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Evaluation of variable parameters in neutrophil function test using chemiluminescence assay

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Summary

Variable parameters in cherniluminescence assay, one of the methods used to assess the functional capacity of neutrophils, were evaluated for suitable adaptation locally. The use of pooled normal human serum as compared to single normal human serum in opsonizing particles for phagocytosis was found to exhibit lower chemiluminescence activity (reduction range of 30%-50%). A similar degree of depression was observed when the particles were opsonized using normal human serum in comparison to that using autologous serum. Different intensity of chemiluminescence was also noted when the opsonized particle used was the Oxford strain of *Staphylococcus aureus* (NCTC 6571) in contrast to a strain of *Staphylococcus aureus* isolated from a patient. The results obtained warrant clinicians to deliver appropriate samples as best they can when the chemiluminescence assay is requested.

Key words: Neutrophil, cherniluminescence.

INTRODUCTION

Polymorphonuclear neutrophils (PMNs) are professional phagocytes that are critically involved in body defence against invading microorganisms.' To capture and destroy foreign materials, PMNs are equipped with a continuous process of phagocytosis which literally means ingestion of particulate material. In phagocytosis, different stages occur: chemotaxis, adherence, ingestion and eventually digestion.

One of the most important events in phagocytosis is the generation of light that occurs during the ingestion phase involving the increased uptake of oxygen, frequently known as respiratory burst.[2,3] This phenomenon of producing low level light by chemical reactions or chemiluminescence can be used to study the effectiveness of phagocytic function in various diseased states.[4,5,6]

Certain important technical aspects to be considered for the measurement of luminol-dependent cherniluminescence have been dealt with.' In this paper, other variable parameters for the chemiluminescence assay were analysed, namely, the effects of using different opsonizing sera and different opsonized particles. The observations recorded are expected to strengthen the recommendations of selecting chemiluminescence phagocytosis assays as routine diagnostic aids in the clinical diagnosis of defects associated with the phagocytic process.

MATERIALS AND METHODS

Preparation of cells

Blood was collected into heparinised tubes by venepuncture from apparently normal healthy individuals. The blood was layered over an equal volume of histopaque and centrifuged at 350 g for 30 minutes. The cell pellet was resuspended into Hank's balanced salt solution (HBSS) before mixing with 6% dextran to allow the red cells to sediment. After 30 minutes, the supernatant was centrifuged leaving the residual red cells and PMNs as pellet. The red cells were lysed in isotonic solution for 5 minutes and the remaining PMNs were washed in HBSS. The cells were finally suspended in phenol red-free HBSS (prf-HBSS). The number of cells was determined using improved Neubauer chamber under phase contrast microscopy.

Opsonization of particles

Zymosan (Sigma) was suspended in phosphate-buffered saline (PBS) to a concentration of 40 mg/ml. The bacteria *(Staphylococcus aureus* NCTC 6571 and a clinical isolate) were grown overnight in nutrient broth, washed twice and

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resuspended in PBS at a concentration of 1.2 X 10^9 cells/ml. Opsonization was performed by adding the required volume and dilution of serum to the appropriate suspension and incubating for 30 minutes at 37°C waterbath. The suspension was centrifuged at 3000g for 10 minutes before the pellet was washed in PBS and finally resuspended in prf-HBSS. The purpose of opsonization is to facilitate the ingestion phase in the phagocytic process since serum contains opsonins: IgG/IgM and complement components. Hence, aberrations in functional activities of serum opsonins can be assessed by neutrophil chemiluminescence assay.

Luminol

Stock solution of luminol (Sigma) was prepared by dissolving lurninol in dimethyl sulphoxide (DMSO, Sigma) at 10^{-2}M. The stock preparation was further diluted in prf-HBSS to the required concentration before use.

Chemiluminescence

The reaction mixture consisted of 0.1 ml cell suspension (10^6 cellslml), 0.2 ml of 10^{-4} M luminol in a polystyrene tube. This was placed in the light-proof chamber of luminometer (LKB1250). A suspension of 0.2 ml opsonized particles was added and the resulting light output in millivolts (mV) was continuously recorded. All constituents of the reaction mixture were kept at 37°C in a water bath and mixed prior to use. Absence or reduced light output could be due to either intrinsic disturbances of leukocytes to undergo respiratory burst or the opsonic activity is defective. The scheme below summarizes the general outcome of chemiluminescence activities:

	Normal neutrophils	Patient neutrophils
Normal human serum	Good chemiluminescence response	*Low or no response indicative of cellular defect
Patient serum	*Low or no response indicates defective opsonization	*Low or no response indicates defective opsonization and/or cellular defects

* The response produced is relative to that of normal cells used during the assessment. Since there are no "standard functional PMNs, the test to be conducted on patient's cells should always include a healthy subject's PMNs as a control.

