LOW LEVEL CHEMILUMINESCENCE OF ALVEOLAR MACROPHAGES

Spectral evidence for singlet oxygen generation

Enrique CADENAS, Ronald P. DANIELE* and Britton CHANCE

Johnson Research Foundation, School of Medicine, G4 and **Cardiovascular-Pulmonary Division, Department of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA*

Received 3 December 1980

1. Introduction

O_2-dependent biochemical reactions of alveolar macrophages are not well known and, in the few species studied, appear quantitatively less than those described for polymorphonuclear leukocytes [reviewed in [1]]. For example, chemiluminescence intensity of alveolar macrophages has been shown to be lower than that from neutrophils. In most studies, photoemission intensity was amplified by the use of luminol [2,3], which chemiluminesces when oxidized. However, luminol-mediated chemiluminescence lacks specificity because luminol can be oxidized by several O_2 intermediates, such as O_2^-, $HO^\cdot$, H_2O_2, and $^1O_2$ (singlet oxygen). On the other hand, spontaneous (not luminol-mediated) low level chemiluminescence of alveolar macrophages has been little studied and offers more reliable information on the generation of highly oxidizing species other than O_2^-, H_2O_2, or $HO^\cdot$. Thus far, this ultra-weak light emission has been reported to be sensitive to superoxide dismutase, catalase and benzoate [4–6], which probably reflect a link between formation of excited chemiluminescent species and production and metabolism of O_2^- and H_2O_2 [7,8].

We describe here alveolar macrophage chemiluminescence, in the resting (spontaneous chemiluminescence) and stimulated (induced chemiluminescence) states. Chemiluminescence was detected by a high sensitivity photon counter, which permitted spectral analysis of the light emission. We also describe the kinetics of macrophage light emission and the dependence of chemiluminescence on O_2 tension of the external medium.

2. Materials and methods

2.1. *Isolation of alveolar macrophages*

Alveolar macrophages were isolated from guinea pigs as in [9]. Cells were suspended in Hanks' buffered solution (HBSS) at $\sim 10^7$ cells/ml and stored on ice until used. Cell viability (>90%) and macrophage purity (87–95%) were determined by trypan blue exclusion and by non-specific esterase staining [10], respectively. Polymorphonuclear leukocytes were always <4%.

2.2. *Photon counting*

Chemiluminescence was measured with a single photon counting apparatus as in [11,12]. Light emission was expressed in counts/s: 1 count/s corresponded to ~10 photons/s allowing an efficiency of 1% for the photon counter. Spectral analysis was performed with cut-off Wratten filters (Eastman-Kodak, Rochester, NY). The filters were attached to a rotating disc, placed between the sample and the light guide, and operated manually to the required position from the outside of a light-tight box. Kodak Wratten filters used were 2A, 2B, 2E, 3, 4, 8, 9, 12, 16, 20, 21, 22, 23A, 24, 25, 29, 92, 70 and 89B. Light transmitted by each filter was expressed as a percentage of the total light detected and corrected for the photomultiplier efficiency according to the manufacturer's specifications (EMI-Gencom, Plainview, NY) [13].

2.3. *Assay conditions*

Chemiluminescence reactions were carried out in a 34 × 35 × 5 mm (11.9 cm^2 surface) cuvette, which was maintained thermostatically at 37°C. Additions to the cuvette containing the macrophage suspension

in HBSS were made from outside the light-tight box by thin polyethylene tubing while the photon counter was in operation. In this way, the reaction was monitored continuously and no chemiluminescence burst was missed. Homogeneous suspension of macrophages along with rapid and even mixing of the added stimulant [concanavalin (con) A from Sigma Chemical Co. (St Louis, MO)] were assured by constant stirring throughout the reaction period.

