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Local Immunity in Patients with Chronic Bronchitis and the Effects of a Bacterial Extract, Broncho-Vaxom®, on T Lymphocytes, Macrophages, Gamma-Interferon and Secretory Immunoglobulin A in Bronchoalveolar Lavage Fluid and Other Variables[1]

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Abstract. In 28 adult patients with nonobstructive chronic bronchitis we investigated components of the immune system of the lower airways and the effects of treatment with Broncho-Vaxom® (BV). An analysis of the washing from bronchoalveolar lavage (BAL) showed, in comparison with healthy controls, an elevation of total cell count ($p = 0.003$) as well as the IgA/albumin values ($p = 0.02$) and a reduction of the macrophage activity ($p < 0.001$) in patients with chronic bronchitis. After BV a reduction in the total cell count ($p = 0.05$), an increase in the helper/suppressor T lymphocyte ratio (due mainly to the reduction in the suppressor cells; $p = 0.04$), a modulation of the IgA/albumin ratio, a stimulation of the impaired alveolar macrophage activity ($p = 0.03$) and increased concentrations of γ-interferon ($p = 0.03$) were found in the BAL fluid of patients with chronic bronchitis. The salivary IgA/albumin ratio remained unchanged, the serum IgE concentration fell ($p = 0.02$) and the urinary IgA concentration rose ($p = 0.002$). Bronchial mucosa lesions, evaluated endoscopically in terms of structural damage, hyperemia and mucus production, were improved ($p < 0.01$). These findings indicate that orally administered BV modulates disordered local and systemic immune functions in patients with chronic bronchitis.

Immunomodulating agents promote the development of resistance to infection by stimulating host responses such as antibody production, generation of cytotoxic cells and phagocytosis [1]. It has been thought that bacterial products are probably the most powerful exogenous immunomodulators [2]; whether extracts or single molecules are used, the common pathway points to macrophage activation as the principal mechanism of their adjuvant activity [1–3]. Killed microbes and bacterial extracts have been used for oral immunization for many years [4], but there is now evidence that in addition to acting as specific antigens [5] they contain factors capable of boosting the body's natural defense mechanisms [2, 6–8].

Broncho-Vaxom® (BV; OM Laboratories, Geneva, Switzerland) is a bacterial extract from eight strains and available commercially in capsules for oral administration. Its therapeutic effectiveness has been demonstrated in placebo-controlled, double-blind trials in adults and children with recurrent respiratory infections [9–15].

The aim of this study was to analyze the immune system of the lower airways in patients with chronic bronchitis and to determine the effects of BV on this system, on IgA production in the salivary glands and urinary tract, on certain systemic immune variables and on bronchial mucosal lesions.

Patients and Methods

Patients, Treatment, Controls

Thirty-eight patients (24 women, 14 men, 17 smokers; age range 19–75 years, mean ± SD 44.8 ± 13.4 years) suffering from chronic bronchitis as defined by the World Health Organization [16] en-

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tered the study on randomly selected dates between 1985 and 1987. No patient was taking corticosteroids or immunosuppressive agents, but 4 took antibiotics and many took mucolytic drugs during the study. Patients with chronic obstructive or interstitial lung disease, severe heart disease, immunologic disorders or malignant disease were not included.

During the first 10 days of each of 3 consecutive months, each patient took 1 BV capsule daily while fasting. BV is a lyophilized extract, obtained by submitting eight microorganisms *(Diplococcus pneumoniae, Hemophilus influenzae, Klebsiella ozaenae, Klebsiella pneumoniae, Neisseria catarrhalis, Staphylococcus aureus, Streptococcus pyogenes* and *Streptococcus viridans)* to progressive alkaline lysis. The resulting preparation is purified by means of clarification and filtration procedures. The final dry preparation contains by weight 35% protein, 8% free amino acids, 10% lipids, 8% nucleotides, 2% carbohydrates and approximately 37% salt. Tests for lipopolysaccharide (endotoxin) contamination, as performed by the *Limulus* amoebocyte lysate assay and pyrogenicity in rabbits, have consistently yielded less than 1 ng of endotoxin/mg bacterial extract [17]. A capsule contains 7 mg of the extract.

There were two control groups: 14 healthy volunteers (8 women, 6 men, 6 smokers; age range 22–64 years, mean 37 ± 14 years) who received no treatment and 10 men (5 smokers; aged 56 ± 20 years) who required repeated bronchoscopy on account of pulmonary neoplasms and who received only symptomatic treatment with mucolytic agents comparable to the symptomatic remedies administered to the BV patients. This group of 10 were used for testing the treatment-independent intraindividual variability of the BAL findings.

