Distribution of immunocompetent cells in the bronchial wall of clinically healthy subjects showing bronchial hyperresponsiveness

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Abstract

Background—Nearly all asthmatic subjects show bronchial hyperresponsiveness, in that the provocative concentration of histamine reducing forced expiratory volume in one second (FEV₁) by 20% (PC₂₀FEV₁) is ≤ 8 mg/ml histamine, and have underlying chronic inflammation of the bronchial wall mediated by T cells. The possible cause and effect relationship between these phenomena remains an enigma. As a proportion of clinically healthy subjects show bronchial hyperresponsiveness, this study was undertaken to determine whether they also show evidence of bronchial inflammation.

Methods—Bronchial biopsy specimens were obtained from 27 clinically healthy subjects with no history of lung disease. Samples were taken perioperatively before elective knee arthroscopy for sports injuries. Specimens were frozen and cryostat sections analysed immunocytochemically with monoclonal antibodies to identify the presence of T lymphocytes, antigen presenting cells, and the expression of HLA-DR. Double immunofluorescence studies were performed with monoclonal antibodies RFD1 and RFD7 to show the relative proportions of RFD1+ RFD7- antigen presenting cells, RFD1- RFD7+ mature phagocytes, and RFD1+ RFD7+ suppressor macrophages. Histological stains were performed to show the presence of eosinophils and mast cells. Three to four weeks after bronchoscopy spirometry was performed on these subjects to record FEV₁, forced vital capacity (FVC), FEV₁/FVC, and forced expiratory flow between 25% and 75% of vital capacity (FEF₂₅₋₇₅). Bronchial hyperreactivity was recorded by determining PC₂₀FEV₁ to histamine.

Results—Nine of the 27 subjects showed bronchial hyperresponsiveness as defined by a PC₂₀FEV₁ of ≤ 8 mg/ml histamine. Segregated subjects with and without bronchial hyperresponsiveness showed no difference in spirometric results. Immunohistological analysis showed no evidence of inflammation in either group. Numbers of T cells, eosinophils, and mast cells were the

same in both groups as was the expression of HLA-DR antigen. No neutrophils were observed in any tissues. Interestingly, reduced numbers of macrophages with the phenotype of antigen presenting cells (monoclonal antibodies RFD1+ RFD7-) were recorded in the subjects with bronchial hyperresponsiveness, who also had a significant increase in the proportion of RFD1+ RFD7+ suppressor macrophages.

Conclusions—Up to 30% of selected clinically healthy subjects may have a PC₂₀FEV₁ of ≤ 8 mg/ml histamine. This physiological trait can exist in the absence of bronchial inflammation. This suggests that bronchial hyperresponsiveness as currently defined is not dependent on immunopathological changes in the bronchial wall and does not necessarily promote even subclinical inflammation.

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Measurement of bronchial reactivity to graded doses of histamine or other stimuli is routinely used to measure bronchial hyperresponsiveness.¹ Since this method was described by Cockcroft *et al* in 1977² the measurement of PC₂₀FEV₁ has been shown to be a valuable variable for monitoring bronchial physiological reactivity, particularly in asthma. Although not specific for asthma, nearly all asthmatic subjects show bronchial hyperresponsiveness, which is currently defined as a PC₂₀FEV₁ of ≤ 8 mg/ml histamine,³ and changes in this variable reflect overall clinical change—for example, after steroid treatment.⁴

Investigations of the immunopathology of the bronchial wall in asthmatic subjects have shown the presence of a chronic peribronchial inflammation characterised by accumulations of T cells, antigen presenting cells, and macrophages.⁵ The increased expression of the CD45RO antigen on the T cells and the increased expression of HLA-DR molecules on the inflammatory cells and the bronchial epithelium that is associated with this T cell dominated inflammation suggest an active acquired cell mediated immune response.⁵ As with the physiological index of bron-

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chial hyperresponsiveness, this inflammatory picture is seen in asthmatic subjects even without acute symptoms.⁶ Measurements of HLA-DR expression in bronchial biopsy specimens of asthmatic subjects and of bronchial hyperresponsiveness in the same patients showed a strong correlation between the immunopathology and pathophysiology.⁵ Furthermore, efficacious treatment with inhaled corticosteroids caused concurrent improvement in bronchial hyperresponsiveness and inflammation.⁷ These results raise the possibility of a cause and effect relation between bronchial hyperresponsiveness and bronchial inflammation. The T cell mediated inflammation in the bronchial wall of asthmatic subjects is present in patients whose condition is stable, it does not necessarily cause clinical symptoms,^{5,6} and a proportion of clinically healthy subjects show bronchial hyperresponsiveness.⁸ We therefore studied clinically healthy subjects to determine whether evidence of bronchial inflammation could be found in non-asthmatic subjects showing bronchial hyperresponsiveness.

