Allergology International (2000) **49**: 125–133

Original Article

Flow cytometric analysis of lymphocytes and lymphocyte subpopulations in induced sputum from patients with asthma

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Abstract

Study objectives were to compare the numbers of lymphocytes and lymphocyte subpopulations in induced sputum from asthmatic patients and from healthy subjects, and to determine the effect of inhaled anti-asthmatic steroid therapy on these cell numbers. Hypertonic saline inhalation was used to non-invasively induce sputum samples in 34 patients with bronchial asthma and 21 healthy subjects. The sputum samples were reduced with dithioerythritol and absolute numbers of lymphocytes and lymphocyte subpopulations were assessed by direct immunofluorescence and flow cytometry. To assess the effect of beclomethasone dipropionate (BDP) on induced sputum, numbers of lymphocytes and lymphocyte subpopulations in sputum also were evaluated after 4 weeks of BDP inhalation treatment in seven asthmatic patients. An adequate sample was obtained in 85.3% of patients with asthma and in 79.2% of the healthy subjects. Induced sputum from patients with asthma had increased numbers of lymphocytes ($P = 0.009$); $CD4^+$ cells ($P = 0.044$); $CD4^+$ cells-bearing interleukin-2 receptor (CD25; $P = 0.016$); and $CD4^+$ cells bearing human histocompatibility leukocyte antigen (HLA)-DR ($P = 0.033$). $CD8^+$ cells were not increased in asthmatic patients. In patients treated with inhaled steroids, numbers of lymphocytes, $CD4^+$ cells, CD25-bearing $CD4^+$ cells and HLA-DR-bearing $CD4^+$ cells in sputum decreased from pretreatment numbers ($P = 0.016$, 0.002, 0.003 and 0.002, respectively). Analysis of lymphocytes in induced sputum by flow cytometry is useful in assessing bronchial inflammation, and activated $CD4^+$ lymphocytes may play a key role in the pathogenesis of airway inflammation in bronchial asthma.

Key words: bronchial asthma, CD4-positive lymphocyte, flow cytometry, induced sputum, interleukin-2 receptor.

Introduction

Asthma now is recognized as an airway inflammatory disorder. Knowledge of cellular mechanisms in the airways of asthmatic patients has been increased by study of specimens from bronchoalveolar lavage (BAL) and mucosal biopsy.[1–5] Eosinophils contribute to bronchial inflammation by releasing granule-derived proteins and inflammatory mediators.[6–9] In addition to eosinophils, T cells may play a key role in the pathogenesis of asthma,[10–12] by producing several cytokines that modulate IgE production by B cells or induce migration and activation of eosinophils.[13,14]

Performing fiber-optic bronchoscopy in patients with asthma to obtain lavage or biopsy specimens sometimes causes severe bronchoconstriction; in one study, 20% of subjects experienced a fall in forced expiratory volume in 1 s (FEV_1) of more than 40% after fiber-optic bronchoscopy.[15,16] Examination of sputum samples is a non-invasive alternative method for studying mechanisms in asthma. Eosinophils and metachromatic cells have been

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Received 9 November 1999. Accepted for publication 21 February 2000.

reported to be present in sputum from asthmatic patients.[17] Pin and coworkers have described a sputum induction method using hypertonic saline for studying airway inflammation in these patients.[18] Other authors have also found sputum induction with hypertonic saline to be useful and relatively safe in patients with asthma.[19–21] Kidney *et al*. have used flow cytometry to examine lymphocytes in sputum and have confirmed the validity and reproducibility of lymphocyte measurements obtained by this method.[22]

We hypothesized that induced sputum from asthmatic patients would contain increased numbers of $CD4^+$ lymphocytes and activated $CD4^+$ lymphocytes, and also that inhaled steroid therapy would decrease the number of such cells in induced sputum. Flow cytometry was used to study overall numbers of lymphocytes and cell numbers in specific lymphocyte subpopulations in induced sputum from asthmatic and healthy subjects. We also studied numbers of lymphocytes and specific lymphocyte subtypes in sputum before and 4 weeks after initiation of inhaled steroid therapy to investigate the effect of the treatment on airway inflammation.