The study was performed with the approval of the local ethical committee and with the informed consent of the participants. There were two reasons to conduct this study without a placebo control group. First, considering that double-blind studies have revealed that about 40–70% of the patients with chronic bronchitis may benefit from BV by reducing acute episodes and the use of antibiotics, the members of the ethical committee were convinced that patients could not be enrolled in such an invasive diagnostic study without getting any information about their personal response. Additionally, there was a logistical argument against a placebo group. Since some immune parameters such as BAL IgA vary extensively from patient to patient, an intergroup comparison would require an unacceptably high number of patients.

Investigations

Three medical examinations were made, the first before the initial administration of BV, the second 3 months later (first follow-up) and the third 6 months later (second follow-up). Each examination included bronchoscopy with BAL and analysis of the BAL fluid, determination of IgA in saliva and urine, analysis of systemic immune functions, estimation of serum IgE concentration, endoscopic assessment of bronchial mucosal lesions, performance of three lung function tests (inspiratory vital capacity, forced expiratory volume in 1 s, and peak expiratory flow) by standard methods [18] and determination of the erythrocyte sedimentation rate. Out of the original 38 patients, 28 underwent the first follow-up examination, and 20 underwent the second. Patients were excluded from evaluation for the following reasons: in 2 patients there was inadequate BV dosage, 8 patients refused a second bronchoscopy and 6 a third bronchoscopy, 1 was lost to follow-up for social reasons and 1 due to a cardiac event after the second bronchoscopy.

BAL Analysis

BAL was performed by instillation of 100 ml of 0.9% saline solution in 5 × 20-ml aliquots into the middle lobe or the lingula.

BAL cells were counted (total and differential count) and their viability determined by the trypan blue exclusion test. Mononuclear cells were separated by density gradient centrifugation with Ficoll-Hypaque. Monoclonal antibodies of the OKT series recognizing the CD3, CD4 and CD8 antigen were used to identify lymphocyte subsets by indirect immunofluorescence.

Albumin, IgA and IgG were measured by single radioimmunodiffusion in agar after concentration of the BAL fluid by ultrafiltration.

The phagocytic activity of alveolar macrophages in the BAL fluid was determined by measuring luminol-enhanced chemiluminescence with an Auto-Biolumat LB 950 (Berthold). The assay mixture contained 0.3 ml of macrophages (1×10^5/ml), 0.1 ml of opsonized zymosan and 0.01 ml of a 0.3-m*M* luminol solution. Resting values were obtained substituting Hank's balanced salt solution for zymosan. Chemiluminescence response was monitored for 6 s at 60-second intervals over a period of 40 min at 37 °C. Results were expressed as the peak rate in counts per minute [19]. This investigation was done in only 10 of the 28 patients with chronic bronchitis. It could not be done in the other 18 patients, because high polymorphonuclear leukocytosis in the BAL fluid interfered with the chemiluminescence reaction.

γ-Interferon was determined in the concentrated BAL fluid of the same 10 patients, in which the alveolar macrophage activity could be estimated, by an immunoradiometric assay using reagents obtained from Centocor [20]. A volume of 0.2 ml of each sample (or control standard material) was incubated in the well of a reaction tray along with polystyrene beads coated with a monoclonal antibody against γ-interferon for 2 h at room temperature. The beads were then washed three times with 1 ml of distilled water, after which 0.2 ml of ^{125}I-monoclonal antibody against γ-interferon was added to each well. The trays were incubated at room temperature for 2 h, the beads washed again and their radioactivity counted with a gamma counter. Human γ-interferon content was expressed as laboratory units per milliliter from a standard curve. The sensitivity of the test was 0.1 unit. Changes were measured as percentage of the pretreatment value.

To correct variations resulting from different dilutions, albumin was used as a reference for all soluble components of the BAL fluid.

Microbiological Analysis of the BAL Fluid. 0.1-ml samples of the BAL fluid were plated on sheep blood agar and chocolate agar. The plates were incubated overnight at 37 °C, and growth of the microorganisms was evaluated semiquantitatively (+ <10; ++ 10–50; +++ >50 colonies per plate). Routine procedures were used to identify the bacterial species and fungi.

All BAL data were analyzed for seasonal variation.

IgA in Saliva and Urine

IgA was evaluated by single radioimmunodiffusion in saliva and in concentrated 24-hour urine specimens.

Systemic Immune Functions

Systemic T cell function was assessed by the multitest method (Mérieux) [21] for assay of delayed cutaneous hypersensitivity to seven recall antigens (candida, diphtheria, proteus, streptococcus, tetanus, trichophyton, tuberculin). Serum IgE was measured by a

Table 1. Pretreatment characteristics of 28 patients with chronic bronchitis treated with BV

Mean age, years	42 ± 14
Sex ratio, men/women	17/11
Duration of disease, years	28 ± 13
Smoker/nonsmoker	9/19
Delayed cutaneous hypersensitivity reduced, %	19
Serum IgE elevated, %	29
Bronchial mucosa structure score	2.0 ± 0.9
Bronchial mucosa hyperemia score	2.1 ± 0.8
Bronchial mucus production score	2.3 ± 0.8
Lung function tests	no evidence of obstruction
Erythrocyte sedimentation rate, mm/h	18 ± 17

standard laboratory enzyme-linked immunosorbent assay technique.