Methods

SUBJECTS

Twenty seven subjects (25 men, two women, age range 21–41) listed for elective arthroscopy were recruited from a sports injury clinic. None of these subjects had asthma or any nasal symptoms as determined from a detailed questionnaire and by normal baseline spirometric results on the day of surgery. None had had any viral infection during the two previous months and none had ever consulted a physician for any lung problem. Eight subjects were smokers and eight were atopic when tested with a panel of eight commercial allergen preparations by skin prick testing. All consented to perioperative bronchoscopy while undergoing knee arthroscopy and the study had the approval of the ethics committee of the James Connolly Memorial Hospital.

Biopsy specimens were taken during January and February and subsequent lung function tests performed during February and March.

BRONCHOSCOPY

Anaesthesia was induced with intravenous thiopentone 3–5 mg/kg and maintained with a mixture of oxygen, nitrous oxide, and enflurane. Subjects were intubated with a disposable size 9 endotracheal tube. Intubating conditions were achieved with suxamethonium 1 mg/kg and perioperative muscle relaxation was maintained with vecuronium titrated to effect. Perioperative analgesia was given as fentanyl 1.0–1.5 µg/kg. Standard monitoring included electrocardiography, blood pressure using an automated cuff, and pulse oximetry. Under the supervision of the anaesthetist a fiberoptic bronchoscope (Olympus BF10) was passed through the endotracheal tube into the trachea. Endobronchial biopsy specimens were removed from adjacent to the right upper

lobe carina under direct vision using spiked cup forceps (Olympus FB22C).

LUNG FUNCTION

Three to four weeks after bronchoscopy lung function was recorded. Baseline spirometry was performed with a computerised Gould 2400 system. The best of three technically acceptable forced expiratory manoeuvres was recorded and the forced expiratory volume in one second (FEV₁), forced vital capacity (FVC), and forced expiratory flow between 25% and 75% of vital capacity (FEF_{25–75}) recorded. A standard bronchial provocation protocol was performed one hour after baseline studies were completed.² After inhalation of nebulised saline control solution, nebulised buffered histamine phosphate was inhaled for two minutes through a face mask using a nebuliser driven by oxygen at 7 l/min. Spirometric results were recorded at 30 s and 90 s and subsequently every 60 s after each dose until FEV₁ fell by 20%, or if FEV₁ value failed to fall the next strength of histamine was given when the FEV₁ returned towards baseline. The initial concentration of histamine was 0.03 mg/ml and this was doubled at successive stages until a concentration of 16 mg/ml was used. The provocative concentration of histamine to reduce FEV₁ by 20% (PC₂₀FEV₁) was calculated from the dose-response curve. To check reproducibility of the PC₂₀FEV₁ measurements a further six normal volunteers (same criteria as selected orthopaedic patients) underwent three procedures to measure bronchial reactivity (same methods as described above) over one month. This study was conducted to control the design of the original study, in which spirometric and bronchial hyperresponsiveness studies were performed one month after bronchoscopy for logistical reasons.

PREPARATION OF BIOPSY SPECIMENS

The endobronchial biopsies were placed in optimum cutting temperature medium (BDH, Poole) on small cork discs and frozen in isopentane cooled in a bath of liquid nitrogen. The frozen tissues were stored in liquid nitrogen for not more than one month. Sections (6 µm thick) were cut on a cryostat maintained at –25°C and transferred to poly-L-lysine coated slides. These were air-dried, fixed in a mixture of chloroform and acetone (1:1), wrapped in cling film, and stored at –20°C until use. At least 40 sections were cut from each biopsy specimen.

HISTOLOGY

Sections from all biopsy specimens were stained with haematoxylin and eosin to show structure and with 0.1% toluidine blue to show mast cells. All these slides were examined by two of us (CP and LWP).

IMMUNOHISTOLOGY

Indirect immunoperoxidase methods were used to reveal specific cell types in the tissues.⁹ T lymphocytes were identified by using a cocktail of monoclonal antibodies