METHODS

Subjects

Subjects recruited for the present study were non-smokers between 20 and 75 years old. Patients seen at Kure Kyosai Hospital between March 1995 and September 1996 who met the criteria for diagnosis of bronchial asthma established by the National Heart, Lung and Blood Institute of the US National Institutes of Health[23] were evaluated. Thirty-four patients (16 women and 18 men, mean age 50.9 ± 13.4 years) were accepted as candidates and underwent sputum induction by hypertonic saline. Clinical and demographic features of subjects with asthma are summarized in Table 1. Patients with asthma were atopic as defined by the presence of serum IgE specific to common aeroallergens (radioallergosorbent test; Pharmacia Diagnostics, Uppsala, Sweden). Of the 34 patients with asthma, 28 were receiving treatment with an inhaled β_2-agonist, 30 with a sustained-release theophylline preparation (daily dose 300–600 mg), eight with inhaled beclomethasone dipropionate (BDP; daily dose 200–800 μg), 11 with prednisone (daily dose, 2.5–40 mg), two with pranlukast hydrate (daily dose 450 mg), one with ketotifen fumarate (daily dose 2 mg), one with ozagrel hydrochloride (daily dose, 400 mg) and one with pemirolast potassium (daily dose, 20 mg). Normal control subjects (nine women and 12 men, mean age 46.2 ± 14.7) were healthy volunteers with no history of allergy or asthma. To assess the effect of BDP on induced sputum, seven of the patients with asthma (four women and three men, mean age 64.4 ± 6.4 years; cases 3, 5 and 25–29 in Table 1) were assessed both before and 4 weeks after BDP inhalation treatment. For at least 3 months preceding the first assessment of induced sputum, none of these seven patients had received any treatment with BDP. After the first assessment, the subjects underwent inhalation treatment with BDP (800 μg/day for 4 weeks), followed by a second assessment. Informed consent was obtained from all subjects. The study was approved by the ethics committee of Kure Kyosai Hospital.

Induction of sputum

Induction of sputum was carried out in the emergency room of Kure Kyosai Hospital with close monitoring by a physician investigator. Peak expiratory flow (PEF) was measured using an Assess peak flow meter (Healthscan Products, Ceder Grove, NJ, USA) before inhalation of hypertonic saline. Patients complaining of wheezing or dyspnea were treated with 30 mg of procaterol hydrochloride by inhalation prior to any saline exposure. Patients with wheezing or dyspnea even after procaterol hydrochloride treatment did not proceed to further steps. Subjects were instructed to rinse their mouths and throats with fresh water just before beginning inhalation of nebulized saline and every 15 min thereafter. Subjects inhaled 3% saline for 60 min via a nebulizer (NE-C11, Omron, Tokyo, Japan) between 9.00 and 10.00 hours. In a preliminary study, we could not obtain a sufficient amount of sputum in some cases using less than 30 min of induction. Inhalation of hypertonic saline for 60 min, in contrast, yielded appropriate sputum samples in most cases. We chose 60 min as the duration of sputum induction. The nebulizer used generates particles with a mean mass median diameter of 10 μm, amounting to an output of 0.3 mL/min. When patients complained of dyspnea or if the physician investigator noted wheezing during inhalation of hypertonic saline, the sputum induction procedure was stopped immediately and subjects then were treated with procaterol hydrochloride. Subjects expectorated saliva and rinsed their mouths just before coughing up sputa. Sputa were collected in a container and kept on ice.

