The relative contribution of bronchoalveolar macrophages and neutrophils to lucigenin- and luminol-amplified chemiluminescence


ABSTRACT: The relationship between differential cell counts and latex-stimulated luminol and lucigenin-amplified chemiluminescence (CL) was investigated by mixing alveolar macrophages (AM) obtained at bronchoalveolar lavage (BAL) with allogeneic peripheral blood neutrophils (PMN) in varying proportions. In 5 non-asthmatic subjects, the mean luminol-amplified CL increased linearly from 2.1 (0.9 SEM) x 10^3 counts per second (cps) with <2% PMN, >96% AM to 47.3 (11.1 SEM) x 10^3 cps with >94% PMN, 0% AM (r=0.996, p<0.001). The regression had a y-intercept indistinguishable from 0 cps, suggesting that luminol-amplified CL exclusively reflected PMN activity. Using the same technique, the mean lucigenin-amplified CL showed a fall from 35 (2.3 SEM) x 10^4 cps with a cell population of >96% AM, <2% PMN to 20 (2.3 SEM) x 10^4 cps with 0% AM, >94% PMN. Both PMN and AM appeared to contribute to lucigenin-amplified CL, with AM contributing approximately 1.7 times as much activity per cell as PMN. Lucigenin-amplified CL appeared to be an appropriate technique for measuring AM activity when the proportion of PMN in mixed cell populations was small. A linear relationship was found between percent PMN count and luminol-amplified CL measured in a mixed BAL cell population from asthmatic subjects (p<0.01) and non-asthmatic controls (p<0.01). The slope of this regression line was significantly greater for subjects with asthma than for control subjects (p<0.01), suggesting a uniform increase in PMN activity in cells obtained from asthmatic airways.


When stimulated, phagocytic cells undergo an acceleration of oxidative metabolism ("the respiratory burst") leading to the generation of unstable oxygen derivatives such as superoxide anion radicals, singlet oxygen and hydroxyl ions [1]. These achieve a stable ground state by the emission of energy as photons (chemiluminescence; CL) [2]. After chemical amplification, this low energy signal can be readily detected and quantified using a photon multiplier [3]. The two chemical amplifiers most frequently employed are luminol (5-amino-2,3-dihydro-1,4-thalazine dione) [4] and lucigenin (10,10' -dimethyl-9' -biacridinium dinitrate) [5]. Luminol-amplified CL has been most widely used and a variety of phagocyte cell types, including peripheral blood neutrophils (PMN) [2], monocytes [6] and tissue and alveolar macrophages (AM) [7], have been studied.

The mechanism of action of luminol-amplified CL has been extensively investigated. It can be generated in a cell-free system by the reaction of hydrogen peroxide with myeloperoxidase (MPO) and a halide [8]. In biological systems, it is inhibited by hydrogen peroxide scavengers such as catalase [9], and by inhibitors of haem-containing enzymes, presumably by their action on MPO [8]. Further evidence for the importance of MPO comes from the observation that MPO-deficient PMN produce very little luminol-amplified CL [10, 11]. Lucigenin, a more recently characterised amplifier of CL, has been used to study a number of cell types including PMN [5] and AM [12]. MPO-deficient PMN produce lucigenin-amplified CL [11] which can be abolished by superoxide dismutase, a specific superoxide scavenger [11]. A cell-free xanthine/xanthine oxidase system, which generates superoxide anion radicals, produces lucigenin-amplified CL, and suggests that lucigenin interacts specifically with superoxide produced by phagocytes and is independent of MPO [13]. Luminol and lucigenin therefore appear to amplify CL.
by different mechanisms and have different specificities for oxygen radicals [5]. WILLIAMS and COLE, studying cells harvested from bronchoalveolar lavage (BAL) fluid, have previously shown an association between PMN counts and luminol, but not lucigenin, amplified CL [14]. This is consistent with the theoretical basis of luminol-amplified CL, as human AM lack MPO [15]. Lucigenin-amplified CL, on the other hand, appeared to be more closely associated with AM numbers [16]. It was therefore suggested that luminol-amplified CL could be used as a measure of PMN activity, but was inappropriate as a measure of AM activity and that lucigenin-amplified CL might be more appropriate as a measure of AM. However, both PMN [17] and AM [16] produce superoxide and should be capable of producing lucigenin-amplified CL. These studies depended on spontaneous differences in PMN counts in subjects with a variety of interstitial lung diseases. No attempts were made to purify or separate the cells, and no account was taken of disease state and the possible associated differences in cell activation. More recently, PMN activity has indeed been shown to be increased in sarcoidosis [18], emphasising the need for studies looking separately at the effects of cell counts and disease activity on luminol- and lucigenin-amplified CL in more homogeneous groups of subjects.

