Airways inflammation in subjects with chronic bronchitis who have never smoked

Mirco Lusuardi, Armando Capelli, Carlo G Cerutti, Elisa L Spada, Claudio F Donner

Abstract

Background — Smoking is the single most common cause of chronic bronchitis but the disease can also occur in non-smokers. Alterations in the lung responsible for the disease, such as oxidant/antioxidant and protease/antiprotease imbalance, have been investigated in smokers. The aim of our study was to evaluate local cellular and soluble factors (albumin, immunoglobulins, proteases, α1-antitrypsin, and transferrin) that may be involved in the development of chronic bronchitis in subjects who have never smoked.

Methods — Sixteen clinically stable patients with chronic bronchitis who had never been smokers were studied and 17 healthy non-smokers served as controls. All subjects underwent bronchoalveolar lavage (BAL). Total and differential cell counts and concentrations of the main proteins (albumin, immunoglobulins, complement fractions, α1-antitrypsin, and transferrin) were measured. Elastase-like activity was assessed in cells and supernatants. To estimate the oxidant burden the release of superoxide anion (O2−) from native cell populations was evaluated.

Results — Recovery of BAL fluid was reduced in older individuals in both the chronic bronchitis and control groups. There was no difference in total cell count, but neutrophil percentage count was higher in those with chronic bronchitis (median (range) 3·5 (1·6–14·2)) than in controls (1·3 (0·5–3·7)). These differences were most pronounced in the first recovery, representative of the bronchial lavage. There was no difference in bronchial epithelial cells. Total proteins and albumin levels were comparable and IgG, IgA, IgM, C3, C4, transferrin and α1-antitrypsin values standardised to albumin did not show any significant differences. No differences in elastase-like levels in supernatants were detected. In cell lysates elastase-like activity × 107 cells (macrophages + neutrophils) was increased in patients with chronic bronchitis (0·25 (0·06–4·3) compared with controls 0·08 (0·03–0·9) μg PPEeq). The release of O2− both at baseline and after opsonised zymosan phagocytosis did not show any differences.

Correlation analysis between FEV1, and BAL fluid data showed a negative correlation only with neutrophils/ml.

Conclusions — Clinically stable non-smokers with chronic bronchitis show no alterations of local immune components, oxidant burden, and free elastase-like activity in BAL fluids, while the content of elastase-like activity in phagocytic cells is increased. As in smokers, bronchial neutrophilia is the most significant cellular modification which correlates with the degree of airflow obstruction.

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Cigarette smoking is considered the major cause of chronic bronchitis in western countries. Nevertheless, recent studies suggest that in non-smokers chronic bronchitis may be associated with other environmental risk factors.1 Local factors in the lung which may play a part in causing the disease have been investigated in smokers. These include oxidant/antioxidant, protease/antiprotease imbalance, and alteration of immune defences,2,3 although a precise index of individual susceptibility to the deleterious effects of smoking has yet to be identified.4 Only a few patients with chronic bronchitis claim never to have smoked in their life. In such cases risk factors other than tobacco smoking have been suggested, including age, an inherited deficiency of antiprotease, defects of immunity with recurrent respiratory infections, environmental factors such as chronic inhalation of irritants (fumes, dusts) in the workplace, and general pollution.5

Bronchial and bronchoalveolar lavage (BAL) have been used to characterise the intraluminal inflammatory components in chronic bronchitis, but these have rarely been compared with histological findings in the bronchial mucosa.6,7 No series has reported only on patients with chronic bronchitis who have never smoked. Smoking induces major modifications of BAL fluid data irrespective of the presence of overt disease, and thus potentially represents a confounding factor.8,9

The aim of our study was to evaluate local cellular and soluble factors potentially involved in the development of chronic bronchitis in subjects who had never been smokers. In particular, we sought to determine whether a local defect of immune components or an increase in oxidant and protease burden may have some importance irrespective of smoking habits.

