Image Cover Sheet

CLASSIFICATION	SYSTEM NUMBER
UNCLASSIFIED	151453

TITLE

OPTIMIZATION OF A PHAGOCYTE MICROPLATE CHEMILUMINESCENT ASSAY

System Number:

Patron Number:

Requester:

Notes:

DSIS Use only:

Deliver to:

J BIOLUMIN CHEMILUMIN 1995; 10: 63–7

Optimization of a Phagocyte Microplate Chemiluminescent Assay

Bill Kournikakis and Maureen Simpson
Medical Countermeasures Section, Defence Research Establishment Suffield, Box 4000, Medicine Hat Alberta, Canada, T1A 8K6

Chemiluminescent assays have been used to quantify phagocytic activity since 1972. In recent years these assays have been adapted to the 96-well microplate format as new luminometers have been developed. In this report we describe the optimization of a lucigenin enhanced phagocyte chemiluminescent assay using a Titertek Luminoskan. Factors such as cell concentration, serum concentration in the opsonization of the zymosan used and lucigenin concentration were all optimized in our assay. In addition we have found that some of the unique features of the Luminoskan, continuous microplate agitation during the assay and microplate temperature control up to 43°C, also significantly enhanced the chemiluminescent response.

Key words: chemiluminescence assay; phagocytosis; monocyte; neutrophil; lucigenin; zymosan

INTRODUCTION

Phagocytic cells, such as macrophages and neutrophils, form an important part of host defences against infection. A number of techniques are in common use for evaluation of the phagocytic response, including microbicidal assays (1,2), radioisotopic detection of labelled bacteria (3), fluorimetric assay (4) and chemiluminescent assay (5–10). The latter assay makes use of the oxygen metabolites involved in the phagocytic response enhanced by chemicals such as lucigenin, which reacts preferentially with O_2^-, and luminol, which reacts preferentially with H_2O_2 in the presence of a peroxidase (6). A comprehensive review of the complex interactions between oxygen metabolites and enhancing agents for chemiluminescence was written by Allen (11).

Although chemiluminescent phagocytosis assays have existed for a number of years, since first reported by Allen *et al.* (12), microplate-based luminometers now permit these assays to be conducted for larger numbers of samples with greater sensitivity (5,13). In this report we describe the optimization of a chemiluminescent phagocytosis assay which utilizes a computer-controlled microplate-based luminometer.

MATERIALS AND METHODS

Cell preparation

Fresh heparinized whole blood from healthy donors was separated by using Mono-Poly resolving media (Flow Laboratories, Irvine, UK). Seven millilitres of whole blood was layered onto 3 mL separation media and centrifuged at 300 *g* for 35 min. The mononuclear and neutrophil fractions were washed with Hank's balanced salt solution without sodium bicarbonate, phenol red, pH 7.4 ($HBSS^-$) (Gibco Laboratories, Grand Island, NY). When necessary, contaminating red blood cells were lysed with distilled water and the remaining white blood cells resuspended in an equal volume of double strength $HBSS^-$ and washed twice with $HBSS^-$. Cells were resuspended in 1–2 mL Hank's balanced salt solution with $CaCl_2$, $MgCl_2$, $6H_2O$, $MgSO_4$ 7 H_2O, pH 7.4 ($HBSS^+$) (Gibco Laboratories, Grand Island, NY). Viable

CCC 0884–3996/95/010063–05

Received 16 December 1993
Accepted (revised) 5 April 1994

cell concentration was determined by trypan blue exclusion on a haemocytometer. Cell viability was greater than 90%. Cells were diluted to required concentration in $HBSS^+$. Cell purity was determined by centrifuging samples onto a glass slide with a Cytospin 2 (Shandon) at 600 rpm for 10 min. The slide was stained with Surestain Wright-Giemsa (Fisher Diagnostic, Pittsburgh, PA). Two hundred cells were counted in triplicate and the percentage of mononuclear cells or neutrophils determined. Purity was normally greater than 99% for neutrophils and ranged from 73% to 92% for mononuclear cells. Contaminants in the mononuclear fraction included red blood cells and some neutrophils.

