

Granulocyte macrophage-colony stimulating factor levels in bronchoalveolar lavage fluid from patients with chronic bronchitis

Mona Hilmy Alrayes, MD* and Entesar Said Ahmad, MD**,

Department of Clinical and Chemical Pathology* and Department of Chest** .
Girls` Faculty of Medicine- Alazhar University

Abstract

Background and objectives: Although inflammatory changes are found throughout the airways of patients with chronic bronchitis (CB), the mechanisms of the pathogenesis of CB are still unclear. The aim of this study was to investigate airways inflammation in patients with and without an exacerbation of CB. Granulocyte macrophage-colony stimulating factor (GM-CSF) levels in serum and bronchoalveolar lavage (BAL) fluid were assayed. **Materials and methods:** 40 CB patients and 10 never smoking, age and sex matched controls were studied. 20 of the CB patients were studied under baseline conditions (B), and 20 during an exacerbation (E) of CB. Bronchoscopy and bronchoalveolar lavage (BAL) with cytological analysis were performed for 7 males from CB(E) group, and 6 males from CB(B) group. The levels of GM-CSF were determined in sera and in BAL supernatants by a solid phase enzyme immunoassay. **Results:** There was high significant elevation of serum GM-CSF in both CB (E) and CB(B) groups in comparison with the control group ($P < 0.0001$ & $P = 0.002$ respectively). Both serum and BAL GM-CSF levels were elevated in CB(E) group in comparison with CB(B) group ($P = 0.0001$ & $P = 0.009$ respectively). Also there was significant elevation of BAL neutrophils, eosinophils and lymphocytes in CB(E) in comparison with CB(B) ($P < 0.05$, $P < 0.01$ and $P < 0.01$ respectively). There was only significant positive correlation between BAL GM-CSF and BAL neutrophils count in CB(E) group, while no significant results were detected in CB(B) group. **Conclusion:** During exacerbations of CB there were changes in the cell populations in BAL of patients consistent with a recruitment of polymorphonuclear leucocytes, eosinophils and lymphocytes in the airway lumen. These recruited different inflammatory cells could work together toward the production of airway abnormalities and lung destruction. The locally elevated levels of BAL GM-CSF might be a cause and /or a result suggesting a role for this cytokine in the inflammatory processes of chronic bronchitis. The elevated levels of serum GM-CSF in chronic bronchitis may be due to the interference of other cytokines, microenvironmental factors in bone marrow and/or other factors.

Introduction

Chronic bronchitis (CB) is a clinical syndrome defined by cough and chronic sputum production occurring on most days of the month for at least 3 months a year during the two years prior to the study. Sputum is defined as expectorated lower respiratory tract secretions and is composed of fluid and cellular components, including macrophages, bronchial epithelial cells and inflammatory cells (Seatta et al, 1997). The major risk factor for the development of CB is cigarette smoking, but the precise

pathogenetic mechanism of chronic sputum production is still unknown. CB is associated with intermittent exacerbations (chronic bronchitis with acute exacerbations [CB(E)]) that present with worsening of the chronic symptoms of productive cough and dyspnea. These exacerbations cause considerable morbidity and in patients with concomitant airway obstruction are major causes of mortality. CB(E) can have one or more of several different etiologies including viral

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infections and atypical bacterial infections (Sethi et al, 2000).

The development of CB seems to be related to inflammatory changes of airway structure. However, the cause and the exact location and type of these changes resulting in altered airway function are not known. Mucosal inflammation is characterized by the recruitment of granulocytes, macrophages and lymphocytes as well as by the shedding of epithelial cells (Linden et al, 1990).

Granulocyte macrophage colony stimulating factor (GM-CSF) is a small glycoprotein produced by monocytes/macrophages, endothelial cells, and epithelial cells. This cytokine stimulates proliferation and maturation of monocytic and polymorphonuclear leukocytes and increases the effector functions of mature leukocytes (Lau et al, 1996). Human GM-CSF is a 127 amino acid polypeptide with a molecular weight of 18-22 KD. The biological effects of GM-CSF are mediated following binding to a high affinity receptor on mature neutrophils, macrophages and eosinophils. Macrophages also secrete GM-CSF in response to stimulation with lipopolysaccharides (LPS) and other immune effectors. In humans, GM-CSF concentrations in serum and alveolar lining have been reported to be in the low pg/ml range. The concentration of GM-CSF in bronchoalveolar lavage (BAL) fluid can be increased in some inflammatory lung conditions such as sarcoidosis, asthma, chronic obstructive pulmonary disease, and usual interstitial pneumonitis (Walker *et al*, 1994).