Methods

STUDY POPULATION

The BAL data from 16 clinically stable, well nourished patients affected by chronic bronchitis were studied. Table 1 gives the demographic data. None had any history of smoking.
Chronic bronchitis was diagnosed according to the MRC standard criteria - that is, the presence of cough and sputum production on most days of the month for at least three months a year in the last two years. Specific risk factors for chronic bronchitis, apart from age (five patients >65 years) were identified in only six patients who had been exposed to inorganic (two patients) or organic (four patients) dusts in the workplace, but not within three years of the start of the study. No clinical or functional evidence of interstitial lung disease was found. A history of recurrent respiratory infections during childhood and/or adolescence was reported by four patients. Significant passive smoking was denied by all the patients. None of the patients with chronic bronchitis had α1-antitrypsin deficiency or systemic immune defects.

Pulmonary function tests included the measurement of forced expiratory volume in one second (FEV1), forced vital capacity (FVC), and forced expiratory flow between 25% and 75% of vital capacity (FEF25-75). In the patients with chronic bronchitis FEV1 measurement was repeated 15 minutes after inhalation of 200 µg salbutamol. The degree of emphysema was not assessed by objective means such as computed tomographic (CT) scanning or measurement of the lung transfer factor (TLCO).

Patients with respiratory disorders other than chronic bronchitis, including asthma, infectious diseases, atopy, immunodeficiency conditions, autoimmune disorders, malignancies or clinically significant cardiovascular, neurological, endocrine and haematological disorders were excluded. Fine cut CT scanning was not available, so a component of bronchiectasis could not be excluded. Only treatment with bronchodilators was permitted. All the patients with chronic bronchitis used inhaled bronchodilators either regularly or on an as needed basis; 11 were on treatment with oral sustained-release theophylline. Patients were not studied within one month of an exacerbation.

Seventeen healthy volunteers who had never smoked served as controls, and their demographic data are summarised in Table 1. Since the controls and the patients with chronic bronchitis were not age matched, the controls were divided into two subgroups of five elderly of median (range) age 63 (55–69) years and eight younger (36 [28–42] years) individuals to study the possible effect of age on BAL data.

The study was carried out under the supervision of the institutional review board at the Medical Center of Rehabilitation in Veruno, Italy, and the subjects gave written informed consent.

<table>
<thead>
<tr>
<th>Table 1 Median (range) demographic and functional data</th>
<th>Controls (n = 17)</th>
<th>Chronic bronchitis (n = 16)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M:F)</td>
<td>12:5</td>
<td>4:12</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>45 (28–69)</td>
<td>65 (49–78)</td>
<td>0.001</td>
</tr>
<tr>
<td>FEV1 (% pred)</td>
<td>90 (81–119)</td>
<td>64 (38–84)</td>
<td>0.0001</td>
</tr>
<tr>
<td>FVC (% pred)</td>
<td>91 (76–120)</td>
<td>94 (69–125)</td>
<td>NS</td>
</tr>
<tr>
<td>FEF25-75 (% pred)</td>
<td>87 (71–117)</td>
<td>54 (26–93)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

BRONCHOALVEOLAR LAVAGE AND PROCESSING OF SPECIMENS

To evaluate local airway inflammatory components BAL was performed using a previously described technique. Three 50 ml boluses of sterile saline warmed to 37°C were instilled through the working channel of a fiberoptic bronchoscope. Each bolus was immediately removed by gentle suction with a syringe and kept separately. Fluids were filtered through a single layer of sterile gauze.

Before centrifugation cytocentrifugation (Cytospin II, Shandon, London, UK) slide preparations of native fluids were made in duplicate for each recovery and stained with May-Grunwald-Giemsa. A minimum of 500 cells per slide were examined at a magnification of ×1000. Cell differential analysis was carried out on the individual and pooled recoveries. Bronchoalveolar cells were separated by centrifugation (Beckman TJ 6, Beckman, Fullerton, California, USA) at 400g for 15 minutes at 4°C. The samples were then resuspended in cell culture medium RPMI 1640 (Boehringer Mannheim GmbH, Mannheim, Germany).

