

QUANTITATION OF LEUKOCYTE CHEMILUMINESCENCE FOLLOWING
PHAGOCYTOSIS: TECHNICAL CONSIDERATIONS USING
LIQUID SCINTILLATION SPECTROMETRY

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Abstract

Electron excitation of oxygen molecules, with release of photons due to electron relaxation, is associated with leukocyte phagocytosis. These events are the result of formation of singlet oxygen and/or superoxide anion, and these, in turn, are unique intracellular microbicidal agents. Rate of photon emission can be monitored in a liquid scintillation counter operated at 25-27°C in the out-of-coincidence mode. Both phagocytic monocytes and polymorphonuclear leukocytes isolated from human blood emitted photons upon engulfment of opsonized zymosan, or yeast. Rate of photon emission was proportional to the concentration of phagocytes present in the sample. Color quenching of photons was noted when contaminating erythrocytes were present in the sample. For this reason, a technique was developed to prepare highly purified preparations of mononuclear and polymorphonuclear leukocytes devoid of erythrocytes. Control studies indicated that chemiluminescence was inhibited if Ca^{++} and Mg^{++} were absent from the test sample. Chemiluminescence appears to be a simple method for determining phagocytosis and intracellular killing on the part of phagocytic monocytes and polymorphonuclear leukocytes present in human blood.

Introduction

Human polymorphonuclear leukocytes (PMNL) bioluminesce upon phagocytosis of bacteria (1). This bioluminescence may be due to the generation of electron excitation states associated with the generation of singlet oxygen ($^1\text{O}_2$) or

to the generation of superoxide anion ($O_2^{\cdot-}$; 1, 2). On the other hand, the molecular explanation for bioluminescence is thought to be unknown by some investigators (3), and there is a great deal of uncertainty as to the relationship between the chemiluminescence and the bactericidal activity of PMNL (3). PMNL from patients with chronic granulomatous disease of childhood fail to produce superoxide anion or hydrogen peroxide after phagocytosis (4). This is associated with a deficiency of bacterial killing (4) and a lack of emission of photons after phagocytosis (2).

A previous report (5) indicates that the emission photon wavelength can be observed as a wide peak of photon emission in the visible region. Singlet oxygen is known to emit photons at distinct peaks of 7030, 6340, 5200 and 4800 Å (6). Studies from our laboratory (unpublished results) suggest that two distinct peaks of photon emission at 4360 and 5500 Å are associated with the chemiluminescence of phagocytosis using human PMNL. These peaks are of a higher energy than the major peak resulting from the relaxation of singlet oxygen, which is 6340 Å (6). Thus, it is unlikely that singlet oxygen is directly responsible for chemiluminescence, although its role as an indirect factor cannot be excluded.

PMNL bactericidal effects are dependent on a halide cofactor (3), and chemiluminescence by phagocytes is also dependent on a halide cofactor (7). Mixture of hydrogen peroxide with hypochlorite, produced by PMNL phagocytosis, is associated with a weak red chemiluminescence thought to be due to the generation of singlet oxygen. This production of chemiluminescence appears to be dependent on the presence of a halide cofactor (Cl^-). Further, the singlet oxygen quencher, 1,4-diazobicyclo [2,2,2] octane (8) inhibits the myeloperoxidase-mediated bactericidal system of the phagocyte (9). Finally, superoxide dismutase will inhibit both chemiluminescence and the bactericidal effects of PMNL phagocytes (2). It has been suggested that superoxide dismutase is present in most mammalian cells to protect them from the toxic effects of radicals produced by the univalent reduction of oxygen (10), but may be compartmentalized in the PMNL so as to provide the PMNL with a unique and potent bactericidal mechanism (11).

Thus, chemiluminescence associated with phagocytosis may be due to the activation of the hydrogen peroxide-halide-myeloperoxidase bactericidal system. Detection and quantitation of such chemiluminescence might therefore be used as a test of phagocytic function. This report

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describes the technical considerations involved in the quantitation of PMNL chemiluminescence using liquid scintillation spectrometry.

Materials and Methods

Liquid Scintillation Counter: A Searle Instrumentation Isocap 300 Scintillation Counter was used for most of the experiments in this study. The counter was operated at ambient temperature (25-27°C) with the coincidence gate bypassed so as to record all photons exciting a single photomultiplier tube. A wide window was used to record all counts.