3. Results

3.1. *Chemiluminescence kinetics of alveolar macrophages*

Alveolar macrophages showed spontaneous chemiluminescence of ~12–15 counts . s^{-1} . 10^6 $cells^{-1}$, which increased after activation of macrophages with concanavalin A (fig.1). After addition of con A there was a lag period of ~1 min, followed by a rapid chemiluminescence burst with the intensity increasing at a rate of 1400–1800 counts . s^{-1} . min^{-1}. The chemiluminescence signal reached a maximum in ~1 min, and then decayed in the form of a triple exponential decay; the first decay phase was rapid and lasted 0.5–1.0 min (rate constant, $k_1 = 0.14$ min^{-1}), and then merged progressively through a transition phase ($k_2 = 0.08$ min^{-1}) into a slow and uniform third phase ($k_3 < 0.001$ min^{-1}). The overall time course of the chemiluminescence reaction (from background level (prior to enhancement of light emission) to a point where decaying chemiluminescence signal reached background level) was ~40–60 min. Both the kinetic pattern (shown in fig.1) and duration of the lag phase were independent of concentration of con A. The inset of fig.1 indicates the dependence of macrophage chemiluminescence on con A concentration and the reciprocal showed an app. K_m for con A of ~0.2 mg/ml.

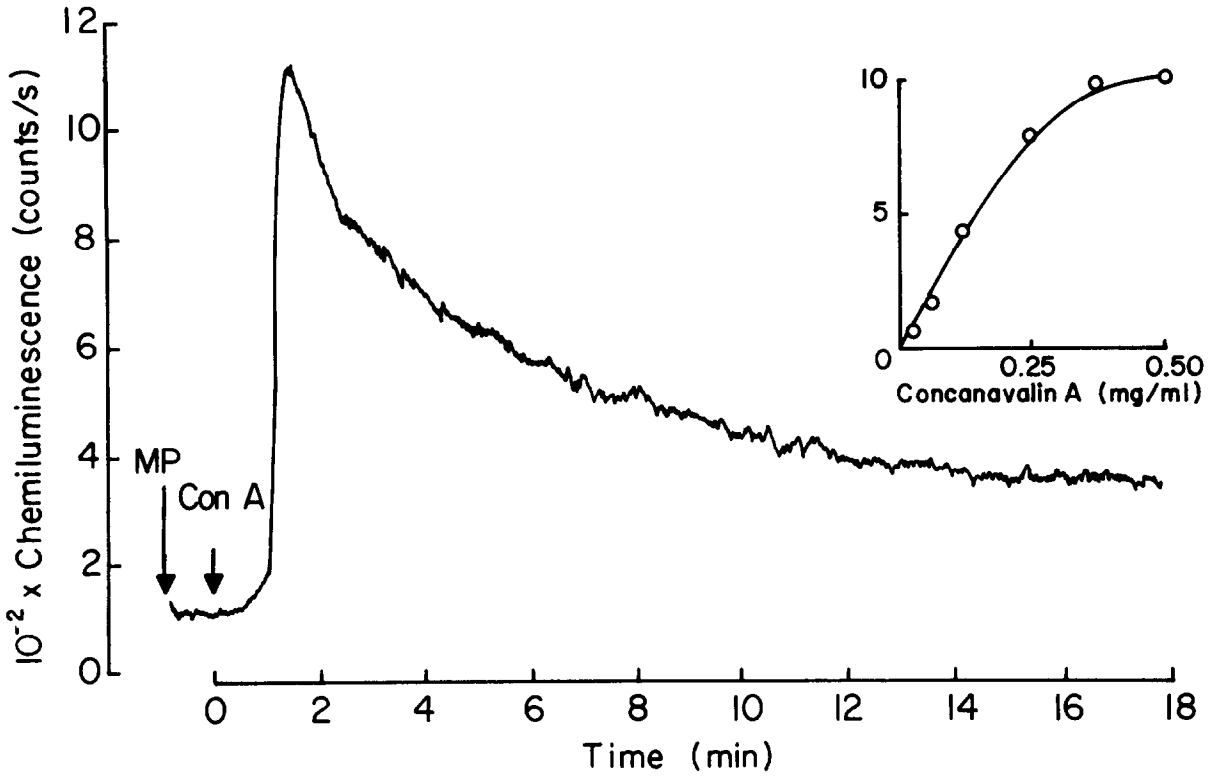

Fig.1. Typical chemiluminescence response of alveolar macrophages activated by con A. Assay conditions were as in section 2. Cells (1.5×10^7) in HBSS buffer were supplemented with 0.37 mg con A/ml. Inset: dependence of macrophage chemiluminescence intensity upon con A concentration; the data plotted reflect the con A-increased counts rate values above the background level. *Abbrevations:* MP, alveolar macrophages; con A, concanavalin A.