Bronchial Mucosal Lesion Score

Bronchial mucosal lesions were assessed endoscopically in the follow-up according to the following score [22], which quantified mucosal structural damage, hyperemia and mucus production: for mucosal structure 0 = normal, 1 = unequal, 2 = slightly swollen, 3 = edematous proliferative; for hyperemia 0 = nil, 1 = slight, 2 = moderate, 3 = marked; and for mucus production 0 = nil, 1 = slight, 2 = moderate, 3 = marked with pus. Indicated score values are the mean of the values which were mostly determined by HPE and independently by a second expert with a teaching endoscope.

Statistical Analysis

The Mann-Whitney test was used for evaluation of group differences, the Friedman or Wilcoxon test for comparison of values before and 3 and 6 months after the start of BV administration, and the Fisher exact tests for testing correlation of pretherapeutic variables to the response status. Circannual changes were calculated by the single cosinor method, and seasonal difference by the Kruskal-Wallis Anova with seasonally pooled data (January–March, April–June, July–September, October–December).

Results

Pretreatment Status

Pretreatment characteristics in the 28 bronchitic patients whose response to treatment with BV was to be evaluated at the first follow-up are summarized in table 1. Delayed cutaneous hypersensitivity as assayed by the multitest system (Mérieux) was below the normal level and serum IgE above the normal level in 19 and 29% of the patients, respectively. Other serum immunoglobulin concentrations were within the normal range. All the patients showed clear evidence of bronchial mucosal inflammation as assessed by the endoscopic score, but absence of signs of irreversible airflow obstruction with the lung function test attested to the early stage of their chronic bronchitis. The erythrocyte sedimentation rate was raised in most of the patients.

The initial total cell count in the BAL fluid was significantly higher ($p = 0.003$) in the patients with chronic bronchitis than in the healthy controls (table 2), chiefly on account of the increased numbers of polymorphonuclear leukocytes in the fluid. On the other hand, the mean ratios of helper to suppressor T lymphocytes (CD4+/CD8+) did not differ significantly between the healthy and the bronchitic groups at the start of the trial.

The BAL IgA content (data not shown) as well as the values for IgA/albumin in the BAL fluid of patients with chronic bronchitis showed great interindividual variation (fig. 1) but were for the most part higher than in the healthy controls ($p = 0.02$; fig. 2, table 3).

Before BV therapy, the mean alveolar macrophage activity was significantly lower ($p < 0.001$) in the 10 bronchitic subjects who could be evaluated than in the 14 healthy controls (fig. 3) and showed less interindividual variation. In the healthy controls it was not influenced by a history of smoking.

The mean γ-interferon content of the BAL fluid in 10 of the bronchitic patients before BV therapy, measured by radioimmunoassay, ranged from 0.01 to 1.2 U/mg albumin (mean 0.368 ± 0.189, median 0.075).

Before BV therapy, a BAL microbiological investigation was conducted in 23 patients. In 6 patients no species were isolated, in 8 patients one and in 9 patients two or three species. *S. viridans* were found in 9 samples, *N. catarrhalis* in 5, *S. aureus* in 4, *Candida albicans* in 3, *Staphylococcus albus* in 2, *Pseudomonas aeruginosa* in 2, *hemolytic Streptococcus serotype B* in 1, *D. pneumoniae* in 1 and *H. influenzae* in 1 sample. In most cases semiquantitative evaluation showed less than 10 colonies/100 µl. Only 2 patients (No. 20 and 38) had more than 50 colonies of *H. influenzae* and *S. aureus*, respectively, in pure culture, which can be regarded as possible causative organisms of an exacerbation of the bronchitis. It is noteworthy that in these 2 cases, as well as in only 1 other case, in which more than 50 colonies of *H. influenzae* were observed at the first follow-up, the increase of bacterial growth was not associated with very high IgA/albumin val-

Table 2. Cellular components of BAL fluid from healthy controls and patients with chronic bronchitis before and after treatment with BV

