Bronchial γδ T-lymphocytes and expression of heat shock proteins in mild asthma

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Bronchial γδ T-lymphocytes and expression of heat shock proteins in mild asthma. I. Fajac, G.L. Roisman, J. Lacronique, B.S. Polla, D.J. Dusser. ©ERS Journals Ltd 1997.
ABSTRACT: Heat shock proteins (HSPs), which are important targets for γδ T-lymphocytes, are thought to play a role in inflammatory and immune diseases. The purpose of this study was to characterize, in asthma, the presence and distribution of αβ and γδ T-lymphocytes and of hsp60, hsp70 and hsp90 in bronchial biopsies, and to seek for a co-localization of γδ T-cells and HSPs.

Ten subjects with mild atopic asthma and nine control subjects underwent fibreoptic bronchoscopy and bronchial biopsies, to which specific monoclonal antibodies and immunohistochemical techniques were applied.

T-lymphocytes present in bronchi both of asthmatic and control subjects were predominantly of the αβ T-cell receptor phenotype (median 642 cells·mm^{-2} (range 85–1,510 cells·mm^{-2}), and 855 cells·mm^{-2} (286–2,424 cells·mm^{-2}), respectively), whereas, γδ T-lymphocytes were always rare (median 26 cells·mm^{-2} (range 0–114 cells·mm^{-2}), and 0 cells·mm^{-2} (0–57 cells·mm^{-2}), respectively). Both in asthmatic and control subjects, bronchial epithelium was positive for hsp60, hsp70 and hsp90. There was no significant difference in the percentages of positive epithelial cells between asthmatic and control subjects. No co-localization of HSPs and γδ T-cells was observed.

Our findings do not support the hypothesis that heat shock proteins and γδ T-cells play an important role in inflammatory and immune responses in mild asthma.
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Heat shock proteins (HSPs) are highly conserved molecules with respect both to function and structure [1, 2]. Numerous HSPs have been identified and are classified according to their molecular weight (*i.e.* hsp 60 kD, hsp 70 kD and hsp 90 kD families). HSPs are typically induced by heat shock, and many of them appear to perform functions that are vital to normal prokaryotic or eukaryotic cells [1, 2]. It has been proposed that HSPs play a role in the processing and/or presentation of antigens, and antibodies or T-cells specific for HSPs have been found in a variety of inflammatory and/or immune diseases [3].

Antigen is recognized by the T-cell antigen receptor (TCR), a heterodimer closely associated with the CD3 complex on the T-cell surface. Most T-lymphocytes express a TCR composed of α- and β-chains [4]. A minor population of T-cells, representing less than 10% of circulating T-lymphocytes in man [5], bears an antigen receptor composed of γ- and δ-subunits [6, 7]. The biological function of γδ T-cells remains to be established, but evidence has been provided that these cells can recognize HSPs, including self-hsp 60 peptides expressed by stressed host cells [3, 8]. It has been proposed that HSP-reactive γδ T-cells may represent a scavenger system capable of recognizing host cells suffering from any insult, such as inflammation. It is, therefore, of interest that a co-localization of HSPs and γδ T-cells has been described in various inflammatory disorders [9–13].

Asthma is a chronic inflammatory disorder, in which a role for γδ T-cells [14–17] and HSPs has been questioned [18]. In patients with asthma, an increased number of γδ T-cells in the bronchoalveolar lavage is debated [14, 16], and an increased expression of hsp70 in bronchial epithelial cells has been reported [18]. Our aim was to determine whether expression both of γδ T-cells and HSPs was increased in bronchial mucosa in patients with mild asthma as compared to control subjects, and to seek for a co-localization of γδ T-cells and HSPs.

Materials and methods

Subjects

Ten subjects with asthma and nine normal control subjects were included in the study. Subjects with asthma met the American Thoracic Society (ATS) diagnostic criteria of asthma [19]. The duration of their asthma ranged from 1–22 yrs. They had symptoms of mild-to-moderate perennial asthma and were clinically stable at the time of the study. They were treated with inhaled β$_2$-agonists alone, used on an as needed basis.