Bronchoscopy and BAL can be used to investigate the airways inflammation associated with CB. Visual inspection of the airways provides presumptive evidence of inflammation (Thompson and Rennard, 1990). BAL provides a direct sample that can be compared with cellular and immunologic components in the vascular circulation. Thus, the recovery of BAL fluid and its components involved directly with CB process permits a much more detailed assessment of new cellular mediators and cytokines participating in the pathologic process (Reynolds 2000).

Abundant neutrophils and mononuclear cells (lymphocytes) with a few of eosinophils are observed in BAL fluids from chronic bronchitis patients (Shimura, 1999). BAL findings differ from sputum findings because the former technique samples mainly the alveolar compartment whereas the latter samples primarily the bronchial compartment. In general, sputum is richer in neutrophils. However, there seems to be good agreement in the number of eosinophils between sputum, BAL, and biopsies (Maestrelli et al, 1995). The results of both sputum induction and BAL seem to be affected by the procedure itself. When repeating sputum induction at daily intervals, the proportion of neutrophils in induced sputum increases. Similarly, the number of neutrophils in BAL is increased 7 and 24 h after performing a BAL. Although these findings do not detract from the value of these procedures, they do highlight the fact that neutrophil migration to the airway lumen can be induced by the sampling procedure (Kips et al, 1995).

Subjects, materials and methods

This study was conducted in The Chest Department, of Alzahraa University hospital. The study included 40 chronic bronchitis patients (CB) and 10 apparently healthy never smoking, age and sex matched controls. 20 of the CB patients were studied under baseline conditions [CB (B)]. They were 16 males & 4 females, their ages ranged from 40-58 years. And 20 CB patients during an exacerbation of bronchitis [CB (E)]. They were 18 males & 2 females, their ages ranged from 42-55 years. All of the patients examined in the present study had smoked cigarettes at some point in their life. Currently smoking or patients who had smoked within 6 months of assessment were excluded. Complete clinical examination, postero-anterior plain X rays and Zeihl Nelson's stain were done to all studied subjects. Sputum inspection of studied patients was characteristically viscous. When held up to the light, pale green or white streaks could often be seen in the mucus. Patients' inclusion and exclusion criteria were

illustrated in table (1). Exacerbations were diagnosed according to criteria from Anthonisen and colleagues. The patient had to have experienced any two of the three major symptoms-increase in dyspnea, sputum purulence, and increased sputum volume in order to be classified as having a CB(E) (Anthonisen et al, 1987).

Bronchoscopy and BAL with cytological analysis were performed for 7 male patients from the CB(E) group before treatment and 6 male patients from the CB(B) group. All studied cases revealed presumptive evidences of inflammation by visual inspection.

Bronchoalveolar lavage: BAL was performed according to the ERS guidelines using a flexible fiberoptic bronchoscope (Olympus, P-20 Olympus, Tokyo, Japan) after local anaesthesia of the upper airway with 2% lidocaine. The bronchoscope was wedged into one of the segmental bronchi of the right middle lobe for lavage and an aliquot of 50 ml sterile saline at body temperature was instilled through the bronchoscope. The fluid was immediately retrieved by gentle suction using a sterile syringe. The entire procedure of instillation and retrieval was repeated three times (a total of 150 ml saline). BAL fluid was placed on ice and processed within 10 minutes after recovery. It was filtered through gauze. BAL fluid was centrifuged at 500g for 5 minutes at 4°C. The protein content of the BAL fluid supernatant was measured chemically by auto-analyzer model Hitachi 902. A part of BAL fluid supernatant was stored at -20 for GM-CSF assay. The cell pellet was suspended in PBS

(phosphate buffered saline) and counted using automated cell counter model Coulter T-660 (Rutgers et al, 2000). Drops of suspended cell pellet were spread onto a glass slide. The resulting slides were dried, fixed, and then stained using Leishman's stain. A manual differential cell counts were determined from 300 cells using a photomicroscope (De Brauwert et al, 2002).

The levels of GM-CSF were determined in sera and in BAL supernatants by a solid phase enzyme immunoassay (ELISA) using Predicta Genzyme kit. Test samples or standards were added to test wells containing immobilized mouse monoclonal antibody to human GM-CSF and incubated. The wells were washed and a direct-labeled HRP-conjugated polyclonal antibody to GM-CSF was added which bound to captured GM-CSF during incubation. After washing, a substrate solution was added producing a blue colour. The reaction was stopped and the blue colour turned yellow. The intensity of the colour was directly proportional to the amount of GM-CSF present. The absorbance was read at 450 nm and a standard curve was constructed to quantitate GM-CSF concentrations in the samples.