	Recovery of BAL fluid %	Total cell count $\times 10^6$	Cell viability %	Macrophages[1]	Lymphocytes[1]	Polymorphonuclear cells[1]	CD4+/CD8+ ratio	CD8+ count $\times 10^6$
Healthy controls (A)	46 ± 9 (14)	4 ± 2 (14)	80 ± 13 (14)	88 ± 8 (14)	8 ± 4 (14)	4 ± 8 (14)	1.09 ± 0.36 (14)	0.19 ± 0.09 (14)
Chronic bronchitis before treatment (B)	49 ± 19 (28)	26 ± 36 (28)	88 ± 32 (28)	46 ± 32 (28)	16 ± 19 (28)	38 ± 34 (28)	1.12 ± 0.43 (22)	1.36 ± 1.79 (22)
3 months after start of treatment (C)	50 ± 16 (28)	16 ± 20 (28)	89 ± 14 (28)	46 ± 28 (28)	16 ± 20 (28)	38 ± 30 (28)	1.38 ± 0.50 (22)	0.74 ± 0.90 (22)
3 months after end of treatment (D)	49 ± 12 (20)	28 ± 54 (20)	86 ± 15 (20)	43 ± 27 (20)	8 ± 4 (20)	49 ± 30 (20)	1.49 ± 1.86 (15)	2.06 ± 4.70 (15)
Figures in parentheses indicate numbers of patients.								
Significant difference between groups:		A/B $p = 0.003$ B/C $p = 0.05$			C/D $p = 0.03$		B/C $p = 0.04$	C/D $p = 0.04$

[1] Expressed in percent of total cells.

ues in the BAL fluid. In these cases, values were 0.5, 0.01 and 0.66. The magnitude of the initial BAL IgA/albumin values was also not influenced by the kind of species found in the BAL fluid. So IgA/albumin values greater than 0.5 were measured in 3/6 patients with no isolates, in 3/8 patients with one isolate and 2/9 patients with a colonization of two or three organisms.

Effects of Broncho-Vaxom

BAL Cytology. At the first follow-up, the total cell count in the BAL fluid was significantly lower ($p = 0.05$) and the mean CD4/CD8 ratio significantly higher ($p = 0.04$; mainly at the expense of a decrease in CD8+ cells) than before treatment (table 2). In the patients whose pretreatment BAL IgA/albumin ratios were below 0.5, the increase in the CD4+/CD8+ T lymphocyte ratio in response to BV therapy was greater than in the whole group, rising from 0.98 ± 0.4 to 1.44 ± 0.6 ($p = 0.01$). After BV therapy, the CD8+ cells again increased significantly ($p = 0.04$).

BAL, Saliva, Serum and Urinary Immunoglobulins. The effects of BV therapy on the IgA/albumin ratios in BAL fluid and saliva, on IgG/albumin ratio in BAL fluid, on IgA concentration in urine and on IgE concentration in serum are summarized in table 3. The only significant change between the mean pretreatment and posttreatment values were in urinary IgA (increase) and serum IgE (decrease at the first follow-up). The urinary IgA remained at a high level in most of the patients after withdrawal of BV.

For a detailed analysis of the effect of BV on pulmonary IgA production, the intraindividual changes were examined (fig. 1) and analyzed into two subgroups of patients (fig. 2). The patients with pretreatment BAL IgA/albumin ratios below 0.5 responded to BV by an increase in the IgA/albumin ratio ($p = 0.003$). The lower the initial value, the greater was the increase. In the patients with initial IgA/albumin below 0.5, BV also increased the BAL IgG/albumin ratio (from 0.27 ± 0.24 to 0.46 ± 0.40; $p = 0.02$ data not shown). Those patients with initial IgA/albumin values above 0.5 either did not respond to BV or had them even decreased. The post-BV treatment level of

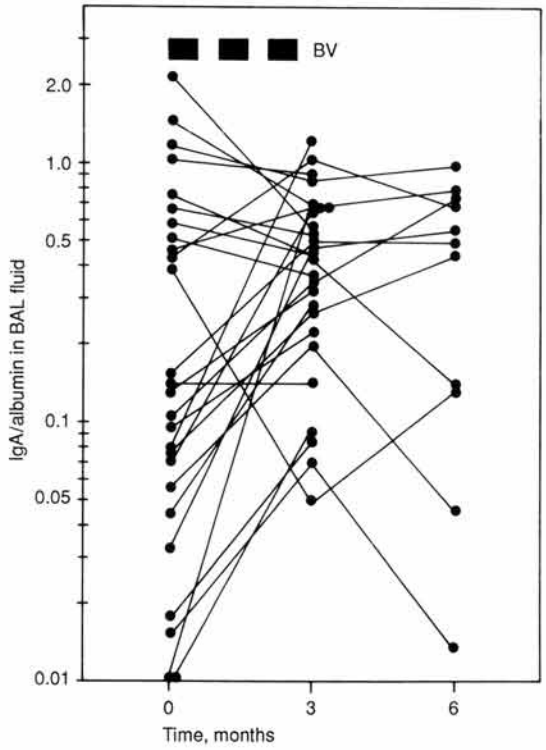

Fig. 1. IgA/albumin ratios in BAL fluid in 26 patients with chronic bronchitis treated with BV for 3 consecutive months and followed up for an additional 3 months. The 18 initially low (< 0.5) BAL IgA/albumin ratios responded to BV with an increase and the 8 initially high (> 0.5) ratios with a decrease.