Reagents

A 1.0 mg/mL solution of zymosan A (Sigma Chemical Company, St Louis, MO) was prepared by boiling zymosan in phosphate buffered saline (PBS, Dulbecco A, Oxoid Limited, UK) for 30 min. Sample was centrifuged for 10 min at 300 *g*, and the pellet resuspended in $HBSS^+$. To opsonize zymosan, the pellet was incubated with human serum for 30 min at 37°C, centrifuged and resuspended in $HBSS^+$ to 1.0 mg/mL. Serum concentrations used were 0% (control), 5%, 10%, 20%, 40%, 60%, 80%, and 100%.

The number of zymosan particles were counted using a haemocytometer. The ratio of zymosan particles : cells used were 5 : 1, 10 : 1, 20 : 1, 40 : 1, and control (with non-opsonized zymosan particles) at a ratio of 10 : 1.

Lucigenin (Sigma Chemical Company, St Louis, MO) was prepared in $HBSS^+$. Concentrations used were 1×10^{-3}, 8×10^{-4}, 6×10^{-4}, 4×10^{-4}, 1×10^{-4}, 8×10^{-5}, 6×10^{-5} and 4×10^{-5} mol/L.

Chemiluminescence assay

Chemiluminescence assays were performed on an unmodified Titertek Luminoskan (ICN Biomedicals, Inc., Huntsville, AL). Titertek fluoroplates, (96 wells, black) were used in the assay (Flow Laboratories, Irvine, UK). One hundred microlitres lucigenin was added to each well. Fifty microlitres neutrophils or monocytes were then added to the appropriate wells. Except where otherwise indicated, the plate was incubated at 37°C for 10 min within the Luminoskan, after which the Luminoskan control program was started by the controlling IBM-compatible personal computer (PC). The Luminoskan was programmed to add 100 μL zymosan to each well to initiate the assay, after which the instrument would scan each well. Measurement times of 2 or 5 s per well were used for the optimization experiments. Raw data from the luminometer was recorded by the controlling PC. The data was subsequently processed by a program written in-house which calculated the means and standard errors of each sample at each time period. These values were imported into SigmaPlot (ver. 5.0) for preparation of graphs for analysis.

RESULTS AND DISCUSSION

The Titertek Luminoskan is a PC-controlled microplate-based luminometer which is capable of measuring chemiluminescent (CL) response. The device incorporates internal reagent dispensers, temperature and agitation controls which allow the CL assay to be semi-automated. A similar assay described by Blair *et al.* (5) made use of a specially modified Amerlite CL reader which was not capable of temperature control nor agitation and required that the plates be transferred between a microplate incubator and the CL reader at 5-min intervals.

The typical CL response by human monocytes

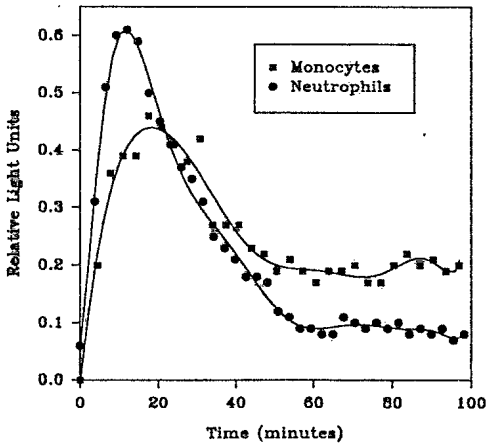

Figure 1. CL response using 5×10^5 cells/well (1×10^7/mL). Each point represents the mean of triplicate wells in a single representative experiment. Unstimulated controls show peak neutrophil response of <0.1 and monocyte response <0.02 (data not shown). Error bars have been omitted for clarity