CD2, CD5, and CD8 (T mix); primed memory T cells by using monoclonal antibody CD45RO¹⁰; and antigen presenting cells by using monoclonal antibody RFD1.¹¹ The distribution of these cells was measured using an image analyser (Seescan Cambridge), with which the area of framed fields in the sections (three per slide) were point counted as positive cells.¹² From each set of sections two were selected at random for each test. Both were examined visually. Representative areas were then selected for measurement with the image analyser. In all sections from all biopsy specimens the variability between selected areas of any one specimen was far less than the variability between specimens. All results given thus quote the range throughout the group (thus reflecting the greatest variability seen). In each measurement the number of cells counted was divided by the area of framed tissue to obtain a figure representing the number of cells per 10⁴ μm². HLA-DR expression was recorded as relative optical density of the reaction product measured with the image analyser.¹² Ten readings of relative absorption were taken from random areas of the lamina propria within a layer under the epithelium that was 10 cells deep. As with the cell counts the variability within the group was far greater than that within a single specimen. Thus ranges within groups are quoted in the results. Proportions of macrophage subsets were determined by double immunofluorescence methods¹³ in which monoclonal antibodies RFD1 and RFD7 were used in combination. By using two immunoglobulin class specific reagents conjugated to fluorescein isothiocyanate and tetramethyl rhodamine isothiocyanate as a combined second layer the relative proportions of RFD1+ RFD7- (antigen presenting cells fluorescing green), RFD1- RFD7+ (phagocytes fluorescing red), and RFD1+ RFD7+ (suppressor cells fluorescing red and green) could be determined.¹⁴ Colour photographs were taken of all areas and the proportion of cell subsets counted from these photographs. In all cases negative controls were performed omitting the specific monoclonal antibodies, and positive reagent controls were performed on sections of human palatine tonsil.

STATISTICS

Results are expressed as medians and ranges for each group. Data were analysed using the non-parametric Wilcoxon signed rank test.

Historical data from asthmatic subjects were also documented. However, this study was not designed to compare normal values with values in asthmatic subjects, so no asthmatic samples were analysed within this investigation. The figures for patients with diagnosed asthma are quoted purely for comparison.

Results

SPIROMETRIC RESULTS AND BRONCHIAL REACTIVITY

Spirometric results were normal in the 27

subjects biopsied. The median (range) % predicted FEV₁ was 100% (67–129%), FVC was 103% (71–129%), and FEF_{25–75} was 97% (57–127%). Median bronchial reactivity to histamine was also normal (12.5 mg/ml), although a broad range was seen including nine subjects who recorded a PC₂₀FEV₁ of 8 mg/ml or less. The 27 subjects were thus segregated into two groups on the basis of bronchial hyperresponsiveness. Nine patients responded to 8 mg/ml or less of histamine (median (range) PC₂₀FEV₁ 5.6 (2–8 mg/ml) and were defined as having bronchial hyperresponsiveness, and the other 18 had a median value of 19.7 (2.4–36) mg/ml (*p* < 0.001) and were defined as not having bronchial hyperresponsiveness. The spirometric values recorded in the two groups showed no significant difference. There were four smokers in each group, three atopic subjects in the group showing no bronchial hyperresponsiveness, and five atopic subjects in the group showing bronchial hyperresponsiveness. None of the physiological or immunohistological parameters studied discriminated between these subjects.

REPRODUCIBILITY

To determine whether PC₂₀FEV₁ readings showed significant variability over a period of four weeks a group of six normal volunteers underwent repeat analysis for histamine reactivity. In four of the six subjects no significant variability occurred over this period. In two cases a doubling dilution variation was recorded (table 1), but in no case did any subject show a shift in bronchial hyperresponsiveness over this time to abnormal values. The subjects with a PC₂₀FEV₁ of ≤8 mg/ml remained stable at this level over the three repeated analyses (table 1).

HISTOLOGY

Haematoxylin and eosin staining of all biopsy specimens showed normal histology. No neutrophils were seen in any specimen. No thickening of the basement membrane was seen. The epithelium itself was disrupted in many specimens and occasionally torn off. This was taken to be artefactual, resulting from the freezing and cryostat sectioning of such small tissue samples. No evidence of inflammation was present. Small numbers of eosinophils, up to 10 per section, were observed but no difference was detected between subjects with and without bronchial hyperresponsiveness. Similarly, toluidine blue staining showed very

Table 1 Reproducibility of PC₂₀FEV₁ (mg/ml histamine) in clinically healthy subjects

Case no	Baseline	Two weeks	Four weeks
1	3.8	4.7	3.2
2	20	12.6	> 16*
3	3.2	2.8	6.3
4	22.9	25.1	12.6
5	8.7	20	25.1
6	26.3	20.9	25.1

*Regression curve too flat to derive accurate figure.