At the preinclusion visit, each subject underwent: interview; skin-prick tests to common airborne allergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, mixed grass pollen, mixed tree pollen, cat and dog danders) (Stallergènes, Fresnes, France); spirometry; and bronchial challenge to methacholine performed according to standard recommendations [20]. None of the asthmatic or control subjects had a respiratory infection for at least 6 weeks before the study, and none were smokers.

All subjects underwent a fibreoptic bronchoscopy 1 or 2 days after the methacholine challenge as described previously [21], and according to the National Institutes of Health guidelines [22]. Bronchial biopsies were obtained from the third bifurcation and subcarinae of the left lower lobe. They were gently extracted from the forceps and immediately frozen in liquid nitrogen or fixed in Bouin's fluid, embedded in paraffin and stained with haematoxylin and eosin or with Luna's reagent specific for eosinophil granular content [23].

The subjects were informed of the risks and purpose of the investigation and gave their written informed consent. The study was approved by the Ethics Committee of Cochin hospital.

Immunohistochemical techniques

Monoclonal antibodies and their respective dilution used to detect T-cells and T-cell subpopulations were: Leu 4 (1/50) (recognizing all CD3+ T-lymphocytes; Becton-Dickinson, Mountain View, CA, USA); βF1 (1/100) (recognizing all αβ T-lymphocytes; T-cell Sciences, Cambridge MA, USA), and TCRδ1 (1/10) (recognizing all γδ T-lymphocytes; T-cell Sciences). Monoclonal antibodies used to detect HSP were: 4B9/89 (1/250) (shown to be specific to the mammalian hsp 60 kD, and kindly provided by G. Rook) [24], SPA-810 (1/1,000) (specific for hsp 72 kD/inducible; Stress Gen, Victoria, Canada), and SPA-830 (1/250) (specific for hsp 90 kD; Stress Gen). An isotype-matched control antibody (Dako, Glostrup, Denmark) was used as a control.

Serial 6 μm thick frozen sections were cut from the cryopreserved tissue blocks, fixed in acetone for 10 min, reacted with appropriate dilutions of monoclonal antibodies for 1 h, and then washed. Positive cells were revealed by reaction with alkaline phosphatase anti-alkaline phosphatase antibody complexes (APAAP kit system; Dakopatts, Glostrup, Denmark) and the fast red enzyme substrate [25]. To test the specificity of the immunohistochemical techniques, monoclonal antibodies were omitted or replaced by an isotype-matched control antibody.

Analysis of results

Positive T-cells and eosinophils present on the whole surface of the biopsy were counted without knowledge of the subject. To determine the surface of tissue evaluated, a grid (ocular test system; Carl Zeiss, Oberkochen, Germany) was inserted in the eyepiece of the microscope. Epithelial cells expressing hsp60, hsp70 or hsp90 and all epithelial cells present on each biospy were

counted. Positive epithelial cells were expressed as a percentage of total epithelial cells. Results are expressed as mean±SEM or as median (minimum-maximum). Comparisons in numbers or percentages of stained cells between asthmatic and control subjects were made using the nonparametric Mann-Whitney U-test. Nonparametric correlations between data were made using the Spearman's rank analysis. A p-value equal to or less than 0.05 was considered to indicate statistical significance.

Results

Clinical characteristics

Clinical characteristics of asthmatic and control subjects are summarized in table 1. Asthmatic subjects (7 males and 3 females) and normal control subjects (5 males and 4 females) did not differ significantly with respect to their age (mean±SEM age: 34±4 and 27±7 yrs, respectively). Skin-prick tests to common allergens were positive in 9 out of 10 asthmatic subjects but in none of the control subjects. The forced expiratory volume in one second (FEV_1) baseline values (mean±SEM) in asthmatic and control subjects were 92±4 and 102±4% of predicted values, respectively. Nine of the 10 asthmatic subjects demonstrated a hyperreactivity to methacholine, with a mean provocative dose of methacholine required to produce a 20% fall in FEV_1 (PD_{20}) of 802±422 μg. None of the control subjects demonstrated hyperreactivity to methacholine.

