Human bronchoalveolar lavage cells and luminol-dependent chemiluminescence

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SUMMARY Human bronchoalveolar lavage cells from several different disease states were examined by the technique of zymosan-stimulated, luminol-dependent chemiluminescence. Light production correlated well with polymorphonuclear leucocyte contamination of the alveolar macrophage suspension but not with lymphocyte contamination. Regression analysis indicated that human alveolar macrophages produce little if any luminol-dependent chemiluminescence. Further investigation of metabolic activity, using measurements of superoxide release, oxygen consumption, and lucigenin-dependent chemiluminescence, showed that 'respiratory burst' activity in alveolar macrophages was stimulated by opsonised zymosan.

Phagocytic cells show increased metabolic activity when their plasma membranes are stimulated by contact with opsonised particles.¹ Oxygen consumption² rises, superoxide anion³ and hydrogen peroxide⁴ are produced, and there are changes in hexose monophosphate shunt activity. Allen *et al.*⁵ described a chemiluminescence that is produced at the time of this 'respiratory burst' by polymorphonuclear leucocytes. Although initially thought to be due to the relaxation of singlet molecular oxygen to its ground state, more recent evidence⁶ suggests that this inherent phagocytic chemiluminescence arises as a result of reactions between the products of oxidative metabolism by the leucocyte and the bacterial constituents.

In a later paper, Allen *et al.*⁷ described the use of luminol - (5 - amino - 2, 3 - dihydrophthalazine - 1,4 - dione)-dependent chemiluminescence to demonstrate the production of reactive species by phagocytosing rabbit alveolar and peritoneal macrophages. Luminol, a cyclic hydrazide, reacts with hydrogen peroxide at alkaline pH to produce the intermediate 3-aminophthalate ion, an electronically excited state which is responsible for the observed light emission. At neutral pH this reaction does not occur, and the precise nature of the reaction involved in the production of phagocytic luminol-dependent chemiluminescence remains unclear. Two different lines of work with polymorphonuclear leucocytes suggest that myeloperoxidase plays a prominent role. First, it has been shown that the reaction is sensitive to

azide, an inhibitor of myeloperoxidase and other haem enzyme activity. Second, Stevens *et al.*⁸ described the virtual absence of luminol-dependent chemiluminescence in the phagocytosing leucocytes from a patient with myeloperoxidase deficiency. They suggested that the residual activity was due to eosinophil contamination of the neutrophil preparation. This contrasts with the only moderate reduction (60%) in inherent phagocytic chemiluminescence demonstrated in myeloperoxidase-deficient patients.⁹

Although there is no doubt concerning the production of luminol-dependent chemiluminescence by polymorphonuclear leucocytes and monocytes, the situation is unclear with regard to alveolar macrophages. In addition to the original observations made by Allen *et al.*⁷ using rabbit alveolar and peritoneal macrophages, there have been reports of its production by sheep and guinea-pig alveolar macrophages.^{10 11}

Because of the benefits of the technique as a means of investigating opsonic and phagocytic metabolic activity,¹² we have examined the production of luminol-dependent chemiluminescence by human alveolar cells obtained by bronchoalveolar lavage. We present data to suggest that human alveolar macrophages produce little if any luminol-dependent chemiluminescence.

Material and methods

CELL PREPARATION

Alveolar macrophages were obtained at broncho-

scopy by bronchoalveolar lavage with bicarbonate-buffered 0.9% saline from patients with a diagnosis of sarcoidosis (11), cryptogenic fibrosing alveolitis (11), asbestosis (3), hypogammaglobulinaemia (1), and extrinsic allergic alveolitis (2) as well as from four normal control subjects. Cells were separated from the lavage fluid by centrifugation at 200 *g* at 4° for 5 minutes and washed twice with medium 199 (4°—Flow Laboratories). They were then suspended in medium 199 with calcium and magnesium, without phenol red, to a concentration of 4×10^5 non-specific esterase positive (NSE)¹³ cells per ml and kept in siliconised glass containers on melting ice until used. Assessment of contamination with cell types other than alveolar macrophages was made on May-Grünwald Giemsa stained cytocentrifuge preparations. Cell viability was quantitated by trypan blue exclusion and exceeded 80% in all cases.

