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Neutrophil Alveolitis in Bronchioloalveolar Carcinoma

Induction by Tumor-Derived Interleukin-8 and Relation to Clinical Outcome

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Tumor infiltrate, predominantly constituted by lymphocytes, may represent an important prognostic factor in bronchioloalveolar carcinoma (BAC), in addition to tumor extension and histological type. In the present study, we determined the presence, the origin, and the prognostic importance of neutrophils that also participate in leukocyte infiltrates of BAC. Neutrophil alveolitis was determined immunohistochemically in both lung biopsies and bronchoalveolar lavage (BAL) fluid samples from 29 patients with histologically proved BAC. The local expression of interleukin (IL)-8 was determined by immunohistochemical and immunoenzymatic techniques. Neutrophil counts were analyzed in relation to the clinical outcome of patients by the Kaplan-Meier method and Cox's univariate and stepwise multivariate models. Lymphocytes and neutrophils dominated the inflammatory cell population in the lower respiratory tract of patients with BAC. Neutrophils were located mainly in the alveolar lumen and seldom in alveolar wall whereas lymphocytes were exclusively present in alveolar wall. A relationship was observed between the number of neutrophils and the level of IL-8 in BAL fluid suggesting the involvement of that chemokine in neutrophil recruitment. The tumor cells were the predominant cells that appeared to express IL-8 by immunolocalization. The presence of increased numbers of neutrophils was significantly associated with a poorer outcome in patients with BAC ($P = 0.02$). In a multivariate analysis, the neutrophil percentage in BAL fluid was an independent predictor of clinical outcome. The risk of death was increased substantially (rate ratio, 5.2; 95% confidence interval, 1.1 to 24.7) among patients with BAL neutrophil percentage of ≥39% (median of the distribution) as compared with the others. In BAC, neutrophils accumulate in the alveolar lumen. Elaboration of IL-8 by tumor cells may be responsible for this event, which is associated with a significantly higher risk of death. ***(Am J Pathol 1998, 152:83–92)***

Lung carcinoma is the first cause of cancer-related death in industrialized countries.[1] Its incidence has been increasing over the last several decades mainly because of the increased occurrence of adenocarcinoma, including bronchioloalveolar carcinoma (BAC).[2–5] BAC is defined as a peripheral and well differentiated adenocarcinoma growing along alveolar walls and sparing bronchial structures. This tumor differs from the other lung adenocarcinomas by salient features such as its occurrence unrelated to tobacco smoking use,[6] its luminal spreading without lymphatic and blood vascular extension,[7] and its marked resistance to both chemotherapy and radiotherapy.[8]

The clinical course of BAC mainly depends on its histological type (ie, mucinous or nonmucinous) and on its extension within the lung, which determines whether surgical resection is possible.[9–11] Thus, outcome is poor in diffuse lesions (less than 10% 5-year survival) as compared with solitary nodules (greater than 60% survival).[9,10] In fact, histological type and pulmonary extension are linked prognosis factors. Indeed, solitary lesions are predominantly nonmucinous tumors whereas diffuse lesions tend to be of the mucinous type.[2,9–11] Irrespective of histological type and pulmonary extension, stroma reaction features also have a prognostic value in BAC. Among them, tumor fibrosis or lymphocytic interstitial infiltration could influence prognosis.[9,10,12,13]

In the present study we described neutrophil alveolitis as a new feature of the BAC stroma reaction. This led us to assess the role of a neutrophil chemoattractant factor,

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Table 1. Clinical and Radiological Characteristics of BAC Patients (n = 29)

	Number of cases	(%)
Clinical symptoms		
Dyspnea	12	(41%)
Bronchorrhea*	8	(28%)
Weight loss	8	(28%)
None	6	(21%)
Radiological aspect		
Aerogenous pattern†	23	(79%)
Solitary nodule	6	(21%)
Radiological extension		
Unilateral	16	(55%)
Bilateral	13	(45%)
TNM classification‡		
T2 N0/N1 M0	5	(17%)
T2 N2 M0	1	(3%)
Tx N0/N1 M0	10	(34%)
Tx N0/N2 M1	13	(46%)

*Bronchorrhea was defined according to its abundance (volume >30 ml per day) and mucoid aspect.
†Aerogenous pattern was defined as diffuse, ill defined pneumonic or multifocal opacities.
‡TNM staging adapted to BAC.[16]

interleukin (IL)-8 in the mechanism of tumor neutrophil recruitment and activation and to determine whether the presence of neutrophils is a predictor of the clinical behavior of BAC.

Materials and Methods

Study Populations

Patients with Bronchioloalveolar Carcinoma (BAC)

Clinical and radiological data of all histologically proved BAC (n = 29) were collected in our chest department from 1986 to 1995. The diagnosis of BAC was based on the following previously published criteria[2,8–12,14]: 1) absence of present or past history of primary bronchogenic adenocarcinoma or extrathoracic malignancies and 2) presence of peripheral well differentiated adenocarcinoma growing along alveolar wall without major modification of the interstitial framework of lung. Histological proof was obtained by open lung biopsies (OLBs; n = 14), transthoracic or transbronchial biopsies (n = 15). Bronchoalveolar lavage (BAL) was performed as a diagnostic tool in 17 patients.[15] Lavage was carried out in the radiologically abnormal segment or lobe. Tumor cell papillary clusters were observed in nine cases. Total and/or differential inflammatory cell counts were provided. Biopsy and BAL specimens were run in parallel for bacterial, fungal, and parasitic microorganisms.