Vials: Kimble low potassium glass scintillation vials were used throughout the study. Vials were kept in the dark prior to use and prescreened for background chemiluminescence prior to each experiment. A single-tube background of <10,000 CPM was considered acceptable.

Leukocyte Preparations: Four different preparations of leukocytes were used in the study (Figure 1). Aseptic siliconized glassware was used throughout.

A mixed preparation of leukocytes and erythrocytes was isolated from heparinized venous blood (10 ml), by diluting the blood to 20% with plasmagel (HTI Laboratories, Buffalo, New York) to increase erythrocyte sedimentation. This was held at room temperature for thirty minutes, the leukocyte-rich supernatant was aspirated and washed three times (400 x G, 10 min) with 10 ml of Hank's balanced salt solution (BSS) (GIBCO, Grand Island, New York) which contained Ca^{++} and Mg^{++} but no phenol red. Following washing, the leukocytes were resuspended in BSS at a concentration of 10^6 PMNL per ml unless otherwise noted. These preparations contained 40-70% PMNL and 30-60% mononuclear leukocytes, with a ratio of approximately 10-100 erythrocytes per leukocyte.

A second method for isolation of mixed leukocytes, devoid of erythrocytes, was developed when we found that the conventional techniques resulted in clumping of leukocytes, and interference with the estimation of the exact concentration of phagocytes and quantitation of chemiluminescence. Methyl cellulose (0.5 ml, 2% w/v in normal saline), was added to 8 ml of heparinized venous blood to enhance erythrocyte sedimentation. Sedimentation was allowed to proceed for 1 hr at room temperature. The leukocyte rich supernatant so obtained was mixed with 2 volumes of warm (37°C) lysing solution (0.155 M NH_4Cl , 10 mM NaHCO_3 , and 0.3 mM EDTA). This mixture was left to

stand for 5 min at 37°C in a water bath, and then centrifuged (400 x G) for 10 min. The leukocyte pellet was gently resuspended in warm lysing solution by drawing the pellet 5 or 6 times into a Pasteur pipette containing lysing solution. This mixture was then incubated for a further 5 min at 37°C, centrifuged (400 x G, 10 min) and the pellet gently resuspended in warm, sterile, normal saline. This preparation was centrifuged (300 x G, 10 min) and the pellet resuspended in saline, centrifuged again (300 x G, 10 min) and the pellet resuspended in warm BSS at a concentration of 1.0×10^6 PMNL per ml. These preparations contained 40-70% PMNL and 30-60% mononuclear leukocytes, of which 60-80% were lymphocytes and the remainder monocytes.

The third preparation, consisting of 98-100% pure mononuclear leukocytes was obtained using an adaptation of the method of Boyum (12). Heparinized venous blood was diluted with an equal volume of saline and carefully layered on a "cushion" of Ficoll-Hypaque. The layered tubes were centrifuged (400 x G, 30 min, 20°C) in a swinging bucket rotor. The leukocyte layer at the plasma-Ficoll-Hypaque interface was aspirated, mixed with 5 volumes of BSS, centrifuged (600 x G, 20 min), gently resuspended in warm BSS (10 ml), centrifuged (400 x G, 20 min), and finally resuspended in warm BSS at a concentration of 1.0×10^6 leukocytes per ml. These preparations contained 98-100% mononuclear leukocytes, 0.2% PMNL, and no erythrocytes. Of the mononuclear leukocytes, 60-80% were lymphocytic by morphological criteria, and the remainder were monocytes.

The fourth preparation, consisting of 93-98% PMNL was obtained using the above methodology, but isolating the PMNL and erythrocytes which had sedimented to the base of the Ficoll-Hypaque containing tubes. The PMNL were seen to form a thin "buffy coat" above the erythrocytes. The buffy coat was aspirated with a Pasteur pipette, mixed with 5 volumes of warm lysing solution, and incubated for 5 min at 37°C. This mixture was centrifuged (400 x G), the pellet gently resuspended in lysing solution, and incubated for 5 min at 37°C before being centrifuged (400 x G) and resuspended in BSS twice more in sequence. The final concentration was 1.0×10^6 PMNL per ml unless otherwise noted, and the preparations contained 93-98% PMNL, and 2-7% mononuclear leukocytes with no contaminating erythrocytes.