3.2. *Oxygen dependence of chemiluminescence*

Fig.2 shows the strict O_2 dependence of light emission of lung macrophages stimulated by con A. There was no light emission in an O_2-free medium. However, maintenance of alveolar macrophages in anaerobic medium for 10–15 min period did not affect their capacity for yielding light emission after restoration of O_2. The app. K_m of macrophages for O_2, reflected by chemiluminescence, is ~90 μM. This low affinity for O_2 might represent an increased adaptation to the relatively high O_2 pressure to which lung macrophages are exposed. On the other hand, strict O_2 dependence of light emission of macrophages after stimulation with con A resembles the requirements for the formation of other O_2 by-products associated with the respiratory burst of alveolar macrophages [7,8].

3.3. *Spectral analysis of chemiluminescence of activated macrophages*

The spectral distribution of the light emission for macrophages activated by con A is shown in fig.3. There are 4 bands a 420–450, 460–480, 570–590 and 630–700 nm, with relative intensities of ~0.16, 0.19, 0.48 and 1.00, respectively. The red light chemiluminescence band gave the greatest contribution (2–5-fold higher) to total photoemission observed. The relative intensities of the 570–590 and 630–700 nm bands remained unchanged in the several spectral analyses performed, whereas relative intensities of the blue–green bands (420–450 and 460–480 nm) varied slightly (±0.04 their relative intensity values) in different preparations.

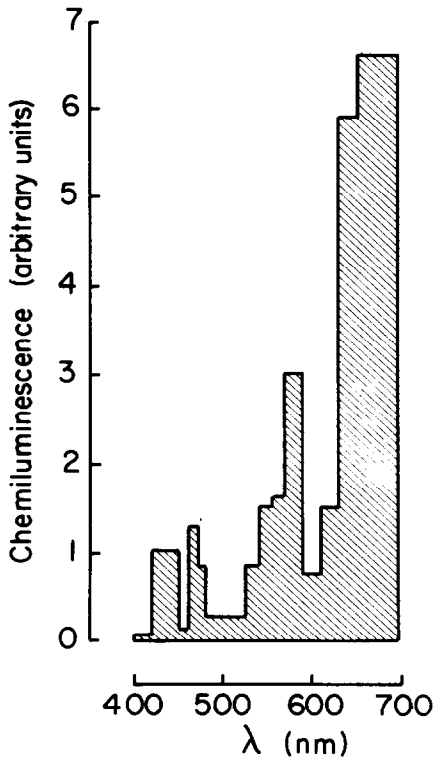

Fig.3. Spectral analysis of light emission of con A-activated alveolar macrophages. The typical spectrum for activated macrophages shown in this figure was corrected for the effect of filters on background level (represented by macrophages in resting state) in addition to the corrections in section 2. Assay conditions as in fig.1.

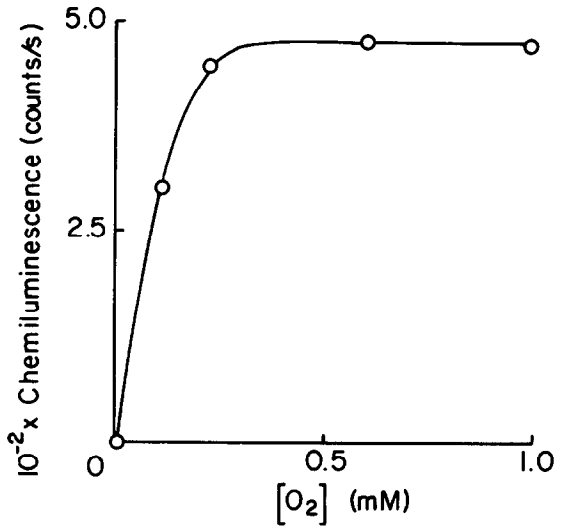

Fig.2. Dependence of the chemiluminescence of con A-activated macrophages on oxygen concentration. Assay conditions as in section 2. Cells (1×10^7) in HBSS buffer were supplemented with 0.37 mg con A/ml to start the chemiluminescence reaction.

