

QUANTIFICATION OF PHAGOCYTTIC CELLULAR CHEMILUMINESCENCE

L.E. McGann, T.D. Horan, T.A. McPherson and A.A. Noujaim
Faculties of Medicine and Pharmacy
University of Alberta and Cross Cancer Institute
Edmonton, Alberta, Canada

ABSTRACT

Highly reactive reduction products of molecular oxygen play a key role in a prime physiological function of phagocytic cells, i.e. microbial killing. Concomitant with oxidant formation, phagocytes exhibit cellular chemiluminescence (CL). We describe a method by which the CL emission from phagocytic cells can be quantified. Polymorphonuclear neutrophils (PMNs) isolated from the peripheral blood of seventy volunteers were phagocytically challenged with opsonized zymosan in the presence of the CL enhancer luminol. Photo emission was detected by a single phototube and the intensity of CL was monitored continuously for a period of 8 minutes. The resulting information was digitized and stored in a laboratory computer for subsequent analysis. The experimental curves are characterized by an initial lag period, an exponential increase in intensity, followed by a maximal response. Because the process of CL associated with phagocytosis is not completely understood, we have described features of the response curve using an exponential function. A least-squares curve fitting technique was used to estimate parameters associated with these three aspects of the response for all the recorded experimental curves. This method of cellular CL quantification may provide an important basis for future comparisons of phagocytic CL in health and disease.

INTRODUCTION

Perturbation of the cellular surface of polymorphonuclear neutrophils (PMNs) by membrane-reactive ligands, such as complemented or immunoglobulin-coated microorganisms initiates a series of cellular metabolic events¹ including a sharp increase in cellular oxygen uptake² and the production of oxygen metabolites such

as superoxide, hydroxyl radicals, and singlet oxygen. These oxygen metabolites are involved in the microbiocidal action of PMNs. During the course of these metabolic events, there is a concomitant cellular emission of photons resulting in chemiluminescence (CL)³ reflecting the non-specific radiative decay of electronic excitation energy. This light production is strictly dependent on cellular respiration, and the detection of photon emission has provided one of the most sensitive analytical techniques for monitoring cellular oxidant production⁴. This has allowed the use of CL analysis to examine mechanisms which control phagocytic oxidant generation, as well as to characterize disorders in phagocytic function since the early 1970's. Many reports on the production of CL in PMNs have appeared over the last decade but there are no methods for the quantitative reporting or comparison of CL responses. In this study we have addressed the problem of quantifying the CL responses of normal PMNs challenged with a phagocytic stimulus in the presence of the quantum enhancer, luminol. The advantage of utilizing a quantum enhancer such as luminol, is that small numbers of cells may be assessed⁵, and furthermore, luminol oxidation is dependent upon the activity of the enzyme myeloperoxidase.

The sequence of biochemical reactions that accompany elevated oxygen consumption of PMNs during the activation process is similar to those involved in phagocyte-mediated microbial destruction⁶, so the quantification of the CL response may assist in the dissection of these processes.

MATERIAL AND METHODS

1. Cell Separation

Peripheral blood (10 mL) collected from human volunteers in heparinized tubes was mixed with 3 mL of Plasmagel (Roger Bellon Laboratories, Neuilly, France) and allowed to sediment at unit gravity for 30 min at 37°C. The leukocyte-rich plasma was layered on a 3 mL Ficoll-Paque (Pharmacia, Upsalla, Sweden) and centrifuged at 400 x g for 30 min at 18-20°C. The PMNs at the bottom of the tubes were collected and the residual erythrocytes were osmotically lysed by exposure of the cell suspension to cold distilled water for 10 sec.

Isolated PMNs were twice washed in cold Hank's balanced salt solution (HBSS), counted and diluted in cold HBSS to a final concentration of 2×10^6 cells/mL.

2. Preparation of Complementized Particles

Zymosan (20 mg) (ICN Pharmaceuticals Incorporated, Life Science Group, Plainview, New York) was washed twice in 5 mL HBSS and the final pellet was resuspended in one mL of fresh human serum. After a 30 min incubation at 37°C with frequent mixing, complementization was terminated by the addition of 4 mL cold HBSS. The treated zymosan was washed once and resuspended in HBSS.

3. Reagents

Luminols (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma 0.5 mg) was dissolved in 50 μ L of dimethyl sulphoxide and diluted with warm HBSS to yield a 2.82×10^{-4} M solution.

4. Chemiluminescence procedure

The reaction mixtures consisted of 50 μ L zymosan suspension and 25 μ L of luminol stock solution. These were placed into the sample changer head of a model 6100 Pico-Lite Luminometer (Packard Instrument Company, Downers Grove, Ill.) in 6 x 50 mm cuvettes and the carousel rotated to bring desired samples into line with the side face photomultiplier tube. The adjustable temperature control of the sample changer allowed all mixtures to pre-equilibrate to 37°C and background readings were set for automatic subtraction. A 25 μ L aliquot of cell suspension was injected with a Hamilton syringe through the light-tight septum of the sample head. Syringe injection into the small volume of the 6 mm cuvette allowed for rapid and complete mixing of each sample. The phototube signal was recorded with a Beckman LS7500 strip chart recorder (0-100 mV range). Chemiluminescent responses were digitized from the strip-chart recordings using a Zeiss MOP-3 digitizer (Carl Zeiss, Inc., West Germany) interfaced to a North Star Horizon laboratory microcomputer (North Star Computers, Berkeley, CA).