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Packed Erythrocytes: Heparinized venous blood was refrigerated at 4°C for 72 hours. The sedimented cells were washed three times in BSS (400 x G, 10 min) and pelleted by centrifugation (400 x G, 10 min). The final preparation consisted of approximately 10^6 erythrocytes per mm^3 .

Particles and Opsonization: *Candida albicans* was prepared from dry yeast (Standard Brands Ltd., Montreal, Canada) by boiling for 10 min in BSS, filtering through sterile gauze, and suspending in BSS at a concentration of 2×10^8 yeast cells/ml. For opsonization, the yeast preparation was diluted to 20% with fresh serum and incubated at 37°C for 30 min with gentle agitation.

Staphylococcus aureus and *Escherichia coli* were prepared from overnight cultures in trypticase soy broth. Bacteria were pelleted by centrifugation, and resuspended in BSS at a concentration of 1.0×10^9 organisms/ml (determined by optical density). For opsonization, the bacteria were pelleted by centrifugation (1,000 x G, 10 min), resuspended in fresh serum (0.5 ml per ml pelleted), and incubated for 30 min at 37°C.

Zymosan was purchased from NCI Pharmaceuticals (Plainview, N.Y.) and suspended in BSS at a concentration of 100 mg/ml. For opsonization, 100 μl of the zymosan mixture was mixed with 0.4 ml serum (final concentration - 20 mg zymosan/ml serum), and incubated for 30 min at 37°C.

Chemiluminescence Assays: Background CPM for each vial used in each experiment was determined by monitoring the chemiluminescence of the vial containing 10 ml BSS with the appropriate concentration of opsonized particles. Vials containing the desired concentration of leukocytes in 10 ml BSS were placed into the counter and the leukocyte background chemiluminescence determined. Particles were then added at a concentration of 1 ml of opsonized *C. albicans*, 100 μl of opsonized *E. coli*, *S. aureus*, or zymosan (unless otherwise noted), and readings in CPM were recorded at 0.2 min intervals for at least 1 min, and at minute intervals thereafter for at least 30-60 min. Representative background readings were <10,000 CPM for empty vials, 11,000-13,000 CPM for vials with 10 ml of BSS, 18,000-25,000 CPM for vials with BSS and leukocytes. The background counts of vials with particles in 10 ml BSS were subtracted from background of vials with BSS alone to determine the net particle background. Particle background was added to the background of vials containing leukocytes in BSS to determine total background, and this figure was subtracted from counts obtained after addition

of particles to leukocytes (CPM over background). Counts above total background were plotted as a function of time.

Results

In preliminary experiments, the first leukocyte preparation, which contained mixed leukocytes and contaminating erythrocytes, was used. Variable results were found, and it was concluded that erythrocyte contamination might be a possible source for quenching of photon emissions. To confirm this, we added increasing amounts of additional packed erythrocytes to 3 of 4 vials containing 10 ml of 10^6 PMNL per ml with a ratio of approximately 10 erythrocytes/leukocyte. One vial received no additional packed erythrocytes, and the other three vials received 100, 200, and 300 μ l of packed erythrocytes respectively. Opsonized *C. albicans* was added to each vial and the chemiluminescence monitored. The results (Fig.2) show that marked quenching of the chemiluminescence response occurred with increasing concentrations of erythrocytes. Thus, it was concluded that leukocyte preparations devoid of erythrocytes were necessary for precise quantitation of chemiluminescence. Varying the method for isolating leukocytes did not alter the ability of the leukocytes to chemiluminesce (Fig. 3). This was shown by studying chemiluminescence of leukocytes isolated by the first method, with those leukocytes separated by the second method after the addition of opsonized zymosan.

Chemiluminescence Response to Different Particles:
The chemiluminescence response of leukocytes isolated by the second method to opsonized and unopsonized *C. albicans* is illustrated in Figure 3. Opsonization is required for a maximal chemiluminescence response, presumably because unopsonized particles are less readily phagocytosed (13). Phagocytosis is also impaired in media devoid of Ca^{++} and Mg^{++} (13). In one experiment, leukocytes were suspended in BSS without Ca^{++} and Mg^{++} and mixed with a preparation of opsonized *C. albicans*, in which the cations had been removed, after opsonization, by addition of 2.5 mg/ml EDTA (disodium ethylenediamine tetraacetic acid). This mixture did not emit photons comparable to the control preparation, consisting of leukocytes in media containing Ca^{++} and Mg^{++} and opsonized *C. albicans* (Fig.4).