The specificities of luminol- and lucigenin-amplified CL suggested by earlier work has unfortunately not been reflected in recent studies, where luminol-amplified CL has been used as a marker of human AM function [19-22]. In this current study, we have re-evaluated the use of luminol and lucigenin in the assessment of the metabolic activity of cells obtained at BAL using defined mixtures of BAL cells with added purified PMN in vitro. We then examined the use of these markers in bronchoalveolar cell populations obtained from a group of asthma patients, with a wide spectrum of disease activity, and a group of control subjects.

**Methods**

**BAL procedure**

All subjects had a clinical indication for fiberoptic bronchoscopy and BAL was performed with their full informed consent and the permission of the Newcastle Ethics Committee. Fiberoptic bronchoscopy (Olympus OT10, Keymed Ltd) was performed following premedication with intramuscular papaveretum (10 mg) and atropine (0.6 mg), using isotonic 1.5% lignocaine for topical anaesthesia. After endobronchial examination, the tip of the bronchoscope was wedged in a subsegmental bronchus of the middle lobe, or lingula, and three 60ml aliquots of sterile phosphate-buffered saline were introduced. After instillation of each aliquot the fluid was immediately aspirated into siliconised glassware at 4°C.

**BAL cell handling**

Aspirated BAL fluid was filtered through a stainless steel mesh (pore size 200 μm) to remove mucus, the volume measured and a total cell count performed by two independent observers using a Neubauer chamber. Cell counts by the two observers were close and not significantly different (geometric mean ratio 0.99). The filtered aspirate was centrifuged (200g, 10 mins, 4°C) and the cell pellet resuspended in a cell medium containing calcium and magnesium, but without azide or phenol red (cell medium 199, Gibco) at a cell concentration of 5 x 10⁶·ml⁻¹. 100 μl aliquots of the resuspending were cytocentrifuged onto glass slides (Shandon 2 Cytospin), stained (May Grunwald Giemsa), a differential cell count performed on 600 cells by the same two independent observers and the mean of the results was found.

**Chemiluminescence assays**

Duplicate 500 μl aliquots of the cell resuspending (250,000 cells) were incubated at 37°C with 900 μl of either 0.1 mM luminol or lucigenin (Sigma) for 10 minutes. 100μl of 5% w/v latex particles (mean diameter 1.09μm, Sigma) were then added and, after mixing, peak stimulated CL was measured using a luminometer (LKB 1250, LKB-Wallac for experiment 1 and Lumac M2010, Sonco Ltd for experiment 2). A reading of 1 mV from our LKB 1250 is equivalent to 7,150 counts per second (cps) from our Lumac M2010 (personal data). The means of duplicate measurements of CL were used throughout, and all CL assays were completed within 3 hours of BAL.

**Measurement of luminol- and lucigenin-amplified CL using a range of PMN proportions generated in vitro**

Five non-asthmatic subjects (median age 40 yrs, range 36-66) with unexplained haemoptysis, normal pulmonary physiology (median FEV₁ 95% predicted, range 70-98%) and BAL cell counts of >95% AM were studied. Three of the subjects were current smokers, three were female, and in none was an endobronchial abnormality found.