Patients were 17 men and 12 women, 63 ± 11 years old (mean ± SD; range, 34 to 81 years). There were 14 smokers and 15 nonsmokers. Clinical and radiological presentations are given in Table 1. Bronchorrhea was defined as mucoid sputum the volume of which was greater than 30 ml per day. We classified the patients (Table 1) according to tumor node metastasis (TNM) staging adapted to BAC[16] in which T_2 is used for a solitary nodule more than 3 cm or multiple nodules less than 3 cm in the same lobe, T_x for diffuse unilateral infiltrate, and M_1 for bilateral nodules or infiltrates. None of the patients had evidence for extrapulmonary metastases at the time of diagnosis.

All cytological and histological samples were taken to ensure diagnosis. After the diagnosis of BAC had been made, paraffin-embedded tissue sections were usually kept and BAL fluid supernatants were stored frozen at −70°C. No additional samples were collected especially for the study. Data were analyzed anonymously.

Control Subjects

We used data from five healthy volunteers evaluated in the course of a previous study (two men and three women; age, 29 ± 4 years; two smokers and three nonsmokers).[17] None had a history of pulmonary or neoplastic disease. BAL was carried out in the middle lobe.

IL-8 and Neutrophil Elastase Detection in BAL Fluid

IL-8 and neutrophil elastase concentrations were directly measured in BAL fluid (n = 9). The presence of ENA-78 and GRO-α in BAL fluid was also evaluated in parallel to IL-8. IL-8, ENA-78, and GRO-α concentrations were determined using a human-specific solid phase sandwich enzyme immunoassay technique (ELISA; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The sensitivity of the ELISA kits permits the detection of IL-8, ENA-78, and GRO-α concentrations in a linear range from 31 to 2000 pg/ml. BAL fluid concentrations of neutrophil-free and complexed elastase were measured using a commercially available immunoenzymatic assay kit (IMAC kit, Merck, Darmstadt, Germany). Results for IL-8, ENA-78, and GRO-α were expressed as nanograms per milliliter of either total BAL fluid or epithelial lining fluid (ELF) volume. Results of elastase concentrations were expressed as micrograms per milliliter. ELF volume was determined using the urea method.[18] Urea was measured by an enzymatic method with urease.[19]

Histological Techniques and Analyses

Samples of lung tumor were fixed in Bouin's solution and then embedded in paraffin or methacrylate. Five-micron sections were stained using hematoxylin-eosin-safran (HES), Masson's trichrome, or periodic acid-Schiff-diastase methods. Standard staining was carried out for evaluation of infectious pathogens in all histological samples.

For 14 patients with OLBs, the histological type of BAC was defined as mucinous (n = 5) and nonmucinous (n = 9) according to Clayton's criteria.[9,10]

Features of the stroma reaction and tumor cells were particularly studied. Inflammatory cells (lymphoid and plasma cells, macrophages, and neutrophils) were identified on morphological criteria, and their localization in the alveolar lumen or the alveolar wall was noted. The lymphoid lineage of mononuclear cells was verified in some experiments by an immunohistochemical method

using anti-CD3 (T lymphocytes), -CD20 or -CD79 (B lymphocytes and plasma cells), and -CD68 (monocyte/macrophage cells) antibodies.[12] Inflammatory cell number was estimated according to a method adapted from previously described techniques.[12,20] Briefly, cells were counted by using a square, cross-hatched grid measuring 1 × 1 mm mounted in a 100× microscopic eyepiece, under a 400× objective. Thirty-two nonoverlapping grid fields were examined on two slides in representative areas of a given tumor (ie, a total of 64 fields per patient). Inflammatory cells obviously present in vessels were not taken into account. Results were expressed as the number of inflammatory cells per field.

Interstitial fibrosis and necrosis were also graded by a semiquantitative method. The interstitial fibrosis was estimated by Sirius red staining and graded from 0 to +++: 0, tumor alveolar wall was not enlarged, as compared with normal alveolar wall, and + for slight, ++ for moderate, and +++ for marked alveolar wall enlargement. The alveolar wall necrosis was estimated by examining all slides containing tumor infiltration (2 to 14 slides per tumor) graded as follows: 0, absence of necrosis; +, area of alveolar wall necrosis approximately equal to a 20× objective field; ++, area of alveolar wall necrosis approximately equal to a 10× objective field; and +++, area of alveolar wall necrosis approximately equal to a 4× objective field.[21]

The angiogenesis was first estimated by identifying vascular sections according to morphological criteria, and two situations were retained: either presence or absence of angiogenesis. The degree of angiogenesis was then quantitatively determined by an immunohistochemical method using anti-CD34 antibody (DAKO, Trappes, France) as previously described.[22] Briefly, a representative section of the tumor was first scanned at low magnification (×40) to identify areas of high neovascularization. Vascular sections, defined as a single cell or clustering cells clearly separated from the others and stained positively with anti-CD34 antibody, were then counted at high magnification (×400) in three consecutive fields of these areas (a field equal to 0.307 mm^2 at ×400). Results were expressed as the average number of capillary sections per field in the three fields.