The chemiluminescence response of leukocytes isolated by the second method to opsonized *C. albicans*, *E. coli*, *S. aureus*, and zymosan is illustrated in Figure 5.

Leukocyte Preparations

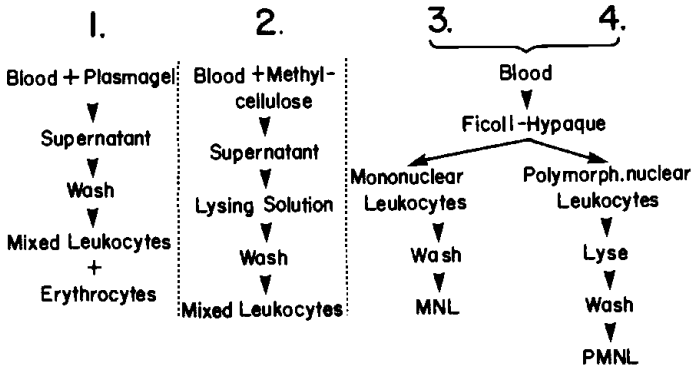


Figure 1. Methods used to isolate leukocytes from heparinized venous blood. MNL - Mononuclear leukocytes

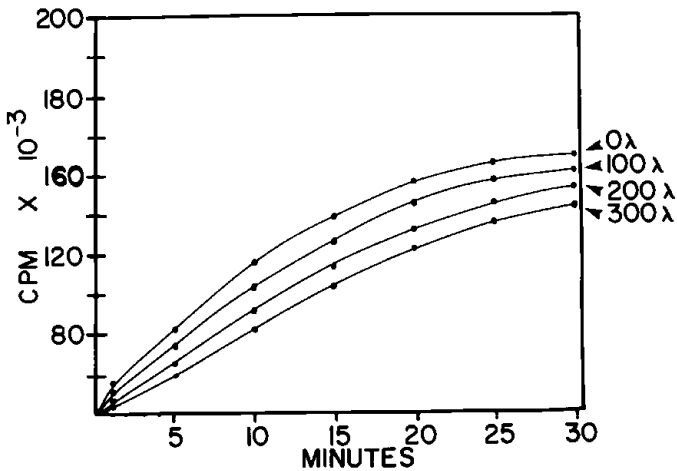


Figure 2. Color quenching of leukocyte chemiluminescence by erythrocytes. Chemiluminescence of leukocytes prepared by method 1 (Fig. 1) to opsonized *C. albicans*, as shown by top line, was markedly reduced by the addition of 100 μl, 200 μl, or 300 μl of packed erythrocytes to leukocyte suspensions.

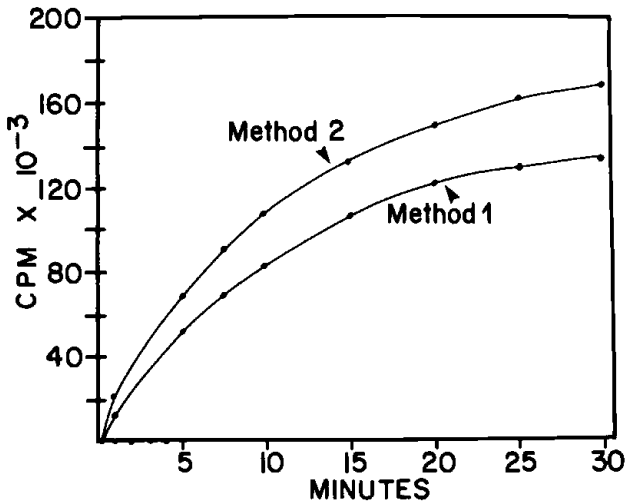


Figure 3. Chemiluminescence response of leukocytes to opsonized zymosan. Leukocytes were prepared from the same blood sample depicted in Figure 2, by method 1, containing contaminating erythrocytes, or by method 2, free from erythrocytes (Fig. 1).