Allogeneic peripheral blood was obtained by venesection and PMN purified using dextran sedimentation (Dextraven 110, CP Pharmaceuticals, Wrexham, UK) (37°C, 15 mins) and Ficoll gradient centrifugation (Histopaque 1077, Sigma) (200 g, 15 mins, 20°C). Cells were washed once, residual erythrocytes lysed (0.2% saline) and differential cell counts performed to check that the resulting population of cells were >94% PMN. The resulting population of purified PMN was resuspended at 5 x 10⁶·ml⁻¹ in phosphate buffered saline.

PMN were added to the BAL cell resuspending to give aliquots of 250,000 cells in 500 μl with varying
proportions of neutrophils. The PMN percentages generated were: <2, 10, 20, 40, 50, 70, and >94. The remainder of the cells in each aliquot comprised resuspended BAL cells. Aliquots were incubated with either 0.1 mM luminol or lucigenin and peak latex-stimulated CL was recorded as above.

Measurement of luminol- and lucigenin-amplified CL in BAL cells from subjects with asthma and control subjects

22 subjects (mean age 47 yrs, range 17–73) with stable asthma, together with 20 control subjects (mean age 53 yrs, range 21–71) with no evidence of airflow obstruction were studied. All subjects were undergoing routine diagnostic bronchoscopy; the indications in the asthmatic subject were streaking (13), undue cough (8), and suspected stridor (1). No asthmatic subjects had evidence of neoplasms, infection nor infarction and none had recognised bronchiectasis. In the control group the indications for bronchoscopy were peripheral radiographic abnormality (9), unexplained haemoptysis (6) or undue cough (5). Four proved to have lung cancer, but on the contralateral side to that lavaged. Ten of the asthmatic subjects were current smokers and ten were male. Of the control group, twelve were current smokers and sixteen were male. Nine of the asthmatic group were on regular inhaled β₂ agonist bronchodilators alone, seven in addition took low dose inhaled steroids (beclomethasone dipropionate <400 μg per day) and six were on no regular medication. No subject had received oral corticosteroids within three months of bronchoscopy or had had a recognised respiratory tract infection within four weeks.

Airway responsiveness to inhaled methacholine (expressed as the dose causing a 20% fall in baseline FEV₁, PD₂0FEV₁) was measured in all subjects four to six days prior to bronchoscopy using a maximum cumulative dose of 6.4mg delivered by an evaluated dosimeter technique [23]. All the asthmatics had a PD₂0FEV₁ of less than 6.4mg while a PD₂0FEV₁ was not attained in any of the control subjects.

BAL and cell preparation were performed and luminol and lucigenin-amplified CL were measured as above.

Statistical analysis

Linear correlation coefficients were calculated by least squares analysis and the slopes of the relationships between luminol-amplified CL and PMN counts for the asthma subjects and control subjects were compared using a modification of the t-test for regression coefficients [24]. P values of <0.05 were taken to be significant.

Results

Luminol- and lucigenin-amplified CL in mixed cell populations generated in vitro

The five control subjects studied had <2% PMN and no eosinophils in their BAL fluid, AM accounting for a median of 97% (range 96–98%) of all BAL cells. The median purity of the PMN preparations from peripheral blood was 96% (range 94–98%), with no eosinophil contamination, and the remaining cells being mononuclear cells.

The effect on both luminol- and lucigenin-amplified CL of an increasing neutrophilia in the mixed cell aliquots is shown in figures 1 and 2A. Luminol-amplified CL increased linearly with increasing PMN number (p<0.001) with an intercept that was indistinguishable from the origin (p=0.60). Lucigenin-amplified CL decreased to 60% of its initial value when the AM were entirely replaced by PMN, i.e., cell for cell AM produced approximately 1.7 times as much lucigenin-amplified CL as did PMN.