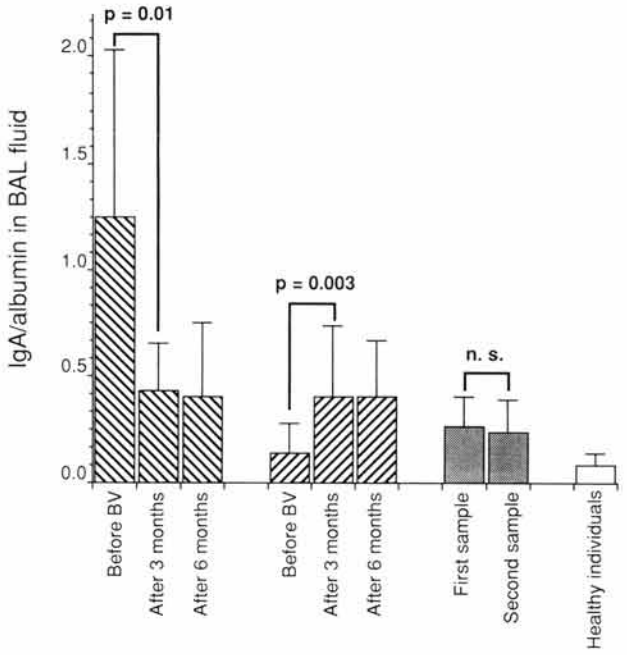

Fig. 2. IgA/albumin ratios in the BAL fluid. ▨ = Eight patients with chronic bronchitis (A) treated with BV on 10 days per month in each of 3 consecutive months and in whom the pretreatment IgA/albumin ratios were high (> 0.5); ▨ = 18 similar patients similarly treated (B), but in whom the pretreatment IgA/albumin ratios were low (< 0.5); ▩ = 10 control patients with pulmonary neoplasms who received symptomatic remedies only (two consecutive samples); □ = 14 healthy subjects. Note the fall in IgA/albumin ratios after BV in group A contrasted with the rise in group B.

Table 3. Immunoglobulin levels in BAL fluid, saliva, urine and serum before and after treatment with BV

	BAL fluid		Saliva	Urine	Serum
	IgA/albumin	IgG/albumin	IgA/albumin	IgA µg/dl	IgE U/ml
Healthy controls (A)	0.08 ± 0.05 (14)	0.14 ± 0.09 (14)	2.5 ± 5.56 (14)	n.d.	n.d.
Chronic bronchitis before treatment (B)	0.48 ± 0.68 (26)	0.44 ± 0.45 (20)	1.56 ± 2.84 (19)	9.08 ± 15.36 (18)	172 ± 288 (24)
3 months after start of treatment (C)	0.41 ± 0.30 (26)	0.51 ± 0.46 (20)	1.39 ± 1.14 (19)	71.93 ± 145.29 (18)	155 ± 293 (24)
3 months after end of treatment (D)	0.40 ± 0.28 (11)	1.01 ± 2.76 (15)	1.19 ± 1.26 (14)	130.03 ± 229.84 (12)	205 ± 393 (14)
Figures in parentheses indicate numbers of patients. n.d. = Not done.					
Significant differences between groups:	A/B p = 0.02			B/C p = 0.002	B/C p = 0.02

IgA/albumin was about the same for both groups. Three months after the withdrawal of BV, the ratios were still at their new, treatment-induced position. In both subgroups of patients, the BAL albumin concentration was not significantly influenced by BV treatment. Therefore the increase in the IgA/albumin values reflects really IgA production and is not only due to alterations in the albumin content of the BAL fluid.

In the patients not given BV, no significant change in BAL IgA/albumin ratios followed the administration of symptomatic remedies similar to those received by the BV patients.

The cellular and immunoglobulin response to BV in the lower respiratory passage could not be predicted by any of the noninvasive investigative methods. No correlation was found between IgA/albumin ratios in BAL fluid and in saliva, nor did age, sex, duration of chronic bronchitis, history of smoking, initial Mérieux multitest scores or season appear to affect changes in the BAL immunoglobulins or in the ratios of helper to suppressor T lymphocytes after BV therapy. No significant changes were detected in the serum concentrations of immunoglobulins other than IgE.

BAL Alveolar Macrophage Activity. Macrophage activity in the BAL fluid was significantly higher ($p = 0.03$) after BV therapy than before in 8 of the 10 patients in whom it was determined. It remained at this elevated level throughout the observation period but did not exceed the normal upper limit at any time (fig. 3).

