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Angiostatic versus angiogenic chemokines in IPF and EAA

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Summary
Background: Extrinsic allergic alveolitis (EAA) and idiopathic pulmonary fibrosis (IPF) share the presence of varying degree interstitial involvement and fibrosis. Vascular changes were often reported to accompany the development of fibrosis.
Objectives: The aim of our study was to examine the differences in angiostatic and angiogenic chemokine milieu in both diseases. Correlations between chemokine levels in bronchoalveolar lavage fluid (BALF), expression of chemokine receptors on CD4+ T cells (CXCR2, CXCR3) in BALF and HRCT pattern of the diseases were investigated.
Methods: Sixteen patients with chronic EAA and 8 with IPF were enrolled to the study. Concentrations of interleukin (IL)-8, epithelial neutrophil activating protein (ENA)-78, interferon-γ-inducible protein (IP)-10 and interferon-inducible T cell alpha chemoattractant (I-TAC) in BALF supernatants were quantified using Fluorokine MultiAnalyte profiling.
Results: There was no significant difference in the BALF chemokine levels between the EAA and IPF group. IL-8 BALF concentrations correlate with the extent of fibrosis in both EAA and IPF ($p < 0.01$). The IP-10 BALF concentrations do not correlate either with the HRCT alveolar or interstitial score and should be evaluated in the relationship with the disease course.
Conclusions: Both IL-8 and ENA-78 probably play a different role in IPF and chronic EAA pathogenesis. While we suggest ENA-78 as the marker of at least partial reversibility of the lung

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impairment in the EAA patients, IL-8 could be rather an indicator of continuous exposition to provoking agent in EAA patients. IL-8 might serve as a potential marker of early phase of IPF.

Introduction

Extrinsic allergic alveolitis (EAA, hypersensitivity pneumonitis) and idiopathic pulmonary fibrosis (IPF) are interstitial lung diseases. They have different etiology and clinical features, but each of them may lead to end-stage fibrosis. EAA and IPF share a common feature — the presence of varying degree of alveolar and interstitial involvement and fibrosis, which may be quantified with the means of high resolution computed tomography (HRCT). Angiogenesis was found to be a critical biological event that accompanies fibroplasia and deposition of extracellular matrix in the lung in the course of pulmonary fibrosis.[1] It is regulated by both angiogenic and angiostatic factors. The large family of CXC chemokines family (four conserved cysteine amino acid residues with the first two cysteines separated by one nonconserved amino acid residue) contains members that exhibit angiogenic and angiostatic activity. Interleukin 8 (IL-8/CXCL8) and epithelial neutrophil activating protein (ENA)-78 (ENA-78/CXCL5) are the angiogenic members. They share the same receptor CXCR2. Angiostatic CXC chemokines include platelet factor-4/CXCL4, monokine induced by interferon (IFN)-γ (MIG/CXCL9), IFN-γ-inducible protein 10 (IP-10/CXCL10) and interferon-inducible T cell alpha chemoattractant (I-TAC/CXCL11). These angiostatic CXC chemokines specifically bind to the receptor CXCR3.[2] Both angiostatic and angiogenic chemokines were documented to have pivotal role in pathogenesis of IPF.[3] Angiostatic and angiogenic chemokines are also known to play important role in the development of fibrosis in EAA patients.[4] The aim of our study was to examine if there is different angiogenic/angiostatic chemokine milieu in the chronic EAA and IPF patients. Since new therapeutical options are approaching (e.g. CXCR2 inhibitor reparixin), knowledge of pathogenetic events leading to lung fibrosis may help us to treat our patients. Correlation between chemokine concentrations or chemokine receptor expression and HRCT score might help us to find new biomarkers, which could help us to monitor degree of lung fibrosis or select patients with unfavourable prognosis.

We investigated correlation between chemokine levels (ENA-78, IL-8, I-TAC, IP-10) in bronchoalveolar lavage fluid (BALF), expression of chemokine receptors on CD4+ T cells (CXCR2, CXCR3) in BALF and HRCT pattern (alveolar versus interstitial HRCT score).

