

Increased bronchoalveolar lavage CD8 lymphocyte subset population in wheezy infants

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Episodes of wheezing are very common in infancy but, despite their high prevalence, their mechanism is still poorly understood. To better understand the airway inflammation of wheezing infants, we examined cells of the bronchoalveolar lavage (BAL), focusing on the phenotype of lymphocytes and macrophages by using cytofluorimetry. Twenty-one wheezers (mean age 15.4 months) and seven non-wheezers (mean age 24.1 months) were studied. BAL was collected at fiberoptic bronchoscopy. Total and differential cell counts were similar in both populations. Eosinophils were not detected in the BAL fluid. The cell-surface markers CD2, CD3, CD4, CD7, CD8, CD19, and CD45 were studied for the lymphocyte sub-population analysis. The cell-surface markers CD14, CD54, CD62L, and human leucocyte antigen (HLA)-DR were studied for the macrophage sub-population analysis. A significant increase in the CD8⁺ lymphocyte population ($p = 0.03$) was observed in wheezers (median 43.1%, 25–75% percentile: 30.1–54.9%), as compared to non-wheezers (median 29.3%, 25–75% percentile: 13.5–34.7%). A significantly ($p = 0.04$) decreased expression of HLA-DR (mean fluorescence intensity [MFI]) was detected in the macrophage population of the wheezers (median MFI, 7,016; range 2135–7986), as compared to non-wheezers (median MFI, 8,369; range: 6478–8860). The results of the present study suggest that viral infection may have induced a CD8⁺ response in BAL cells.

**Bernard Arnoux¹, Jean Bousquet¹,
Monique Rongier¹,
Pierre Scheinmann² and
Jacques de Blic²**

¹INSERM U454 – IFR3, Hôpital Arnaud de Villeneuve, 34295 Montpellier Cedex 5,

²Service de Pédiatrie, Hôpital Necker Enfants Malades, Paris, France

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Bernard Arnoux, INSERM U454 - IFR3, Hôpital Arnaud de Villeneuve, 34295 Montpellier Cedex 5, France

Tel.: (+33) 4 67 33 59 32

Fax: (+33) 4 67 63 28 55

E-mail: arnoux@montp.inserm.fr

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Episodes of wheezing are very common in infancy but, despite their high prevalence, the relationship between these episodes and the progression to asthma has not been completely established (1). Moreover, infantile asthma is a heterogeneous disease in which it is difficult to differentiate between viral infections and atopy (2).

Airways inflammation has been demonstrated in infants with severe recurrent wheezy bronchitis, even during symptom-free intervals (3,4). However, there are few data on the bronchoalveolar lymphocyte populations, which play a role in the development of inflammatory reactions in lung tissue (5–7). Inflammation is present within the airways of virtually all asthmatic adults (8) and involves an array of alterations in tissues,

cells, and mediators. Eosinophils, lymphocytes (CD4⁺ T cells), and mast cells, as well as structural and other inflammatory cell types, are often found in increased numbers and/or are activated. In order to better understand the airway inflammation of wheezy infants, we examined cells of the bronchoalveolar lavage (BAL), focusing on the phenotype of alveolar lymphocytes and macrophages.

Materials and methods

Patients

Twenty-one wheezers (12 boys and nine girls, mean age 15.4 months, range 7–49 months)

were included. The severity and/or frequency of wheezing requiring oral steroid courses (more than once a month) and/or persistent respiratory symptoms between acute episodes and poor response to anti-asthmatic treatment, led to a flexible bronchoscopy to identify airways abnormalities.

Bronchoscopy was performed following an acute exacerbation, and inhaled corticosteroids were stopped at least 2 weeks prior to performing the bronchoscopy. At the time of flexible bronchoscopy, the oxygen trans-cutaneous saturation before examination was >95%. Some infants (11/21) had a personal or familial history of atopy (Table 1). Research analysis was carried out after informed consent was obtained from the parents of the children. Owing to ethical reasons, it was impossible to study healthy children. Therefore, as control subjects, seven children without a past or present history of wheezing and without history of personal or familial atopy, constituted the control group. These children (four boys and three girls; mean age 24.1 months, range 8–36 months) underwent bronchoscopy for investigation of cystic fibrosis (one child), sickle cell disease (one child), suspicion of an inhaled foreign body (one child), and recurrent pulmonary infections (four children). Only patients without anatomic or dynamic abnormality (especially tracheo-bronchomalacia) were included in the study.