BAL γ-Interferon. The γ-interferon content of the BAL fluid, measured as a percentage of pretreatment value, was found to have increased significantly at the first follow-up after BV therapy ($p = 0.03$; fig. 4).

Bronchial Mucosal Lesions. At the first follow-up the endoscopic bronchial mucosal lesions were improved in nearly all the patients, with a highly significant reduction of the mean structure score from 2.0 ± 0.9 to 1.2 ± 0.8 ($p = 0.003$), of the hyperemia score from 2.1 ± 0.8 to 1.3 ± 1.0 ($p = 0.001$) and of the mucus production score from 2.3 ± 0.8 to 1.5 ± 0.9 ($p = 0.005$).

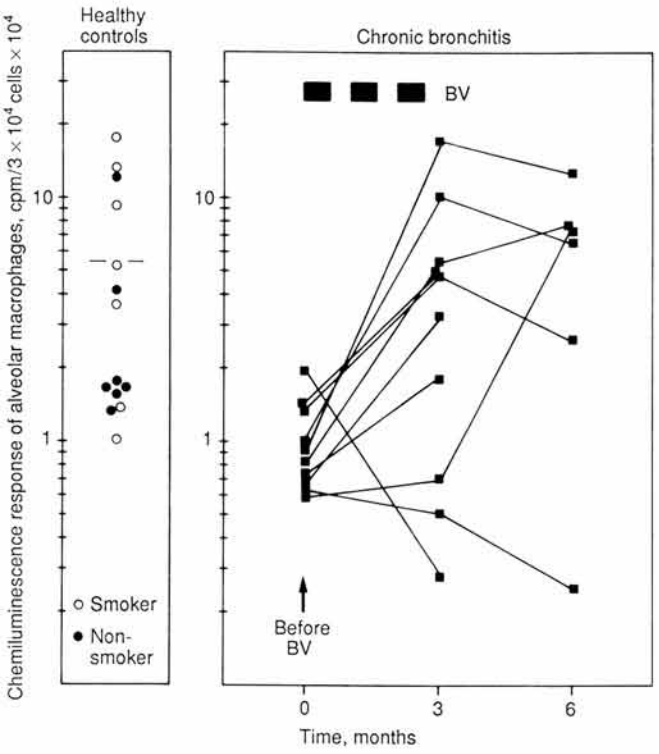

Fig. 3. Alveolar macrophage activity measured by chemiluminescence in BAL fluid from 14 healthy untreated controls and from 10 patients with chronic bronchitis treated with BV for 3 consecutive months and followed up for an additional 3 months. Before BV, mean macrophage activity was lower in the bronchitic subjects than in the controls ($p < 0.001$); after BV, macrophage activity was significantly above the pretreatment level in 8 of the 10 bronchitic patients in whom it was estimated ($p = 0.03$). Results expressed as the peak rate in counts per minute.

Microbiological Studies

Microbiological studies of BAL fluid before and after BV therapy treatment could be performed in 19 patients. In 8 patients a quantitative reduction or even a disappearance of colonized and causative organisms could be observed without any antibiotic treatment (table 4). In case No. 20 *H. influenzae* was quantitatively reduced from +++ to ++, and in case No. 38 *S. aureus* could no longer be isolated at the first follow-up. Generally a reduction in the number of colonies was not correlated to an increase in BAL IgA. Seven patients showed no significant changes in colonization. There was only 1 patient who showed an exacerbation from the microbiological point of view during the first 3 months. In this case the first follow-up BAL fluid yielded *H. influenzae* ++, where as from the initial BAL fluid only *S. viridans* + and *C. albicans* + could be isolated. Interestingly, the bronchoscopic assessment of the mucosa lesions demonstrated no exacerbation of the inflammation in this case. Here too, IgA/albumin levels did not seem to be related to bacterial growth.