Polymorphonuclear leucocytes were separated from the peripheral venous blood of five healthy donors. After removal of the mononuclear cell layer after gradient centrifugation of defibrinated blood on Ficoll-Trisil (SG 1-077), the polymorphonuclear leucocytes were isolated by sedimenting erythrocytes with dextran (MW 110 000) and treating with 0.83% tris ammonium chloride. The cells were washed twice and resuspended in medium 199. May-Grünwald Giemsa stained cytocentrifuge preparations showed more than 95% purity of polymorphonuclear leucocytes in all cases.

OPSONISATION

Pooled human serum from five apparently healthy donors, which was stored at -70° in aliquots, was used for opsonisation of zymosan (Sigma); 400 μ l of serum was added to 1900 μ l zymosan suspension (10 mg/ml medium) and incubated at 37° for 30 minutes. The particles were then washed twice and resuspended in phosphate-buffered saline to an approximate concentration of 1.3×10^9 /ml.

CHEMILUMINESCENCE

Chemiluminescence was measured in a photometer (Luminometer 1250, LKB Wallac) using cylindrical polystyrene cuvettes (Sterilin) at 37°. One hundred microlitres of opsonised particles was added to the reaction vial containing 500 μ l cell suspension and 900 μ l 10^{-5} M luminol (or 10^{-4} M lucigenin) with 0.1% gelatin. The light generated was recorded on a chart recorder (LKB Wallac), and evaluation was based upon the chemiluminescence reading 12 minutes after the addition of opsonised particles.

Luminol (Sigma) was dissolved in dimethylsulphoxide at a concentration of 10^{-2} M and then diluted to 10^{-5} M with HEPES buffered Hanks's balanced

salt solution (HBSS), pH 7.4. Lucigenin (dimethylbiacridinium nitrate, Sigma) was dissolved in HBSS.

OXYGEN CONSUMPTION

Oxygen consumption was measured polarographically using a Clarke-type O₂ electrode (Rank Brothers) at 37°; 2.7 ml of cell suspension (alveolar macrophages: 10^6 NSE positive cells/ml; polymorphonuclear leucocytes: 10^6 cells/ml) in medium 199 with 0.1% gelatin was allowed to equilibrate in the chamber before the addition of 200 μ l opsonised zymosan. Oxygen consumption was then monitored during the next 20 minutes.

The results for alveolar macrophage oxygen consumption are expressed both as the total oxygen consumption (nmol/ 10^6 NSE positive cells/min) for the lavage cells and after correction for polymorphonuclear leucocyte contamination using the mean values as obtained for purified polymorphonuclear leucocytes from peripheral blood (Table 2).

SUPEROXIDE RELEASE

Superoxide release was determined by the superoxide dismutase-inhibitable reduction of cytochrome C. Alveolar macrophages (4×10^5 cells/ml) in medium 199 with 0.1% gelatin were incubated for 15 minutes at 37° with 13 μ mol/l ferricytochrome C (horse heart type VI, Sigma) in the presence and absence of 50 μ g superoxide dismutase. The amount of reduced cytochrome C in the cell-free supernatants was determined spectrophotometrically at 550 nm and the superoxide generation was calculated using the extinction coefficient of 21×10^3 /cm/m.¹⁴

The results for alveolar macrophage superoxide release are expressed both as the total superoxide release (nmol/ 10^6 NSE positive cells/min) for the lavage cells and after correction for polymorphonuclear leucocyte contamination using the mean values as obtained for purified polymorphonuclear leucocytes (Table 2).

Results

The number of contaminating polymorphonuclear leucocytes (PMN) (Table 1; Figs 1 and 2) and lymphocytes (Table 1) in the lavage fluid was quantitated, and the results were correlated with the chemiluminescence. The zymosan-stimulated, luminol-dependent chemiluminescence correlated well with the number of PMN ($r = 0.98$; $P < 0.001$; Fig. 1, Table 1) but not with the lymphocytes ($r = -0.01$; Table 1). With few PMN there was little response to zymosan despite the presence of 2×10^5 alveolar macrophages (AM). To ensure that this was not due to a failure of opsonised zymosan to stimulate the 'respiratory burst' in AM, other means of investigat-

Table 1 Correlation* between contaminating cell count and chemiluminescence

Contaminating cells ($\times 10^5$ /vial)	Chemiluminescence (mV)		
	Luminol + cells + zymosan	Lucigenin + cells + zymosan	Lucigenin + cells
	$\bar{x}$ = 6.93 SD = 13.81 n = 32	$\bar{x}$ = 6.40 SD = 4.68 n = 24	$\bar{x}$ = 1.22 SD = 1.83 n = 19
PMN			
$\bar{y}$ = 1.39 SD = 3.26 n = 32	r = 0.98		
$\bar{y}$ = 1.46 SD = 3.63 n = 24		r = 0.73	
$\bar{y}$ = 1.77 SD = 4.04 n = 19			r = -0.12
Lymphocytes			
$\bar{y}$ = 0.42 SD = 0.57 n = 32	r = -0.01		
$\bar{y}$ = 0.42 SD = 0.60 n = 24		r = -0.16	
$\bar{y}$ = 0.49 SD = 0.66 n = 19			r = -0.25