Bronchoscopy

Bronchoscopy was performed according to a method previously described in detail (3,4,9). Briefly, children were premedicated with atropine and sedated with midazolam, then upper and lower airway anesthesia was obtained with 2% and 0.5% lidocaine, respectively. The bronchofiberscope Olympus BF3C30 (Olympus Winter IBE GMBH, Hamburg, Germany) was wedged into a sub-segmental bronchus of the right lower lobe, and prewarmed sterile saline, 4 ml/kg body weight, was instilled in 10-ml aliquots and withdrawn by gentle aspiration. The first aliquot was retained for viral analysis (respiratory syncytial virus [RSV], parainfluenza [PI], influenza, adenovirus, cytomegalovirus), which was carried out using immunofluorescence techniques, and the remaining recovered fluid was immediately filtered through two layers of sterile gauze, collected into sterile polypropylene vials (Falcon 50 ml; Becton-Dickinson, 38800 Le Pont de Claix, France) and kept on ice until centrifugation.

Analysis of BAL

The subsequent aliquots of BAL lavage, prepared as described above, were pooled, then BAL enumeration was performed and a differential cell count was conducted, on 200 cells, on cytocentrifuged slides (Cytospin; Shandon Ltd, Cheshire, UK) stained with May-Grünwald Giemsa. The phenotype of alveolar lymphocytes and macrophages was assessed using cytofluorometric methods.

Analysis of cell-surface markers

Alveolar cells were washed and concentrated to 10⁶ cells/ml in phosphate-buffered saline (PBS) by two consecutive centrifugations (300 g for 10 min at 4°C). Cell samples (100 µl) were incubated for 15 min at 4°C with 10 µl of the following conjugates: CD45-PerCP (clone 2D1) together with CD2-phycoerythrin (PE) (clone S5.2), CD3-PE (clone SK7), CD4-PE (clone SK3), CD7-fluorescein isothiocyanate (FITC) (clone M-T701), CD8-PE (clone SK1) or CD19-PE (clone SJ25C&) (antibodies from Becton-Dickinson) for lymphocyte sub-population analysis. Macrophage surface markers were analyzed after incubation of the cells with 10 µl of CD14-FITC (clone MfP9), alone or with 10 µl of CD54-PE (clone LB-2), CD62L-PE (clone SK11) or HLA-DR-PE (clone L243) (10). The isotype control antibody was obtained from the same source as the specific antibody (immunoglobulin G [IgG]₁ or IgG_{2a}; Becton-Dickinson). Cell-surface marker fixation was performed using 2 ml of lysing solution (Becton-Dickinson). The cells were then washed twice with PBS, re-suspended in 250 µl of PBS, and stored at 4°C until analysis. Cell-surface markers were analyzed on a fluorescence-activated cell sorter (FASCalibur; Becton-Dickinson) using CellQuest software (Becton-Dickinson). Lymphocytes were gated first by forward/side-scatter parameters and then gated according to CD45-PerCP expression and side-scatter parameters. Combination of the two gates gave the lymphocyte population data. Expression of the other lymphocyte surface markers was studied in this population and analyzed on 10⁴ lymphocytes. Results are expressed as percentage of the CD45⁺ population. The macrophage population was gated by forward/side-scatter parameters and then according to CD14-FITC expression and side-scatter parameters. The expression of other macrophage surface markers was studied on this population and analyzed on 10⁴ macrophages. Results are expressed as percentage of

