with ( $\Delta$ ,  $\blacktriangle$ ) stimulation with tetanus toxoid. Individual and median values are presented.  $\circ$ ,  $\Delta$ : asymptomatic smokers;  $\bullet$ ,  $\blacktriangle$ : chronic bronchitis patients.

For IL-2, IL-4 and TGF- $\beta$ , all values except one were below the detection limit of the assays. These cytokines were therefore not included in the analysis and tabulation.

#### NK-cell activity assay

The median fractions of lysed target cells were 20% (range 1–46%) and 14% (2–38%) in the CB and AS groups, respectively. The difference was not statistically significant.

### Discussion

In the present study we investigated different aspects of the immune system in a well-defined group of patients with CB and recurrent infectious exacerbations. Our aim

was to search for possible changes in the immune system associated with recurrent infectious exacerbations in patients with CB, and not attributable to smoking itself. Our results indicate that systemic and local immunity in CB patients with recurrent infectious exacerbations does not in any major way differ from that of asymptomatic smokers when smoking status is comparable. However, detailed analysis revealed some interesting findings.

The results of the serum Ig analyses indicate a possible connection between recurrent infectious exacerbations and lower levels of IgG<sub>3</sub> in smokers with CB, although no correlation was found between the number of exacerbations and IgG<sub>3</sub> levels. An over-representation of IgG<sub>3</sub> deficiency has previously been reported in patients with recurrent respiratory infections [29, 30], but it appears to be more common among nonsmoking patients than among smokers with CB and recurrent infectious exacerbations [30]. In studies of patients with chronic obstructive pulmonary disease (COPD), IgG<sub>1</sub> and IgG<sub>2</sub> deficiencies seem to be more frequent than IgG<sub>3</sub> deficiency [31, 32]. Results from these studies are difficult to compare with those of the present study, due to differences in patient selection. However, the possible connection between IgG<sub>3</sub> deficiency and recurrent infectious exacerbations in smokers with CB needs further attention.

When comparing lymphocyte phenotypes in blood and BAL from CB patients with those from asymptomatic smokers, few if any indications of pathological changes related to susceptibility to infection were found in our CB patients. However, results of the flow cytometry analysis demonstrate major differences in the distribution of lymphocyte phenotypes between BAL and blood, with a higher fraction of T-cells, predominantly of the memory type (expressing CD45R0), and a lower fraction of B-cells in BAL compared with blood. These findings are in accordance with earlier findings in both smokers and nonsmokers [33, 34]. Furthermore, confirming and extending earlier observations [34], we found a much higher expression of common activation markers on T-cells in BAL compared with blood, with the expression of CD25 comparatively less pronounced than that of CD69 and HLA-DR. It appears as if the transformation of naive systemic T-cells (expressing CD45RA) to memory T-cells lining the alveolar surfaces coincides with an increase in surface expression of some activation markers, *i.e.* CD69 and HLA-DR, but not CD25, suggesting preactivation but not necessarily proliferation.

Results from previous studies on the effect of smoking on the proliferative response of PBMCs to mitogenic or antigenic stimulation are not conclusive [17, 35–37]. Proliferative responses of PBMCs from patients with CB have been investigated by SCHÖNFELD *et al.* [38] and were found to be decreased to both mitogens (PHA, Concanavalin A) and an antigen (tetanus toxoid). However, no account of smoking status was given in their report. We found a significantly higher response to diphtheria toxoid in the CB group, but no differences for the other stimulants. Our results do not support the suggestion made by SCHÖNFELD *et al.* [38] that patients with CB have impaired cell-mediated immunity.

The only difference in cytokine production found between our study groups was a significantly higher level of stimulated TNF- $\alpha$  production in the CB group. This is in line with several reports indicating a dysregulation of



TNF- $\alpha$  production and release in patients with inflammatory airways disease. Significantly higher levels of TNF- $\alpha$  have been demonstrated in induced sputum from patients with COPD compared with both smoking and nonsmoking controls [39]. Furthermore, in two recent reports comparing weight-losing and weight-stable COPD patients, higher serum TNF- $\alpha$  levels [40] as well as higher stimulated blood monocyte production of TNF- $\alpha$  [41] were found in the weight-losing groups. Smoking alone has also been shown to influence TNF- $\alpha$  production. Increased production of TNF- $\alpha$  in lipopolysaccharide (LPS)-stimulated PBMCs from smokers as compared to those from nonsmokers has been reported [42], while on the other hand LPS-stimulated alveolar macrophages (AMs) from smokers seem to have impaired ability to release TNF- $\alpha$  compared to AMs from nonsmokers [43].

To conclude, in this extensive investigation of different aspects of immune function, we found only minor differences between smokers with chronic bronchitis and recurrent infectious exacerbations and asymptomatic smokers with comparable smoking habits. If major differences had been found, an extension of the present study comparing chronic bronchitis patients with and without recurrent infectious exacerbations and with similar smoking habits would have been of interest. Not only smoking but also presence of chronic bronchitis could then be ruled out as confounding factors. In light of the predominantly negative results of the present study, such an investigation seems unwarranted, with the possible exception of further study of immunoglobulin G subclasses and regulation of tumour necrosis factor- $\alpha$  production and release.

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