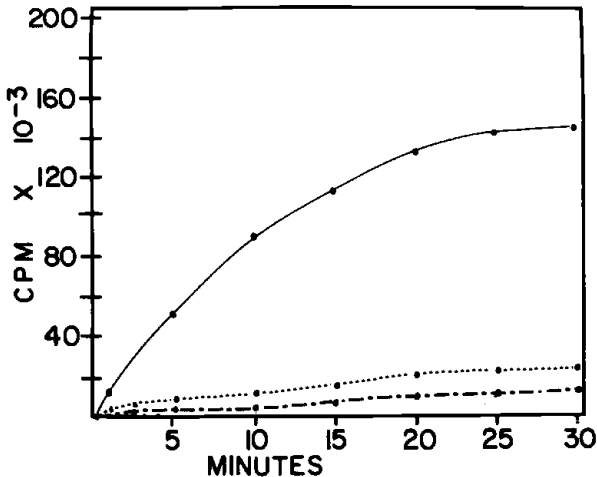


Figure 4. Inhibition of phagocyte chemiluminescence response to *C. albicans* (—) by use of media devoid of cations (.....), or by use of unopsonized particles (-----). In the latter two cases, microscopic examination of the incubation mixture revealed markedly decreased phagocytosis of particles by phagocytes.

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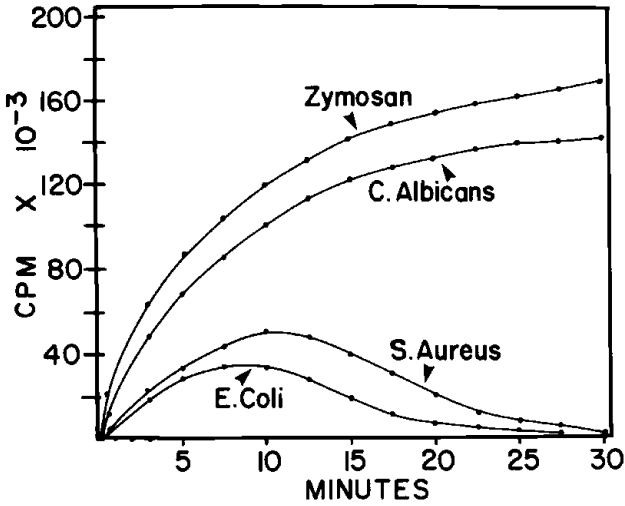


Figure 5. Chemiluminescence response of phagocytes elicited by opsonized zymosan, *C. albicans*, *S. aureus*, or *E. Coli*. Leukocytes were prepared as described in method 2 (Fig. 1).