BIOCHEMICAL ANALYSIS

Proteins and protein fractions

Supernatants were immediately frozen at −80°C and assays were performed within 15 days. The levels of albumin, IgG, IgA, IgM, C3, C4, α1-antitrypsin, and transferrin in the recovered BAL fluid samples were assessed using a modified immunoturbidimetric method (URIN-PAK immuno MICROALB for albumin; SERA-PAK immuno for the other substances; Miles Italiana, Cavenago Brianza, Milan, Italy). All BAL fluid samples were unconcentrated. The immunoturbidimetric reaction required a 30 minute incubation for all the assays; the absorbance readings were performed at 340 nm using a COMPUR M 2000 CS2 spectrophotometer (Bayer Diagnostic-Electronic, Munchen, Germany). All samples were tested in duplicate.

Elastase-like activity

The elastase burden was evaluated on supernatants (free elastase-like activity) and lysates of the entire cell population (potentially releasable elastase-like activity) using a soluble synthetic substrate, Succinyl-Ala-Ala-Ala-p-Nitro-Anilide (SAPNA), according to a modification of the method of Bieth et al.13 In brief, the substrate solution contained SAPNA 50 mg/dl (final concentration) in Tris buffer 0.2 M + CaCl2 50 mM, pH 8.0, + dimethylsulphoxide 10% (v/v) + Triton X-100 0.003% (v/v) (all reagents from Sigma Chemicals, St Louis, Missouri, USA). The working solution, composed of 1.9 ml of the substrate solution and 100 µl of the sample, was incubated for 24 hours at 37°C. The absorbance readings were performed at 405 nm using a COMPUR M 2000 CS2 spectrophotometer (Bayer Diagnostic-Electronic, Munchen, Germany). All samples were tested in duplicate.

Calibration curves were produced with pan-
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Table 2 Median (range) cell counts and differentials in the three sequential and pooled BAL fluid recovery volumes

<table>
<thead>
<tr>
<th></th>
<th>1st recovery</th>
<th>2nd recovery</th>
<th>3rd recovery</th>
<th>Pooled recoveries</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>CB</td>
<td>Controls</td>
<td>CB</td>
</tr>
<tr>
<td>Cells/ml × 10⁶</td>
<td>1.3 (0.5-4.1)</td>
<td>1.3 (0.3-4.6)</td>
<td>2.1 (0.5-5.5)</td>
<td>1.8 (0.7-4.8)</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>87 (78-97)</td>
<td>90 (82-97)</td>
<td>90 (80-98)</td>
<td>NS</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>79 (46-40)</td>
<td>88 (77-97)</td>
<td>90 (72-96)</td>
<td>88 (76-94)</td>
</tr>
<tr>
<td>Basophils (%)</td>
<td>83 (1.2-17.4)</td>
<td>88 (1.3-17.0)</td>
<td>90 (1.0-18.4)</td>
<td>NS</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>2.1 (0.9-5.7)</td>
<td>10.3 (2.0-21.8)</td>
<td>0.8 (0.2-2.4)</td>
<td>1.7 (0.5-8.4)</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>0.2 (0.2-9)</td>
<td>0.3 (0.1-4)</td>
<td>0.2 (0.2-9)</td>
<td>0.3 (0.1-4)</td>
</tr>
<tr>
<td>Basophils (%)</td>
<td>0 (0-0.6)</td>
<td>0 (0-0.2)</td>
<td>0 (0-0.4)</td>
<td>0 (0-0)</td>
</tr>
</tbody>
</table>

CB = chronic bronchitis.

The demographic and functional data of patient and control subjects are shown in table 1. Pulmonary function tests in the patients with chronic bronchitis showed mild to moderate airways obstruction. Reversibility after inhaled salbutamol was always <15%. The groups were not matched for age. The comparison of the two control subgroups showed a significant difference in recovery of BAL fluid (oldest 69 (63-98) ml v youngest subjects 93 (74-107) ml, p=0.02) which was not related to differences in respiratory function (in both groups the mean FEV₁ was 91% predicted). Considering the whole case series, recovery of BAL fluid was reduced in patients with chronic bronchitis (70 (42-108) ml compared with the control group (86 (63-114) ml, p=0.02). However, when the five elderly control subjects and the patients with chronic bronchitis were compared, there were no differences in BAL fluid recovery. The highly significant difference in FEV₁, between controls and patients with chronic bronchitis (table 1) suggests that age was the main factor responsible for the reduction in recoveries.