IL-8 Immunolocalization in Tissue Samples

The expression of IL-8 was tested by immunohistochemistry in OLB tissue (n = 14), in transbronchial and transthoracic biopsies (n = 15), and in BAL cells (n = 5). The A549 human BAC cell line[23] (American Type Culture Collection, Rockville MD) served as control.[24] The tissue sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol. Cytospin BAL cell slides and A549 cell monolayers were fixed in 70% ethanol at 4°C for 20 minutes and then dried for 1 hour. All samples were processed for immunochemical study according to a previously described technique.[24] Sections incubated with a polyclonal rabbit anti-human IL-8 antibody (Peprotech, Rocky Hill, NJ) were reacted with a biotinylated secondary antibody (DAKO) and peroxidase-conjugated streptavidin (DAKO). Positive cells were revealed by reaction with the subtrate chromogen (diaminobenzidine and hydrogen peroxide). In tissue sections obtained from OLB, IL-8 immunoreactivity was graded from 0 to +++, by comparison with the anti-carcinoembryonic antigen (CEA) (DAKO) staining intensity of tumor cells (0, no expression of IL-8, and +, ++, and +++, IL-8 staining intensity less, similar, and more than CEA expression, respectively).

Statistical Analysis

Comparisons between cases and controls for quantitative and qualitative variables were made using the Mann-Whitney nonparametric test and Fisher's exact test, respectively. Data are presented as the mean ± SEM and *P* value. Spearman's ρ coefficient was used for correlation studies between quantitative variables. For semiquantitative analysis, findings expressed as 0 to +++ were distributed in only two groups because of the small size of the sample: a group referred to as low grade for 0 and + and a group referred to as high grade for ++ and +++. Quantitative variables, namely, the percentage of neutrophils in BAL and the density of vascular section, were also coded as dichotomous (low level and high level), and the cut-off values were the medians of distributions (39% neutrophils and 59 capillary sections per field, respectively). The Kaplan-Meier method was used for computing survival probabilities. Cox's proportional hazards regression univariate model was used for computing risk ratios. A stepwise multivariate selection algorithm was used to determine which variables were independently and significantly associated with survival. Because of the limited number of patients, no more than three variables were submitted to the models. For the three submitted variables, *P* value, risk ratio, and its 95% confidence interval were tabulated. The two-tailed level of significance for all tests was 0.05. Data were processed by StatView and Survival tools F-4.11 (Abacus Concepts, Berkeley, CA).

Results

Neutrophil Alveolitis Is Observed in BAC

Total BAL cell number was increased in patients with BAC as compared with control subjects. This resulted mainly from a dramatic increase in total (252 ± 85 *versus* 2 ± 0.4 cells/μl; $P = 0.047$) and differential (37 ± 7% *versus* 1.2 ± 0.2%; $P = 0.031$) neutrophil counts (Table 2). In 10 of 17 patients, the neutrophil percentage was more than 30% (Table 2). Neutrophils were morphologically unaltered, and no evidence of bacterial infection was observed (Figure 1A). Macrophage and lymphocyte counts did not differ between patients and controls (Table 2).

The degree of the neutrophil alveolitis was not related to tobacco use, clinical symptoms, radiological features, or histological type of the tumor. In addition, there was no correlation between blood and alveolar neutrophil counts (data not shown). Finally, there was no significant association between the presence or the absence of tumor cells in BAL fluid and the extent of neutrophil alveolitis.

Table 2. Cytological Analysis of BAL from BAC Patients (n = 17) and Controls (n = 5)

Patient	Cells/μl*	Macrophages		Lymphocytes		Neutrophils	
		%	Number per μl*	%	Number per μl*	%	Number per μl*
5	ND	14	ND	13	ND	69	ND
9	ND	18	ND	2	ND	80	ND
10	ND	85	ND	15	ND	0	ND
12	ND	2	ND	4	ND	94	ND
15	ND	90	ND	7	ND	3	ND
17	ND	58	ND	12	ND	30	ND
18	310	28	87	5	15	67	208
19	1070	29	310	0	0	70	749
20	820	59	484	2	16	39	320
21	600	81	486	19	114	0	0
22	160	86	138	6	10	6	10
23	470	37	174	1	5	61	287
24	1760	49	862	2	35	39	686
25	210	99	208	1	2	0	0
26	1620	69	1118	9	146	22	356
27	94	89	84	4	4	7	7
29	240	53	127	2	5	45	108
Mean ± SEM	704 ± 57	56 ± 7	399 ± 105	6 ± 1	32 ± 15	37 ± 7	252 ± 85
Controls							
Mean ± SEM	173 ± 57	90 ± 3	161 ± 56	8 ± 3	9 ± 2	1.2 ± 0.2	2.0 ± 0.4
P value†	0.047	0.015	NS	NS	NS	0.031	0.047

ND, not determined; NS, not significant.
*Count per microliter of total BAL fluid.
†By Mann-Whitney test.

Results of histological studies confirmed the presence of neutrophils in the tumor areas (Table 3). By contrast, neutrophils were absent from the adjacent sections of normal lung (12 ± 4 *versus* 0.4 ± 0.15 cells per power field in tumor area *versus* normal lung tissue; $P = 0.0004$). In tumor areas, no bacterial infection was associated with neutrophil alveolitis as evidenced histologically. All evaluated tumor sections were also infiltrated by lymphocytic cells, which represented more than 90% of the mononuclear cell infiltrate as assessed by immunohistochemical analysis (30 ± 6 *versus* 2 ± 1 cells per power field in tumor area *versus* normal lung tissue; $P = 0.0005$; Table 3). Neutrophils were mainly located in the alveolar lumen whereas lymphocytic cells infiltrated only the tumor alveolar wall (Figure 1B). Neutrophils were sometimes also present facing denuded alveolar wall (Figure 1C). This phenomenon was associated with a shedding of tumor cells into the alveolar lumen. In general, the basal membrane was unaltered, although necrosis of alveolar wall was occasionally present. In these areas, a significant positive correlation was observed between the number of neutrophils invading the tumor alveolar wall and that of neutrophils present in the alveolar lumen ($\rho = 0.59$; $P = 0.032$). The number of neutrophils was higher in the mucinous type of BAC than in the nonmucinous type (18 ± 0.4 *versus* 8 ± 2.8; $P = 0.027$). No other histological feature, including angiogenesis, was correlated with the extent of neutrophil alveolitis (Table 3).