RESULTS AND ANALYSIS

The set of all the response curves in Figure 1 show that the CL may be characterized by an initial lag period, an exponential-like increase and a maximal response. The general features of the chemiluminescent response are summarized in Figure 2. Since the process of CL is not understood well enough to allow development of a mathematical model, we selected an exponential curve to describe two primary features of the curves - the time constant associated with the increase in intensity, and the asymptotic maximal response. The experimental data was fitted to an exponential curve of the form

$$I = a (1 - e^{-b (t - t_0)}) \quad (1)$$

where I is the intensity of CL at time t, t_0 is the duration of the initial lag period, a and b are the parameters used to characterize the curves. Parameter a is the asymptotic maximal response, and b is the time constant. Analysis of the responses from the 70 normal volunteers resulted in these values for the parameters: $a = 77.7 \pm 1.4$ mV (mean \pm standard deviation), $b = 0.4 \pm 0.14$, and $t_0 = 0.8 \pm 0.2$ min. The distributions of parameters a and b are plotted in the histograms shown in Figure 3 and in a correlated plot of both parameters in Figure 4.

DISCUSSION

The curves in Figure 1 represent the range of responses for normal volunteers, and illustrate the dilemma in attempting to compare normal and altered CL responses using qualitative criteria. Ideally, a mathematical model of the process of CL would summarize the current knowledge and serve as a tool for further development and application of the phenomenon, but the nature of the process is poorly understood and there is insufficient information on which to base a model. This does not preclude the use of this sensitive technique to study cellular oxidant production, so a simple exponential function was selected to describe the elementary features of the response curves.

The values of parameters a and b which are used to describe the

EXPERIMENTAL DATA

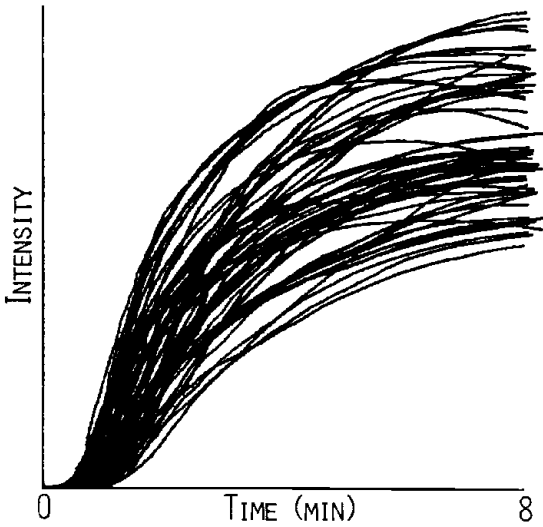


Figure 1. Intensity of chemiluminescence from human polymorphonuclear cells as a function of time after mixing with zymosan. Data is from 70 normal volunteers.

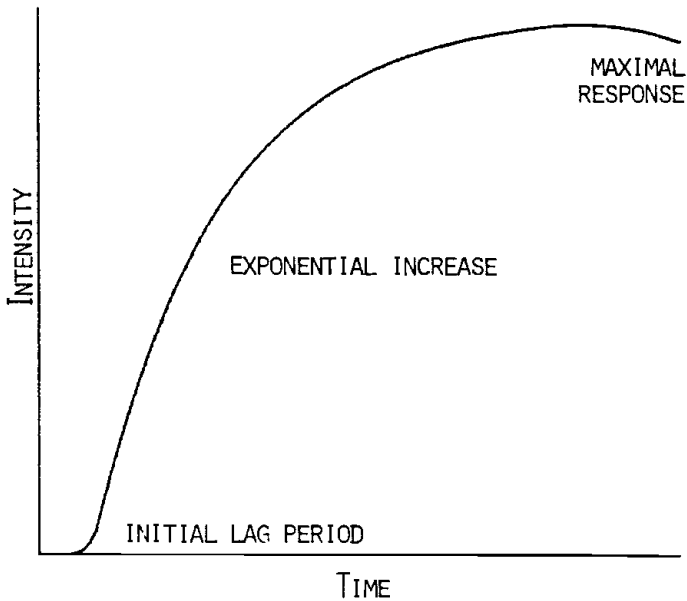


Figure 2. A summary of the general features of the chemiluminescent response.

