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The Role of CD8+ Th2 Lymphocytes in the Development of Smoking-Related Lung Damage

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There is increasing evidence for the existence of different subsets of CD8+ T lymphocytes in humans, according to their cytokine secretion profile. Resistance or susceptibility to infections and the outcome of some inflammatory processes may depend on the lymphokine profile which predominates. We show one consequence of the switching of a host CD8+ T cell response from the Th1 effector function to the Th2 pattern in relation to the exposure to a common toxicant and its pathogenetic implications. Chronic obstructive bronchitis is a pulmonary disease characterized by airway inflammation with predominance of CD8+ T lymphocytes, mucus hypersecretion, repeated airway infections, and decline in lung function. Though smoking-related, it affects only a portion of smokers. The results of this study, comparing the functional characteristics of CD8+ T cell clones from smokers with the disease, unaffected smokers and healthy individuals, indicate that the smokers who have a predominance of CD8+ T lymphocytes of the Th2 phenotype may be predisposed to develop more severe smoking-induced lung damage, with chronic airway inflammation, repeated infections and persistent airflow obstruction.

For unknown reasons, only a proportion of heavy cigarette smokers develop chronic obstructive bronchitis (COB) (1-3). Smoking lowers the ratio between CD4+ and CD8+ in peripheral blood (PB) and in the lung (4,5), but only the smokers with chronic bronchitis have airway inflammation with a predominance of CD8+ T cells (6,7), particularly those showing airflow limitation. These subjects have repeated bouts of infections with acute exacerbations of the disease. Since cytotoxic CD8+ T lymphocytes represent a major defense against pathogens by production of interferon-γ (IFN-γ) and cytolytic activity (8,9), the CD8+ T cells of these patients may have different functional characteristics (9) that instead favour infections and lung damage.

To test this hypothesis, we analyzed and compared the patterns of lymphokines produced by PB and lung clones of CD8+ T cells from smokers with COB, smokers without COB and healthy controls.

MATERIALS AND METHODS

Subjects. The group of smokers with COB was composed of 18 patients with a smoking history of greater than 10 pack/years. Chronic bronchitis was defined as the presence of cough productive of morning sputum for at least 3 months a year during the two years preceding the study (10). Airflow limitation was taken as forced expiratory volume in 1 second (FEV_1) < 80% of predicted with less than 15% increase after the inhalation of 400 μg of the bronchodilator salbutamol, and as reduced ratio between FEV_1 and the forced vital capacity (FVC) (10). Persistent airflow limitation was confirmed after bronchoscopy by repeating FEV_1 measurements after a formal 2-week course of oral prednisolone. These patients had never suffered from allergic diseases, or any pulmonary disease other than the clinical syndrome diagnosed as COB, or any systemic disease involving the lung. They had been free of acute upper respiratory tract infections in the month preceding the study and none had received glucocorticoids or antibiotics over the same period of time. The control groups of smokers and non-smokers were respectively composed of 16 and 14 subjects. The smokers had a smoking history of greater than 10 pack/years. All these subjects had never suffered from allergic diseases or any pulmonary disease, and they did not have systemic diseases affecting the lung. They had been free of acute upper respiratory tract infections in the month preceding the study and had not taken any drug over the last two weeks. Their FEV_1 was > 80% of predicted. The 3 groups were matched for gender and age, and the 2 groups of smokers were also matched for numbers of cigarette smoked and duration of smoking. All the tested subjects had routine blood analysis, electrocardiography and chest radiography. The patients who showed emphysema were excluded. The study was approved by the appropriate institutional review boards and each subject gave informed consent.

Isolation and of lung and PB CD8+ T lymphocytes. Lung lymphocytes were obtained by bronchoalveolar lavage during fiberoptic

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TABLE 1

Demographic and Clinical Characteristics of the Subjects

	Healthy subjects	Smokers without COB	Smokers with COB
Age, years, median (range):	48 (41-55)	46 (39-57)	52 (43-61)
Gender, M/F:	8/6	9/7	10/8
Smoking history, -Pack/years, median (range):	0	42 (18-84)	47 (15-96)
FEV_1 % predicted, mean ± SD:	102.3 ± 12.9	97.2 ± 9.4	60.6 ± 10.3*
Post-salbutamol FEV_1 % increase, mean ± SD:			3.4 ± 2.2
FEV_1/FVC, mean ± SD:	0.85 ± 0.04	0.79 ± 0.07	0.55 ± 0.09*
FEV_1/FVC, % predicted, mean ± SD:	101.8 ± 6.3	93.7 ± 10.1	68.4 ± 11.2*