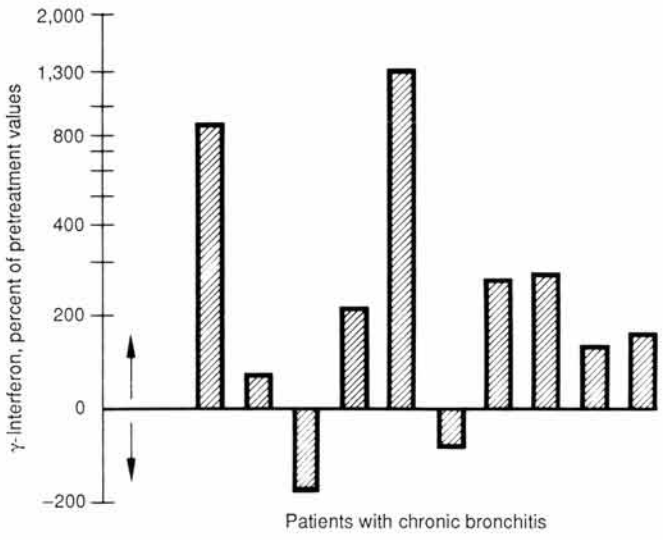

Fig. 4. γ-Interferon concentrations in BAL fluid from 10 patients with chronic bronchitis who had received BV on 10 days per month during each of 3 consecutive months. Changes are indicated as percentages of the pretreatment values which, measured by radioimmunoassay, ranged from 0.01 to 1.2 U/mg albumin (mean 0.368 ± 0.189, median 0.075). There is a significant increase in γ-interferon production ($p = 0.03$).

Erythrocyte Sedimentation Rate

The erythrocyte sedimentation rate fell from 18 ± 17 to 11 ± 12 mm/h ($p = 0.03$).

Seasonal Variation

No statistically significant seasonal or circannual variation in any BAL fluid variable was found before BV therapy. Only in the patients who received BV in the July–December period was an increase in the CD4/CD8 T lymphocyte ratio found (1.12 ± 0.35 to 1.72 ± 0.48; $p = 0.03$), whereas in the first half of the year no significant responses of the helper/suppressor T cell ratios (1.10 ± 0.53 to 1.12 ± 0.28; $p = 0.34$) to BV therapy was seen.

General

No significant changes were detected during follow-up in the albumin levels in the BAL fluid, the saliva or the urine, in the results of the Mérieux multitest assay of delayed cutaneous hypersensitivity, in serum levels of C3 and C4 complement proteins, in total and differential blood counts or in the results of lung function tests. Mild nausea as a side effect attributable to BV was observed in 1 of the 28 patients.

Table 4. BAL IgA in patients showing a reduction in the colonized and causative organisms without any antibiotic treatment

Patient No.	Isolated organisms in the BAL fluid				BAL IgA/albumin	
	before BV treatment		3 months after start of BV treatment		before BV treatment	3 months after start of BV treatment
	species	colonies	species	colonies		
7	*N. catarrhalis*	+		none	0.01	0.67
	S. viridans	++				
	C. albicans	+				
13	*P. aeruginosa*	+		none	0.07	0.67
	S. viridans	++				
18	*S. aureus*	++		none	0.74	0.42
	P. aeruginosa	+				
20	*H. influenzae*	+++	*H. influenzae*	++	0.50	0.36
28	*N. catarrhalis*	+		none	0.38	0.03
	S. viridans	+				
29	*N. catarrhalis*	+				
	S. viridans	+	*S. viridans*	+	0.02	0.06
	S. aureus	++				
30	*N. catarrhalis*	+		none	3.75	0.08
	S. viridans	+				
	S. albus	+				
38	*S. aureus*	+++		none	0.01	0.48

Discussion

The work presented in this report has provided evidence that the bacterial extract BV, when administered orally to patients with chronic bronchitis, increases cellular and humoral immune parameters in the lung and the mucosa-associated lymphoid tissues of the urinary tract and may additionally reduce systemic IgE production.

Ethical and logistical arguments did not permit a placebo control group in our study. The reliability of the results, however, is ascertained by comparing these with those of other studies and those of a non-placebo control group, by the kinetics of the observed effects and by exclusion of possibly interfering factors. The effects on T lymphocyte subsets, macrophage activities, serum IgA and γ-interferon levels are confirmed by the findings of several other studies investigating the action of bacterial extracts in vitro and in vivo. An elevation in plasma interferon levels and an increase in the helper/suppressor T blood lymphocyte ratio were for example also observed in patients treated with a mixed bacterial vaccine derived from *S. pyogenes* and *Serratia marcescens* [23]. BV, as indicated, also contains extracts of *S. pyogenes*. As γ-interferon is a potent macrophage-activating factor [8, 24], our observations that BV stimulates alveolar macrophage activity (fig. 3) and raises γ-interferon production in the same patients (fig. 4) are consistent with each other. The chemiluminescence test, by which we measured alveolar macrophage activity, records the macrophages' oxidative metabolism, which increased during phagocytosis [19, 25]. Increased phagocyte activation has also been found after addition of BV to cells in vitro [17, 26]. Evidence that BV has an effect on secretory IgA synthesis has also been shown in another trial conducted with healthy subjects and which also demonstrated an effect of BV treatment on the MALT apparatus as evidenced by the significant increases in salivary IgA with respect to baseline values, remaining at high levels for several months in the patients who received a second treatment course [27].