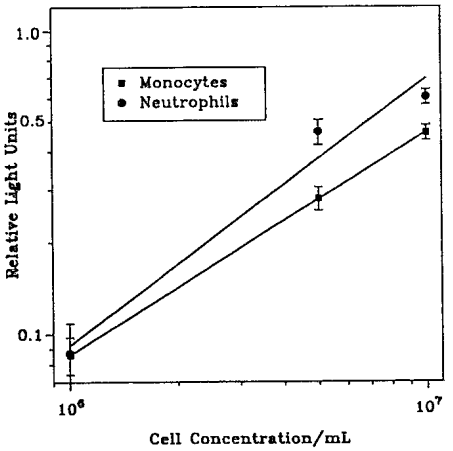

Figure 2. The effect of cell concentration on peak height of CL response. Each points represents the mean and standard error of three replicates in a single experiment. Similar results were obtained in three other experiments

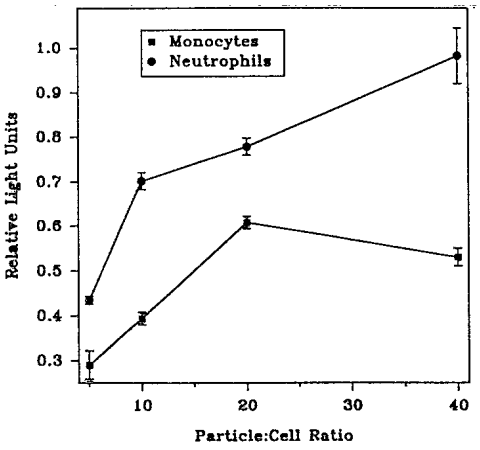

Figure 4. The effect of variation of the particle to cell ratio on the peak height of the CL response. Each point represents the mean and standard error of three replicates in a single experiment. Similar results were obtained in two other experiments

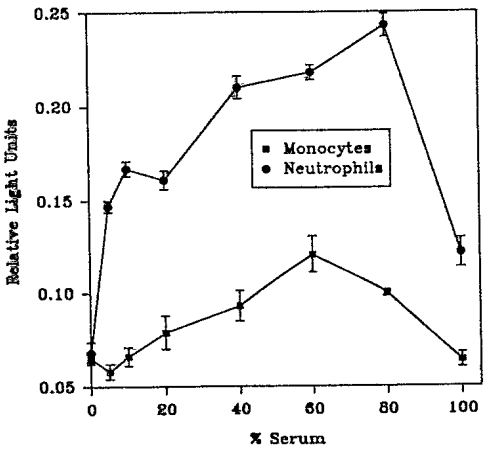

Figure 3. The effect of the serum concentration used to opsonize zymosan particles on the CL peak height. Each point represents the mean and standard error of six replicates in a single experiment. Similar results were obtained in four other experiments

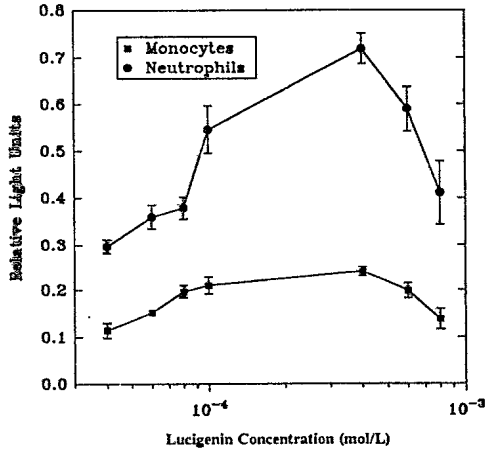

Figure 5. The effect of variation of lucigenin concentration on the peak height of the CL response. Each point represents the mean and standard error of three replicates in a single experiment. Similar results were obtained in three other experiments

and neutrophils is shown in Fig. 1. The response seen is similar to that described by Blair *et al.* (5) in that the neutrophil CL response peak is greater than the monocyte peak. Control responses were negligible. The peaks we have shown are more clearly resolved, similar to results obtained by Robinson *et al.* (7) who used a scintillation counter for chemiluminescent detection. The peak height of the chemiluminescent response was selected as the factor on which the assay would be optimized, although the initial slope and area under the curve could also be utilized in analysing the CL response.