Cellular components were not influenced by age. Cell counts were superimposable (table 2). Viability was similar in the two groups: controls 98 (91-99)%, chronic bronchitis 95 (92-98)%. The differential cell counts revealed significant differences in the percentage of neutrophils (table 2) and absolute values (chronic bronchitis 5.2 (2.6-31.9) ml × 10⁶, controls 2.1 (1.1-8.5) ml × 10⁶, p<0.001). Although these differences were accounted for mainly by the first recovery, representative of the bronchial environment, a significantly higher percentage of neutrophils was also present in the second and third recoveries from the patients with chronic bronchitis (table 2). The number of macrophages was significantly reduced in the first recovery sample (table 2). Basophil cell counts were significantly reduced in the subjects with chronic bronchitis (table 2). Surprisingly, the percentage of bronchial epithelial cells in the first recovery was similar in the patients with chronic bronchitis (1.8 (0.5-9.5)) and the control subjects (2.3 (0-5-3.5)).

Absolute, but not standardised values of protein components tended to be influenced by age but not significantly. Total proteins and

Phagocytosis

To obtain data that would most closely reflect findings in vivo the entire BAL cell population was used for the measurement of phagocytosis. The BAL cells were rinsed three times in phosphate buffered saline (PBS) and then suspended in RPMI 1640 + 10% fetal calf serum (FCS, Boehringer Mannheim GmbH, Mannheim, Germany). Viability was assessed using the trypan blue dye exclusion test. Superoxide anion release was assessed both at baseline and after opsonised zymosan phagocytosis as superoxide dismutase (SOD) inhibitable reduction of ferricytochrome C according to a modified Bellwitt’s assay. Four sterile test tubes labeled 1A, 1B, 2A, 2B were prepared for each cell suspension with 400 μl of a Krebs Ringer phosphate (KRP) buffer solution containing 0.25 mM cytochrome C and 5 mM glucose. Ten μl SOD solution (2.5 mg/ml; 3000 U/mg protein) were added to tubes 1B and 2B. Tubes 2A and 2B contained zymosan, 1 g/l, opsonised with group AB human serum (10%) in a thermostat bath at 37°C for 15 minutes to evaluate the phagocytic activity. Tubes 1A and 1B, containing no zymosan, were used to evaluate the basal production of superoxide anion. Following the addition of 100 μl of alveolar cell suspension the tubes were incubated in a water bath for 10 minutes at 37°C with continuous shaking (about 100 rpm). The reaction was stopped with 2 ml of ice cold KRP buffer. The tubes were centrifuged at 1500g for 10 minutes. The absorbance of cell-free supernatants was measured at 550 nm and data were multiplied by the dilution factor and divided by the extinction coefficient (μmol/l) of cytochrome C determined at 550 nm (0.0189).

The difference between the values of O₂⁻ production with and without zymosan is reported as a phagocytic index. O₂⁻ production was expressed in nM O₂⁻/10⁶ 10 minutes.

**Statistical analysis**

All data are reported as median (range). Differences in BAL fluid data were evaluated according to the Mann-Whitney U test as the distribution of data did not appear to be normal. Probability values greater than 8.5% were considered statistically significant. The Spearman’s rank correlation coefficient was used to study correlations between FEV₁ and BAL fluid data. Analyses were performed using a microcomputer (Macintosh LC) and Statview+Graphics software (Abacus Concept).
Table 3  Median (range) biochemical data from BAL fluid supernatants