Immunohistochemical findings

A tissue surface of 0.14 mm² (0.08–0.26 mm²) and 0.10 mm² (0.03–0.21 mm²) was evaluated in asthmatic and control subjects, respectively.

TCR expression by bronchial T-lymphocytes

Both in asthmatic and control subjects, numerous CD3+ T-lymphocytes were noted, infiltrating the epithelium and the submucosa. No significant difference in the number of CD3+ T-lymphocytes was observed between asthmatic and control subjects (1,011 cells·mm⁻² (85–1,738 cells·mm⁻²), and 997 cells·mm⁻² (286–3,000 cells·mm⁻²), respectively; p>0.05).

In asthmatic and control subjects, CD3+ T-lymphocytes expressing αβ TCR infiltrated both the epithelium and the submucosa. No significant difference in the number of αβ TCR+ T-cells was observed between asthmatic and control subjects (642 cells·mm⁻² (85–1,510 cells·mm⁻²), and 855 cells·mm⁻² (286–2,424 cells·mm⁻²), respectively; p>0.05) (fig. 1).

In asthmatic and control subjects, γδ T-lymphocytes were seen both in the epithelium and the submucosa, and no preferential location of γδ T-lymphocytes in or near bronchial epithelium was observed. Although there was a trend to an increased number of T-cells expressing γδ TCR in asthmatic subjects (26 cells·mm⁻² (0–114 cells·mm⁻²)) as compared to control subjects (0 cells·mm⁻² (0 to 57 cells·mm⁻²)), γδ T-cells were always

Table 1. – Clinical characteristics of asthmatic and control subjects

Ss No.	Age yrs	Sex	Skin-prick tests*	Duration of asthma yrs	FEV1 % pred	PD20 µg
Asthmatic subjects						
1	25	M	+	15	101	2141
2	24	M	+	20	86	74
3	66	M	+	6	67	126
4	22	M	+	17	94	3596
5	32	F	+	19	81	71
6	28	M	+	10	116	NR
7	53	F	-	1	84	33
8	33	M	+	15	86	1032
9	24	M	+	16	106	106
10	31	F	+	22	101	35
Control subjects						
11	23	F	-	-	103	NR
12	26	M	-	-	94	NR
13	23	M	-	-	94	NR
14	24	F	-	-	105	NR
15	27	M	-	-	94	NR
16	44	F	-	-	107	NR
17	25	M	-	-	107	NR
18	26	F	-	-	84	NR
19	23	M	-	-	125	NR

*: nine allergens were used: *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, mixed grass pollen, mixed tree pollen, three different mixed weed pollens, cat and dog danders. Ss: subjects; M: male; F: female; FEV1: forced expiratory volume in one second; % pred: percentage of predicted value; PD20: provocative dose of methacholine required to produce a 20% fall in FEV1; +: positive; -: negative; NR: not reactive.

rare and no significant difference (p=0. 11) was observed between asthmatic and control subjects (fig. 1). Expressed as a percentage of total T-cells, γδ T-lymphocytes represented less than 7% of CD3+ T-lymphocytes in nine of the ten asthmatic subjects and in all of the nine control subjects (table 2).

