QUANTITATIVE ASSESSMENT OF HUMAN NEUTROPHIL FUNCTION BY CHEMILUMINESCENCE

J.J. Capone and P.C. Brennan
Packard Instrument Company
2200 Warrenville Road
Downers Grove, Illinois 60515

ABSTRACT

Biochemical mechanisms resulting in chemiluminescence are reviewed. Several practical applications of these mechanisms are described concerning their use in clinical research and hematology. Chemiluminescent data from isolated cells correlated with the other multiparameter evaluations and supported the hypothesis that chemiluminescence provides a reliable quantitative assay for neutrophil function. Data derived from whole blood suggest that chemiluminescence may be useful in screening large numbers of patients for possible neutrophil defects.

INTRODUCTION

Allen et al.[1] were the first to demonstrate that polymorphonuclear neutrophils (PMN) emitted light during phagocytosis. This was correlated with increased oxygen consumption via the hexose monophosphate shunt (HMP). Babior[2] and Allen[3] reviewed the reactions of this "respiratory burst", and it is now believed that a number of free radicals and electronically excited molecules which occur as intermediates can, either by themselves or by interaction with cellular components, yield chemiluminescence (CL) by the emission of a photon. These intermediates include superoxide anion ($.O_2^-$), singlet oxygen ($^1O_2$), hydroxyl radical (OH.), hydrogen peroxide (H_2O_2) and hypochlorite ($O\,Cl^-$).

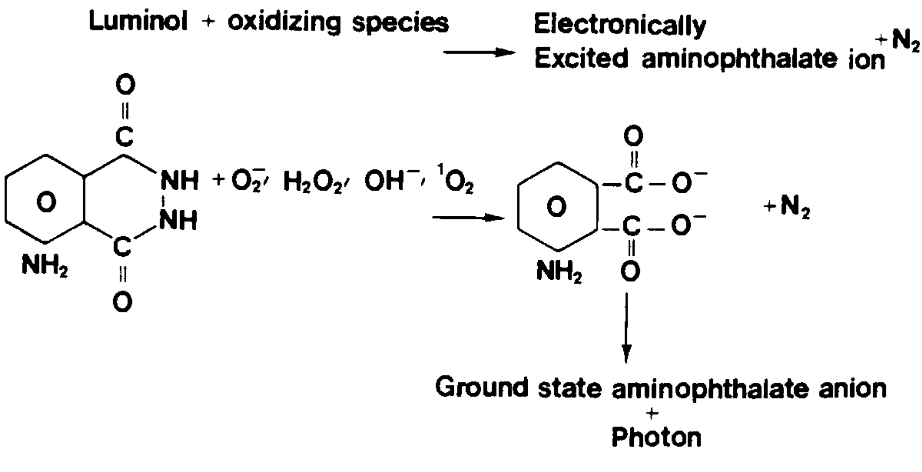
A schematic of the respiratory burst may be depicted as:

$$\text{PMN + Stimulus} \longrightarrow \text{Elevated HMP Shunt Activity} \longrightarrow \text{Elevated NADPH}$$

$$\text{NADPH} \xrightarrow[O_2]{\text{NADPH-Oxidase}} O_2^- \xrightarrow[Cl^-]{\text{Myeloperoxidase}} {}^1O_2 \text{ or other excited oxygen forms}$$

The inherent or "native" CL associated with these reactions is a consequence of relaxation of electronically excited states to ground state by the emission of a photon. The amount of "native" CL generated is very low and was detected in early studies using liquid scintillation counters (LSCs) operated in the out-of-coincidence mode[1,4]. Although native CL of PMN can be detected in LSCs, the amount of CL generated by phagocytic cells other than PMN, such as alveolar and peritoneal macrophages and circulating monocytes, can not.

To overcome this difficulty, Allen and Loose[5] introduced the use of luminol (5-amino-2,3-dihyro-1,4-phthalazinedione) which yields CL when reacted with a variety of oxidizing agents. In this reaction, luminol is oxidized to the electronically excited aminophthalate ion that relaxes to ground state by photon emission as shown below.