Table 1 Characteristics of patients with asthma

Case no.	Gender	Age	IgE (RAST score) HD	IgE (RAST score) DF	Type	%PEF	Severity	Age at onset (years)	Duration of symptoms	Medications
1	F	49	2	2	A	98.7	Severe	48	12 mo	B,T,I,P
2	F	40	< 1.0	< 1.0	NA	61.4	Moderate	30	10 yr	B,T
3	F	55	< 1.0	< 1.0	NA	26.0	Severe	54	1 yr	B
4	M	50	2	< 1.0	A	83.7	Intermittent	50	1 mo	B,T,P
5	F	68	4	4	A	73.5	Moderate	64	4 yr	B,T
6	F	61	< 1.0	< 1.0	NA	70.5	Severe	55	6 yr	B,T,I,P
7	M	26	ND	ND	ND	ND	Moderate	10	16 yr	B
8	M	28	ND	ND	ND	83.9	Mild	7	16 yr	B,T,K
9	M	52	4	4	A	78.8	Moderate	49	3 yr	B,T,I
10	F	54	ND	ND	ND	88.7	Mild	33	21 yr	T,I
11	F	60	2	2	A	39.6	Severe	32	28 yr	B,T,P
12	F	37	6	6	A	29.0	Severe	21	16 yr	B,T
13	F	46	< 1.0	< 1.0	NA	25.6	Severe	37	9 yr	B,T,I,P
14	M	68	< 1.0	< 1.0	NA	87.9	Severe	15	5 yr	B,T,P
15	M	37	ND	ND	ND	117.8	Severe	20	18 yr	B,T,P
16	M	46	< 1.0	< 1.0	NA	102.2	Mild	45	13 mo	T,I
17	F	53	3	4	A	88.9	Intermittent	52	13 mo	T
18	M	61	4	4	A	38.6	Severe	34	27 yr	B,T,P,Pe
19	F	48	< 1.0	< 1.0	NA	28.6	Severe	27	21 yr	B,T,I,Pr
20	F	42	ND	ND	ND	98.5	Intermittent	42	3 mo	B
21	M	64	< 1.0	< 1.0	NA	81.2	Mild	62	18 mo	B,T
22	M	45	< 1.0	< 1.0	NA	69.8	Severe	39	6 yr	B,T,P
23	F	63	< 1.0	< 1.0	NA	100.3	Mild	63	3 mo	B,T,Pr
24	F	55	< 1.0	< 1.0	NA	84.0	Mild	52	3 yr	T,O
25	F	64	< 1.0	< 1.0	NA	71.1	Moderate	59	5 yr	T
26	M	69	4	4	A	69.0	Severe	28	41 yr	B,T,P
27	F	69	< 1.0	< 1.0	NA	100.3	Mild	64	5 yr	B,T
28	M	70	< 1.0	< 1.0	NA	43.1	Severe	42	28 yr	B,T
29	M	56	5	5	A	74.2	Moderate	11	46 yr	B,T
30	M	40	3	4	A	114.2	Intermittent	38	2 yr	B
31	M	24	5	5	A	96.5	Mild	24	3 mo	B,T
32	M	36	5	5	A	63.9	Moderate	33	3 yr	B,T,I
33	M	63	5	4	A	87.3	Mild	56	7 yr	T
34	M	29	5	5	A	133.6	Intermittent	27	2 yr	B,T,P

HD, house dust; DF, *Dermatophagoides farinae*; A, atopic; NA, non-atopic; ND, not determined; PEF, peak expiratory flow; yr, years; mo, months; B, inhaled beta-agonist; T, theophylline; I, inhaled beclomethasone dipropionate; P, prednisone; K, ketotifen fumarate; O, ozagrel hydrochloride; Pe, pemirolast potassium; Pr, pranlukast hydrate.

Processing of sputum cells

Sputum was diluted approximately 40-fold with phosphate-buffered saline (PBS) containing 0.01 mol/L dithioerythritol (Sigma Chemical Co., St Louis, MO, USA) and 0.1% sodium azide. Diluted sputa were shaken for 2 h on ice and then passed through a cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ, USA) to remove unsolubulized sputum components and debris. Sputum cells were centrifuged at 900 *g* for 20 min at 4°C. Cells were washed twice with PBS and resuspended in 1 mL of PBS containing 2.5% bovine serum albumin (Sigma Chemical Co.) and 0.1% sodium azide. During the course of processing, cells were kept at 4°C.