Expression of HSP

In all bronchial biopsies from asthmatic and control subjects, epithelium was the only positive location for hsp60, hsp70 and hsp90. When stained, epithelial cells were diffusely positive, suggesting an intracytoplasmic distribution of HSP (fig. 2).
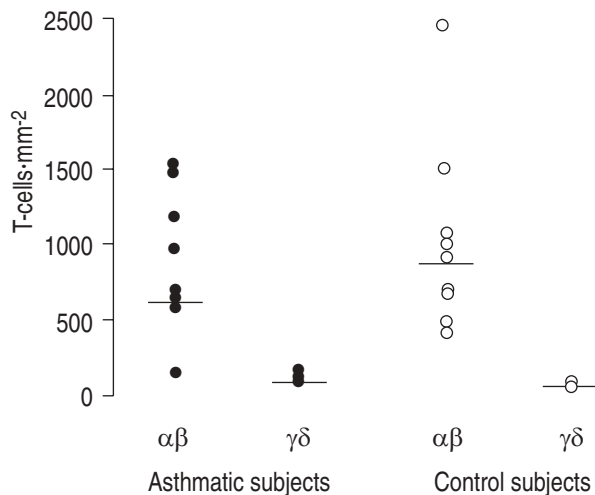

Fig. 1. – Individual values of bronchial αβ and γδ T-lymphocytes in asthmatic (●) and control (○) subjects. Horizontal bars represent the medians.

Expressed as a percentage of total epithelial cells, the numbers of epithelial cells expressing hsp60, hsp70 or hsp90 were not different between asthmatic and control subjects (table 2). For each HSP tested, there was a great variability in the percentage of positive epithelial cells

Table 2. – Individual values of bronchial γδ T-lymphocytes and epithelial expression of HSPs in asthmatic and control subjects

Ss No.	γδ TCR+ cells % of T-cells	Expression of HSP by epithelial cells % of epithelial cells		
		hsp60	hsp70	hsp90
Asthmatic subjects				
1	6.0	86	100	23
2	1.6	30	74	66
3	5.7	74	81	60
4	0	30	100	36
5	10.8	0	50	0
6	0	0	40	0
7	0	0	0	26
8	2	40	10	0
9	2.1	100	0	20
10	2.3	0	80	10
Median	2.2	30	62	21
Control subjects				
11	5.0	0	50	0
12	0	90	38	30
13	5.7	6	76	31
14	6.2	74	100	0
15	0	67	98	10
16	0	70	50	20
17	5	0	100	100
18	0	0	0	0
19	2.1	100	0	20
Median	0	6	50	10

Ss: subjects; TCR: T-cell receptor; HSP: heat shock protein.
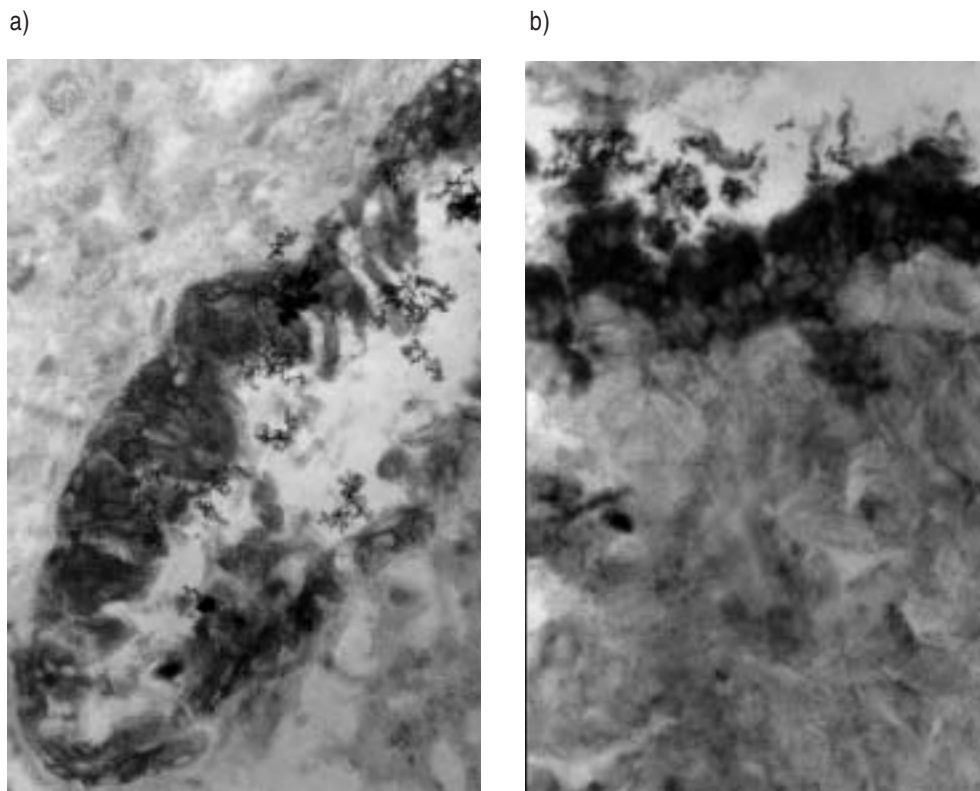
a)   b)