Material and methods

Study design

Twenty-four patients with mean age of 62 years (range 27—81 years) were prospectively enrolled to the study — after signing informed consent approved by Ethics committee of the Institute for Clinical and Experimental Medicine and Faculty Thomayer Hospital. None of the patients included in this study had previously been treated with steroids. A diagnosis of idiopathic pulmonary fibrosis/usual interstitial pneumonitis (IPF/UIP) was established in 8 patients. Four patients were unable to undergo lung biopsy for different reasons and they were diagnosed IPF by the use of clinical criteria (ATS/ERS joint statement criteria).[5] Sixteen patients were diagnosed chronic EAA. There are no generally accepted diagnostic criteria for EAA.[6] Diagnostic criteria applied in our department include a history of exposition to suspect antigen, symptoms, physical findings, radiographic abnormalities, pulmonary function, immunologic tests, and BAL results. A lung biopsy specimen is required when the diagnosis is not definite (2 EAA patients in our study). Basic spirometry investigation (flow-volume spirogram, machine ZAN 100 Messgerate GmbH) and diffuse lung capacity investigation (single breath method, machine ZAN 300 Messgerate GmbH) were performed in all subjects.[7] A control group could not be included in the study because of ethical reasons.

All patients underwent bronchoscopy including BAL with the cytologic and cytometric examination of BALF and HRCT of the chest. BALF supernatants were frozen at — 80 °C and processed later.

Methods

Bronchoalveolar lavage (BAL), cytologic and cytometric analysis of BAL fluid

BAL was performed according to the technical recommendations and guidelines for the standardization of BAL procedures.[8] Briefly, BAL was performed with a flexible bronchoscope (Olympus, Olympus Optical CO, Ltd., Japan) wedged into segmental or subsegmental middle lobe bronchus. Five 50 ml aliquots of sterile 0.9% saline solution were instilled and than aspired. First two doses were processed for differential cell counts, with the use of May-Grünwald and Giemsa-Romanowsky stains. The third dose of BALF underwent cytometric investigation. Mononuclear cells were stained with a commercially available conjugated and unconjugated mAbs that belonged to the Becton Dickinson series. They included: CD3, CD4, CD8, HLA-DR and isotype matched controls. The BALF specimens were analysed in the cytometer Cytomics FC 500 (Beckman Coulter) using the CXP software (Beckman Coulter). Fourth and fifth doses of BALF were immediately centrifuged at 250 $\times$ *g* for 5 min at room temperature to remove most of the cells. The supernatant was centrifuged again at 500$\times$g for 10 min at room temperature to produce completely cell-free fluid. Then the supernatants were frozen at —80 °C and they were processed together later.

Assay

Concentrations of IL-8, ENA-78, IP-10 and I-TAC in BALF supernatants were quantified using Fluorokine MultiAnalyte

Table 1 Demographic and clinical characteristics of the patients.

	EAA	IPF
Number of patients	16	8
Gender (men/women)	3/13	6/2
Smokers/nonsmokers	2/14	1/7
FVC (L)	2.1 ± 0.2	3.1 ± 0.3
FVC (% pred)	72.2 ± 4.7	86.3 ± 6.9
FEV_1 (L)	1.7 ± 0.2	2.5 ± 0.2
FEV_1 (% pred)	72.7 ± 4.4	88.6 ± 7.4
FEV_1/FVC	83.4 ± 1.2	82.4 ± 1.9
Tlco (% pred)	47.1 ± 3.4	44.8 ± 8.1
BALF recovery (ml)	24.6 ± 1.9	26.8 ± 3.0

Values are expressed as mean ± SEM. FVC % pred: Forced vital capacity % predicted; FEV_1 % pred: Forced expiratory volume within the first second % predicted; Tlco % pred: carbon monoxide transfer factor % predicted.

profiling (RD systems, Minneapolis) and Luminex 100™ (Luminex Corporation), as described previously.[9] A standard curves were created for each chemokine. Detection limit for ENA-78 was 3.60—6635 pg/mL, for IL-8 0.32—2789 pg/mL and for IP-10 0.15—2267 pg/mL and for I-TAC 0—842 pg/mL. Intra- and inter-assay variability of the assays was provided by the manufacturer.

HRCT scoring system (Table 2)

HRCT scans were performed using the Somatom Sensation Four (Siemens, Germany) scanner. They were evaluated by an experienced radiologist using the modified scoring system by Gay S.[10] The radiologist scored alveolar and interstitial scores at four levels of the right and left lung. These scores were summed for each patient and the average interstitial and alveolar scores were calculated.