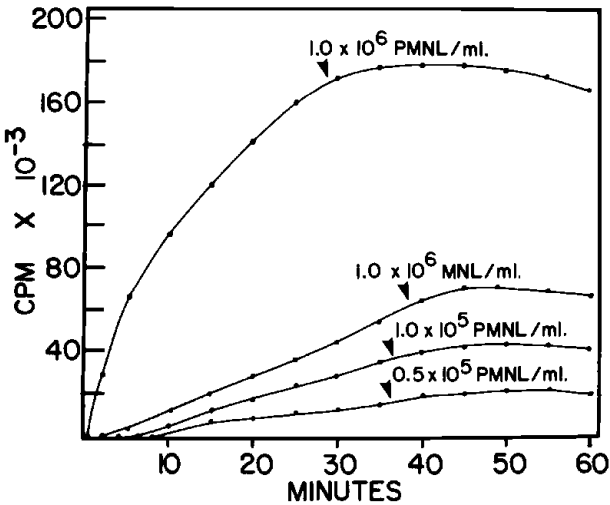


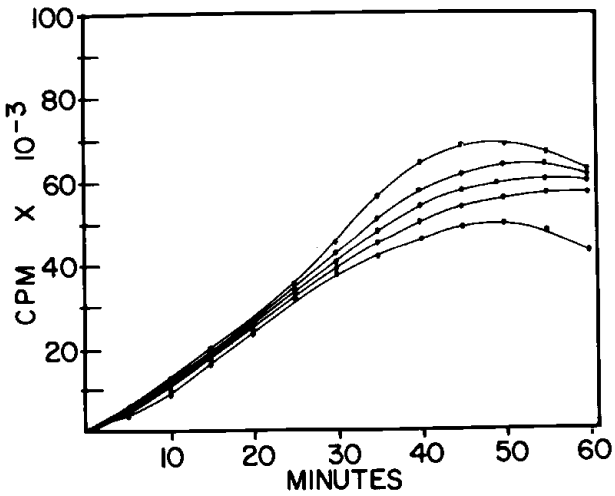
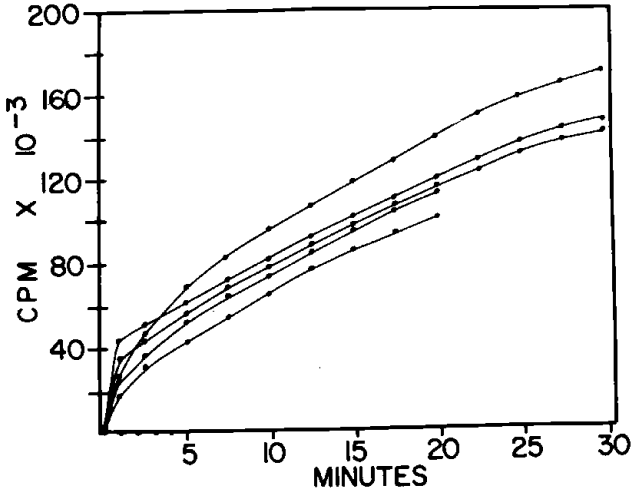
Figure 6. Chemiluminescence response by PMNL and mononuclear leukocytes isolated by methods 3 and 4 (Fig. 1). Leukocytes were suspended in BSS at the concentrations indicated, and tested using opsonized zymosan.

The response to bacteria was short-lived, whereas the response to *C. albicans* and zymosan lasted for up to 30 min and then fell gradually. A possible explanation for this might be that the larger yeast particles are subject to a continual attack by antimicrobial systems, whereas the smaller bacteria are phagocytosed, killed and digested more quickly.

Monocyte Chemiluminescence: The mechanism for bacterial killing by human monocytes might be similar to that of PMNL (14). However, no published information is available concerning the chemiluminescence of monocytes. In our hands, mononuclear leukocytes, isolated by the third method, containing <3% PMNL contamination, emitted photons in response to *E. coli* and zymosan. In an attempt to ensure that contaminating PMNL did not cause this response, chemiluminescence response of suspensions of PMNL (1.0×10^5 and 0.5×10^5 per ml) isolated by the fourth method, were studied, after the addition of opsonized zymosan (Fig. 6). A PMNL suspension of 1.0×10^5 would be equivalent to a 10% PMNL contamination of the monocyte suspension, while a suspension of 0.5×10^5 PMNL would be equivalent to a 5% PMNL contamination. Neither of these concentrations of PMNL gave sufficient photon emission to account for the observed monocyte response. The response of 1.0×10^6 PMNL/ml isolated from blood of the same individual as the above mononuclear leukocyte suspension is also shown in Figure 6 for comparison. PMNL responded more quickly and with greater intensity than the mononuclear leukocytes. However, the majority of the mononuclear leukocytes in the suspension were nonphagocytic lymphocytes, and therefore the response in terms of CPM per cell cannot be directly compared.

Variability: The day-to-day, and individual-to-individual, variability of chemiluminescence response of PMNL and monocytes was studied. The chemiluminescence response to opsonized zymosan of PMNL and mononuclear leukocytes, isolated from the blood of five different healthy individuals and assayed on one day, is shown in Figures 7 and 8. The response of mononuclear leukocytes showed little variability for the first 30 min, but the response of PMNL showed much greater variability. However, it was not thought that this variability in PMNL response would preclude the use of chemiluminescence for quantitative comparisons in a clinical setting. The variability of the response to opsonized zymosan by PMNL and mononuclear leukocytes isolated from the blood of one

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Figures 7 and 8. Variability in the chemiluminescence response by PMNL (Fig. 7) and mononuclear leukocytes (Fig. 8) to opsonized zymosan. Leukocytes were isolated by methods 3 and 4 (Fig. 1) from the blood of five healthy individuals, and tested on the same day. Note the difference in axis scale of Figure 6 as compared to Figure 7.

healthy individual on three consecutive days was minimal (Figs. 9 and 10).