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Chronic bronchitis</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total proteins (mg/dl)</td>
<td>8.5 (5.0-11.8)</td>
<td>9.2 (5.0-17.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Albumin (mg/dl)</td>
<td>3.0 (0.9-7.5)</td>
<td>3.2 (1.3-9.0)</td>
<td>NS</td>
</tr>
<tr>
<td>IgG/albumin</td>
<td>0.2 (0.01-0.5)</td>
<td>0.26 (0.05-1.26)</td>
<td></td>
</tr>
<tr>
<td>IgA/albumin</td>
<td>0.09 (0.004-0.7)</td>
<td>0.17 (0.018-0.6)</td>
<td>NS</td>
</tr>
<tr>
<td>IgM/albumin</td>
<td>0.08 (0.01-0.14)</td>
<td>0.04 (0.02-0.16)</td>
<td>NS</td>
</tr>
<tr>
<td>C3/albumin</td>
<td>0.034 (0.001-0.22)</td>
<td>0.04 (0.0-0.13)</td>
<td></td>
</tr>
<tr>
<td>C4/albumin</td>
<td>0.012 (0.007-0.01)</td>
<td>0.023 (0.004-0.06)</td>
<td>NS</td>
</tr>
<tr>
<td>α1-antitrypsin/albumin</td>
<td>0.033 (0.002-0.52)</td>
<td>0.031 (0.008-0.09)</td>
<td></td>
</tr>
<tr>
<td>Transferrin/albumin</td>
<td>0.125 (0.015-0.25)</td>
<td>0.071 (0.004-0.18)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Figure 1  Box whisker plots of the elastase-like activity (ELA) in bronchoalveolar lavage fluids in non-smoker patients with chronic bronchitis compared with normal controls. Since the data are not normally distributed the plots show the median, the interquartile range, and the 10th and 90th percentiles. Individual data points which lie outside the 10th and 90th percentiles are shown individually. See text for levels of significance.

Figure 2  Box whisker plots of the O2 release from the naive bronchoalveolar lavage cell population at baseline and after phagocytosis with opsonised zymosan in non-smoker patients with chronic bronchitis and normal controls. Since the data are not normally distributed the plots show the median, the interquartile range, and the 10th and 90th percentiles. Individual data points which lie outside the 10th and 90th percentiles are shown individually.

Discussion

This study was conducted to evaluate and compare the characteristics of BAL fluids in a group of subjects with chronic bronchitis who had never smoked and a group of non-smoking healthy controls.

The two groups were not matched for age and the mean age of the bronchitic group was significantly higher than that of the controls. In spite of this disparity, the only result clearly influenced by age was the amount of fluid recovered. Interestingly, bronchial obstruction did not have a major effect on reduced recovery volumes, at least in patients with mild to moderate airflow limitation. A previous study also found that bronchial obstruction was not associated with significantly lower recovery volumes.

The finding of similar cell numbers in the subjects with chronic bronchitis and the controls strongly suggests that the disease itself is not responsible for an increase in cells. The lavage fluid obtained from the subjects with chronic bronchitis differed in cellular proportions by an average 3-4 fold increase in neutrophils. The magnitude of increase was always greatest at the first recovery and lowest at the third, and the increase in percentage of neutrophils in the first recovery accounted for the reduced percentage of macrophages. Similar alterations in cell differentials have been described in smokers. Cigarette smoking is associated with a steady increase of neutrophils in the airways but, according to our results, chronic bronchitis is characterised by intraluminal neutrophilia irrespective of smoking. Smoking is one, but by no means the only, factor to cause airways inflammation associated with heightened recruitment of neutrophils. As
found in smokers with chronic bronchitis, there was a significant correlation between the number of neutrophils and the degree of bronchial obstruction. In clinically stable bronchitic patients, the number of neutrophils in the BAL fluid may serve as an index of the severity of bronchial obstruction. Such information would be useful in clinical studies — for example, for evaluating the local efficacy of anti-inflammatory treatment.

The part played by neutrophils in the pathogenesis of chronic bronchitis remains elusive. Basic alterations in peripheral neutrophil functions have been described in chronic obstructive lung disease, and a number of peripheral neutrophil functions may be increased in chronic bronchitis.18 It would also be interesting to evaluate the release of chemotractant agents from the mucosal mononuclear cells which predominate over the other types of inflammatory cells in the bronchial walls of smokers,22 with a view to elucidating the features of activation.19 Inactivation of chemotactic factor inhibitor has also been described, but in association with tobacco smoke.23

What then is the status of alveolar macrophages in patients with chronic bronchitis who have never smoked? Macrophages were not increased, and were morphologically identical to those in the controls when studied by optical microscopy. Functional studies of the entire cell population (O2 release and phagocytosis) were not consistent with a state of activation; however, chemotactic factors were not directly assessed.