Analysis

BMDP-PC 90 statistical software was used for statistical analysis. As for the statistical methods, Spearman's nonparametric correlation test (r) was applied. p levels <0.05 were regarded as significant. Mean values of chemokines in BALF, expression of CXCR receptors on CD4+ T cells and HRCT scores in the different groups were compared by the Kruskal—Wallis test. Data are expressed as a mean ± SEM.

Results

The demographic and clinical characteristics of the patients are shown in Table 1.

BALF differential cell counts and expression of chemokine receptors CXCR3 and CXCR2 on CD4+ T cells are shown in Table 3. We found no significant difference in BALF differential cell counts including CD4+/CD8+ T cells ratio and CXCR3 as well as CXCR2 expression on CD4+ T cells between chronic EAA and IPF patients. BALF chemokine levels are shown in the Table 4. There was no significant difference in the BALF chemokine levels between the EAA and IPF group. Mean HRCT alveolar score in the EAA group was 2.5 ± 0.4. HRCT alveolar score in the IPF group was significantly lower (1.0 ± 0.4, $p < 0.01$). HRCT interstitial score in EAA group (1.9 ± 0.33) did not significantly differ from that in IPF group (2.6 ± 0.46).

There was a negative correlation between HRCT alveolar score and CD4+/CD8+ T cells ratio in BAL fluid in EAA group ($p < 0.01$) (Fig. 1), while we observed no such correlation in the IPF group. HRCT interstitial score in EAA patients positively correlated with the percentage of neutrophils in BALF (0.05) (Fig. 2) and the IL-8 BALF concentration ($p < 0.01$) (Fig. 3).

There was a positive correlation between the IL-8 BALF concentration and HRCT alveolar score ($p < 0.01$) (Fig. 4) and between HRCT interstitial score and percentage of neutrophils in BALF in IPF patients ($p < 0.01$) (Fig. 5). We found no correlation between CXCR2 expression on BALF CD4+ T cells and HRCT interstitial as well as alveolar score in IPF group. We have not observed any correlation between the percentage of neutrophils and the concentrations of IL-8 and ENA-78 in the BALF of IPF and EAA patients.

There was no correlation between HRCT scores and both expression of CXCR3 on CD4+ T cells and BALF angiostatic chemokine concentration in EAA and IPF group.

Table 2 Evaluation of HRCT scans, the alveolar and interstitial scoring system.

Alveolar score	Interstitial score
0 No alveolar disease	0 No interstitial disease
1 GGO involving 0-4% of the lung parenchyma	1 Interlobular septa thickening, no discrete honeycombing
2 GGO involving up to 5—24% of the lung parenchyma	2 Honeycombing involving up to 0—24% of the lung parenchyma
3 GGO involving 25—49% of the lung parenchyma	3 Honeycombing involving 25—49% of the lung parenchyma
4 GGO involving 50—74% of the lung parenchyma	4 Honeycombing involving 50—74% of the lung parenchyma
5 GGO involving 75—100% of the lung parenchyma	5 Honeycombing involving 75—100% of the lung parenchyma

Alveolar score — inflammatory activity of the disease = GGO — glass ground opacity. Interstitial score — fibrotic changes, predominantly seen in the chronic forms of EAA and in IPF = linear opacities, honeycombing.

Table 3 BALF differential cell counts and expression of chemokine receptors CXCR3 and CXCR2 on CD4+ T cells.

	EAA	IPF
AM	51.6 ± 7.8	51.8 ± 13.3
Ly	24.6 ± 6.6	30.0 ± 10.8
PMN	19.0 ± 7.3	13.8 ± 8.1
Eos	4.8 ± 2.0	5.1 ± 3.5
CD4/CD8	2.1 ± 0.6	2.3 ± 0.4
CXCR3	5.3 ± 1.6	1.5 ± 0.6
CXCR2	3.5 ± 1.2	0.74 ± 0.2

Values are expressed as mean ± SEM. AM — alveolar macrophage, Ly — lymphocyte, PMN — polymorphonuclear, Eos — eosinophil. No significant differences were found in all above mentioned parameters between both groups.