Fluorescence spectra of alveolar macrophages in the resting state (emission peaks at 432 and 484 nm, with excitation at 419 and 472 nm) did not vary in the activated state after addition of con A (not shown).

4. Discussion

4.1. *Induced chemiluminescence: macrophages in activated state*

The 570–590 and 630–700 nm bands of the O_2-dependent light emission of macrophages stimulated by con A correspond with the dimol emission of $^1O_2$ in several electronic excited and vibrational states:

$[^1\Delta g]$ $[^1\Delta g]$ (1,0) for 580 nm, and $[^1\Delta g]$ $[^1\Delta g]$ (0,0) and $[^1\Delta g]$ $[^1\Delta g]$ (0,1) for 634 and 703 nm, respectively [14–16]. Although the fluorescence spectrum between resting and stimulated macrophages did not differ significantly, it is possible that the blue–green photoemission bands (420–450 and 460–480 nm) are due to decay of fluorescence species (432 and 484 nm) inherent in the macrophages suspension and independent of the activation process. Because there is no light emission spectrum of macrophages in resting state to subtract from the one in the stimulated state, this hypothesis is difficult to confirm. On the other hand, both the 570–590 and 630–700 nm bands seem to be derivative of the activation process; no fluorescence emission was observed in these wavelengths.

In spite of the association of chemiluminescence with oxidizing species formed during the respiratory burst [4–6] and the present support for possible $^1O_2$ generation, more evidence is needed in order to formulate a mechanism for light emission. However, the appearance of $^1O_2$ raises the possibility that $^1O_2$ might play a role in lung's macrophage defense against airborne bacteria.

4.2. *Spontaneous chemiluminescence: macrophages in resting state*

Spontaneous chemiluminescence of alveolar macrophages in the absence of a stimulant (~12–15 counts . s^{-1} . 10^6 $cells^{-1}$) may also be important, since standard values for this light emission might establish a reference for defining abnormal or diseased states. At present, however, this spontaneous photoemission is difficult to characterize, at least in terms of spectral distribution, due to the low counts and the large number of cells required.

Acknowledgements

This research was supported by USPHS grant HL-SCOR-15061 and by a Research Career Development Award to R. P. D.

References

[1] Johnston, R. B. jr (1978) Fed. Proc. FASEB 37, 2759–2764.
[2] Allen, R. and Loose, L. D. (1976) Biochem. Biophys. Res. Commun. 69, 245–252.
[3] Hatch, G. E., Gardner, D. E. and Mengel, D. B. (1978) J. Exp. Med. 147, 182–195.
[4] Beall, G. D., Repine, J. E., Hoidal, J. R. and Rasp, F. L. (1977) Infec. Immun. 17, 117–120.
[5] Miles, P. R., Lee, P., Trush, M. A. and Van Dyke, K. (1977) Life Sci. 20, 165–170.
[6] Miles, P. R., Castranova, V. and Lee, P. (1978) Am. J. Physiol. 235, 103–108.
[7] Holian, A. and Daniele, R. P. (1979) FEBS Lett. 108, 47–50.
[8] Holian, A. and Daniele, R. P. (1981) J. Appl. Physiol. in press.
[9] Dauber, J. H. and Daniele, R. P. (1978) Am. Rev. Resp. Dis. 117, 673–684.
[10] Li, L. Y., Lam, K. W. and Yani, L. T. (1973) J. Histochem. Cytochem. 21, 1–12.
[11] Boveris, A., Cadenas, E., Reiter, R., Filipkowski, M., Nakase, Y. and Chance, B. (1980) Proc. Natl. Acad. Sci. USA 77, 347–351.
[12] Cadenas, E., Boveris, A. and Chance, B. (1980) Biochem. J. 187, 131–140.
[13] Cadenas, E., Boveris, A. and Chance, B. (1980) FEBS Lett. 112, 285–288.
[14] Seliger, H. H. (1960) Analyt. Biochem. 1, 60.
[15] Khan, A. U. and Kasha, M. (1970) J. Am. Chem. Soc. 92, 3293–3300.
[16] Inaba, H., Shimizu, Y., Tsuji, Y. and Yamagishi, A. (1979) Photochem. Photobiol. 30, 169–175.