To estimate the relationship between AM number and lucigenin-amplified CL, a value for the PMN-derived CL was subtracted from the total for each aliquot. It was assumed that the lucigenin-amplified CL from PMN was linearly related to PMN number as previously described [5] and additive to the AM contribution. On this basis the lucigenin-amplified CL calculated to be derived from the AM in the admixtures was correlated against AM number (p<0.001), as in figure 2B.
subjects (median 86%, range 35-95%), due to increased numbers of lymphocytes in the group of asthmatic subjects (median 78%, range 35-95%).

Luminal-amplified CL and AM numbers was not able to be produced by both neutrophils and alveolar macrophages, with the latter accounting for about 1.7 fold as much CL per cell as the neutrophils. Each point represents the mean (SEM) for the 5 subjects. The family tree represents the proportion of total lucigenin-amplified CL produced by the 250,000 cells to be accounted for by the varying numbers of neutrophils, assuming a linear relationship between neutrophil numbers and lucigenin-amplified CL. (5).

Each regression was linear (asthmatic subjects, r=0.993, p<0.001; controls, r=0.48, p>0.10). The slope of the regression for the asthmatic group was significantly steeper than that for the controls (p<0.01).

Lucigenin-amplified CL in asthmatic and control subjects

A significant linear relationship between lucigenin-amplified CL and AM numbers was not detected in the control group (r=0.14, p>0.10). This may have been due to the high AM percentage values obtained in most subjects, exceeding 80% in 16 of 20 subjects (median 86%, range 50-96%).

There was a wider range of AM percent in BAL found in the group of asthmatic subjects (median 78%, range 35-95%), due to increased numbers of lymphocytes

Fig. 2. (a) The latex-stimulated lucigen-amplified chemiluminescence (CL) produced by aliquots of 250,000 cells, generated as for figure 1, was found to be produced by both neutrophils and alveolar macrophages, with the latter accounting for about 1.7 fold as much CL per cell as the neutrophils. Each point represents the mean (SEM) for the 5 subjects. The assumed line represents the proportion of total lucigenin-amplified CL produced by the 250,000 cells to be accounted for by the varying numbers of neutrophils, assuming a linear relationship between neutrophil numbers and lucigenin-amplified CL (5). (b) The latex-stimulated lucigen-amplified chemiluminescence (CL) attributed to the alveolar macrophages in each aliquot of 250,000 cells has been calculated by the subtraction of the assumed line in figure 2A from the total lucigenin-amplified CL produced by the mixed cell populations. It was linearly proportional to the percentage of alveolar macrophages (r=0.983, p<0.001).

Luminol-amplified CL in asthmatic and control subjects

The median PMN count in the asthmatic group was 3% (range 0.5-10%), and in the control subjects was 2% (range 0.5-8%). The relationship between luminol amplified CL and PMN counts in BAL fluid from asthmatics and controls are shown in Fig. 3. Eosinophils were not detected in the BAL fluid from 16 (73%) of the asthmatic group. The remaining 6 (27%) had <1% eosinophils, and the relationship between luminol-amplified CL and percent PMN was not appreciably altered when the total granulocyte count, i.e. PMN plus eosinophils, was used instead. Eosinophils were not found in the BAL fluid from control subjects.

Each regression was linear (asthmatics, p<0.01; controls, p<0.001) with an intercept that was indistinguishable from the origin (asthmatics, p>0.90; controls p>0.09). However, the slope of the regression for the asthmatic group was significantly greater than that for the controls (p<0.01).