Discussion

The chronic form of EAA is sometimes difficult to be distinguished from IPF. In advanced diseases, where fibrotic changes predominate, the IPF and EAA histology patterns are both represented with usual interstitial pneumonitis (UIP).[11] UIP consists of a temporal heterogeneity of lung tissue histological changes. Areas of fibroblastic foci resulting from recent active fibrosis and honeycombing cysts representing older fibrotic lesions are both present. This suggests that the lung have been subjected to multiple hits. While in IPF neither the initial event nor the other harmful events are known, in EAA appropriate inhalation antigen is clearly responsible for the injury and perpetuation of fibrosis.

The aim of the presented study was to document, if there are any differences in BALF chemokine and chemokine receptor profiles between these disorders. We examined BALF levels of I-TAC/CXCL11, IP-10/CXCL10, IL-8/CXCL8 and ENA-78/CXCL5 of patients with chronic EAA and IPF. We investigated a relationship between the levels of chemokines in BALF, expression of appropriate chemokine receptors on CD4+ T cells and HRCT score.

Chronic EAA was found to differ from the subacute and acute form of the disease. It has been widely accepted, that low CD4+/CD8+ T cells ratio in BALF is lower in acute and subacute forms of EAA.[6] Chronic EAA was revealed to exhibit normal or higher CD4+/CD8+ T cells ratio in the BALF. Our results correspond with this observation, since we demonstrated negative correlation between HRCT alveolar score and CD4+/CD8+ T cells ratio in the BALF of chronic EAA patients.[12]

IL-8 as well as ENA-78 was reported to be involved in the IPF pathogenesis. Both belong to the angiogenic chemokines

Table 4 BALF chemokine levels.

	EAA	IPF
IP-10	137.9 ± 93.0	56.5 ± 41.4
I-TAC	10.0 ± 9.0	0.7 ± 0.4
IL-8	96.7 ± 30.7	44.6 ± 14.5
ENA-78	54.0 ± 11.9	74.6 ± 27.0

Values are expressed in pg/L as mean ± SEM.

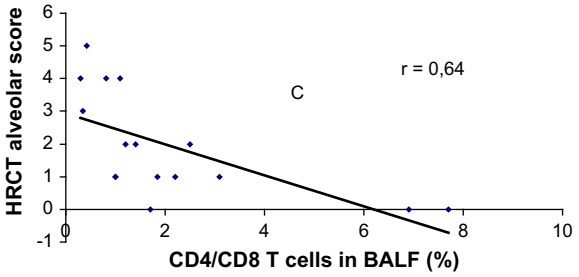

Figure 1 Negative correlation between HRCT alveolar score and CD4+/CD8+ T cells ratio in BAL fluid in EAA group.

family. It is believed that angiogenesis plays an important role in the development of lung fibrosis.[13] IL-8 was also reported to play an important role in pathogenesis of EAA.[4] To our knowledge, no studies concerning the role of ENA-78 in the development of EAA have been published to date. We found no significant difference in the IL-8 and ENA-78 BALF concentrations of patients with EAA and IPF, even though higher BALF concentrations of IP-10, I-TAC and IL-8 in EAA patients and higher ENA-78 BALF concentration in IPF group are indicated. This finding may document, that both IL-8 and ENA-78 play role in the development of lung fibrosis in chronic EAA patients.

IL-8 is the main chemotactic factor for neutrophils and ENA-78 plays pivotal role in angiogenesis.[14] There are few reports in the literature concerning EAA and angiogenesis. Pathological angiogenesis occurs during chronic inflammation, which is radiologically quantified by the means of HRCT alveolar score. Since EAA patients have significantly higher HRCT alveolar score than the IPF group (2.5 ± 0.4 versus 1.0 ± 0.4, $p < 0.01$), we may speculate that EAA patients display angiogenic activities mediated through ENA-78/CXCR2 axis in the early inflammatory phase of disease, while angiogenesis need not play the major role during the development of irreversible lung fibrosis. We suggest ENA-78 as the marker of at least partial reversibility of the lung impairment in the EAA patients.