Table 2 Immunohistological analysis in clinically healthy subjects with and without bronchial hyperresponsiveness. Values are medians (ranges)

Group	T cells (no/10 ⁴ μm ²)	RFD1+ cells (no/10 ⁴ μm ²)	HLA-DR (relative optical density)
Bronchial hyperresponsiveness:			
Present (n = 9)	0.2 (0–3.3)	0.1 (0–0.69)	0.04 (0–0.04)
Absent (n = 18)	0.56 (0–1.2)	0.34 (0–2.8)	0.06 (0–0.17)
p value	NS	< 0.05	NS

Table 3 Proportions of macrophage subsets in 14 clinically healthy subjects and according to bronchial hyperresponsiveness*

Group	RFD1+	RFD7+	RFD1+ RFD7+
Total (n = 14)	11 (3–19)	44 (17–65)	46 (27–75)
Bronchial hyperresponsiveness:			
Present (n = 5)	7 (3–13)	30 (17–42)	64 (48–75)
Absent (n = 9)	11 (4–19)	54 (41–65)	34 (27–44)
p value†	NS	< 0.05	< 0.001

*In each case median (range) of all positive cells with specific phenotype is given as a percentage:

$$\frac{\text{Specific phenotype}}{((\text{RFD1+}) + (\text{RFD7+}) + (\text{RFD1+ RFD7+}))} \times 100$$

†Comparing those with bronchial hyperresponsiveness with those without.

small numbers (1–3 per section) of mast cells in all samples irrespective of bronchial hyperresponsiveness.

Frozen tissue and sections are, however, inappropriate to study these granulocytes, which are best preserved by better fixation and embedding. Differences in these populations may thus exist, which were not detectable in this study (see discussion).

IMMUNOHISTOLOGY

Immunohistological studies of bronchial biopsy specimens failed to detect any difference between subjects with and without bronchial hyperresponsiveness in terms of the numbers and distribution of T cells and the expression of HLA-DR (table 2). The numbers of RFD1+ macrophage-like cells were, however, significantly reduced in specimens taken from subjects with bronchial hyperresponsiveness. All results (irrespective of bronchial hyperresponsiveness) were 5–10 fold lower than previous data obtained from asthmatic subjects in whom the median figures for T cells, D1+ cells, and DR expression were 8.9, 2.2, and 1.6 respectively.⁷

In 14 subjects immunofluorescence studies were performed with a combination of the monoclonal antibodies RFD1 and RFD7 to discriminate subtypes of macrophages. The proportions of RFD7+ RFD1+ suppressor cells were significantly increased in the group with bronchial hyperresponsiveness (table 3). This increase occurred at the expense of the RFD7+ RFD1– population, whose proportion was significantly reduced.

Discussion

Our study of 27 clinically healthy subjects with no history of any clinically significant lung disease shows that up to 30% of this population were bronchially hyperresponsive as defined by a PC₂₀FEV₁ of ≤ 8 mg/ml histamine. As all these subjects regularly participated in sporting activities they may be

considered a selected population, and our results do not necessarily indicate the prevalence of bronchial hyperresponsiveness in the general population.

Nevertheless, the total absence of any evidence of bronchial inflammation in the subjects showing bronchial hyperresponsiveness suggests that bronchial hyperresponsiveness is not dependent on bronchial inflammation. Furthermore, the presence of bronchial hyperresponsiveness need not in itself cause any inflammatory change in the bronchial wall. Interestingly, even the subject who had abnormal spirometric results (67% predicted FEV₁) showed no bronchial hyperreactivity or any evidence of airway inflammation.

Although bronchial hyperresponsiveness has been described as a cardinal feature of asthma,³ it is not specific for this disease.¹⁵ Indeed, bronchial hyperresponsiveness defined as an exaggerated response to histamine inhalation may be found in a proportion of the healthy, non-asthmatic population.² The accepted cut off point of 8 mg/ml histamine may be too high.¹⁶ Our results support this and suggest that the term bronchial hyperresponsiveness may need to be redefined. It is notable that two of the six subjects used to check reproducibility of results in the lung function laboratory consistently responded to concentrations of histamine lower than 8 mg/ml. Hyperresponsiveness in any given subject might therefore be identified only when a change from baseline values is seen.

Interestingly, the difference in bronchial hyperresponsiveness that segregated the original group of 27 subjects existed in the absence of any difference in spirometric values between the two groups. This observation supports recent data showing that bronchial hyperresponsiveness and spirometry show different kinetics of recovery in patients with acute asthma.¹⁷ As significant correlations have been observed between bronchial hyperresponsiveness and inflammation in asthmatic patients,⁵ how do the healthy subjects in our study remain clinically normal and show no evidence of bronchial inflammation in the face of an apparent physiological defect? One possible clue emerges from the observation that the proportion of cells with the phenotype of suppressor macrophages is raised in these subjects. As previous studies have identified a unique balance of macrophage subsets at mucosal surfaces,¹⁴ increases in this putative suppressor population may reflect a response of the local immunological network to raised bronchial hyperresponsiveness that is aimed at further suppressing acquired T cell reactivity in the bronchial wall and thus reducing the risk of inflammation. This possibility is under further investigation. Other possibilities, such as changes in the local eosinophil population, which may be associated with bronchial hyperresponsiveness,¹⁸ clearly exist. Our study shows, however, that bronchial hyperresponsiveness can exist in the absence of any evidence of T cell mediated inflammation in the bronchial wall.

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