The practical significance of this is that luminol-amplified systems have been used by a number of investigators to study defects of phagocytic function[6-10]. CL in these systems was measured in LSCs operated in the coincidence mode. Andersen and Brendzel[11] pointed out several disadvantages to the use of LSCs, among them that the environment in LSCs is not optimal for CL production. Packard

Instrument Company has manufactured a luminometer which detects and counts CL in a controlled environment. We have also developed standardized luminol-amplified reagents to stimulate cells from normal donors and from patients with a variety of clinical conditions and compared the results with conventional measures of PMN function.

EXPERIMENTAL

Isolation of PMN

Peripheral blood was obtained by venipuncture in EDTA Vacutainer tubes. Five ml of the anticoagulated blood was carefully layered on 4 ml of a modified Ficoll-Hypaque gradient as described by Ferante and Thong[12]. After centrifugation the PMN layer was collected and diluted 1:3 in Hank's balanced salt solution (HBSS) without phenol red or bicarbonate. The cells were centrifuged again in HBSS and then ice-cold buffered 0.84% ammonium chloride (10 ml) was added to the cell pellet to lyse contaminating erythrocytes. The PMN were then washed twice in HBSS. The final cell pellet was adjusted to 2×10^6 cells/ml in HBSS containing 5% fetal calf serum. This procedure routinely yielded a preparation containing >95% viability as judged by trypan blue dye exclusion. Cell suspensions were stored on ice and used within four hours of isolation.

Reagents for CL

Two lyophilized reagents were used to stimulate PMN CL. Thirty minutes before use they were reconstituted with purified water. The first reagent was a standardized opsonized zymosan A preparation (ZAP) containing luminol (Baker Chemical Co.). This reagent was used to test phagocytic activity in both whole blood and isolated PMN. The second reagent was a polyacrylamide bead (Bio-Rad) preparation to which IgG and luminol were chemically bound. This preparation (BB) was used to test isolated cells only.

CL Assay

Assays were performed at 37°C in a PICOLITE™ luminometer. Fifty µl of whole blood diluted 1:3 in HBSS was mixed with 200 µl reconstituted ZAP, placed in the PICOLITE and the tubes counted every

10 min for 30 sec for a total of 40 min. Isolated cells were tested by mixing 100 µl of cell suspension with 50 µl ZAP or BB and counted as above.

After the 40 min incubation a drop of 0.5% methylene blue was added to the BB tubes and the percentage of cells which had engulfed beads determined.

<u>NBT Dye Reduction and Bactericidal Assay</u>

The reduction of nitro blue tetrazolium (NBT) was performed according to the method of O'Donnell et al.[13]. The bactericidal test was a modification of the method of Tan et al.[14] using *Staphyloccus aureus* A as the challenge organism.

RESULTS AND DISCUSSIONS

Figure 1 shows typical CL results of the whole blood screening assay with ZAP using blood from normal adult donors and from four patients with chronic granulomatous disease (CGD). Clearly CL quickly detects potential CGD. Figure 2 shows the CL response with ZAP of isolated cells from two families with CGD patients. Also included are the curves from an unaffected brother and father from one family and the mothers in both families (presumed carriers) as well as the sister of patient 1. As expected, the CGD patients showed no response above background and the mothers showed about one-half the normal response. Since the sister of patient 1 also showed half the normal response, we suspect that she is a carrier. Figure 3 shows the results using BB reagents on isolated cells from the same individuals. The mother of patients 2 and 3 diagnosed as having CGD as well as the father of 2 and 3 show a nearly normal result. This difference from the response observed for ZAP reagents has not been explained but clearly demonstrates the need for proper selection of reagents to demonstrate disease carriers.