TABLE 1: **Peak chemiluminescence produced by normal PMNs using two different types of opsonizing sera: normal human serum (NHS) and pooled normal human serum (PNHS). Zymosan was used as the opsonized particles**

Donor sample	Peak chemiluminescence (mV) Opsonization with NHS	Opsonization with PNHS	% reduction
1	102.3	50.0	51.0%
2	105.9	55.0	48.1%
3	109.9	72.0	34.5%
4	124.0	70.0	43.5%
5	137.0	96.0	30.0%

RESULTS

The use of pooled normal human sera (PNHS) as the opsonising serum was compared to that of a single normal human serum (NHS). The peaking time was similar for both types of sera used but the peak chemilurninescence was higher with NHS when compared to PNHS (Table 1). The range of reduced chemiluminescence was between 30% to 51%. All of the chemiluminescence activations recorded displayed similar pattern as that shown in Figure 1.

The effect of light emission when opsonization using autologous serum (AS) was also observed in comparison to that using NHS. In this particular work, opsonization using AS seemed to result in a better light output as compared to NHS. There was no difference in the peaking time between these two groups of sera (Figure 2). As can be seen, a great difference was detected in the peaking activity with an almost similar range

TABLE 2: **Peak chemiluminescence produced by normal PMNs using autologous (AHS) and normal human sera (NHS) to opsonize zymosan particles.**

Donor sample	Peak chemiluminescence (mV) Opsonization with AHS	Opsonization with NHS	% reduction
6	196.0	93.0	52.6%
7	90.0	45.0	50.0%
8	216.0	124.0	42.6%
9	63.0	34.0	46.0%
10	26.0	16.0	38.5%

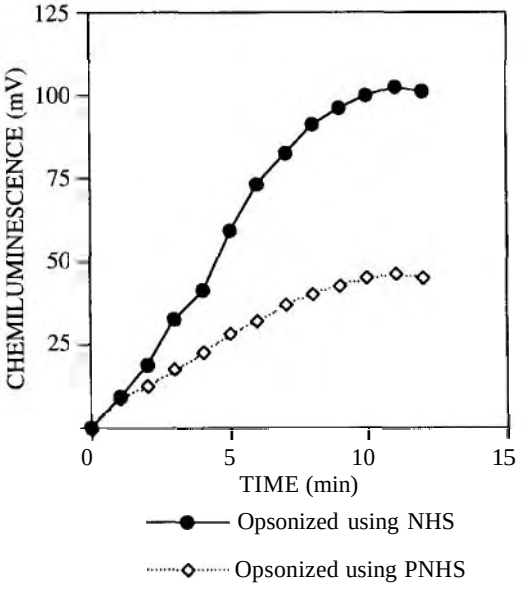

FIG. 1: Graphic presentation of a chemiluminescence activity by normal PMNs. Zymosan particles were opsonized with NHS (normal human serum) or PNHS (pooled human serum).

of reduced activity as that of earlier experiments comparing the use of PNHS to NHS (Table 2).

The opsonized *Staphylococcus aureus* strain isolated from a patient, resulted in a higher chemiluminescence response than the opsonized NCTC 6571 strain as presented graphically (Figure 3). The percentage of reduction varied from 9.7% to *37.8%* (Table 3).