*Correlation coefficient (r) for the relation between number of contaminating polymorphonuclear leucocytes (PMN) or lymphocytes, and the chemiluminescence response (mV) obtained 12 minutes after addition of reaction mixtures to vials using luminol (10^{-5} M) or lucigenin (10^{-4} M) as substrate and opsonised zymosan (100 μ l) as stimulating particle.

The number of contaminating cells of both types added to the reaction vial in 500 μ l medium containing 2×10^5 alveolar macrophages is expressed as the mean ($\bar{y}$) and standard deviation (SD). Chemiluminescence is expressed as the mean ($\bar{x}$) and SD. n represents the number of experiments, and gelatin (0.1%) was present in all cases.

ing phagocytic oxidative metabolism were used.

Zymosan-stimulated, lucigenin-dependent chemiluminescence also correlated ($r = 0.73$; $P < 0.001$, Table 1) with the PMN contamination of the lavage fluid, but, as can be seen from Fig. 2, a significant response was obtained even with low PMN counts. Furthermore, when the lavage specimen containing 1.8×10^6 PMN/ 2×10^5 AM was disregarded in the statistical analysis, luminol-dependent chemiluminescence correlated strongly with PMN count ($r = 0.91$; $P < 0.001$) while lucigenin-dependent chemiluminescence did not ($r = 0.47$; $P < 0.05$). With luminol no response was produced on the addition of cells to reaction vials in the absence of particles, but with lucigenin there was a small response which did not correlate with PMN count (Table 1).

Superoxide release and oxygen consumption were measured in some cases with low PMN contamination (mean = 0.37×10^5 PMN/ 2×10^5 AM; SD 0.32; n = 12; Table 2). In addition to a total value

Fig. 1 Relation between luminol-dependent chemiluminescence (CL) response and polymorphonuclear leucocyte (PMN) contamination of alveolar macrophage (AM) suspension. To measure chemiluminescence 100 μ l opsonised zymosan suspension was added to reaction vials containing 500 μ l AM suspension (4×10^5 NSE positive cells/ml) and 900 μ l luminol (10^{-5} M). Equation for regression line: $CL = (4.16 \times PMN) + 1.14$.

Fig. 2 Relation between lucigenin-dependent chemiluminescence (CL) response and polymorphonuclear leucocyte (PMN) contamination of alveolar macrophage (AM) suspension. To measure chemiluminescence 100 μ l opsonised zymosan suspension was added to reaction vials containing 500 μ l AM suspension (4×10^5 NSE positive cells/ml) and 900 μ l lucigenin (10^{-4} M). Equation for regression line: $CL = (0.94 \times PMN) + 5.03$.

for the lavage cells, the results were corrected for PMN contamination using values obtained for purified PMN. Zymosan stimulated the release of superoxide and consumption of oxygen, and this could not be accounted for by PMN.

Discussion

Following the original description by Allen *et al.*⁵ phagocytic low-level chemiluminescence has been used as a measure of metabolic activity in polymorphonuclear leucocytes and has proved to be a

Table 2 *Superoxide release and oxygen consumption by human alveolar macrophages before (resting) and after (stimulated) addition of zymosan*

		Resting	Stimulated
Alveolar macrophages	Total	0.09 (SD 0.06; n = 9)	0.51 (SD 0.28; n = 12)
Superoxide release (nmol/10 ⁶ AM/min)	Corrected	0.09 (SD 0.06; n = 9)	0.36 (SD 0.31; n = 12)
Alveolar macrophages	Total	4.65 (SD 1.58; n = 8)	6.99 (SD 2.8; n = 9)
Oxygen consumption (nmol/10 ⁶ AM/min)	Corrected	4.63 (SD 1.58; n = 8)	6.31 (SD 2.77; n = 9)
Polymorphonuclear leucocytes			
Superoxide release (nmol/10 ⁶ PMN/min)		0.03 (SD 0.01; n = 5)	0.96 (SD 0.28; n = 5)
Polymorphonuclear leucocytes			
Oxygen consumption (nmol/10 ⁶ PMN/min)		0.04 (SD 0.01; n = 5)	2.54 (SD 0.93; n = 5)

The results are expressed as the mean, and the number of subjects examined (n) and the standard deviation (SD) are shown. In addition to the total result obtained, a corrected figure for alveolar macrophages is given, which takes account of the PMN contamination of each individual alveolar lavage specimen, using the mean figure as shown for PMN.

