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Comparison of Three Different Methods in the Assessment of Neutrophil Function

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Summary

Three different methods to measure the oxidative respiratory burst of neutrophils were performed. Of the three, the chemiluminescence technique was observed to be the most sensitive among them. The strong statistical correlation and an acceptable agreement between chemiluminescence with that of the killing assay provides evidence for using the chemiluminescence assay as an alternative method of detecting gross defects of neutrophil respiratory burst killing assays.

Key Words: Chemiluminescence, Neutrophil function test

Introduction

The main functions of neutrophils in the host include resistance to infectious diseases or immunity, and production of inflammation, necrosis and fever[1] The action of neutrophils as an essential agency for protection of the host is best evidenced by an enormous increase in susceptibility to sepsis of persons afflicted with agranulocytosis and chronic granulomatous disease[2,3]. Thus, there is a need to analyse neutrophil function when severe or repeated infections occur in patients. One of the functional assays that would assist in understanding the nature of such clinical entities is the respiratory burst killing assay.

Simple methodology is usually used, for example the nitroblue tetrazolium test (NBT) and the candidacidal assay (CA). The chemiluminescence assay (CL) which was introduced later than the aforementioned tests has been considered to be an alternative method[4]. However, its effectiveness in assessing neutrophil functions has to be evaluated and simultaneously compared to NBT and CA.

This paper reports the comparison between these 3 methods, with a different approach, in the search for confirming the advantages of employing CL in the laboratory diagnosis of neutrophil defects.

Materials and Methods

Cells preparation

Neutrophils were isolated from the heparinized blood of pediatric (age <12 years old) patients and age-matched normal controls. The latter group consisted of healthy children prior to undergoing elective minor surgery. All the 18 patients reported in this study had recurrent bacterial infections as the major clinical

manifestation. Five milliliters of blood was layered over an equal volume of lymphocyte separation medium (Nycomed), centrifuged at 350 g for 30 minutes and the upper layers discarded. The bottom layer containing erythrocytes and neutrophils was resuspended in 5 ml of Hanks Balanced Salt Solution (HBSS, Flow) before mixing with 6% dextran solution. After allowing the red cells to sediment for 30 minutes, the supernatant was centrifuged. Contaminating erythrocytes were lysed in hypotonic solution of 0.83% Tris-buffered ammonium chloride and the remaining neutrophils were subsequently resuspended in phenol-red free-HBSS (prf-HBSS). Final cell concentration was adjusted to 1 x 10^6 cells/ml for all the three methods. The viability of the cells obtained was >98% as checked by trypan blue exclusion staining.

Chemiluminescence assay

A stock solution of luminol (Sigma) was prepared by dissolving luminol in dimethyl sulphoxide (DMSO, Sigma) at 10^{-2} M. The stock preparation was further diluted in prf-HBSS to the required concentration before use. The reaction mixture consisted of 0.1 ml cell suspension (10^6 cells/ml), 0.2 ml of 10^{-4} M luminol as the light amplifier and 0.1 ml of 10^{-4} M phorbol myristate acetate (PMA, Sigma) as the stimulant in a polystyrene tube. This was placed in the light-proof chamber of the luminometer (LKB1250). The resulting light output in millivolts (mV) was continuously recorded at 10 seconds intervals. All constituents of the reaction mixture were maintained at 37°C in a water bath prior to use.

Candidacidal assay

The killing assay done was a modified version of the method introduced by Lehrer & Cline[5]. One hundred microliters of leukocyte suspension at 1 x 10^7 cells/ml were mixed with 0.1ml *Candida albicans* (3 x 10^8 cells/ml) suspension in the presence of 40% serum and incubated at 37°C in a waterbath for 1 hour. Cells were then fixed in 2.5% glutaraldehyde, stained with methylene blue and finally observed microscopically for dead and live *Candida albicans* within the leukocytes. A total of 200 blastospores were counted, recording both viable cells (that excluded the dye) and dead yeasts (blue stained), and obtaining a figure for the percentage of dead yeasts.

NBT assay

The NBT reduction assay was performed according to the specified instructions in the kit (Sigma) with a slight modification. The cells were incubated with or without PMA for 15 minutes in the 37°C waterbath and continued for an additional 15 minutes at room temperature. Smears of fixed cells made were eventually treated with Wright's stain. A total of 200 cells were counted microscopically. Those neutrophils showing formazan deposits were recorded as positive, and the figure used for the test was obtained by substracting the PMA stimulated positive cells from those of the unstimulated cells (without PMA).