Neutrophil Alveolitis Is Associated with High IL-8 Levels in BAL Fluid

The neutrophil alveolitis observed in BAC led us to identify neutrophil chemotactic factors in BAL fluid. Among them, IL-8 has been considered as a major mediator implicated in the initial attraction and activation of neutrophils, especially in the lung.[25] Immunoreactive IL-8 was detectable in nonconcentrated BAL fluid in six of the nine evaluated specimens (5.63 ± 1.83 ng/ml of ELF) whereas it was undetectable in the BAL fluid of the five controls ($P = 0.013$; Figure 2A). IL-8 concentrations in BAL fluid correlated with total alveolar cell counts ($\rho = 0.81$; $P = 0.003$) and alveolar neutrophil numbers and percentages ($\rho = 0.72$; $P = 0.009$, and $\rho = 0.74$; $P = 0.007$, respectively; Figure 2B). They also correlated positively with alveolar macrophage numbers ($\rho = 0.60$; $P = 0.030$). No correlation was observed between IL-8 concentrations and alveolar lymphocyte counts in BAL fluid. To assess the involvement of other C-X-C chemokines in the alveolar recruitment of neutrophils, we determined the BAL concentrations of ENA-78 and GRO-α. Immunoreactive ENA-78 and GRO-α were detected, respectively, in nine (0.39 ± 0.16 ng/ml of total BAL fluid; range, 0.30 to 1.34) and three (0.09 ± 0.50 ng/ml of total BAL fluid; range, 0 to 0.49) BAL fluids from patients, whereas they were undetectable in BAL fluid from controls.

Neutrophil elastase levels in ELF were higher in BAC patients as compared with control subjects (6.46 ± 2.28 *versus* 0.93 ± 0.33 ng/ml ELF; $P = 0.005$; Figure 2C) and correlated with neutrophil counts ($\rho = 0.60$; $P = 0.045$). Finally, elastase and IL-8 levels in BAL fluid were highly correlated ($\rho = 0.87$; $P = 0.0025$; Figure 2D).

IL-8 Is Produced by Tumor Cells of BAC

Cells containing IL-8 were identified by immunohistochemical techniques. IL-8 staining was observed in tumor cells from all of the 14 OLB sections studied (Figure 1D). The staining, which was homogeneous and cytoplasmic, was almost entirely blocked by the incubation of