Measurements

Primary outcome measurements

Specific binding of monoclonal antibodies was analyzed by direct immunofluorescence using a two-color combination of fluorescein isothiocyanate (FITC) and phycoerythrin (PE). Negative control samples of cells incubated with isotype-matched irrelevant FITC- and PE-labeled monoclonal

antibodies were included in each run. Briefly, 100 μL aliquots of sputum cell suspension were incubated with 10 μL of relevant FITC- or PE-labeled antibody for 30 min in the dark on ice. The following two-color combinations (FITC/PE) were used: CD8/CD4, CD25/CD4, human histocompatibility leukocyte antigen (HLA)-DR/CD4 and CD45/CD14 (Ortho Diagnostic Systems, Raritan, NJ, USA). After cells were washed once, two-color immunofluorescence analysis was performed with a Cytoron Absolute flow cytometer (Ortho Diagnostic Systems). An appropriate gate was drawn around the lymphocyte population, as defined by forward scatter and side scatter characteristics in the peripheral blood.

CD45, also known as the leukocyte common antigen, is a surface glycoprotein expressed on cells of all hematopoietic lineages except for mature erythrocytes. CD14 is expressed on monocytes, macrophages and activated granulocytes. Cell populations that were CD45 positive and CD14 negative were defined as lymphocytes. Amounts of contaminating monocytes, macrophages and debris in sputum were estimated by this combination of CD14 and CD45. In most samples, we found sufficient numbers of lymphocytes for study and small numbers of monocytes or macrophages. For samples in which the amount of contamination within the lymphocyte-related gate interfered with analysis of lymphocyte subpopulations, all data were discarded. Absolute lymphocyte count was performed using the Ortho-Count Calibration Kit (Ortho Diagnostic Systems). The cytometer used delivers precise volumetric aliquots of samples for analysis. The volume delivered can be calibrated so that accurate and precise absolute cell subset determinations can be made. Briefly, four microparticle suspensions were used to calibrate and verify the counting functions of the cytometer. The constant flow rate was calibrated from the known number of particles per unit volume; then the counts in the other three suspensions were determined to verify calibration and counting linearity.[24] We used this method to study the absolute number of lymphocytes, $CD4^+$ cells, CD25-bearing $CD4^+$ cells, HLA-DR-bearing $CD4^+$ cells and $CD8^+$ cells. Positive cells were calculated excluding non-specific staining and assessed using isotype control antibodies directed against an irrelevant antigen.

Secondary outcome measurements

Differential cell counts were performed on cytocentrifuge preparations using cell suspension and centrifugation at 300 *g* for 10 min. After fixation in 99.5% ethanol solution for 5 min, sputum samples were air dried and stained with May-Giemsa. More than 400 cells were counted by scanning several fields of view. Samples containing more than 10% squamous cells were considered inadequate and discarded.

Statistical analysis

All data are presented as the mean ± SD. Student's *t*-test was used to assess the differences between healthy and asthmatic subjects. Cell counts before and after treatment with BDP inhalation were compared using a paired *t*-test; *P* values less than 0.05 were considered statistically significant.

RESULTS

Safety and success of the method

In two patients with asthma, inhalation of saline caused slight wheezing that subsided quickly after inhalation of a bronchodilator. Of the 34 patients with asthma, adequate samples were obtained from 29 patients (16 women and 13 men, mean age 53.1 ± 12.1 years) corresponding to cases 1–29 in Table 1. Samples were inadequate in the five other patients with asthma (14.7%). Of the 21 healthy subjects, adequate samples were obtained from 16 (seven women and nine men, mean age 46.3 ± 15.8 years), while five (23.8%) had inadequate samples.

Comparison of lymphocytes and lymphocyte subpopulations between patients with asthma and healthy subjects

Direct immunofluorescence and flow cytometry were used to quantitate lymphocytes and lymphocyte subpopulations in sputum samples. As shown in Table 2, absolute numbers of lymphocytes, $CD4^+$ cells, CD25-bearing $CD4^+$ cells and HLA-DR-bearing $CD4^+$ cells were significantly higher in induced sputum from patients with asthma than from healthy subjects. However, the number of $CD8^+$ cells in induced sputum from patients with asthma was not significantly higher than in healthy subjects.