Statistical analysis

Data were expressed as mean ± SD. Student's t test used to detect the difference. Spearman correlation was tested, a probability (P) value <0.05 was considered-significant.

Results

Table (1): Criteria of chronic bronchitis (CB) groups.

Inclusion criteria	Exclusion criteria
1- The presence of chronic bronchitis.	1- A history of asthma or atopy.
2-Absence of asthma or bronchiectasis.	2-Use of chronic oral or parenteral steroids.
3- Ability to comply with monthly clinic visits.	3- History of bronchiectasis, lung cancer, TB, or congestive heart failure.
4-Absence of immunosuppression or other life-threatening illness.	4-Current smoking or history of having smoked within 6 months of assessment.

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Table (2): Comparison between chronic bronchitis (E) and control groups.

	CB(E) (n=20) mean ± SD	Control (n=10) mean ± SD	P	Sig
Serum GM-CSF pg/ml	6.6 ± 3.2	1.1 ± 0.4	0.0001	HS

CB: chronic bronchitis E: exacerbation Sig: significance HS: highly significant

There was high significant elevation of serum GM-CSF in CB (E) group in comparison with the control group (table 2).

Table (3): Comparison between chronic bronchitis (B) and control groups.

	CB(B) (n=20) mean ± SD	Control (n=10) mean ± SD	P	Sig
Serum GM-CSF pg/ml	2.2 ± 0.9	1.1 ± 0.4	0.002	S

CB: chronic bronchitis B: basal condition Sig: significance S: significant

There was significant elevation of serum GM-CSF in CB (B) group in comparison with the control group (table 3).

Table (4): Comparison between CB(E) and CB(B) groups.

	CB (E) BAL (n=7) mean ± SD	CB(B) BAL (n=6) mean ± SD	P	Sig
Serum GM-CSF pg/ml	6.6 ± 3.2	2.2 ± 0.9	0.0001	HS
BAL GM-CSF pg/ml	18.2 ± 2.0	10.0 ± 3.4	0.009	HS
BAL neutrophil x10 ³ /ml	3.7 ± 0.7	1.6 ± 0.4	<0.05	S
BAL eosinophils x10 ³ /ml	1.5 ± 0.1	0.7 ± 0.1	<0.01	S
BAL lymphocytes x10 ³ /ml	13.5 ± 2.1	5.9 ± 1.1	<0.01	S
BAL total protein gm/dl	0.2 ± 0.03	0.19 ± 0.01	>0.05	NS

CB: chronic bronchitis E: exacerbation B: basal condition Sig: significance

BAL: bronchoalveolar lavage HS: highly significant S: significant NS: non significant

The chronic bronchitis during exacerbation group showed high significant elevation of both serum GM-CSF & BAL GM-CSF with only significant elevation of BAL neutrophils, eosinophils and lymphocytes in comparison with the chronic bronchitis under basal condition group (table 4).

Table (5): correlation between BAL GM-CSF and other studied parameters in CB(E) group.

	r	P	significance
Serum GM-CSF pg/ml	0.37	>0.05	NS
BAL neutrophils x10 ³ /ml	0.6	<0.05	S
BAL eosinophils x10 ³ /ml	0.1	>0.05	NS
BAL lymphocytes x10 ³ /ml	0.15	>0.05	NS

There was only significant positive correlation between BAL GM-CSF and BAL neutrophils count in CB(E) group (table 5).

Table (6): correlation between BAL GM-CSF and other studied parameters in CB(B) group.

	r	P	significance
Serum GM-CSF pg/ml	0.45	>0.05	NS
BAL neutrophils x10 ³ /ml	0.3	>0.05	NS
BAL eosinophils x10 ³ /ml	0.1	>0.05	NS
BAL lymphocytes x10 ³ /ml	0.1	>0.05	NS

No significant correlation was detected between BAL GM-CSF and any of the studied parameters in CB(B) group (table 6).

Discussion

Chronic bronchitis is characterized by inflammatory changes in the bronchial tissue and by recurrent bronchitis exacerbations (Balbi et al, 1997). Acute exacerbation of chronic bronchitis is an important disease affecting millions of patients with CB. The use of antibiotics in such patients is controversial, as the etiology of acute exacerbation is complex, including inhalation of environmental irritants, discontinuation of medications, deviation from diet, viral infections and atypical bacterial infections. Although the use of antibiotics in patients with acute exacerbation has been reported to be beneficial, it is clear that not every episode of acute exacerbation needs antimicrobial therapy. Furthermore, injudicious use of antibiotics has led to increasing bacterial resistance, resulting in ineffectiveness of commonly used antibiotics (Watanakunakorn 2000).