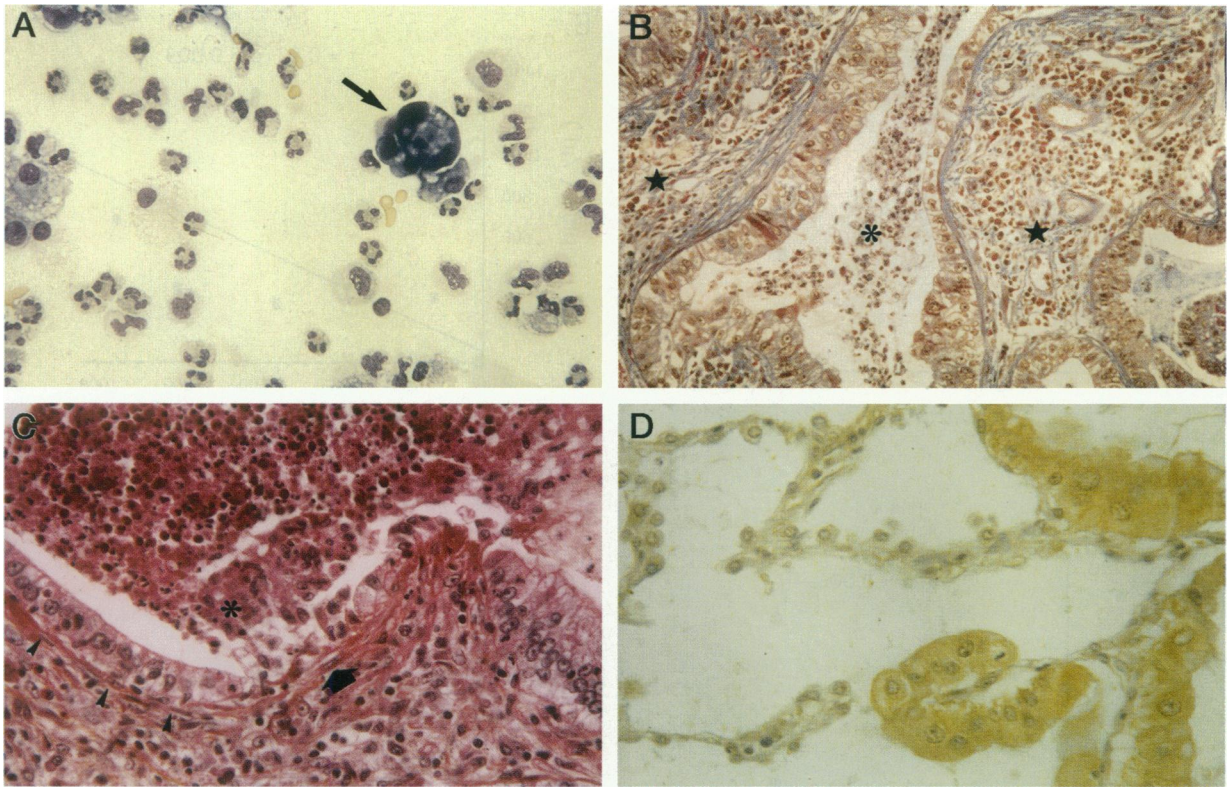

Figure 1. Cytological and histological aspects of BAC. **A:** Neutrophil alveolitis associated with tumor cell clusters (**arrow**) in BAL recovered from BAC patients (Giemsa; magnification, ×400). **B:** Representative features of stroma reaction present in most BAC (Masson's trichrome; magnification, ×200). Note that lymphoplasmacytic cells (**stars**) are located in the tumor alveolar wall whereas neutrophils (**asterisk**) are in the alveolar space. **C:** Neutrophil alveolitis facing denuded tumor alveolar wall (**arrow**) (hematoxylin-eosin-safran; magnification, ×400). Note the presence of a tumor cell cluster shed in the alveolar lumen (**asterisk**) and the absence of alteration of basal membrane (**arrowheads**). **D:** IL-8 immunoreactivity in lung tissue from a patient with BAC (magnification, ×400). Cytoplasm of tumor cells were stained brown by polyclonal IL-8 antibody (right side) whereas normal alveolar epithelial and interstitial cells were negative (left side).

IL-8 polyclonal antibody with an excess of recombinant IL-8 before its deposition on the tissue sections. IL-8 expression was sometimes observed in inflammatory cells, particularly in macrophages associated with the tumor rather than in macrophages present in alveolar lumen from the adjacent normal lung. A minority of neutrophils and plasma cells also contained IL-8. Normal alveolar epithelial cells, endothelial cells, and fibroblasts did not exhibit any IL-8 immunostaining (Figure 1D). Analysis of BAL cytospin slides (n = 5) and transthoracic or transbronchial biopsy specimens (n = 15) confirmed the IL-8 immunostaining of tumor cells. This contrasted with

Table 3. Histological Findings in Open Lung Biopsies from BAC Patients (n = 14)

Case	Histological types	Alveolar neutrophils (cells/field)*	Interstitial neutrophils (cells/field)*	Interstitial lymphocytes (cells/field)*	Angiogenesis (capillary sections per field)*	Tissue IL-8 expression grading†
1	NM	4 (0)	1 (0)	39 (2)	69 (6)	+
2	NM	3 (0)	1 (0)	11 (2)	121 (19)	++
8	M	6 (0)	0 (0)	54 (6)	29 (3)	++
10	NM	1 (0)	0 (0)	12 (1)	41 (4)	+
11	M	20 (0)	1 (0)	14 (2)	26 (6)	+++
13	NM	0 (0)	0 (0)	14 (1)	112 (6)	++
16	NM	1 (0)	0 (0)	12 (1)	47 (4)	+
19	M	32 (0)	13 (1)	77 (6)	93 (11)	++
22	M	9 (1)	1 (0)	9 (1)	45 (4)	+++
25	NM	51 (1)	11 (0)	65 (7)	54 (2)	+
26	NM	4 (0)	1 (0)	57 (7)	118 (26)	++
27	NM	7 (0)	1 (0)	31 (4)	61 (13)	++
28	NM	4 (0)	1 (0)	26 (4)	57 (1)	+
29	M	23 (1)	0 (0)	6 (1)	97 (16)	+

M, mucinous type; NM, nonmucinous type.

*Results are expressed as mean from 64 fields for neutrophil and lymphocyte counts and from 3 fields for angiogenesis. In parentheses are given the SEM for each patient count.

†IL-8 immunoreactivity was graded from 0 to +++, by comparison with the anti-carcinoembryonic antigen staining intensity of tumor cells (see Materials and Methods).