Calculations

To calculate the percentage depression of neutrophil function in CL assay, the following formula was used:

$$\frac{\text{Peak reading from patient cells}}{\text{Peak reading from normal control}} \times 100 = N$$

$$N - 100 = \%\ \text{depression}$$

The degree of depression in the NBT and candidacidal tests was determined using similar formula by replacing the peak readings of CL with the percentages of each particular tests obtained. All the tests were done in duplicate.

Statistical analysis

Spearman's rank correlation, r_s, was used to indicate existence of positive correlation in the investigation and eventually the significance of the correlation. To assess the degree of agreement between the methods employed in the study, statistical analysis as proposed by Altman and Bland[6] was adapted.

Results

A comparison of chemiluminescence activity and the candidacidal assay showed an equivalent percentage of reduction in the neutrophil function in the patients as exhibited by the simple plot of the results of one method against the other (Fig. 1). These calculated values were derived from the peak chemiluminescence readings and the percentage of killed organisms. Statistically, the Spearman's rank correlation of r_s =

0.99 showed an excellent relationship which was significant at $P < 0.01$. However, according to Altman and Bland[6] a plot of the difference between the methods against their mean is more informative (Fig. 2). In measuring the agreement of both methods, the 'limits of agreement' was calculated to be -8.87 and 17.53. Thus, the chemiluminescence reading could be 8.87% below or 17.53% above the candidacidal assay reading. The 95% confidence interval for the lower limit of agreement was -14.55 to -3.19. For the upper limit of agreement, the 95% confidence interval was 11.85 to 23.21. From a laboratory standpoint, these calculated intervals are relatively small, hence the degree of agreement is acceptable.

By contrast, the Spearman's rank correlation of r_s = 0.63 was still significant ($P < 0.01$) when CL was compared to that of NBT assay (Fig. 3). Nevertheless, a plot of the difference between the two methods against the average measurement (Fig. 4) displays considerable lack of agreement between these two assay systems. The statistical analysis done resulted in the 'limits of agreement' to be -3.48 and 58.6. The value of the 95% confidence interval for the lower limit of agreement was 9.90 to 16.86 whilst for the upper limit of agreement, the 95% confidence interval was 45.22 to 72.0. These wide intervals therefore, would be unacceptable for laboratory purposes.