In the present study there was significant elevation of serum GM-CSF in both CB(E) and CB(B) in comparison with the control group (P=0.0001 and P=0.002 respectively). Also serum GM-CSF was significantly elevated in CB(E) when compared with CB(B) group (P=0.0001). Balbi et al (1997) reported that patients with chronic bronchitis, as a whole, had significantly increased levels of BAL GM-CSF compared to control subjects, and similar levels of serum GM-CSF. Also they found that both serum and BAL levels of GM-CSF were markedly increased in chronic bronchitic patients with an exacerbation, as compared with patients under baseline conditions. The data of the present study showed that BAL GM-CSF was insignificantly correlated with serum

GM-CSF in both CB groups (P<0.05). These results might indicate that BAL GM-CSF was locally produced by the inflammatory cells within the airways and lung parenchyma rather than being passively transferred from the serum. Erduran et al (1997) found that the mean serum GM-CSF levels in patients with acute pulmonary infection (non-repeaters) were significantly higher than patients with recurrent pulmonary infections (repeaters) in the acute period of infection, but serum GM-CSF levels of both groups were not different in the recovery period. In addition, they reported that the serum GM-CSF levels of both repeaters and non-repeaters in the acute period of infection were higher than those in the recovery period. The elevated levels of serum GM-CSF in both CB(E) and CB(B) in the present study in comparison to the control may be due to the interference of other cytokines, microenvironmental factors in bone marrow and/or other factors.

There is increasing evidence that CB is associated with chronic inflammation in the airways and lung parenchyma. The data of the present study revealed high significant elevation of BAL GM-CSF with only significant elevation of BAL neutrophils, eosinophils and lymphocytes in CB(E) group when compared with CB(B) group (P=0.009, P<0.05, P<0.01 and P<0.01 respectively). These data might indicate that, during exacerbations of CB there were changes in the cell populations in BAL of patients consistent with a recruitment of polymorphonuclear leucocytes in the airway lumen. The increased levels of GM-CSF would suggest a role for this cytokine in the inflammatory processes

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of chronic bronchitis. Rinsho (1999) found that abundant neutrophils and lymphocytes with a few of eosinophils were observed in BAL fluids from chronic bronchitis patients, while abundant neutrophils and macrophages were seen in BAL fluids from emphysema patients. Seatta et al (1997) reported that the location of neutrophils within the bronchial glands is crucial for activation of the secretory function of gland cells, and therefore for induction of the chronic sputum production in subjects with chronic bronchitis.

Aaron et al (2001) reported that patients with clinically stable CB were known to have increased numbers of neutrophils and macrophages in their sputum and BAL fluid relative to normal subjects, suggesting that neutrophil recruitment and activation might play a role in the pathogenesis of chronic airflow disease.

Di Stefano et al (1996) found that the inflammatory changes in the large airways of subjects with CB had shown a predominance of lymphocytes in the airway wall and a predominance of neutrophils in the airway lumen. This discrepancy had led to the hypothesis that the inflammation in the lumen may differ from that in the bronchial wall in patients with CB. The results of the study done by Saetta et al (1997) showed an increased number of neutrophils in the bronchial glands of subjects with CB, providing evidence for a neutrophilia not only in the airway lumen but also in the airway wall of these subjects, contributing to a better understanding of the apparent discrepancy between luminal and parenchymal findings in this disease.

Cosio and Guerassimov (1999) findings in CB with exacerbations were of interest. They reported that large numbers of neutrophils and eosinophils were found in both sputum and bronchial biopsies. Since exacerbations were most often secondary to an infection so this was not surprising. They explained that, possibly the earliest inflammatory reaction involves the neutrophil, followed by the alveolar macrophages in all the epithelial surfaces of the lung. These cells would in time damage epithelial cells and the interstitial protein

structures (elastin, collagenase, proteoglycans, etc.). These proteins could be processed by dendritic cells and macrophages into peptides with antigenic potential that could be recognized by T cells initiating T cell activation and proliferation. These activated T cells, could recruit other cells such as neutrophils and even eosinophils to the site of inflammation. Thus, all these different inflammatory cells could work together toward the production of airway abnormalities and lung destruction. Wells et al (1998) reported that neutrophils were linked to the morphological extent of disease in patients with lung lesion and eosinophils were more closely linked to functional impairment than neutrophils.

In the present study there was only significant positive correlation between BAL GM-CSF and BAL neutrophils count in CB during exacerbation group ($P < 0.05$). On the other hand no significant results were detected in CB under basal conditions. These results might indicate that BAL GM-CSF could play a role in the recruitment of neutrophils in the large airways of subjects with CB during exacerbations.