Note. One pack/year is one pack of cigarettes smoked per day for one year. * $p < 0.01$ versus healthy subjects and smokers without COB.

bronchoscopy under local anaesthesia, according to our protocol (11), which is based on published international guidelines (12). The tip of the bronchoscope was wedged into a segmental bronchus of the lower lobe of one lung. Sterile isotonic saline solution, warmed to body temperature, was used for bronchoalveolar lavage. Four aliquotes of 50 ml were instilled into a subsegmental bronchus and the bronchoalveolar lavage fluid (BALF) was aspirated through the suction channel of the bronchoscope, collected in chilled suction traps, pooled, and held at 4°C. BALF cells were washed, resuspended in RPMI 1640 medium (GIBCO Laboratories, Paisley, Scotland) and antibiotics and centrifuged on a Ficoll/Hypaque density gradient. T lymphocytes were successively enriched from the mononuclear cells by rosetting with neuraminidase-treated sheep red blood cells followed by repeated density-gradient separations on Ficoll/Hypaque, as described (13). More than 95% of the cells isolated by this procedure were T lymphocytes as determined by immunospecific labeling with a monoclonal antibody to the CD3 antigen (Ortho Diagnostics, Raritan, Florida). CD8+ T lymphocytes were then enriched by negative selection with the use of an anti-CD4 monoclonal antibody (Ortho Diagnostics) and magnetic beads attached to an anti-mouse IgG (Advanced Magnetics, Boston, Massachusetts) (11). 10^6 cells were incubated with 1 μg of anti-CD4 for 30 minutes at 4°C, washed and then reincubated with the IgG-coupled magnetic beads for 30 minutes at 37°C. The bead-cell complexes were extracted from the unlabeled cells in a magnetic field. The purity of the final population of CD8+ T cells was greater than 99%, as demonstrated by immunospecific labeling with the anti-CD8 monoclonal antibody (Ortho Diagnostics). PB mononuclear cells were isolated from heparinized venous blood by using density-gradient centrifugation on Ficoll/Hypaque. These cells were resuspended in RPMI 1640 medium with antibiotics, and processed as reported for BALF mononuclear cells to obtain a final population of greater than 99% pure CD8+ T lymphocytes.

CD8+ T lymphocyte clones. PB and BALF CD8+ T cells were cloned using a limiting dilution analysis (14). Cells were seeded onto 60-well Terasaki tissue-culture plates in serial two-fold dilutions from 16 to 1 lymphocytes per well, together with 10^4 irradiated human splenocytes as feeder cells. The cells were cultured in RPMI 1640 medium (GIBCO Laboratories) containing 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 15% human AB serum (complete medium) and supplemented with 1 μg/ml phytohemagglutinin (PHA) (Sigma Chemicals Co., St. Louis, Missouri) and 100 U/ml IL-2 (Cetus, Emeryville, California). Cultures were refed after 7 days with fresh medium containing 1 μg/ml PHA and 50 U/ml IL-2, and feeder cells. After an additional incubation period of 7 days, growing clones were identified under an inverted microscope and cloning efficiency was calculated. Clones from wells showing a probability of clonality ≥ 0.8 were reincubated with 1 μg/ml PHA and 50 U/ml IL-2 for 12-14 days to obtain sufficient cells for further analysis. The CD8+ phenotype was confirmed by immunospecific labeling.

Analysis of cytokine release. Cytokine analysis was performed blind on panels of 6 randomly selected clones from 6 subjects extracted at random from each group. Cells were stimulated by CD3 cross-linking (15). To do this, they were incubated for 24 hours in 24-well culture plates coated with a goat anti-mouse IgG and in presence of 5 μg/ml mouse anti-CD3 monoclonal antibody (Ortho Diagnostics) (16) (10^5 cells per well in 200 μl complete medium). The supernatants were tested for the cytokines IFN-γ, interleukin (IL)-4, IL-5 and IL-6 by enzyme-linked immunosorbent assay with specific antibodies (Pharmingen, San Diego, California), using commercially available recombinant cytokines (Genzyme Co., Boston, Massachusetts) for standardization and complete medium as negative control.