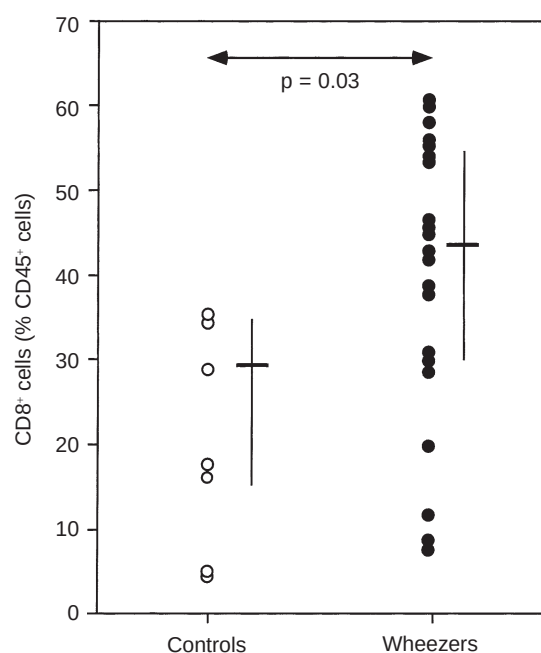


Fig. 1. Expression of CD8 by bronchoalveolar lymphocytes from wheezy vs. non-wheezy infants. $p=0.03$, Mann-Whitney U -test. Median values and 25th and 75th percentiles are shown.

the CD14⁺ population. Moreover, the mean fluorescence intensity of CD62L, CD54, and human leucocyte antigen (HLA)-DR was analyzed on alveolar macrophages.

Statistical analysis

Data are expressed as median values with 25–75th percentiles. The comparison between wheezing and non-wheezing infants was analyzed by using the Mann-Whitney U -test. The significance was set at $p < 0.05$. All analyses were conducted using the statistical package statview 4.5.

Results

No viral infection was detected by immunofluorescence in wheezers or non-wheezing infants at the time of analysis. A statistical difference ($p = 0.03$) in age was observed between the two groups. The difficulty of obtaining BAL cells from healthy subjects, during the same time-period as BAL was obtained from wheezy infants, explains this difference between our study groups.

Fluid recovery averaged 65% (range 40–80%) and did not differ statistically between groups. The number of cells recovered by BAL was similar in the two populations (Table 2). No statistically significant differences in the differential cell count in BAL cells were detected

between wheezy and non-wheezy patients (Table 2). However, a statistically significant increase ($p = 0.03$) in the CD8⁺ lymphocyte population was observed in wheezers (mean 43.1%, range 30.1–54.9%) as compared to non-wheezers (mean 29.3%, range 13.5–34.7%) (Table 3, Fig. 1). No statistically significant differences in the expression of the other lymphocyte cell-surface markers were found. Moreover, the CD4/CD8 ratio was similar in both groups (Table 3). The expression of CD54, CD62L, and HLA-DR surface markers on CD14⁺ alveolar macrophages was identical in the two groups of subjects (Table 4). However, a significant ($p = 0.04$) decrease in the mean fluorescence intensity (MFI) expression of HLA-DR was detected in the macrophage population of the wheezers (median 7,016, range 2,135–7,986 MFI arbitrary units), as compared to non-wheezing subjects (median 8,369, range 6,478–8,860 MFI arbitrary units). No relationship was detected between any of the parameters analyzed (BAL recovery, number of cells, cell-surface markers) and the age of the subject, in relation to either familial or personal atopy.

Discussion

Previous publications (6,11) have reported data on cell populations in BAL obtained from healthy children or control infants. The average age of children in our non-wheezy group was younger than in those of the two other reports, 24 months vs. 31 or 97.5 months, respectively. If we analyze the fluid recovery and differential cell count of BAL cells, similar values are reported in the 'healthy' groups of these three reports. However, like Marguet et al. (6) we did not observe any relationship between cytology findings and age, as was reported for lymphocytes in their earlier publication (11). Despite the lack of information about a healthy control group of the same age as our wheezy group, we made comparisons between our two groups.

The results of the present report show that the major difference between wheezy and non-wheezy infants is the presence of increased numbers of CD8⁺ T cells in the group of wheezers. The other lymphocyte surface markers were similar to values previously reported (6,7,11). This excludes the expression of CD3 in our two groups, which is lower than the value reported by Reidler et al. (11). The broad age-range of their group (from 3 months to 10 years) could be the reason for such a difference. However, the absence of details on

Table 1. Clinical data of the children

No.	Gender	Age (months)	Familial and/or personal atopy
Wheezers			
1	F	22	+
2	M	9	–
3	F	49	–
4	M	18	+
5	F	8	+
6	M	10	–
7	M	39	–
8	M	7	+
9	F	19	–
10	F	13	+
11	M	10	–
12	F	9	+
13	M	26	–
14	M	7	–
15	F	10	+
16	M	7	+
17	M	7	+
18	F	11	+
19	F	10	–
20	M	15	–
21	M	17	+
Non-wheezers			
1	M	36	–
2	M	20	–
3	F	8	–
4	F	36	–
5	M	26	–
6	F	23	–
7	M	20	–

the age repartition restrains any further analysis.