Nevertheless, pathogenesis of lung fibrosis and angiogenesis is complex and cannot be attributed only to the CXCR2/CXCR2 ligand axis. This may explain, why no correlation between BALF CXCR2 expression on CD4+ T cells and HRCT interstitial as well as alveolar scores was founded in both groups. Although it was documented, that blockade of CXCR2 receptor inhibits angiogenesis and the pro-angiogenic cytokines production and decreases IL-8-induced endothelial cell

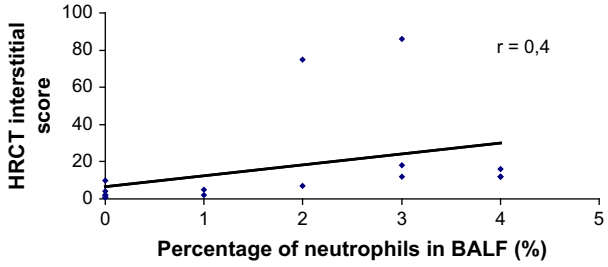

Figure 2 Positive correlation between percentage of neutrophils in BALF and HRCT interstitial score in EAA patients.

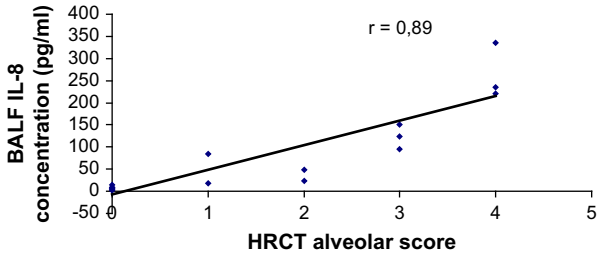

Figure 3 Positive correlation between HRCT alveolar score and the IL-8 BALF concentration in EAA patients.

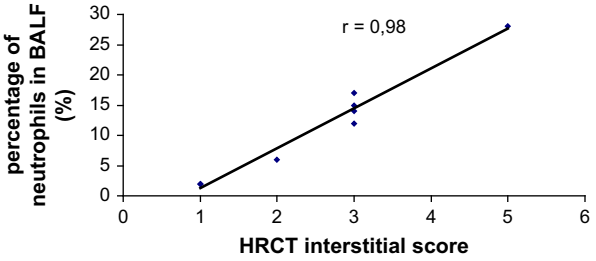

Figure 5 Positive correlation between HRCT interstitial score and percentage of neutrophils in BALF in IPF patients.

activation, an effect on neutrophils does not appear to account for the CXCR2 blockade effects in lung fibrosis pathogenesis. There are different ways how neutrophils participate in the lung fibrosis developement.[15,16] We found a positive correlation between percentage of neutrophils in BALF and HRCT interstitial score in EAA as well as in the IPF group. Neutrophils, which are also involved in the pathogenesis of lung fibrosis, show an exaggerated release of oxygen radicals when activated. The imbalance in production of oxidant/antioxidant agents has been proposed as one of the mechanisms of injury in IPF.[17] Nevertheless, there are not any widely accepted prognostic criteria based on BALF cell differential counts including neutrophil count in IPF.[18] Activated neutrophils loaded with matrix metalloproteinase 9 (gelatinase B) and collagenase-2 were found to play role in lung damage and fibrotic response in chronic EAA patients. Moreover, higher BALF neutrophil count was documented to be a sign of irreversible lung fibrosis in EAA patiens.[19] Our results correspond with the previously reported observations and document the important role of neutrophils in IPF and EAA development. The conclusive role of neutrophils in the pathogenesis of IPF and EAA needs to be established.

We have not observed any correlation between the neutrophil cell count and the BALF IL-8 concentrations of IPF and EAA patients. We suppose, that this may be caused by other chemokines involved in the neutrophil chemotaxis (e.g. CXCL1/keratinocyte-derived chemokine (KC), CXCL2/monocyte-inhibitory protein-2 (MIP-2), and CXCL5/LPS-induced chemokine (LIX)).[20]

Positive correlation was documented between the IL-8 BALF concentration and HRCT alveolar score in the IPF group. It was showed, that the areas of ground glass seen on HRCT scans usually represent fine lung fibrosis in IPF patients. Minor alveolar involvement in IPF group is reflected with the lower HRCT alveolar score observed in these patients. We found no study investigating longitudinal IL-8 production in IPF patients. It is possible, that the IL-8 expression may fluctuate in time. We may speculate that the IL-8 expression may vary in the early and advanced phases of IPF. Further studies with more patients will help us to find, if IL-8 might serve as a potential marker of early phase of IPF.