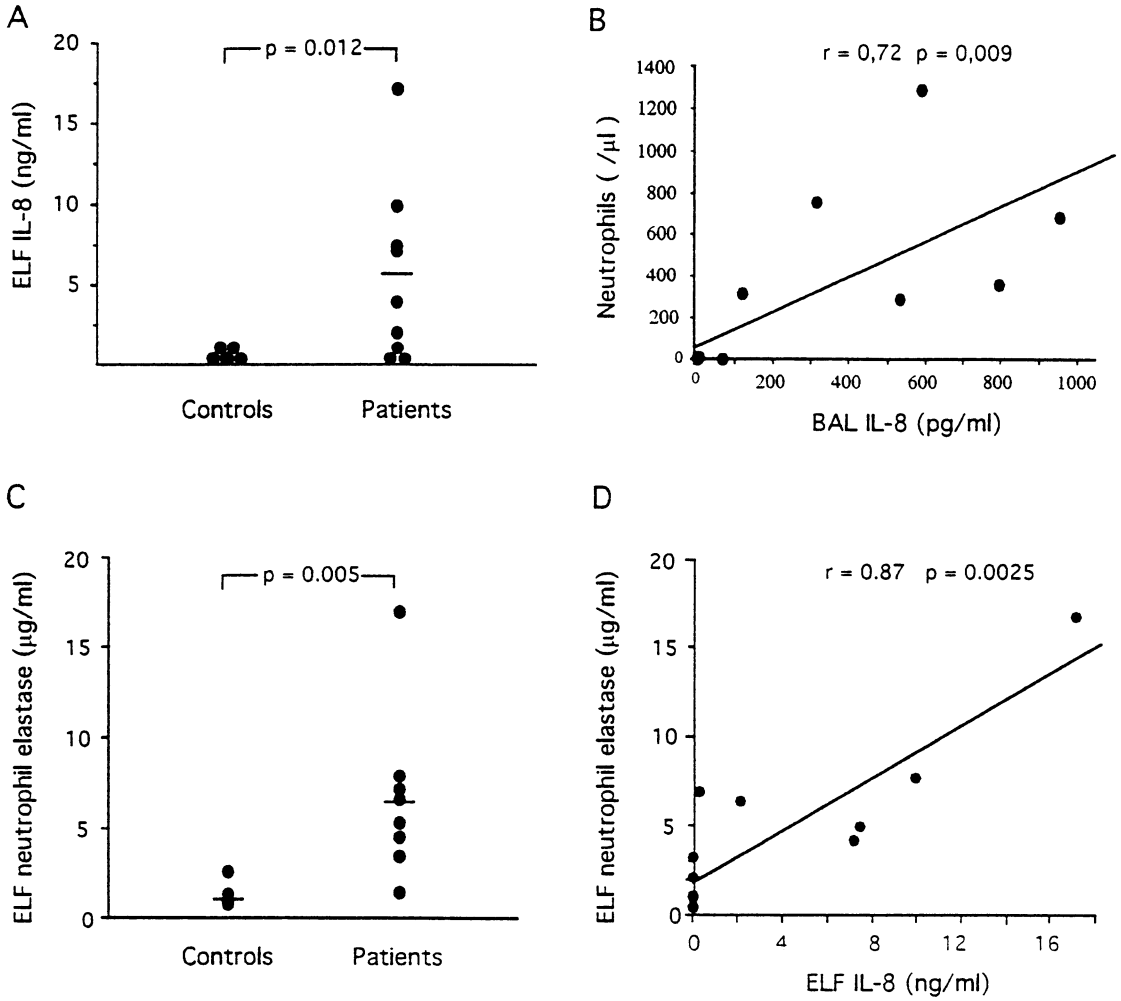

Figure 2. Concentrations of IL-8 (**A**) and neutrophil elastase (**C**) in ELF from BAC patients and controls and correlations between IL-8 concentrations and either neutrophil counts (**B**) or elastase concentrations (**D**) in BAL.

the absence or faint expression of IL-8 in macrophages and neutrophils. Finally, the IL-8 staining intensity (graded from 0 to +++) in tissue did not correlate with the different components of the stroma reaction quantitatively evaluated in OLB (Table 3).

Neutrophil Alveolitis Is Associated with a Poor Outcome in Patients with BAC

We evaluated the prognostic value of IL-8-associated neutrophil alveolitis as compared with prognostic factors described in previously published series of BAC. In our series, the overall 5-year survival rate was 15.3% with a median survival time of 10 months.

First, we conducted a univariate analysis. The patients with a high level of neutrophil alveolitis (more than 39% of neutrophils in BAL fluid, median of distribution) had a median survival time of only 3 months compared with 30 months for the patients with a low level (Figure 3A). The risk ratio was 5.2 for patients with a high level (95% confidence interval, 1.1 to 24.7; $P = 0.02$; Table 4). The prognostic impact of other clinical, radiological, and immunohistological factors that was studied by the univariate analysis of survival is given in Table 4. The prognosis was not significantly linked to TNM staging, even when clinical behavior was worse for M_1 tumors than for T_x and T_2 tumors, respectively ($P = 0.09$). Interestingly, a high grade of alveolar wall necrosis and a high degree of angiogenesis (>59 capillary sections per field, ie, the median value) were or tended to be associated with a poorer outcome (Figure 3, B and C) with a risk ratio of 5.4 and 6.4, respectively (95% confidence interval, 1.0 to 30.6 ($P = 0.03$) and 0.6 to 63.7 ($P = 0.07$), respectively). Finally, a high grade of IL-8 immunostaining in tumor cells also correlated with a poorer outcome (Figure 4D) with a risk ratio of 7.2 (95% percent confidence interval, 0.84 to 62.4; $P = 0.04$).

Second, BAL neutrophil percentage was submitted to a stepwise forward Cox regression model to establish whether the poor outcome associated with neutrophil alveolitis depended on other prognostic factors. This model included clinical and radiological data. Because OLBs were not performed in all patients with BAL, histological data could not be taken into account. In this