Fig. 2. – Expression of heat shock protein, hsp 70, by bronchial epithelium: a) in an asthmatic subject; and b) in a control subject. Monoclonal antibody specific for hsp70 was applied and positive cells were revealed by reaction with alkaline phosphatase-anti-alkaline phosphatase antibody complexes. The whole surface of epithelial cells is intensely positive.The same pattern of staining is observed in asthmatic and in control subjects (light microscopy; internal scale bar = 10µm).

between the subjects, either asthmatic or control. Similarly, for each subject, either asthmatic or control, there was a great variability in the percentage of positive epithelial cells between the three HSPs, therefore making it unlikely that the expression of HSP that was observed resulted from a "technical stress" due to the bronchoscopy procedure or the methacholine challenge (table 2). However, the pattern of HSP expression by epithelial cells was similar in the different sections examined from the same patient.

No co-localization of $\gamma\delta$ T-cells and HSPs was observed. There was also no correlation between the percentages of $\gamma\delta$ T-cells and the percentages of epithelial cells expressing either hsp60, hsp70 or hsp90 (table 2).

The number of eosinophils infiltrating the epithelium was greater in asthmatic subjects (0.65 cells·mm^{-1} (0– 1, 333 cells·mm^{-1})) than in control subjects (0 cells·mm^{-1} (0–43 cells·mm^{-1})) (p<0.01). There was also a trend to an increased number of eosinophils infiltrating the submucosa in asthmatic patients (1.7 cells·mm^{-1} (0–32 cells·mm^{-1})) as compared to control subjects (0 cells·mm^{-1} (0–4.7 cells·mm^{-1})) (p=0.06). There was no correlation between the percentages of epithelial cells expressing either hsp60, hsp70 or hsp90 and the numbers of eosinophils infiltrating the epithelium or the submucosa (p>0.05 for each correlation).

Discussion

In this study evaluating the TCR phenotype of infiltrating T-lymphocytes and the expression of HSPs in bronchial biopsies of asthmatic and control subjects, we have shown that: 1) both in asthmatic and control subjects, most T-lymphocytes express the $\alpha\beta$ TCR, whilst $\gamma\delta$ T-lymphocytes are rare; 2) hsp60, hsp70 and hsp90 are expressed in bronchial epithelium of control subjects, and there is no increase in expression of HSPs in bronchial epithelium of asthmatic subjects; 3) no co-localization of lymphocytes bearing $\gamma\delta$ TCR and HSPs was observed either in asthmatic or control subjects.

Most bronchial T-cells expressed the $\alpha\beta$ TCR, and $\gamma\delta$ T-cells were rare both in asthmatic and control subjects. In control subjects, the present results are in accordance with our previous study characterizing the TCR phenotype of bronchial T-lymphocytes in normal human bronchi obtained by surgery [26]. In asthma, no previous study has characterized the TCR phenotype of T-lymphocytes infiltrating the bronchi. Studies performed using bronchoalveolar lavage cells in asthmatic subjects have reported conflicting results for the number of $\gamma\delta$ T-lymphocytes. WALKER et al. [14] found no difference in the number of $\gamma\delta$ T-cells between asthmatic and control subjects, which is in accordance with our finding in the bronchial mucosa. Conversely, SPINOZZI et al. [16] recently reported an increased number of $\gamma\delta$ T-cells in asthmatic patients. These different results might be due to the study populations, since more severe asthmatic patients (FEV1 <70%) were included in the study by SPINOZZI et al. [16]. That infiltration of $\gamma\delta$ T-cells into the airway mucosa may vary with the severity of the disease was further emphasized in the study of PAWANKAR et al. [27], who reported an increased number of $\gamma\delta$