Lucigenin-amplified CL in asthmatic and control subjects

A significant linear relationship between lucigenin-amplified CL and AM numbers was not detected in the control group (r=0.14, p>0.10). This may have been due to the high AM percentage values obtained in most subjects, exceeding 80% in 16 of 20 subjects (median 86%, range 50-96%). There was a wider range of AM percent in BAL found in the group of asthmatic subjects (median 78%, range 35-95%), due to increased numbers of lymphocytes (median 18%, range 2-59%), but no significant relationship was found between lucigenin-amplified CL and AM numbers (r=0.48, P>0.10). Neither was a relationship found when the data for both groups was pooled (r=0.32, p>0.10).
Discussion

This study has shown a close association between increasing PMN numbers and luminol-amplified CL for cells stimulated with latex. The close linear relationship with an intercept at the origin suggests that luminol-amplified CL exclusively reflects granulocyte activity in mixed BAL cell populations in these study groups and was not influenced by the presence of AM. The findings are consistent with the suggestion that only phagocytes containing myeloperoxidase, or an alternative peroxidase, produce luminol-amplified CL [10, 11, 25]. MPO makes up some 5% of PMN dry weight [2] while peroxidase staining is usually absent from AM [15]. In acute situations, such as following airway antigen challenge newly recruited monocytic cells may also contain peroxidase [26]. However, in stable asthmatics similar to those in this study we have subsequently confirmed the absence of peroxidase staining in AM (unreported observation).

Interpretation in this study was simplified by the absence of eosinophils in most of the cell samples assayed, even from the asthmatics. Both eosinophils and PMN possess peroxidase activity and are capable of producing luminol-amplified CL [25]. Luminol-amplified CL would therefore be expected to measure the sum of PMN and eosinophil activity and changes in luminol-amplified CL should be a useful measure of granulocyte activation in mixed cell populations obtained at BAL from subjects with lung disease.

When stimulated with latex, both AM and PMN produce lucigenin-amplified CL. Lucigenin-amplified CL is linearly related to PMN numbers when these are obtained from peripheral blood [5]. Subtracting a value for the lucigenin-amplified CL likely to have been produced by PMN in our mixed cell populations disclosed a linear relationship between lucigenin-amplified CL and AM number. The data suggested that cell for cell AM produce approximately 1.7 times as much lucigenin-amplified CL as PMN. Except in some cases of interstitial lung disease or infection, PMN and eosinophils generally constitute only a small percentage of cells in BAL populations and would not be expected to make an appreciable contribution to lucigenin-amplified CL. Thus, under most circumstances lucigenin-amplified CL would appear to be an appropriate marker of AM activity in BAL fluid.

Compared with peripheral blood or animal models, relatively few cells can be obtained at BAL in humans and techniques such as CL which do not require large numbers of cells are particularly valuable. Using luminol and lucigenin as amplifiers, the activity of two major cell types can be measured without the need for cell separation which leads to significant cell losses and may in itself cause alterations in cell functional activity [27]. The use of these techniques, however, demands that they be thoroughly evaluated. Failure to do this has resulted in their inappropriate use and misleading conclusions. Despite the theoretical objections and the previous experimental evidence, several recent studies have used luminol-amplified CL as a measure of human AM activity [19-22]. A major purpose of this study was to clarify the continuing ambiguity.

A linear relationship was demonstrated not only between the percentage of PMN in cell populations generated in vitro by the addition of allogenic PMN but also when the proportion of PMN in BAL fluid varied spontaneously, both in control and asthmatic subjects. Our results are therefore similar to those of Williams and Cohn [12, 16] who measured zymosan-stimulated luminol- and lucigenin-amplified CL in BAL cells. Latex is now also widely used as a stimulus with a number of cell types, including peripheral PMN [28] and AM [18]. Unlike most particulates latex does not require opsonisation and phagocytosis to be an effective stimulus, but acts in a similar way to soluble, surface-active stimuli such as phorbol 12-myristate 13-acetate [29]. Its use in this long-term study meant that 'batch variation' of the stimulus, demonstrated for zymosan [28], or variation due to an opsonisation procedure was not a problem. The production of oxygen radicals may however vary with the stimulus used [29] and while our results agree with those obtained using opsonised zymosan, it cannot necessarily be concluded that identical findings would be obtained if other stimuli were to be used.