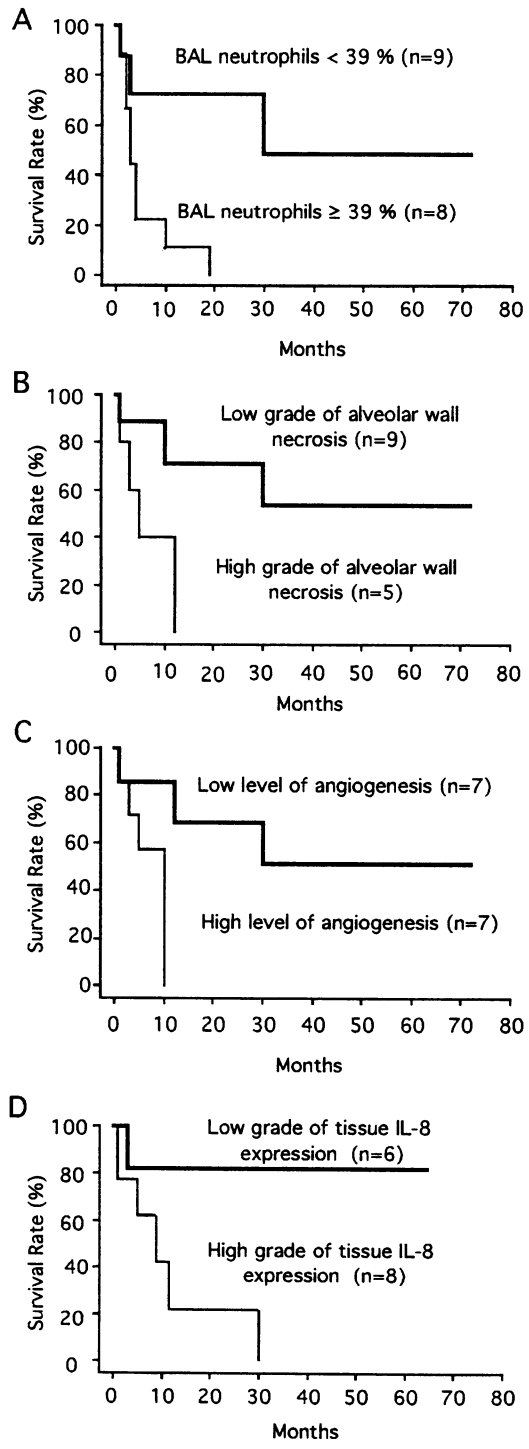

Figure 3. Kaplan-Meier analysis showing cumulative rates of survival in BAC patients according to the neutrophil percentage in BAL (**A**), the grade of alveolar wall necrosis (**B**), or the degree of angiogenesis (**C**) in tumor and the IL-8 immunostaining intensity of tumor cells (**D**). The median of BAL neutrophil percentages distribution (39%) and of angiogenesis degrees (59 capillary sections/field) determined the cut-off value for the two groups (high and low level). Patients with an elevated BAL neutrophil percentage (>39%) had a significantly less favorable outcome than patients with a lower percentage (P = 0.04). Patients with a high grade of alveolar wall necrosis had less favorable outcome than those with low grade (P = 0.03). Patients with a high degree of angiogenesis (>59 capillary sections/field) tended to have less favorable outcome than those with lower degree (P = 0.07). Patients with a high grade of IL-8 immunostaining had a less favorable outcome than those with lower grade (P = 0.045).

model, BAL neutrophil percentage was the only variable independently associated with survival (Table 5).

Discussion

The present study provides direct evidence that neutrophils are present in the alveolar lumen of BAC. It also suggests that neutrophils are locally recruited and activated at least partly by tumor-cell-derived IL-8. It finally shows that the degree of neutrophil alveolitis is a predictor of the clinical behavior of BAC.

Cell analysis of BAL fluid recovered from patients with BAC clearly demonstrated the presence of neutrophil alveolitis (Table 2). This alveolitis was confirmed by histological studies that demonstrated the presence of neutrophils in alveolar lumen beside that of lymphocytic cells in alveolar walls (Table 3). That such a neutrophil alveolitis has not been previously described in BAC might be due to several reasons. First, in general, BAL fluid is recovered from patients to identify tumor cells shedding into the alveolar lumen and, hence, to achieve diagnosis. So far, little attention had been paid to the immune/inflammatory cells also recovered. Second, because histological studies evaluated the stroma reaction in BAC,[9,10,12,13] these analyses focused on cell modifications within the alveolar wall and not within the alveolar lumen.

IL-8 is the best known member of the C-X-C family of peptides that are able to attract and activate neutrophils. This chemokine has been implicated in the neutrophil alveolitis observed in a wide range of pulmonary diseases, such as bacterial pneumonia, pulmonary fibrosis, and adult respiratory distress syndrome.[25] The strong correlation observed between the number of neutrophils and the concentration of IL-8 in BAL fluid from patients with BAC strongly suggests a role for this cytokine in the local recruitment of neutrophils. The observation that IL-8 concentrations in ELF correlated with neutrophil elastase concentrations indicates that the detected immunoreactive cytokine may have *in vivo* a biological activity on the recruited neutrophils. Indeed, elastase is a serine protease that is specifically secreted by neutrophils in response to very low IL-8 concentrations.[25] The possibility that other tumor-derived C-X-C chemokines also participate in neutrophil alveolitis could not be excluded as ENA-78 and GRO-α were also recovered in large amounts in BAL fluid from some of our patients.

It is reasonable to hypothesize that BAL fluid IL-8 is mainly released from tumor cells as IL-8 was detected by immunohistochemistry in tumor cells from all of the 14 tested OLBs and neutrophils were present only in tumor areas and not in the adjacent normal lung tissue. Furthermore, the intense and homogeneous cytoplasmic staining of tumor cells suggests that these cells produced IL-8 rather than that they engulfed extracellular IL-8. These findings are in line with the observation that BAC-derived A549 cells produce IL-8 *in vitro*.[24] A similar IL-8 staining of tumor cells has also been described in some cases of primary lung adenocarcinoma.[26] The possibility that other cell types, especially macrophages, might partici-

Table 4. Univariate Survival Analysis from BAC Patients

Variable	Patients at risk	Deaths	Median survival (months)	*P* Value	RR (95% CI)
Bronchorrhea					
No	21	12	15	0.01	3.1 (1.2–8.0)
Yes	8	8	3		
Surgery					
Yes	14	7	15	0.04	2.5 (1.0–6.4)
No	15	13	4		
Radiological aspect					
Solitary nodule	6	2	30	0.06	3.6 (0.8–15.8)
Aerogenous aspect	23	18	5		
Radiological extension					
Unilateral	16	9	15	0.07	2.2 (0.9–5.4)
Bilateral	13	11	4		
BAL neutrophils*					
Low level	8	3	30	0.02	5.2 (1.1–24.7)
High level	9	9	3		
Histological type					
Non mucinous	9	2	NA	0.04	4.8 (0.9–25.0)
Mucinous	5	5	10		
Alveolar wall necrosis†					
Low grade	9	3	NA	0.03	5.4 (1.0–30.6)
High grade	5	4	5		
Angiogenesis*‡					
Low level	7	3	NA	0.07	6.4 (0.6–63.7)
High level	7	4	10		
Tissue IL-8 expression†					
Low grade	6	1	NA	0.04	7.2 (0.8–62.4)
High grade	8	6	10		

The *P* value was obtained by log rank test, and Cox's model was used for computing risk ratio with 95% confidence interval (CI). RR, risk ratio; NA, not applicable, as more than 50% of the patients survived.