A treatment-independent variation of BAL IgA by an exacerbation of infection could be excluded, since an exacerbation was observed in only 1 patient and was not associated with a marked increase in the BAL IgA/albumin value (it increased from 0.45 to 0.66). Also, the fact that the controls who had received no BV showed no variation of BAL IgA levels in two consecutive BALs and the findings that BAL IgA, urinary IgA and alveolar macrophage activity were still elevated 3 months after the termination of BV therapy support the interpretation that the changes of BAL IgA/albumin are really BV related and not caused by a treatment-independent undulation of these parameters.

The post-BV increases in BAL IgA and BAL IgG in patients with initially low BAL IgA levels, and of urinary IgA in all the patients, do not seem to be an expression of a specific antibody response to antigens of the bacteria used for the production of BV. Indirect evidence for this conclusion is based on two findings. First, the reduction of BAL fluid organisms was not correlated to an increase in BAL IgA (table 4), and second the stimulation of IgA production was generally higher as expected in the case of specific antibody response. These results together with those obtained by others [7] in the murine system indicate that the effect of BV on immunoglobulin synthesis is mainly a polyclonal B cell activation.

The fall in serum IgE concentration ($p = 0.02$; table 3) noted by us in response to BV in patients in whom at the onset of the trial this value was normal or elevated is consistent with the result of a double-blind study conducted by other workers [28]. A hypothetic pathway by which this effect on IgE synthesis could be mediated, may be an influence of BV on the complex isotype-specific regulation of the IgE response [29]. That BV may influence allergic reactions also by mechanisms other than the IgE response is shown in in vitro studies with leukocytes of subjects with bronchial asthma, in which BV was able to inhibit both the direct and the antibody-dependent allergic autocytotoxicity response [30].

The absence of seasonal or circannual variation in the immune function of the lower respiratory tract before immunomodulative treatment in the bronchitic subjects may reflect a reduced capacity of the bronchitic bronchial mucosa to adapt to seasonal change. The statistically significant fall in CD8+ cells with the consequent rise in the CD4+/CD8+ ratios observed in the second half of the year in the BAL fluid of bronchitic subjects treated with BV may be due either to restoration by BV of physiologic seasonal variation in bronchial immune function or to a specific circannual 'chronoeffectiveness' of this agent [31].

All these pleiotropic effects of BV on pulmonary defense mechanisms seen in our study are unspecific in the sense that they are not directed against specific

antigens, but they are important for natural resistance to infections in chronic bronchitis. They are also involved in the inflammation reactions of the bronchial mucosa and seem to be therefore responsible for healing of bronchial mucosa inflammation as shown in a recent study [32]. In this study the BAL findings were compared with the mucosa grading score. It reveals that beside a high total cell and polymorphonuclear leukocyte count also a high CD8+ lymphocyte count and high IgA/albumin values correlate with the grade of inflammation. The positive influence of BV on these BAL parameters may therefore explain the significant reduction of bronchial mucosa lesions observed in nearly all patients of our study and the clinical benefits (reduced rate of bronchitis exacerbation, lower antibiotic prescription and decrease in the severity of cough and expectoration) documented in double-blind protocols [11, 12]. In this context it is remarkable that also after oral immunization with killed *H. influenzae* in chronic obstructive lung disease no increase in specific antibodies to *H. influenzae* in saliva was measured, though a tenfold reduction in the incidence of infection in comparison with a placebo control was observed [33]. The fact that orally administered BV acts on both the bronchial and urogenital mucosal compartments highlights the primary role of the intestine in mucosal defense and raises the question whether the unspecific cellular and humoral actions of BV are mediated by similar cellular pathways as they are described for the specific IgA response after oral immunization [34, 35].

Hitherto unknown are the molecular substances which are responsible for the wide range of host responses influenced by BV in vivo and in vitro [36]. Several components of bacterial cell walls, such as the peptidoglycan derivative muramyl dipeptide, lipopeptides and lipopolysaccharides can 'in fact' work as biological response modifiers [2, 7, 37]. Recent in vitro studies led to us suppose that membrane glycolipoproteins of molecular weights between 5 and more than 100 kD, which show a high in vitro activity as measured by hexose monophosphate shunt in mouse macrophages, are the active components of BV [17]. A participation of lipopolysaccharides (endotoxin) in the observed effects of BV is excluded for many reasons. The endotoxin concentration was found to be extremely low. The antibiotic polymyxin B, which blocks lipopolysaccharides by forming a complex with the lipid A moiety of endotoxin, was unable to inhibit either the metabolic or the functional activities elicited by BV in murine macrophages [17]. Furthermore, extracts of the three gram-positive bacteria which do not contain lipopolysaccharides show in vitro activities similar to gram-negative bacterial extracts on macrophages and lymphocytes [unpubl. data]. However, the clinically active agents of bacterial extracts will have to be defined in more detail in future research before optimal dosage schedules and combinations of these promising natural products can be determined.

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