The percentage of B lymphocytes reported in the literature was similar to our data.

The percentage of alveolar macrophages that expressed CD54, CD62L, and HLA-DR was identical in our two groups of infants. Nevertheless, a decrease in the MFI of HLA-DR in the wheezer population was observed. This measure represents the density of HLA-DR molecules on the alveolar macrophage surface. Usually after a cellular activation, an increased expression of HLA-DR might be expected (13). It would seem that in specific infectious situations (14), expression of HLA-

DR on monocytes could be decreased for a few months. The lack of information concerning previous viral status does not allow any significant conclusion to be made in our group. Grigg et al. (5) showed that alveolar macrophages from very young children without respiratory pathology express less HLA-DR than older children. This was considered to be attributable to the immaturity of alveolar macrophage function in early childhood. However, this result was obtained by comparison of HLA-DR expression obtained from six of 11 infants younger than 2 years of age to five of 18 children older than 2 years of age (range 24–207 months). The absence of clear information on the age of the subjects prevents further comparison with our data.

The main finding of our study was an increased number of CD8⁺ cells in the BAL of wheezy infants. Some authors have investigated BAL lymphocyte subset populations (6,7,12). In wheezy infants, Marguet et al. (12) found a lower proportion of CD8⁺ cells and a higher CD4/CD8 ratio in wheezy infants than in children with asthma. The median CD4/CD8 ratio obtained in healthy children was 0.6, as previously reported (9) and as observed in the control group of this study. If the CD4/CD8 ratio decreased in the wheezy patients of the present study, the difference did not reach statistical significance. We observed only an increased number of CD8⁺ cells.

The T-cell hypothesis of chronic asthma is based on the concept that the disease is driven and maintained by the persistence of a specialized subset of chronically activated T memory cells. These are sensitized against an array of allergenic, occupational or viral antigens, which home to the lung after appropriate antigen exposure or viral infection (15). Allergens induce a CD4⁺ T helper (Th) cell response, whereas viruses recognize CD8⁺ T cytotoxic (Tc) cells. CD8⁺ T cells can differentiate into two effector phenotypes – Tc1 and Tc2 – which secrete cytokine patterns similar to those of the Th1 and Th2 phenotypes (16,17). CD8⁺ T cells

Table 2. Bronchoalveolar lavage (BAL) cellularity

	Wheezy	Non-wheezy	p-value
Total cells/10 ³ /ml	345 (227–472)	380 (275–410)	NS
Epithelial cells (%)	5.5 (2.7–8.6)	5.1 (2.8–8.2)	NS
Lymphocytes	8 (2–10)	5 (2–6)	NS
Macrophages	86 (75–92)	87 (81–89)	NS
Neutrophils	7 (3–14)	5 (3–9)	NS
Eosinophils	0 (0–0)	0 (0–0)	NS

Data are expressed as median (25th and 75th percentiles). Statistical analysis was performed by using the Mann–Whitney *U*-test.

Table 3. Expression of alveolar lymphocyte CD45⁺ membrane markers in wheezy and non-wheezy infants

	Wheezy	Non-wheezy	p-value
CD2	74.7 (62.6–81.8)	50.2 (40.4–60.7)	NS
CD3	25.5 (17.2–40.7)	27.1 (12.1–35.8)	NS
CD4	17.2 (12.9–23.9)	13.3 (8.5–27.3)	NS
CD7	63.2 (50.2–73.8)	54.8 (26.5–64.8)	NS
CD8	43.1 (30.1–54.9)	29.3 (13.5–34.7)	0.03
CD19	3.4 (1.6–6.7)	4.5 (1.9–26.6)	NS
CD4/CD8	0.43 (0.29–0.57)	0.68 (0.48–0.87)	NS

Data are expressed as median (25th and 75th percentiles). Statistical analysis was performed by using the Mann–Whitney *U*-test.

have the ability to lyse virus-infected target cells and to produce high levels of interferon- γ (IFN- γ) (18). More recently, it has become clear that CD8⁺ T cells can produce Th2 cytokines, contributing to their effector function (16). Both subsets are cytotoxic via the perforin and Fas pathways. Both subsets induce inflammation with similar cellular infiltrates. However, the analysis of cells obtained by bronchial lavage of infants with acute RSV infection showed low numbers of CD8⁺ cells detected, their proportion being consistently lower than CD4⁺ cells (19).