Changes in lymphocyte profile in induced sputum after treatment with inhaled BDP

Four weeks after starting BDP inhalation, significant decreases were noted for several cell types: the number

Table 2 Cells in induced sputum from healthy subjects and patients with asthma

Patient no.	Squamous cell contamination (%)	Differential cell count				Cell count				
		Lym. (%)	Mac. (%)	Eos. (%)	Neu. (%)	Lym.* ($\times 10^4$)	CD4† ($\times 10^4$)	CD25/CD4‡ ($\times 10^4$)	HLA-DR/CD4§ ($\times 10^4$)	CD8 ($\times 10^4$)
Healthy subjects										
1	0.8	3.3	44.4	0.0	52.3	1.39	1.08	0.18	0.53	0.23
2	0.9	1.6	25.2	3.5	69.7	2.72	1.65	0.17	0.74	0.79
3	0.7	3.1	38.3	3.6	55.0	5.49	3.75	0.27	2.05	0.94
4	1.5	0.6	62.1	2.8	34.5	0.23	0.18	0.05	0.13	0.04
5	8.6	1.4	60.2	2.1	36.3	1.18	0.23	0.10	0.16	0.18
6	1.7	1.3	38.8	0.7	59.2	5.21	2.15	0.62	0.72	0.61
7	3.0	0.8	30.6	4.4	64.2	2.27	1.12	0.16	0.59	0.19
8	2.1	3.0	73.7	0.0	23.3	2.43	1.29	0.40	0.91	1.14
9	4.2	1.6	39.6	1.5	57.3	1.28	1.12	0.45	0.61	0.12
10	2.3	1.7	34.3	0.4	63.6	6.75	2.60	0.74	0.86	2.77
11	0.2	1.9	31.8	3.7	62.6	3.00	2.29	0.56	1.57	0.68
12	0.4	2.1	40.8	1.8	55.2	6.05	4.84	0.97	2.65	0.91
13	4.5	1.9	61.0	2.1	35.0	1.94	1.24	0.41	1.02	0.16
14	0.8	2.3	59.1	0.7	37.9	14.05	5.15	1.03	2.25	1.58
15	4.3	2.5	64.3	1.0	32.2	3.55	1.70	0.10	0.80	0.15
16	9.0	0.6	19.3	0.0	80.1	0.23	0.13	0.01	0.05	0.03
Mean	2.8	1.9	45.2	1.8	51.2	3.61	1.91	0.39	0.98	0.66
SD	2.7	0.8	16.0	1.5	16.0	3.44	1.53	0.32	0.77	0.73
Patients with asthma										
1	1.0	2.9	5.3	86.0	5.8	15.49	5.69	0.34	3.19	4.19
2	1.0	0.9	3.7	65.5	30.0	1.08	0.62	ND	0.45	0.14
3	4.7	1.8	16.3	49.4	32.6	10.55	2.84	0.97	1.76	2.03
4	0.6	2.5	50.1	28.1	19.3	5.35	3.16	1.07	2.14	0.40
5	0.6	1.3	29.6	64.7	4.3	9.28	2.32	0.52	1.31	0.53
6	0.5	2.8	12.9	15.8	68.6	7.56	7.17	2.38	6.47	0.23
7	4.3	2.5	17.6	36.6	43.3	9.73	2.67	0.81	2.04	0.18
8	2.9	1.5	62.6	10.5	25.4	2.55	1.60	0.34	1.08	0.43
9	2.0	1.4	32.0	17.5	49.1	3.60	1.53	0.41	0.99	0.29
10	0.5	1.4	33.6	58.9	6.1	1.74	1.47	0.44	1.18	0.19
11	0.5	1.2	39.3	24.9	34.6	4.38	1.70	0.24	1.15	0.60
12	0.2	2.2	2.6	54.6	40.5	34.98	5.20	0.81	3.01	3.91
13	0.6	1.5	35.4	13.7	49.4	7.35	1.02	0.33	0.65	0.64
14	0.7	1.5	7.4	6.8	84.3	9.35	2.03	0.62	0.98	5.79
15	1.1	2.4	49.5	2.8	45.2	46.32	11.68	0.97	4.60	8.06
16	0.7	3.0	64.8	9.5	22.6	37.50	19.26	4.64	9.57	5.33
17	0.9	4.0	15.8	57.6	22.5	13.40	6.67	2.68	2.06	1.13
18	1.2	1.0	10.0	41.9	47.0	10.40	6.19	1.91	3.92	1.30
19	0.2	1.7	14.0	49.2	35.1	11.24	3.35	1.12	2.46	1.48
20	1.5	5.0	82.1	8.2	4.7	12.10	5.32	1.06	2.13	1.97
21	0.1	5.1	23.3	55.1	16.5	52.53	27.91	5.81	18.93	13.15
22	0.1	2.3	6.0	26.2	65.5	61.41	25.20	5.27	11.69	14.71
23	5.8	1.2	11.8	53.2	33.8	5.27	1.75	0.55	1.07	0.55
24	1.8	2.8	8.4	54.2	34.6	15.82	2.47	0.92	1.29	0.35
25	4.4	2.1	66.5	17.3	14.1	2.98	1.43	0.44	0.91	0.48
26	3.7	2.1	12.5	52.9	32.5	3.60	1.39	0.32	0.82	0.29
27	8.8	0.6	13.8	19.3	66.3	3.36	1.42	0.80	1.07	1.31
28	5.6	3.9	10.2	72.6	13.2	21.61	4.37	0.83	3.67	2.02
29	2.4	2.6	12.9	20.8	63.7	8.29	4.48	1.07	2.58	0.39
Mean	2.0	2.2	25.9	37.0	34.8	14.79	5.58	1.34	3.21	2.49
SD	2.2	1.1	21.9	21.9	21.2	16.01	6.95	1.50	3.99	3.74