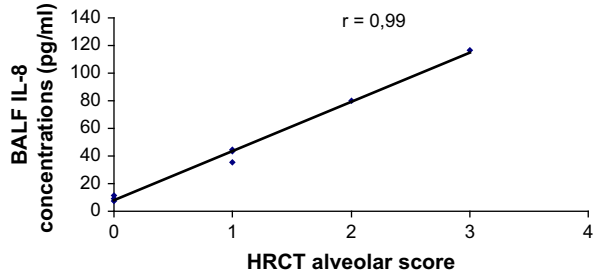

Figure 4 Positive correlation between HRCT alveolar score and the IL-8 BALF concentration in IPF group.

There was a positive correlation of HRCT interstitial score and IL-8 BALF concentration in the EAA group, but no such correlation was proven in the IPF group. It was documented, that airborne endotoxin exposure in cotton workers leads to IL-8 concentration increase in nasal lavage fluid.[21] It is possible, that the exposition to inhalation agents leads to simultaneous IL-8 increase in BALF of EAA patients. These patients are known to be in continual low-level exposure to inhalant antigens. From time to time the exposure to provoking agent may rise. This may explain the observed correlation between IL-8 BALF concentration and interstitial HRCT score. On the other hand, the above mentioned histopathological character of lesions observed in IPF patients gives evidence for the discontinuity of injurious moments. We suggest IL-8 as an indicator of continuous exposition to provoking agent in EAA patients.

IP-10 and I-TAC are two angiostatic chemokines, which share the same receptor CXCR3. The importance of CXCR3 ligand/CXCR3 axis documented the study of Strieter et al., who postulated, that potential defect in IPF pathogenesis may be related to the inappropriate CXCR3/CXCR3 ligand response to injury.[22] Their study has shown, that I-TAC is the only chemokine from the angiostatic group in IPF, which is actually stimulated by INF-γ and thus mainly attenuates the fibrogenetic process. We found very low BALF I-TAC concentration in both groups. Low levels of I-TAC in BALF were documented also by Nishioka et al. They found 10-fold-lower I-TAC than of IP-10 levels in BALF and serum of sarcoidosis patients.[23] I-TAC has 100-fold higher affinity to CXCR3 when compared with the other CXCR3 ligands,[24] thus we cannot rule out its effect in fibrotic lung tissue even in these low concentrations.

I-TAC was documented to play an important role in development of acute and subacute EAA and granuloma formation.[25] To our best knowledge there is no study concerning the role of I-TAC in chronic EAA development, where granulomas are not the main pathologic feature. Although we have found no difference between I-TAC BALF concentrations in IPF and EAA patients, we cannot exclude the influence of I-TAC even in concentrations under the detection limit, which can affect our observation. Because of very low BALF I-TAC concentration in investigated patients, we did not try to find any correlations with other

investigated parameters, since the acquired data have been distorted.

We documented no correlation of the BALF IP-10 concentration and either HRCT alveolar or HRCT interstitial score in both groups. Previously documented differences in the levels and time related patterns of CXCR3 ligands may influence our results.[26]

Investigated patients had different extent of lung fibrotic and alveolar changes and they differed also in the disease duration. They were in different stages of the disease and the production of IP-10 may thus vary.

We conclude, that both IL-8 and ENA-78 probably play a role in chronic EAA pathogenesis, but this role might be different. While we suggest ENA-78 as the marker of at least partial reversibility of the lung impairment in the EAA patients, IL-8 could be rather an indicator of continuous exposition to provoking agent in EAA patients. As for IPF group, IL-8 might serve as a potential marker of early phase of IPF. Furthermore, we suppose, that the neutrophils play a similar role in the lung fibrosis development in both IPF and EAA. The I-TAC BALF concentrations are very low and generally under the detection limit of multiple cytokine analysis. IP-10 production is the same in both studied groups, nevertheless it does not influence the radiological pattern of either disease.

Conflicts of interest

All authors declare that they have no financial relationship with any commercial entities that have an interest in the subject matters or material included in our manuscript.

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