The reasons explaining the differences in the number of CD8⁺ T cells between wheezing and non-wheezing infants are probably related to viral infection. It has been shown (20) using a mouse model of RSV-induced lung eosinophilia that lung CD8⁺ cells are recruited during respiratory syncytial infection. The infants in the present study were investigated some time after experiencing any acute episode but it is probable that their wheezing episodes were the result of viral infections, particularly of RSV infection (21). CD8⁺ T cells play an important role in the host defense responses against viral infection (22). Therefore, the presence of an increased number of CD8⁺ T cells may represent the consequence of recent or repeated viral infection(s) and support the concept of excessive lymphocyte-mediated cytotoxic activity after a viral infection. On the other hand, allergen triggers are probably not involved, but virus may enhance aeroallergen sensitization (23). Although it has been found that selective CD8⁺ T cells accumulate in the lungs of patients with allergic asthma after allergen broncho-provoca-

tion (24), the results of this study do not agree with previous data on BAL and biopsies in adults, which did not find an increased number of CD8⁺ cells (25–28).

The consequences of an increased number of CD8⁺ cells in wheezing infants are not completely understood. Although CD8⁺ T cells have been regarded as mainly having cytotoxic properties against viruses and other cells, it appears that they can regulate immunoglobulin E (IgE) synthesis (29). It has been postulated that viral infections may protect against asthma and allergies during childhood (26), and this may be related to the potent inhibition of antigen-specific CD8⁺ T cells to inhibit IgE responses (30). On the other hand, it has been shown that CD8⁺ T cells activated by viruses can switch to a phenotype (Tc2-type CD8⁺ cells) that produces Th2 cytokines (and interleukin-5 [IL-5] production), secretes less IFN- γ , and induces airway eosinophilia (31). We did not test this hypothesis in the present study, by intracellular detection of cytokine, owing to the small number of cells available in our samples. These Tc2-type CD8⁺ T cells have been found in BAL (32) and bronchial biopsies from asthmatics (33). These results may explain the link between viral infections and bronchial asthma, as this switch to Tc2-type CD8⁺ T cells and IL-5 secretion may not only exacerbate asthma by recruiting eosinophils into the lungs, but may also delay viral clearance from the host.

In conclusion, we have shown that in ‘normal’ control subjects, the fluid recovery, differential cell type, and lymphocyte and macrophage subsets reported in the literature are similar in children in the age-range of 7–49 months. Such

Table 4. Expression of alveolar macrophage CD14⁺ membrane markers from wheezy and non-wheezy infants

	Wheezy	Non-wheezy	p-value
CD54	63.7 (36.6–94.5)	58.2 (15.3–95.2)	NS
CD62L	10.6 (2.0–52.3)	62.0 (12.6–92.5)	NS
HLA-DR	98.4 (96.4–99.5)	98.6 (92.1–99.7)	NS

Data are expressed as median (25th and 75th percentiles). Statistical analysis was performed by using the Mann–Whitney *U*-test.

an observation justified the analysis performed in the present study, comparing our control group with the wheezy patient group, and particularly because the age-range was similar in our two groups, 7–49 months in wheezy patients and 8–36 months in the control, non-wheezy group. Detection of an increased number of CD8 lymphocytes in wheezy patients is in agreement with the data previously published in an animal experimental model of viral infection. However, the absence of information about the exact date of infection of those infants leads only to speculation regarding an association between an increased number of CD8⁺ lymphocytes and the potential role of viral infection. Sequential BAL cell analysis could reveal more detailed pathophysiological information on the relationship between the modulation of this cell type and the date of viral infection. However, for ethical reasons, such a study must be performed on animal models and extrapolated to human disease.

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