T-cells in the nasal mucosa of patients with perennial allergic rhinitis as compared to patients with seasonal allergic rhinitis outside the pollen season. In the present study, the absence of an increased number of γδ T-cells in bronchial biopsies performed in asthmatic subjects does not rule out the possibility that an increased number of γδ T-cells may exist either in deeper layers of the airway wall or in more distal bronchi. It is also of interest that the presence of γδ T-cells appears to be restricted to early stages of infection or inflammation [9, 28, 29]. In the present patients, the duration of asthma, which was at least 1 year, might have been too long for bronchial infiltration of γδ T-cells to persist.

In asthmatic subjects, an epithelial expression of all the HSPs studied was found, and was intense for hsp70. However, an epithelial expression for all the HSPs studied was also observed in control subjects, and there was no significant difference between asthmatic and control subjects. Concordant findings have been reported previously (S.T. Holgate, personal communication); however, VIGNOLA et al. [18] found a significant increase of hsp70 expression in asthmatic subjects. This finding arose from the fact that no expression of hsp70 was observed in control subjects [18]. The discrepancies between the findings of the present study and the absence of HSP expression in control subjects reported by VIGNOLA et al. [18] is not likely to be due to inclusion criteria or differences in methodology, since they were similar in both studies. However, our findings in normal human bronchi are in accordance with previous reports [30, 31]. In contrast to the present study, BONAY et al. [31] reported only a weak reactivity of bronchial epithelial cells for hsp60. This might be due to the monoclonal antibody they used, ML30, since doubt has been cast upon its reliability as a probe for human hsp 60 [24].

It has been suggested that HSPs could play a role in inflammatory and immune diseases, including diseases with eosinophilic inflammation, such as asthma [18]. It has also been reported that HSP expression in asthma is correlated with the severity of asthma and the eosinophilic infiltrate [18]. The present patients formed a homogeneous group with mild asthma, in which the correlation between the expression of HSPs and the severity of the disease could not be verified. However, since no correlation was found between the expression of HSPs and the eosinophilic infiltrate, and since no increased bronchial expression of HSPs was found in mild asthma as compared to control subjects, further studies, including studies in more severe asthma, are needed to assess the possible role of HSPs in asthma.

Evidence has accumulated that γδ T-cells can recognize autologous HSPs and participate in immune reactions in inflammatory diseases [3, 32, 33]. This was emphasized by several reports of co-localization of γδ T-cells and HSPs in inflammatory disorders [9–13]. However, although an epithelial expression of HSPs was observed in the bronchi of the present asthmatic patients, γδ T-cells were rare and there was no co-localization of γδ T-cells and HSPs. As has been emphasized previously [10], our findings suggest that the expression of HSPs, in itself, is not a sufficient condition for a cell to become the target of γδ T-cells. It should also be stressed that the severity of the disease in the present patients might have been too mild to observe a γδ

T-cell infiltration co-localized with HSPs. However, it is difficult to perform fibreoptic bronchoscopy in more severe stages of asthma. Therefore, our conclusions are only valid for mild-to-moderate asthmatic subjects during a stable period.

In conclusion, we have demonstrated that, although hsp60, hsp70 and hsp90 are expressed in bronchial epithelium in mild asthma, γδ T-cells are rare and are not co-localized with heat shock proteins. Our findings do not support the hypothesis that heat shock proteins and autologous heat shock protein reactive γδ T-cells play an important role in inflammatory and immune responses in mild asthma.

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