Conclusion

During exacerbations of chronic bronchitis there are changes in the cell populations in bronchoalveolar lavage of patients consistent with a recruitment of polymorphonuclear leucocytes, eosinophils and lymphocytes in the airway lumen. The locally elevated levels of BAL GM-CSF might be a cause and /or a result suggesting a role for this cytokine in the inflammatory processes of chronic bronchitis. Recruited neutrophils and eosinophils may release increased amounts of inflammatory mediators capable of damaging the bronchial tissue. Further studies to clarify whether GM-CSF participates in the pathogenesis of neutrophil mediated lung diseases such as acute respiratory distress syndrome and collagen vascular disease are recommended.

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مستويات العامل المنشط لمستعمرة الخلية الملتزمة الكبيرة المحببة فى سائل الغسيل الحويصلى الشعبى فى مرضى الإلتهاب الشعبى المزمن

د.منى حلمى الرئيس* و د.إنتصار سيد أحمد**

قسمى الباثولوجيا الإكلينيكية والكيميائية* و الأمراض الصدرية** كلية طب البنات-
جامعة الأزهر.

بالرغم من وجود تغيرات إتهابية خلال الممرات الهوائية فى مرضى الإلتهاب الشعبى
المزمن فإن ميكانيكية تطور هذا المرض لا تزال غير واضحة.

هدف البحث: يهدف البحث إلى فحص إتهاب الممرات الهوائية فى مرضى الإلتهاب الشعبى
المزمن الغير مصحوب و المصحوب بإشتداد. وكذلك قياس مستويات العامل المنشط لمستعمرة
الخلية الملتزمة الكبيرة المحببة (ج م- ك س ف) فى كل من المصل و سائل الغسيل الحويصلى
الشعبى.

مواد و طرق البحث: تمت الدراسة على ٤٠ من مرضى الإلتهاب الشعبى المزمن و ١٠ من
الأصحاء الذين لم يسبق لهم التدخين و المتوافقين فى العمر و الجنس كمجموعة ضابطة. و كان
٢٠ من مرضى الإلتهاب الشعبى المزمن فى حالة مستقرة بينما كان ٢٠ منهم فى حالة إشتداد. وقد
تم عمل منظار شعبى و غسيل حويصلى شعبى (ب ال) مع تحليل خلوى لعدد ٧ من رجال
مجموعة حالة الإشتداد و ٦ من رجال مجموعة الإستقرار. كذلك تم قياس مستويات ج م-ك س ف
فالمصل وفى طفو ب ال بواسطة القياس المناعى الإنزيمى للهيئة الصلبة.

النتائج: أظهرت النتائج إرتفاعا هاما فى ج م- ك س ف فى المصل فى كل من مجموعتى الإلتهاب
الشعبى المزمن المستقرة و المشتدة عند مقارنتهم بالمجموعة الضابطة. كذلك كان هناك إرتفاعا
فى مستوى ج م- ك س ف فى كل من المصل و ب ال فى مجموعة الإشتداد عند مقارنتها
بالمجموعة المستقرة. أيضا كان هناك زيادة هامة فى الخلايا المتعادلة و الخلايا الحمضية و
الخلايا الليمفاوية فى ب ال مجموعة الإشتداد عند مقارنتهم بالمجموعة المستقرة. و أظهر البحث
علاقة موجبة هامة بين ج م- ك س ف و عدد الخلايا المتعادلة فى سائل ب ال فى مجموعة
الإلتهاب الشعبى المزمن المشتدة.

الإستنتاج: نستنتج من البحث أن هناك تغيرات فى أقوام الخلايا فى الغسيل الحويصلى الشعبى فى
مرضى الإلتهاب الشعبى المزمن أثناء الإشتداد و أن هذا التغيير يتمشى مع تجنيد كرات الدم
البيضاء متعددة أشكال النواة و الخلايا الحمضية و الخلايا الليمفاوية فى تجويف الممرات الهوائية.
و أن هذه الخلايا الإلتهابية المختلفة و المجددة ممكن أن تعمل معا للإحداث اضطرابات فى
الممرات الهوائية و تدمير للرئة. كما أن الزيادة الموضعية فى مستوى ج م- ك س ف فى ب ال
هؤلاء المرضى قد يكون سبب أو نتيجة مقترحا بذلك دورا لهذا المحرك الخلوى فى إتهابات
الشعب الهوائية المزمنة. و قد تكون زيادة ج م- ك س ف فى مصل مرضى الإلتهاب الشعبى
المزمن نتيجة لتدخل محركات خلايا أخرى أو عوامل بيئية فى نخاع العظم أو عوامل أخرى.