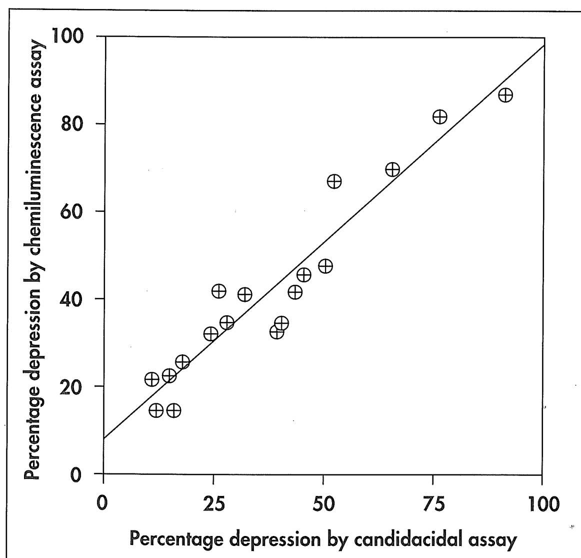

Fig. 1: Scatterplot of percentage of depression with CL and CA methods

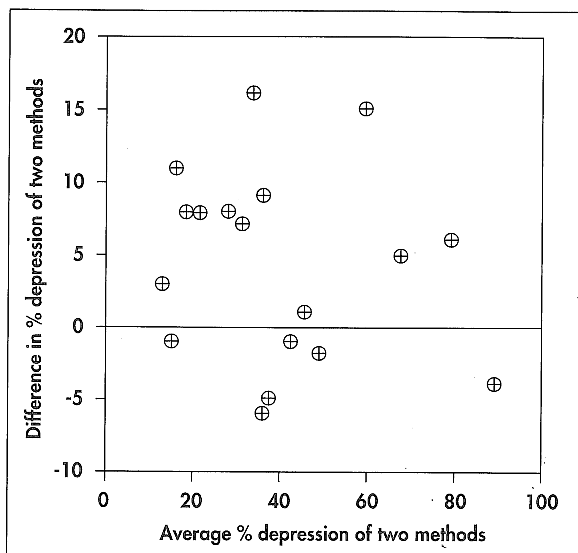

Fig. 2: Difference (CL-CA) against the mean of the two methods (CL+CA)/2

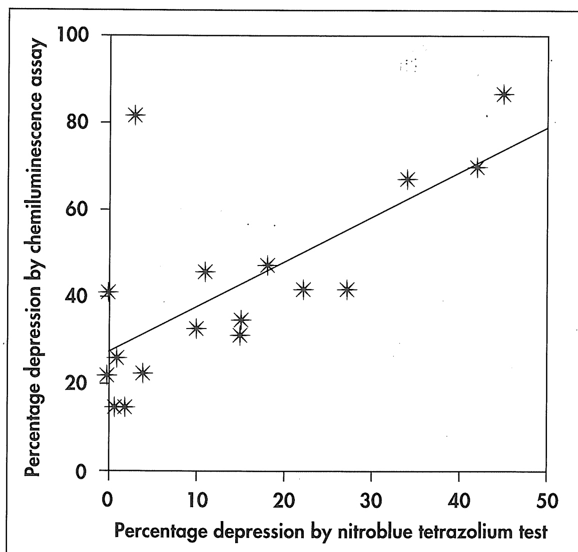

Fig. 3: Scatterplot of percentage of depression with CL and NBT methods

Discussion

Methods of assessing the phagocytic capability of neutrophils have been introduced and improved for several years[7]. One of the assays that has been cited to be a versatile technique is chemiluminescence. This sensitive screening method for the oxidative metabolism

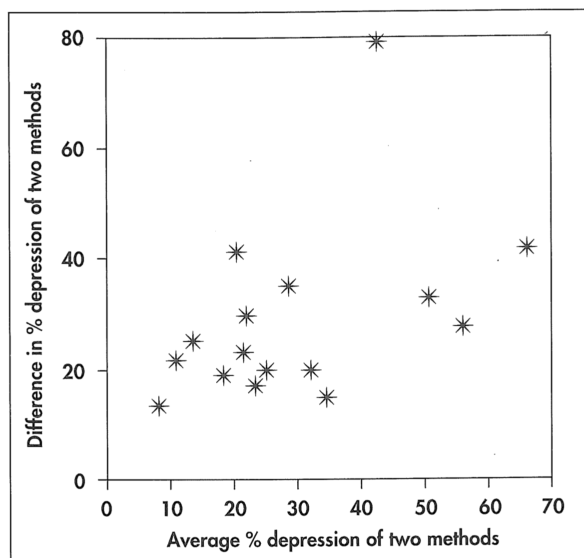

Fig. 4: Difference (CL-NBT) against the mean of the two methods (CL+NBT)/2

of phagocytic cells has been considered to be more useful than the killing assay or the NBT test. In one of the study reported earlier, the results obtained did not show a complete correlation between the measurement of the CL response and susceptibility of bacteria to be phagocytosed[8]. Hence, a direct comparative study of the different assay systems in the process of recommending CL as the alternative method in evaluating phagocytosis was lacking.

The work conducted thus far showed that CL is as good or better in analysing phagocytic function than the earlier methods, namely the NBT and candida killing assays. The strong positive correlation obtained with that of candidacidal assay using Spearman's rank correlation and a good agreement following the proposed method of analysis in the comparison of two methods of measurement, shows that CL assay presents an indirect reflection on the capability of phagocyte to kill. This seems to be acceptable since the agonist PMA used is an exogenous activator of protein kinase C, one of the key factor responsible in the activation of neutrophils function[9]. Any detectable dysfunction on the oxidative burst when using PMA would therefore indicate the probable defect in killing activity of phagocytes as the protein kinase C fails to be functionally operative.

On the other hand, even though the pattern of CL activity among the patients and normal individuals inspected was comparable to that of NBT, the degree of depression from both techniques applied were markedly different. The CL assay being quite sensitive may have been able to detect a much smaller degree of abnormality. Nitroblue tetrazolium screening test has been the test of choice in laboratory diagnosis of certain diseases for example chronic granulomatous disease (CGD).

Nevertheless, the work presented seems to portray the superiority of CL in assaying the killing potential of neutrophils as compared to the NBT test. The moderate degree of correlation and lack of agreement exhibited between NBT and the CL system greatly strengthened the need to consider introducing CL as a possible diagnostic tool.

Other advantages offered by CL are that of its simplicity and rapidity as compared to killing and NBT assays. Presence of unforced technical errors can be quickly corrected while doing the CL assay since the reading is being displayed allowing quicker decision making in maintaining the performance of the test. As for the other two methods, any technical faults can only be detectable after completion of the whole procedure. Results recorded from CL are automated but the other two tests are subjective especially for those medical laboratory technologists who may be unfamiliar with the determination of killed organisms or the identification of deposited formazan crystals.

Thus, the subjectivity of CA and NBT generally will lead to greater variations in the results obtained while maintaining their reproducibilities unlike that of CL which gives smaller variations between assays performed either by the same operator or different operator. In spite of its relatively high cost of maintaining the machine, chemiluminescence assay should be used in the investigation of defective neutrophil functions. The method has been extended to help in diagnosing CGD and other infectious diseases in this laboratory.

Acknowledgement

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