Discussion

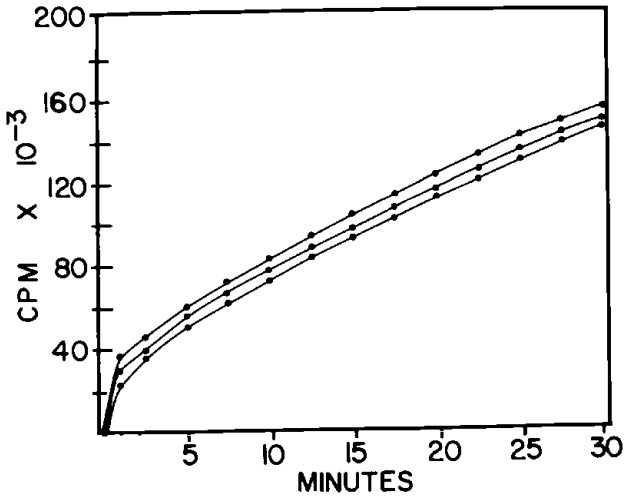
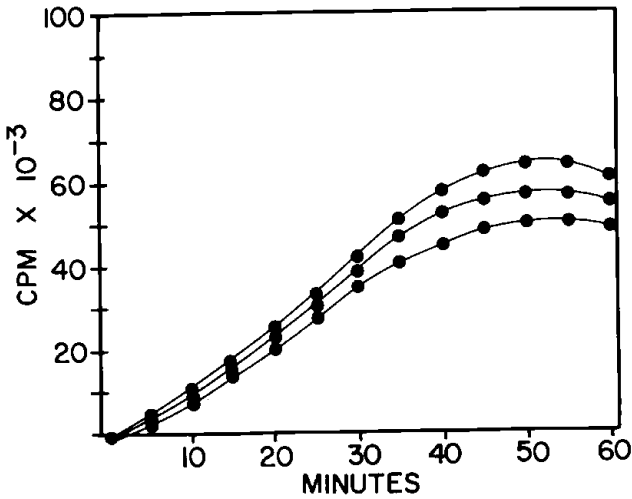
Chemiluminescence of human PMNL has previously been reported (17). However, few studies have commented upon the importance of the quenching effect of contaminating erythrocytes on chemiluminescence, and none, to our knowledge, have considered the contribution of contaminating mononuclear leukocytes to PMNL chemiluminescence. The studies reported here demonstrate that precise and consistent chemiluminescence quantitation is difficult, if not impossible, if heterogeneous populations comprising PMNL, mononuclear leukocytes and contaminating erythrocytes of varying numbers are used as samples. As reported here, methods are available for the isolation of relatively homogeneous suspensions of PMNL and mononuclear leukocytes, which are virtually devoid of contaminating erythrocytes. Such homogeneous suspensions are particularly useful for studying chemiluminescence of phagocytic cells, and should have clear-cut clinical application - particularly, if phagocyte chemiluminescence proves to be a measurement of the rate and total microbicidal capacity of phagocytes. In addition, the chemiluminescence technique, using the methods described herein, represents an important advance in terms of accuracy, ease, and consistency when compared to conventional quantitative bactericidal assays.

Stossel (13) indicates criteria which must be met in order to assert that a particular assay measures phagocytosis and intracellular killing. These include:

- (1) Inhibition of the reaction at ice bath temperatures
- (2) Demonstration that the rate of particle ingestion decreases as particle load increases
- (3) Demonstration that no reaction occurs at zero time at physiological temperatures.

Presently, we are performing experiments to meet these criteria, as well as to determine whether chemiluminescence parallels bactericidal activity as assayed by conventional methods. In addition, we are also investigating the effect of various metabolic and bactericidal inhibitors on the chemiluminescence response of human phagocytes in an attempt to delineate the biochemical nature of the chemiluminescence assay.

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Figures 9 and 10. Variability in the chemiluminescence response by PMNL (Fig. 9) and mononuclear leukocytes (Fig. 10) to opsonized zymosan. Leukocytes were isolated by methods 3 and 4 (Fig. 1) from the blood of one healthy individual on three consecutive days.

Acknowledgements

This work was supported in part by the Research Committee of the Provincial Cancer Hospitals Board, Alberta, the Dr. Mervin Laskin Fellowship in Cance (DE), and by the MRC of Canada (Grant MA-5904).

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