Lym., lymphocytes; Mac., macrophages; Eos., eosinophils; Neu., neutrophils. Cell population designations including slashes (/) indicate positivity for both markers. *$P = 0.009$, †$P = 0.044$, ‡$P = 0.016$, §$P = 0.033$.

Table 3 Peak expiratory flow and cells in induced sputum before and after treatment with BDP

Subject no.	Total cells ($\times 10^6$)		PEF (%)		Differential cell count (%)								Cell count ($\times 10^4$)							
					Lym.		Mac.		Eos.		Neu.		Lym.		CD4		CD25/CD4		HLA-DR/CD4		CD8	
	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
1	7.14	1.24	73.5	90.9	1.3	0.7	29.6	77.0	64.7	16.2	4.3	6.1	9.3	0.9	2.32	0.24	0.52	0.03	1.29	0.11	0.53	0.07
2	5.86	1.12	26.0	76.6	1.8	3.3	16.3	30.2	49.4	54.2	32.6	12.3	10.5	3.7	2.84	0.99	0.97	0.34	1.76	0.62	2.03	0.71
3	1.42	1.23	71.1	86.3	2.1	1.2	66.5	54.2	17.3	13.2	14.1	31.5	3.0	1.5	1.38	0.76	0.43	0.20	0.88	0.49	0.48	0.32
4	2.57	1.90	69.0	59.8	1.4	1.3	12.6	27.8	53.3	15.7	32.7	55.2	3.6	2.5	1.39	0.78	0.32	0.24	0.82	0.55	0.29	0.20
5	5.54	5.04	43.1	84.1	3.9	2.3	10.2	28.1	72.6	29.2	13.2	40.3	21.6	11.6	4.37	3.43	0.83	0.46	3.67	2.72	2.02	2.57
6	5.60	4.01	100.3	97.7	0.6	0.5	13.8	27.4	19.3	7.1	66.3	65.0	3.4	2.0	1.42	0.66	0.80	0.08	1.07	0.44	1.31	0.51
7	3.19	2.52	74.2	80.1	2.6	2.0	12.9	23.6	20.8	12.1	63.7	62.3	8.3	5.0	4.48	3.37	1.07	0.69	2.58	2.13	0.39	0.53
Mean	4.47	2.44	65.3	82.2	2.0	1.6	23.1	38.3	42.5	21.1	32.4	39.0	8.5	3.9	2.60	1.46	0.71	0.29	1.72	1.01	1.01	0.70
SD	2.08	1.54	24.0	12.1	1.1	1.0	20.2	19.9	23.1	16.1	24.6	23.6	6.5	3.7	1.36	1.34	0.28	0.23	1.06	1.00	0.77	0.85
P value	NS		NS		NS		NS		< 0.05		NS		< 0.05		< 0.01		< 0.01		< 0.01		NS	