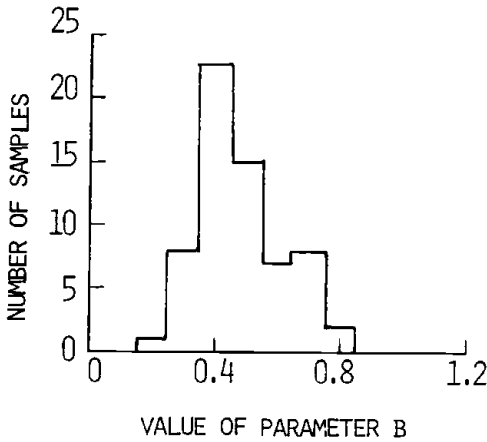
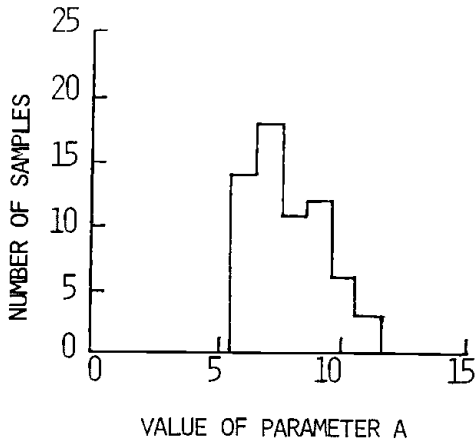


Figure 3. The chemiluminescent response of each sample was fitted to an exponential curve of the form $\text{Intensity} = A(1 - e^{-b(t - t_0)})$ and distributions of the values of parameters A and B plotted in the histograms.

curves vary independently, so neither parameter alone is representative of a sample. The characteristics of the panel of normal volunteers were therefore presented as a correlated plot of both parameters. This representation illustrates the range of values

for normal cells, and forms a template for comparison of altered or diseased states. Cells with abnormal combinations of these parameters would fall outside the range defined for normal cells. The scatter of the data within the normal range verifies that the two parameters are indeed independent. A primary result of this method is the reduction of a complicated CL response to a pair of parameters which can be used as a basis for comparison.

CORRELATED PLOT OF THE EXPERIMENTAL DATA

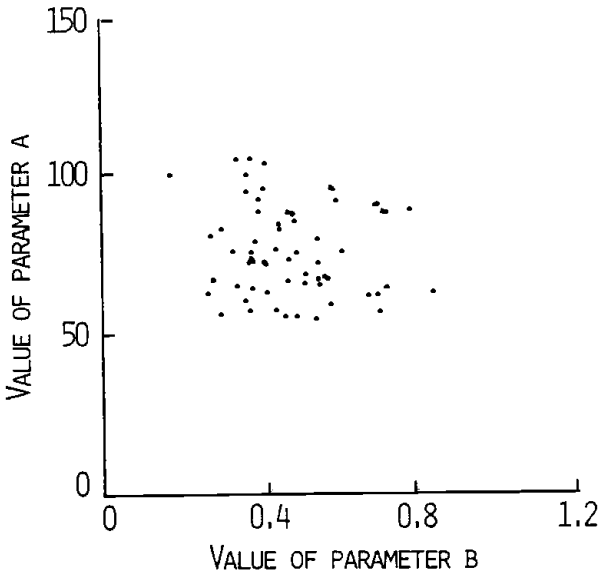


Figure 4. Correlated plot of the exponential parameters a and b associated with the chemiluminescent response of polymorphonuclear cells from normal volunteers.

CONCLUSIONS

1. An exponential curve can be used to reduce a complicated chemiluminescent response to a pair of parameters which can be used as a basis for comparison.
2. At least two parameters are required to adequately describe the chemiluminescent response.
3. A correlated plot of the exponential parameters is a convenient way to visually compare a sample with a control panel.

REFERENCES

1. J.C. Whitin, C.E. Chapman, E.R. Simons, M.E. Chovaniec and H.J. Cohen, "Correlation between membrane potential changes and superoxide production in human granulocytes stimulated by phorbol myristate acetate: evidence for defective activation in chronic granulomatous disease", *J. Biol. Chem.* 255, 1874-1878, 1980.
2. C. Baldrige and R. Gerard, "The extra respiration of phagocytosis", *Am. J. Physiol.* 103, 235-236, 1933.
3. R. Allen, R. Stjernholm, and R. Steele, "Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity", *Biochem. Biophys. Commun.* 47, 679-684, 1972.
4. H. Seliger, "Single photon counting and spectroscopy of low intensity chemiluminescent reactions" in "Liquid Scintillation Counting, Recent Applications and Development", (C. Peng, D. Horrocks and E. Alpen, eds.), Academic Press, New York, 281-319, 1980.
5. T. Horan, T. McPherson and A. Noujaim, "Application of liquid scintillation spectrometry in the evaluation of neutrophil function" in "Liquid Scintillation Counting, Recent Applications and Development" (C. Peng, D. Horrocks and E. Alpen, eds.), Academic Press, New York, 321-340, 1980.
6. T. Horan, D. English and T. McPherson. Association of neutrophil chemiluminescence with microbicidal activity. *Clin. Immunol. Immunopathol.* 22, 259-269, 1982.