An association between AM numbers and lucigenin-amplified CL has previously been shown in BAL using subjects with a variety of different diseases [16]. We were unable to reproduce this. However, the limited range of AM percentage counts found in our control subjects may partially explain the lack of a relationship in this group. BAL samples from subjects with different diseases, yielding considerably wider ranges of AM counts, have previously been used in the evaluation of assays of cell function, but their use in confirming linearity may not be appropriate. We have previously demonstrated an association between airway responsiveness to methacholine and degree of AM activation [30], which probably explains our inability to define a relationship between AM number and lucigenin-amplified CL in both the asthmatic group alone, and also when pooled with the control data.

There was a significantly greater slope to the regression line relating PMN numbers and luminol-amplified CL for asthmatics than for control subjects. This suggests that in asthma PMN function is enhanced, but the degree of cell activation would seem to be uniform over what was a wide spectrum of clinical disease activity [30]. This is consistent with the demonstration of an enhanced production of oxygen radicals from peripheral PMN in asthmatics, when measured directly [17, 31]. However the state of activation of cells in peripheral blood need not necessarily reflect the inflammatory changes occurring in the airways in asthma, at least to the extent that might be expected for airway cells obtained directly at BAL. This uniform increase in airways PMN activation across a wide range of disease activity suggests a response of the PMN to the presence of the disease state rather than its severity, but this interpretation is uncertain. It does, however, again caution against the use of
cells from subjects with different diseases in drawing methodological conclusions about a technique such as CL. Unless the degree of activity within cells is uniform, their use in elucidating the relationship between strength of a signal such as CL and cell number may lead to misleading results.

Acknowledgements: This work was supported by a grant from the Asthma Research Council. We are grateful for the secretarial assistance of Miss Catherine Phillips.

References


RÉSUMÉ: Les relations entre les décomptes cellulaires différentiels et la chemoluminescence (CL) amplifiée par le luminol stimulé au latex ou la lucigenine, ont été investiguées en mélangant des macrophages alvéolaires (AM) obtenus pendant un lavage broncho-alvéolaire (BAL) avec des neutrophiles du sang périphérique allongénique (PMN) en proportions diverses. Chez 5 sujets non asthmatiques, la CL moyenne amplifiée au luminol augmente de façon linéaire de 52.1 (0.9 SEM) × 10⁵ cps avec < 2% de PMN, et >96% d’AM, jusqu’à 47.3 (11.1 SEM) × 10⁵ cps avec >94% de PMN et 0% de AM (r=0.996, p<0.001). La régression a une interception-y indifférenciable de 0 cps, suggérant que CL amplifiée par luminol reflète de façon exclusive l’activité polymorphonucléaire. En utilisant la même technique, la CL moyenne amplifiée par lucigenine montre une chute de 35 (2.3 SEM) × 10⁵ cps en présence d’une population cellulaire de plus de 96% de AM, et de moins de 2% de PMN, jusqu’à 20 (2.3 SEM) × 10⁵ cps avec 0% de AM, et plus de 94% de PMN. A la fois PMN et AM semblent contribuer à la chemoluminescence amplifiée par la lucigenine, AM y contribuant environ 1.7 fois plus par cellule que PMN. La chemoluminescence amplifiée par lucigenine parait être une technique appropriée pour mesurer l’activité des AM, quand la proportion de PMN dans les populations cellulaires mixtes est petite. On a trouve une relation linéaire entre le décompte de PMN en pour cent et la CL amplifiée par luminol mesurée dans une population cellulaire mixte du lavage alvéolaire chez des patients asthmatiques (p<0.001) ou chez des contrôle non asthmatiques (p<0.001). La pente de cette ligne de régression est significativement plus marquée pour les sujets asthmatiques que pour les contrôle (p<0.01), ce qui suggère une augmentation uniforme de l’activité PMN dans les cellules obtenue dans les voies aériennes de sujets asthmatiques.