Statistical analysis. Demographic and pulmonary function data were expressed as mean ± SD, and compared by analysis of variance and the Student's *t* test. The distribution of the other data was skewed. They were therefore expressed as medians and ranges, and compared by non-parametric statistics, using Kruskal-Wallis analysis and the Mann-Whitney *U* test.

RESULTS

The demographic and clinical characteristics of the patients and control subjects are reported in Table 1. The smokers with a clinical history of COB had persistent airflow obstruction, as indicated by a FEV_1 lower than 80% of predicted and improving less than 15% after the inhalation of a bronchodilator, and by a reduced ratio between FEV1 and FVC. The lung function parameters of the smokers without symptoms of COB were comparable to those measured in the healthy subjects.

In order to delineate different functional subsets of CD8+ T lymphocytes, these cells were isolated from PB and BALF (purity > 99%) and cloned. To examine the pattern of lymphokine production, a panel of randomly selected clones from 6 randomly selected subjects in each group were stimulated for 24 hours by antigen receptor triggering, using a monoclonal antibody to CD3, and the supernatants were collected for measurement of immunoreactive IFN-γ, IL-4, IL-5, and IL-6. The production of these cytokines is shown in Tables 2 and 3, representing median and range values from 6 PB clones and 6 BALF clones from each subjects. The majority of the clones from healthy subjects and smokers without COB secreted large amounts of IFN-γ and detectable amounts of IL-6 (Th1 secretion

TABLE 2

Cytokine Release from PB CD8+ T Cell Clones

Subject No.	IFN-γ	IL-4	IL-5	IL-6
Healthy subjects				
1	550 (234-970)	<1 (<1)	<0.5 (<0.5)	361 (127-596)
2	830 (320-1158)	<1 (<1)	<0.5 (<0.5)	121 (32-276)
3	475 (<0.1-830)	<1 (<1-21)	<0.5 (<0.5-88)	75 (<0.8-304)
4	696 (315-1283)	<1 (<1)	<0.5 (<0.5)	148 (49-372)
5	427 (13-998)	<1 (<1-161)	<0.5 (<0.5-59)	316 (<0.8-709)
6	125 (<0.1-415)	<1 (<1-98)	<0.5 (<0.5-117)	68 (<0.8-198)
Smokers without COB				
1	216 (41-423)	<1 (<1-84)	<0.5 (<0.5-42)	169 (<0.8-315)
2	168 (35-320)	<1 (<1)	<0.5 (<0.5)	84 (9-243)
3	815 (621-1350)	<1 (<1)	<0.5 (<0.5)	212 (72-357)
4	368 (<0.1-850)	<1 (<1-273)	<0.5 (<0.5-28)	108 (<0.8-360)
5	108 (<0.1-981)	8 (<1-79)	12 (<0.5-93)	96 (<0.8-294)
6	726 (291-1107)	<1 (<1)	<0.5 (<0.5)	404 (171-659)
Smokers with COB				
1	25 (<0.1-118)	<1 (<1-1507)	<0.5 (<0.5-281)	4 (<0.8-36)
2	19 (<0.1-146)	138 (<1-602)	96 (<0.5-446)	6 (<0.8-144)
3	86 (<0.1-397)	<1 (<1-246)	<0.5 (<0.5-551)	72 (<0.8-203)
4	<0.1 (<0.1-23)	741 (<1-1027)	257 (<0.5-614)	<0.8 (<0.8-51)
5	<0.1 (<0.1-102)	532 (<1-1824)	132 (<0.5-721)	<0.8 (<0.8-30)
6	35 (<0.1-244)	115 (<1-586)	79 (<0.5-382)	<0.8 (<0.8-62)
p value vs control groups	<0.005	<0.05	<0.05	<0.01

Note. Median U/10^5 cells/ml (range).

profile). IL-4 and IL-5 immunoreactivity was low or below the detection limit of the assays. By contrast, most of the clones from smokers with COB secreted appreciable amounts of IL-4 and IL-5 but little or no IFN-γ and IL-6 (Th2 secretion profile), particularly those derived from BALF lymphocytes (Table 3).