PEF, peak expiratory flow; BDP, beclomethasone dipropionate; Lym., lymphocytes; Mac., macrophages; Eos., eosinophils; Neu., neutrophils; B, before treatment; A, after treatment. Cell population designations including slashes (/) indicate positivity for both markers.

of lymphocytes decreased from 8.53 ± 6.54 to $3.89 \pm 3.67 \times 10^4$ ($P = 0.016$); $CD4^+$ cells decreased from 2.60 ± 1.36 to $1.49 \pm 1.34 \times 10^4$ ($P = 0.0023$); CD25-bearing $CD4^+$ cells decreased from 0.70 ± 0.28 to $0.29 \pm 0.23 \times 10^4$ ($P = 0.0026$); and HLA-DR-bearing $CD4^+$ cells decreased from 1.72 ± 1.05 to $1.01 \pm 0.99 \times 10^4$ ($P = 0.0022$). No significant changes were noted in total number of cells, 4.47 ± 2.08 versus $2.44 \pm 1.54 \times 10^6$, or $CD8^+$ cells, 1.01 ± 0.77 versus $0.70 \pm 0.85 \times 10^4$ (Table 3).

Discussion

Several types of inflammatory cells, including eosinophils, mast cells, and T lymphocytes, have been implicated in the chronic inflammatory process in the airway in asthma.[12,25] An increased number of activated T cells has been observed, not only in peripheral blood,[11] but also in BAL fluid from patients with asthma.[26] Increased numbers of CD25-positive cells also are found in bronchial biopsy specimens from patients with asthma.[12] Robinson and coworkers have found that asthma is associated with increased lymphocytic expression of mRNA for interleukin (IL)-2, IL-3, IL-4, and IL-5, and for granulocyte–macrophage colony-stimulating factor (GM-CSF).[13] Interleukin-4, IL-5 and GM-CSF support the survival, growth, differentiation and accumulation of eosinophils, which release several mediators and proteins that contribute to mucosal and bronchial hyperresponsiveness.[27]

Inhalation of hypertonic saline aerosol is a well-known stimulus causing bronchoconstriction in patients with asthma.[28,29] In two patients, inhalation of saline using the present method caused slight wheezing that was controlled by the inhalation of a β_2-agonist. Caution and emergency treatment provisions are essential when non-isotonic solutions are inhaled. Wong and Fahy have recommended measurement of peak flow at regular intervals during sputum induction procedures involving hypertonic saline aerosols.[28] In the present study, hypertonic saline inhalation was carried out in our hospital's emergency room to facilitate management of any possible sudden attack of asthma. During sputum induction, symptoms were monitored closely by a physician investigator and the procedure was stopped if the patient complained of wheezing or dyspnea.

Although we do not know the mechanisms by which lymphocytes are recruited into sputum by hypertonic saline inhalation, previous studies have concluded that

this method of induction produces sufficient and appropriate sputum for cellular analysis.[18,19,21,30] One important aspect of obtaining a proper sample is exclusion of saliva. We asked subjects to rinse their mouths thoroughly just before coughing up sputum during the induction. Although we tried to exclude salivary contamination as much as possible, a certain number of squamous cells was always observed in the induced sputum, suggesting the presence of some saliva. More than 99% of the cells in saliva reportedly are squamous cells;[19] because the sputum samples that we studied contained less than 10% squamous cells, we are confident that the amount of saliva was not sufficient to affect lymphocyte numbers.