Peak height was directly related to cell concentration (Fig. 2), an observation also noted by Blair *et al.* (5). The monocyte CL response was directly related to the increasing concentration of serum used to opsonize zymosan to a peak at 60% while the neutrophil response peaked at 80% serum concentration before falling (Fig. 3). These peaks were

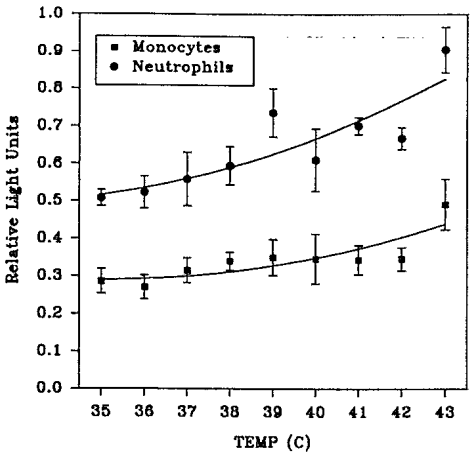

Figure 6. The effect of temperature on the peak height of the CL response. Each point represents the mean and standard error of 4–8 experiments

associated with a greater initial slope and total CL (area under the curve).

Variation of the zymosan particle to cell ratio demonstrated an increase in chemiluminescent response for neutrophils with an increase in the ratio up to 40 : 1, while the macrophage response peaked at 20 : 1 ratio and fell slightly thereafter (Fig. 4). The effect of lucigenin concentration was shown to peak at 4×10^{-4} mol/L for monocytes and neutrophils (Fig. 5) although results for 1×10^{-4} and 8×10^{-4} are also acceptable for monocytes.

The Luminoskan is equipped with a mixer function which is capable of continually agitating the microtitre plate during the assay. In all cases tested the use of the mixer option provided greater initial slope, peak heights and total CL for both monocytes and neutrophils (data not shown), in some cases exceeding a twofold increase in peak height. Blair *et al.* (5) attempted to agitate assay plates by manually tapping the plate several times between readings; however this manual approach to plate agitation did not enhance the CL response.

The Luminoskan is also capable of controlling the temperature of the assay from ambient to 43°C. Variation of the temperature from 35°C to 43°C showed an increase in peak height for neutrophils and monocytes (Fig. 6). Chemiluminescent assays are normally conducted at 37°C (5,6,14). The 78% increase in neutrophil CL peak height between 37 and 43°C was significant (t-test, $p = 0.0003$), as was the 56% increase in peak height for monocytes (t-test, $p = 0.025$). These increases were also associated with a greater initial slope of the CL reaction and total CL response.

Using the optimized assay, CL profiles were conducted using cells from 13 healthy volunteers with both 2 and 5 s measurement times. Peak heights for monocytes ranged from 0.097 to 0.153 and 0.133 to 0.251 respectively. Peak heights for neutrophils range from 0.221 to 0.640 and 0.387 to 1.006 for the 2 and 5 s measurements, clearly showing the variations in response by different individuals. There was no apparent correlation between the monocyte and neutrophil peaks for each individual (data not shown).

Intra-assay variation with this CL assay is low, as seen by the error bars in Figs 2 to 5. Inter-assay variation is generally greater as seen in Fig. 6. We believe that this is a result of having used cells from a variety of volunteers who show different CL responses. We have also observed that when using cells from the same individual the peak CL response can vary by a factor of two on a day-to-day basis (data not shown). Additionally, we have found that if three separate assays are conducted on the same day using cells collected from one individual there is no significant difference in the CL peaks (one way ANOVA, $p > 0.05$).

Work is now under way to determine whether the initial CL kinetic response can be related to peak height. This may allow total assay time to be shortened considerably.

In summary, we have optimized a microtitre plate based chemiluminescent assay which makes use of a PC-controlled luminometer. The assay is semi-automated and capable of evaluating the CL response of up to 96 samples plus controls at one time. The assay is useful in evaluation of the phagocytic response of cells based upon the CL response. Although optimized for use with human phagocytic cells, the same assay has also proved successful in measuring the CL response from porcine and murine phagocytes (work in progress).