The proportion of PB clones expressing IL-4 and IL-5 was (median, range) 50%, 33.3-83.3% in smokers with

TABLE 3

Cytokine Release from BALF CD8+ T Cell Clones

Subject No.	IFN-γ	IL-4	IL-5	IL-6
Healthy subjects				
1	744 (415-1182)	<1 (<1)	<0.5 (<0.5)	328 (61-607)
2	507 (321-984)	<1 (<1)	<0.5 (<0.5)	97 (<0.8-224)
3	421 (107-690)	<1 (<1-95)	<0.5 (<0.5-14)	134 (<0.8-296)
4	982 (502-2140)	<1 (<1)	<0.5 (<0.5)	41 (6-158)
5	821 (14-1208)	<1 (<1-102)	<0.5 (<0.5-37)	282 (<0.8-839)
6	954 (67-2306)	<1 (<1-48)	<0.5 (<0.5-3)	216 (<0.8-402)
Smokers without COB				
1	456 (<0.1-831)	<1 (<1-13)	<0.5 (<0.5-75)	238 (<0.1-496)
2	342 (117-815)	<1 (<1)	<0.5 (<0.5)	121 (83-331)
3	912 (284-2016)	<1 (<1)	<0.5 (<0.5)	415 (162-703)
4	409 (199-907)	<1 (<1-104)	<0.5 (<0.5-26)	227 (65-508)
5	615 (<0.1-1993)	<1 (<1-125)	<0.5 (<0.5-71)	119 (<0.8-310)
6	981 (322-2711)	<1 (<1)	<0.5 (<0.5)	104 (23-445)
Smokers with COB				
1	<0.1 (<0.1-150)	974 (<1-1815)	284 (<0.5-416)	<0.8 (<0.8-36)
2	<0.1 (<0.1-32)	415 (<1-738)	315 (<0.5-591)	<0.8 (<0.8-11)
3	<0.1 (<0.1-133)	608 (<1-912)	268 (<0.5-662)	<0.8 (<0.8-55)
4	<0.1 (<0.1-94)	327 (<1-540)	412 (<0.5-813)	<0.8 (<0.8-24)
5	<0.1 (<0.1-17)	812 (<1-2229)	309 (<0.5-698)	<0.8 (<0.8-120)
6	46 (<0.1-382)	549 (<1-797)	181 (<0.5-327)	24 (<0.8-203)
p value vs control groups	<0.001	<0.001	<0.001	<0.005

Note. Median U/10^5 cells/ml (range).

COB versus 8.3%, 0-33.3% in healthy non-smokers ($p < 0.01$) and 8.3%, 0-50% in smokers without COB ($p < 0.01$). These differences were even more evident when comparing BALF clones, since 66.6% (50-83%) of those derived from smokers with COB produced IL4 and IL-5 versus 8.3% (0-16.6%) in healthy non-smokers and 8.3% (0-33.3%) in smokers without COB ($p < 0.001$ for both comparisons).

The predominance of CD8+ T cell clones secreting IL-4 and IL-5 in patients with COB was not merely due to cigarette smoke, because the proportion of these clones in smokers without COB was comparable to that observed in healthy subjects who never smoked, and the two groups of smokers only differed for the presence of the clinical syndrome diagnosed as COB.

DISCUSSION

The increased frequency of CD8+ T lymphocytes producing IL-4 and IL-5 instead of INF-γ in the T cell repertoire of patients with COB, and their compartmentalization in the lung , are novel findings that improve our understanding of the pathogenesis of this disease.

Those cells are similar to the non-cytotoxic CD8+ T lymphocytes of the Th2 phenotype previously described in humans (15).

The switch towards a Th2 response may have several consequences on the outcome of airway infections and inflammatory processes. Reduced ability of producing INF-γ is known to prevent the elimination of pathogens (8,9) and, although IL-4 release represents a necessary condition for the suppressor activity of non-cytotoxic CD8+ T cells (15) by enhancing antibody production, it also down-regulates some steps of cell-mediated responses that are required for host protection (8,9,15), including the function of CD4+ T cells. In addition, one study in transgenic mice that over-express IL-4 selectively in the lung (17) has demonstrated enhanced mucus glycoprotein synthesis and alterations in the mucin release that may be similar to those present in smokers with chronic bronchitis. Finally, CD8+ T cell-derived IL-5 may contribute to explain (18) the increased number of eosinophils in the bronchial tissue of patients with COB and their activation (19,20).

In the context of a low CD4+/CD8+ ratio induced by cigarette smoke *per se* (4,5), the predominance of CD8+ T lymphocytes producing IL-4 and IL-5, instead of the protective cytokine IFN-γ, may render some smokers more susceptible to the toxic effects of cigarette smoke by favouring chronic airway inflammation, uneffective elimination of the infectious agents and the resulting decline in lung function.

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