*These variables were coded as dichotomous; the cut-off value was the median of the distribution.

†Alveolar wall necrosis and tissue IL-8 expression were initially coted in four classes, 0 to +++, and three classes, + to +++, respectively, according to intensity and then expressed for statistical analysis in two groups: low grade for 0 and + and high grade for ++ and +++.

‡Angiogenesis was expressed in number of capillary sections per field.

pate in the alveolar production of IL-8 cannot be excluded. Indeed, a positive correlation was observed between IL-8 concentrations in BAL fluid and macrophage numbers. Additionally, a faint IL-8 immunostaining of macrophages was occasionally observed, whereas normal type II epithelial cells, endothelial cells, smooth muscle cells, and fibroblasts were not stained.

The severity of neutrophil alveolitis was associated with a very poor prognosis as the risk ratio of death for the patients with the highest neutrophil percentages in BAL was 5.2 (95% confidence interval, 1.1 to 24.7; $P = 0.02$). Neutrophils recruited in the alveolar space and activated by IL-8 may play a direct role in the clinical outcome of BAC through the release of many mediators. For instance, neutrophil elastase, which exhibits a secretagogue activity toward airway epithelial cells,[27] might be responsible for bronchorrhea. In patients with advanced BAC, this hypersecretion results in asphyxia by drowning. In this context, bronchorrhea was found to be a prognostic factor dependent on BAL neutrophil percentage, which also correlated with high BAL elastase levels. Similarly, mediators produced by neutrophils (ie, neutrophil elastase and reactive oxygen intermediates)[28] might promote alveolar wall necrosis, a feature also associated with a poorer outcome in our series (Table 4). Alternatively, these mediators could favor shedding and dispersion of tumor cells throughout the alveolar space by inducing deformation of the cytoskeleton and redistribution of integrin receptors from the basolateral to apical cell surface, two mechanisms involved in epithelial cell motility.[28–30] Indeed, neutrophils facing denuded alveolar wall were observed, and in these cases, isolated tumor cells or cell islets were also present within the alveolar lumen.

Table 5. Multivariate Survival Analysis by Stepwise Cox Regression from BAC Patients

Model	*P* Value	RR (95% CI)
High level of BAL neutrophils*	0.04	5.2 (1.1–24.7)
Bronchorrhea	0.65	
Radiological aerogenous pattern	0.60	

The *P* value was obtained by log rank test, and Cox's model was used for computing risk ratio with 95% confidence interval (CI). RR, risk ratio.

*BAL neutrophils were coded as dichotomous; the cut-off value was the median of the distribution.

Besides its involvement in neutrophil recruitment and activation, IL-8 could participate directly in tumor progression by two mechanisms: the proliferation of tumor cells and/or the induction of stroma reaction (angiogenesis, fibrosis, or inflammatory cell infiltration). No prognostic value was associated with sclerosing fibrosis and interstitial lymphocytic infiltrate, although these components of the stroma reaction have been recently sug-

gested to predict the course of nonmucinous BAC.[9,10,12,13] Even though IL-8 has been shown to have a mitogenic activity on human melanoma cell lines,[31] Arenberg et al[32] did not abrogate the proliferation of BAC-derived A549 cells by using anti-IL-8 neutralizing antibodies. Thus, this role of IL-8 in the progression of BAC is unlikely. Alternatively, it has been suggested that IL-8 may promote non-small-cell lung cancer spreading by its potent angiogenetic properties, favoring oxygen and nutrient diffusion to the tumor. Smith et al[26] have found large amounts of IL-8 in primary lung carcinoma homogenates. These homogenates had an *in vitro* angiogenetic activity that was partially blocked by the use of anti-IL-8 neutralizing antibodies. Furthermore, passive IL-8-immunization of tumor-implanted SCID mice resulted in more than 40% reduction in tumor size, with a decrease in tumor-associated vascular density and angiogenetic activity.[32] These data further substantiated that the presence of high levels of angiogenesis was associated with a higher risk of death for BAC patients (6.4; 95% confidence interval, 0.6 to 63.7). That angiogenesis had a poor clinical prognostic value in our series but did not correlate with IL-8 staining intensity is not contradictory with the results of previous studies mentioned above. First, even if IL-8 exhibits angiogenic activity, a large number of other factors produced by tumor cells or cells from the host stroma reaction could also regulate angiogenesis.[33] Second, BAC is a well differentiated adenocarcinoma, characterized by the integrity of the basal membrane and the persistence of tumor cell polarization.[7,8] Thus, tumor-derived IL-8 could interact preferentially with cells present in the alveolar lumen rather than with cells in the vascular bed and hence could affect the outcome of BAC patients favoring tumor cells spreading along the alveolar space rather than through the vascular bed.[7,8]

In summary, our results support the notion that neutrophils participate in tumor infiltrates of BAC and suggest that tumor production of IL-8 might be crucial for the migration and the activation of these cells. Because the increased number of neutrophils in tumors is associated with a significantly poorer outcome, the development of therapies that attenuate the production of IL-8 by BAC might be beneficial for these patients.

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