The increase in proteases in cells could, theoretically, cause damage to the bronchial mucosa. However, the quantity of elastase-like activity released into fluids was similar in the patients with chronic bronchitis and the healthy controls. In the light of the characteristics of extracellular proteolytic processes, proteases stored in resting phagocytic cells may be more important than the aliquot released into the alveolar lining fluid which can easily be neutralised by α1-antitrypsin.19 25

Superoxide anion, another important cause of damage to the mucosal cells, was increased both at baseline and after phagocytic stimulation. This increase was not significant despite the augmentation of viable neutrophils. Mucus in the airways can inhibit macrophage phagocytosis.29 Bronchodilator therapy should also be taken into account, as methylxanthines and β2 agonists have anti-inflammatory effects not only in vitro but also in vivo.15 27 The comparison of BAL fluid data from the patients who were taking theophylline and those who were not revealed no differences. On the other hand, other factors may have triggered macrophage and neutrophil activities. Secretions in patients with chronic bronchitis are frequently contaminated by bacteria28 whose products can stimulate phagocytic cells.

The shedding of epithelial cells can be held as a marker of damage. In keeping with others,30 we observed no increase in ciliated epithelial cells in BAL fluid samples. The differences between the percentage of ciliated cells identified in this study and previously reported are likely due to the technique, for example, and enhanced response to chemotactic factors and extracellular proteolysis.19 Recent data from patients with chronic bronchitis showed that increased expression of adhesion molecules in the bronchial mucosal vessels was associated with tissue neutrophils,31 but we do not know whether they are the cause or the effect of the anatomical and functional damage. Thompson et al found that epithelial cells from the submucosal glands in the large airways of bovines can release increased quantities of neutrophil chemotactic factor when stimulated by endotoxin.21 Functional studies in smokers have shown that alveolar macrophages release more chemotactic factors responsible for neutrophil recruitment.14 It would also be interesting to evaluate the release of chemotactic agents from the mucosal mononuclear cells which predominate over the other types of inflammatory cells in the bronchial walls of smokers,22 with a view to elucidating the features of activation.23 Inactivation of chemotactic factor inhibitor has also been described, but in association with tobacco smoke.23

Although the interpretation of biochemical data in BAL fluid remains controversial,30 chronic bronchitis does not seem to be associated with a quantitative defect of IgA secretion. Furthermore, the cellular immune defence mechanisms, and phagocytosis in particular, as demonstrated by the superoxide anion release after challenge with opsonised zymosan, were not depressed in our patients with chronic bronchitis. Even in smokers the data on lung immune defences and their clinical relevance are controversial.10

Our data on immune defences in patients with chronic bronchitis who had never smoked agree with previous observations that, with the exception of a limited number of subjects who are affected by inherited disorders of definite immune components, chronic bronchitis is not associated with a defect but rather with an enhancement of local immune defences in response to a failure of other non-immune defence mechanisms such as cough and mucociliary clearance.31 It is unlikely that an absolute defect in local immune defences plays a crucial part in the pathogenesis of chronic bronchitis or in recurrent exacerbations. Functional defects are more plausible25 but need to be substantiated by further studies.
In summary, although only a few patients with chronic bronchitis are non-smokers, the characterization of intraluminal cells and soluble factors compared with normal controls without the confounding effect of smoking can lead us to conclude that local immune defences (Ig, phagocytosis) are not significantly different, the global oxidant burden is not increased, and the elastase-like activity content of alveolar phagocytic cells is increased, but the component released in the alveolar milieu is within the normal range. The increase in protease burden may be an important cause of mucosal damage even in clinically stable patients. Finally, bronchial neutrophilia was the most significant characteristic in our patients and correlated with airways obstruction. Thus, at the bronchial level luminal signs of inflammation in patients with chronic bronchitis correlate with functional impairment, irrespective of smoking habits.

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