For measurement of superoxide release 4×10^5 alveolar macrophages in medium with 0.1% gelatin were incubated for 15 minutes at 37° with 13 μ mol/l ferricytochrome C in the presence or absence of 50 μ g superoxide dismutase.

For oxygen consumption measurements, 2.7 ml of cell suspension (10⁶ AM/ml) with 0.1% gelatin was allowed to equilibrate in the oxygen electrode chamber at 37° before addition of 200 μ l opsonised zymosan.

useful means of detecting serum opsonic defects. Initial attempts at using the same technique with macrophages proved unsuccessful, and, partly for this reason, the cyclic hydrazide luminol was introduced into the system to act as a substrate for oxidation by the reactive species produced at the time of phagocytosis, thus increasing the light production. Since then the method has proved to be a useful technique when using polymorphonuclear leucocytes.

Stevens *et al.*⁸ demonstrated that polymorphonuclear leucocytes from a patient with myeloperoxidase deficiency produced very little luminol-dependent chemiluminescence on phagocytosis and suggested that the residual activity derived from eosinophils, which have an alternative peroxidase. Furthermore, it is known that azide, an inhibitor of myeloperoxidase and other haem enzyme activity, abolishes the luminol-dependent chemiluminescence response, suggesting an important role for myeloperoxidase in its production.

Alveolar macrophages from several different species¹⁵⁻¹⁷ have been shown to have either no peroxidase activity or low activity restricted to the nuclear envelope, rough endoplasmic reticulum, and Golgi apparatus. For this reason, it might be expected that the production of phagocytic, luminol-dependent chemiluminescence by alveolar macrophages would be unlikely or at most very weak. Allen *et al.*⁷ used the technique with rabbit alveolar macrophages, and although they recorded polymorphonuclear leucocyte contamination they did not attempt to remove the cells. They observed that the response was less than 1% of that obtained when equivalent numbers of peripheral polymorphonuclear leucocytes were used. In other studies, sheep and guinea-pig cells have been examined, again with low-level responses compared to polymorphonuclear

leucocytes, despite relatively high luminol concentrations.^{10,11}

Our results, using lavage cells from patients with a variety of clinical conditions, show a strong positive correlation ($r = 0.98$) between the light response and the number of contaminating polymorphonuclear leucocytes. Because of the degree of correlation and the very low response in the presence of only small numbers of polymorphonuclear leucocytes, it was evident that the production of light by the alveolar macrophages was negligible under the conditions used. Furthermore, other techniques of assessing 'respiratory burst' activity showed that this lack of response was not the result of a failure of opsonised zymosan to stimulate the 'respiratory burst'. Measurements of oxygen consumption and superoxide production on the addition of opsonised zymosan to alveolar macrophages contaminated by small numbers of polymorphonuclear leucocytes showed increased activity in excess of that produced by equivalent numbers of peripheral blood polymorphonuclear leucocytes.

Lucigenin-dependent chemiluminescence has been used as a means of assessing polymorphonuclear leucocyte metabolic activity, and we have found it to be useful in monitoring the release of superoxide during alveolar macrophage adherence.¹⁸ Stimulation with opsonised zymosan produced a lucigenin-dependent chemiluminescence response that did not correlate strongly with polymorphonuclear leucocyte contamination. In contrast to the situation found when using luminol as substrate, there was a significant response using lucigenin when only very few polymorphonuclear leucocytes were present.

In view of these findings we feel that there is a need for caution when interpreting the results obtained using luminol-dependent chemiluminescence for the assessment of alveolar macrophage

function. Although it is possible to obtain highly purified populations of alveolar macrophages from some species,¹¹ this has been difficult with human alveolar macrophages. We have not proved that human alveolar macrophages in disease states or in normals do not produce luminol-dependent chemiluminescence but we have shown that, if they do, it is negligible compared to that produced by contaminating polymorphonuclear leucocytes. Furthermore, even in the presence of less than 1% contamination by polymorphonuclear leucocytes, it is doubtful whether results obtained from alveolar macrophages could be successfully interpreted.

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