Recognition of cells in a May–Grünwald-Giemsa stained sputum smear with a light microscope is time-consuming and sometimes difficult.[30] Furthermore, the differential count of lymphocytes in sputum can be as low as 2%.[17,18,21] Such factors impede accurate determination of lymphocyte numbers in sputum by routine light microscopy. The method that we describe here is the first to allow easy and precise determination of absolute numbers of lymphocytes and lymphocyte subpopulations in sputum. Direct immunofluorescence is used for flow cytometry with a count calibration kit. Pin and other researchers have studied differential white cell counts in induced sputum from healthy and asthmatic subjects, finding an increased percentage of eosinophils in samples from asthmatic patients but no significant difference in the percentage of lymphocytes (absolute numbers were not determined).[18,19] We used absolute number to assess lymphocytes in sputum, because a differential count of cells may not accurately depict actual changes of cells in sputum. For example, in a differential count a prominent increase of eosinophils in sputum could mask an absolute increase of sputum lymphocytes, because the eosinophils could misleadingly lower the differential count of lymphocytes. The discrepancy in sputum lymphocyte numbers between previous reports and the present study is as yet unexplained, but it may be related to differences in methods used to assess lymphocyte number or simply to the difficulty of counting cytologically stained sputum lymphocytes by light microscopy. Differences in sputum induction and processing also may have contributed to differences in lymphocyte numbers. In our method, sputum was induced by inhalation of the same concentration of saline (3%) for the same period (60 min) by all subjects, and we analyzed all sputum induced during the induction procedure instead of selecting mucus plugs within the sputum for analysis.

CD25 is a useful marker of acutely activated lymphocytes; its expression is transient and disappears quickly in the absence of continued stimulation.[31] In contrast, HLA-DR antigen is expressed late after antigen stimulation. We found overall numbers of lymphocytes, as well as $CD4^+$ cells and activated $CD4^+$ cells, to be increased in sputa from patients with asthma compared with numbers in sputa from healthy subjects. Our results complement others demonstrating increased numbers of lymphocytes, $CD4^+$ and $CD8^+$ cells in bronchial biopsy specimens from patients with asthma.[12,32] In studies where flow cytometry has been used to analyze BAL cells from patients with asthma, increased expression of CD25 among $CD4^+$ cells, as well as degree of activation of $CD4^+$ lymphocytes, correlated with severity of symptoms and degree of bronchial hyperresponsiveness. Kidney *et al*. have used flow cytometry to examine lymphocyte subpopulations in sputum, finding that sputa from patients with asthma contains an increased proportion of B lymphocytes and activated helper T cells.[22] Although the difference was not statistically significant, the number of $CD8^+$ cells in patients with asthma was higher than in healthy subjects in the present study. Increased numbers and activation of $CD8^+$ cells have been described in BAL fluid from patients with intrinsic asthma,[26] but involvement of $CD8^+$ cells in bronchial asthma remains unclear.

Corticosteroids have several anti-inflammatory actions. In addition to inhibition of cytokine production,[33–36] these agents inhibit expression of CD25 and rapidly reverse induced CD25 expression by T lymphocytes *in vitro*.[37] Inhaled corticosteroids have been shown to reduce bronchial hyperresponsiveness and improve pulmonary function in patients with asthma.[38,39] In the present study, the mean percentage PEF in seven patients with asthma increased from 65.3 ± 24.0 to 82.2 ± 12.1 after 4 weeks of treatment with BDP inhalation, a difference that was not statistically significant. Reductions in T cell numbers and activation status in peripheral blood, bronchial mucosa and BAL fluid after corticosteroid therapy have been reported.[11,40] Significant decreases in numbers of lymphocytes, $CD4^+$ cells and activated $CD4^+$ cells observed in induced sputum following BDP treatment are in agreement with these reports. In our study, although the change was not significant, the total number of cells in sputum was also reduced after 4 weeks of BDP treatment. In this sense, the effect of BDP may not be wholly specific to lymphocytes or lymphocyte subpopulations in sputum.

In summary, we have demonstrated the feasibility of flow cytometry for analysis of lymphocytes and lymphocyte subpopulations in induced sputum samples. We believe that further studies of pathophysiologic changes, such as lymphocytic expression of mRNA for cytokines and production of cytokines by lymphocytes in relation to lymphocyte surface markers, are necessary to understand the role of lymphocytes in the inflammatory process of bronchial asthma.

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