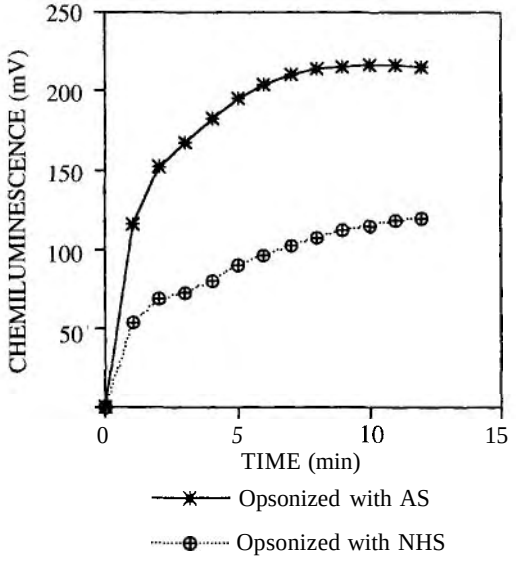

FIG. 2: Graphic presentation of a chemilurninescence response from normal PMNs using zymosan opsonized either with AS (autologous serum) or NHS (normal human serum).

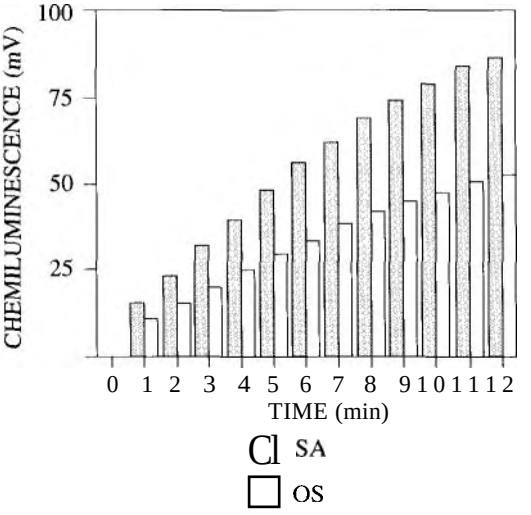

FIG. 3: Chemiluminescence of normal PMNs following the addition of opsonized *Staphylococcus aureus* clinical isolate (SA) or opsonized S. *aureus* NCTC 6571 (OS). Both particles were opsonized with autologous serum.

DISCUSSION

Various tests of neutrophil function have been used in the assessment of suspected immunodeficiency.[8] Obviously respiratory burst killing assays are the methods of choice in evaluating phagocytic activation. Techniques used to screen phagocytic processes include the killing test, nitroblue tetrazolium test (NBT) and the recently employed chemiluminescence method. Even though chemiluminescence assay has been accepted in certain health centers as the method choice for assessing phagocytic function, the test is yet to be used widely in Malaysia. The

TABLE 3: Peak chemiluminescence produced by normal PMNs using a clinical isolate of *Staphylococcus aureus* and *S. aureus* NCTC *6751* as the opsonized particles. The particles were opsonized with autologous serum

Donor sample	Peak chemiluminescence (mV) *Staphylococcus aureus*	NCTC 6571	% reduction
11	90.0	56.0	37.8%
12	66.0	50.0	24.2%
13	134.0	121.0	9.7%
14	145.0	117.0	19.3%
15	192.0	158.0	17.7%

evaluation of the chemiluminescence assay was carried out to study the feasibility of adapting the assay system for use in this country. Variable parameters that have not been analysed before were studied; comparisons using different types of sera for opsonization and different strains of S. *aureus* to be opsonized were made.

Though PNHS has been cited to be a suitable opsonizing agent? it was found that better results were obtained using just NHS from a single source. The former, a combination of sera from different sources, may contain a number of soluble factors that can possibly suppress PMN functions. In cases where autologous serum is available, this should be used in preference to NHS to opsonize the particles being phagocytosed. The results shown in Table 2 point to the advantage of employing the patient's own serum. Undoubtedly if the patient's autologous sera were to be used, the chemiluminescence assay would be better since opsonic activity of the patient's serum can be simultaneously evaluated as outlined in the materials and methods section.

Standard laboratory organisms such as the Oxford strain of *Staphylococcus aureus* (NCTC *6571)* have been frequently used in doing bacterial killing assays to assess PMN function. A culture isolate from the patient to be investigated for defective neutrophil function would however be more appropriate for the assay. Besides resulting in high chemiluminescence activity (Table 3), it would also indirectly indicate if such infective organisms will survive or be destroyed when encountered with the professional phagocytes *in vivo.*

The choice of chemiluminescence as a method to assess phagocytic function seems to be acceptable in local health centres since it is considered to be relatively simple with easy operating equipment. Currently, a study is being undertaken to compare the use of chemiluminescence to that of the traditional ways of testing phagocytic function namely the killing and nitroblue tetrazolium tests.

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