REFERENCES

1. Leigh PCJ, Van Den Barselaar MTh, Van Furth R. Kinetics of phagocytosis and intracellular killing of *Staphylococcus aureus* and *Escherichia coli* by human monocytes. Scand J Immunol 1981;13:159–74.
2. Marodi L, Leijh P, Van Zwet R. A micrometho1 for the separate evaluation of phagocytosis and intracellular killing of

Staphylococcus aureus by human monocytes and granulocytes. J Immunol Methods 1983;57:353–61.
3. Verhoef J, Peterson RK, Quie PG. Kinetics of staphylococcal opsonization, attachment, ingestion and killing by human polymorphonuclear leukocytes: a quantitative assay using ^{3}H-thymidine labelled bacteria. J Immunol Methods 1977;14:303–11.
4. Oben JA, Foreman JC. A simple quantitative fluorometric assay of *in vitro* phagocytosis in human neutrophils. J Immunol Methods 1988;112:99–103.
5. Blair AL, Cree IA, Beck JS, Hastings MJG. Measurement of phagocyte chemiluminescence in a microtitre plate format. J Immunol Methods 1988;112:163–8.
6. Pauksens K, Sjölin J, Venge P. Chemilulminescence of polymorphonuclear leukocytes and whole blood during acute bacterial infection. Scand J Infect Dis 1989;21:277–84.
7. Robinson P, Wakefield D, Breit SN, Easter JF, Penny R. Chemiluminescent response to pathogenic organisms: normal human polymorphonuclear leukocytes. Infect Immun 1984;43:744–52.
8. Allen RC, Stevens D. The circulating phagocyte reflects the *in vivo* state of immune defense. Curr Opin Infect Dis 1992;5:389–98.
9. Davies B, Edwards SW. Chemiluminescence of human bloodstream monocytes and neutrophils: an unusual oxidant(s) generated by monocytes during the respiratory burst. J Biolumin Chemilumin 1992;7:229–38.
10. Dahlgren C, Follin P, Johansson A, Lock R, Orselius K. Localization of the luminol-dependent chemiluminescence reaction in human granulocytes. J Biolumin Chemilumin 1989;4:263–6.
11. Allen RC. Phagocytic leukocyte oxygenation activities and chemiluminescence: a kinetic approach to analysis. Methods Enzymol 1986;133:449–93
12. Allen RC, Stjernholm RL, Steele RL. Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. Biochem Biophys Res Commun 1972;47:679–84.
13. Maly F-E, Vittoz M, Urwyler A, Koshikawa K, Schleinkofer L, De Weck AL. A dual microtiter plate (192 sample) luminometer employing computer-aided single photon imaging applicable to cellular luminescence and luminescence immunoassay. J Immunol Methods 1989;122:91–6.
14. Cree IA, Blair AL, Beck JS. Use of a microtitre plate chemiluminescence reader to study surface phagocytosis by human monocytes. J Biolumin Chemilumin 1989;3:71–4.

#151453

NO. OF COPIES NOMBRE DE COPIES	COPY NO. COPIE N°	INFORMATION SCIENTIST'S INITIALS INITIALES DE L'AGENT D'INFORMATION SCIENTIFIQUE
1	1	DAG
AQUISITION ROUTE FOURNI PAR ▶ DRES		
DATE ▶ 09 May 95		
DSIS ACCESSION NO. NUMÉRO DSIS ▶		

DND 1158 (6-87)

National Defence / Défense nationale

PLEASE RETURN THIS DOCUMENT TO THE FOLLOWING ADDRESS:

DIRECTOR
SCIENTIFIC INFORMATION SERVICES
NATIONAL DEFENCE
HEADQUARTERS
OTTAWA, ONT. - CANADA K1A 0K2

PRIÈRE DE RETOURNER CE DOCUMENT À L'ADRESSE SUIVANTE:

DIRECTEUR
SERVICES D'INFORMATION SCIENTIFIQUES
QUARTIER GÉNÉRAL
DE LA DÉFENSE NATIONALE
OTTAWA, ONT. - CANADA K1A 0K2