Assistance with a study of an accidental exposure to chlorinated organics led us to study some drug effects on isolated neutrophils (PMN). Figures 4 and 5 show representative curves from a cohort of patients occupationally exposed to dioxin. Note there are both low and high responders. The response is qualitatively identical for both

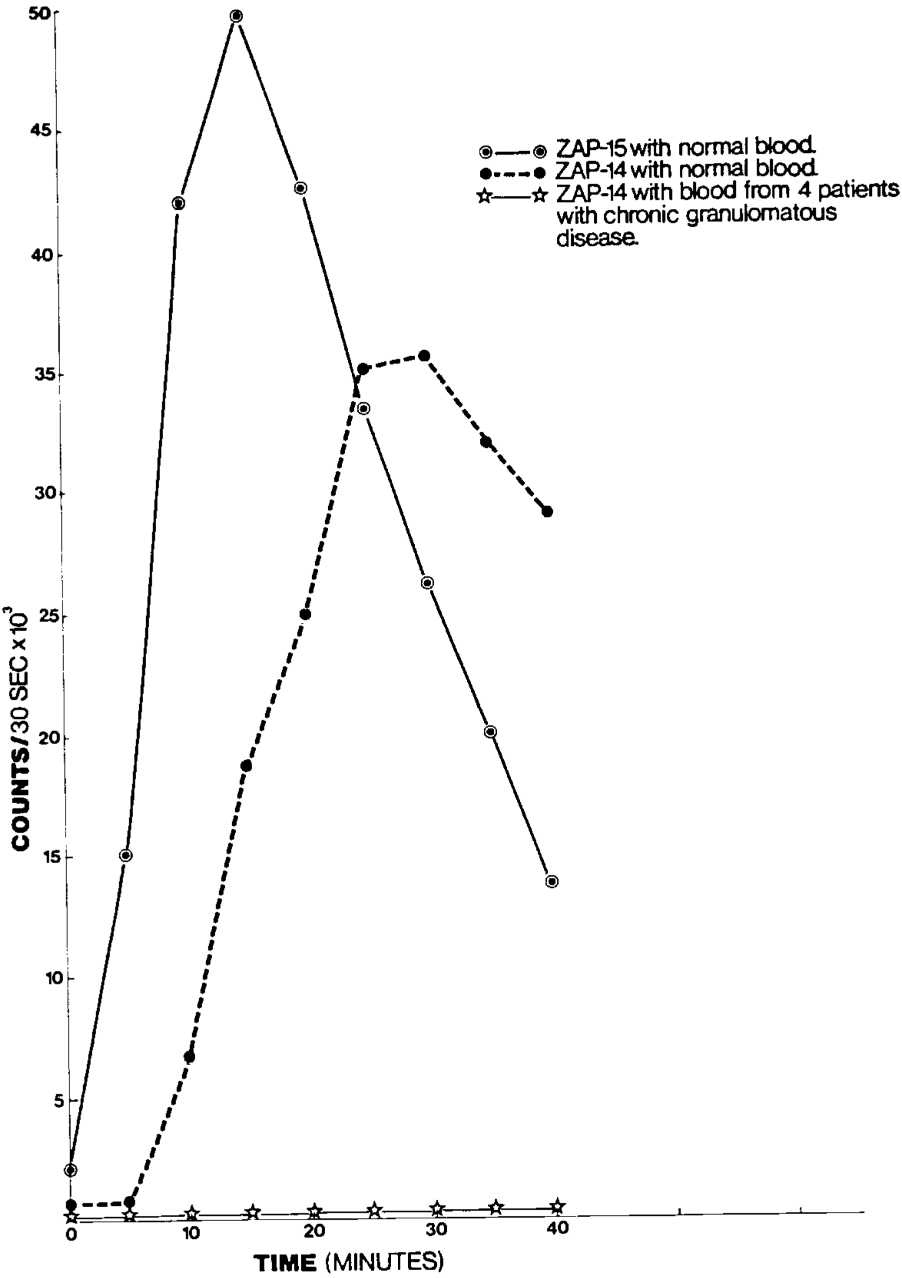


Figure 1. Representative whole blood screening assay with two preparations of zymosan A-protein (ZAP).

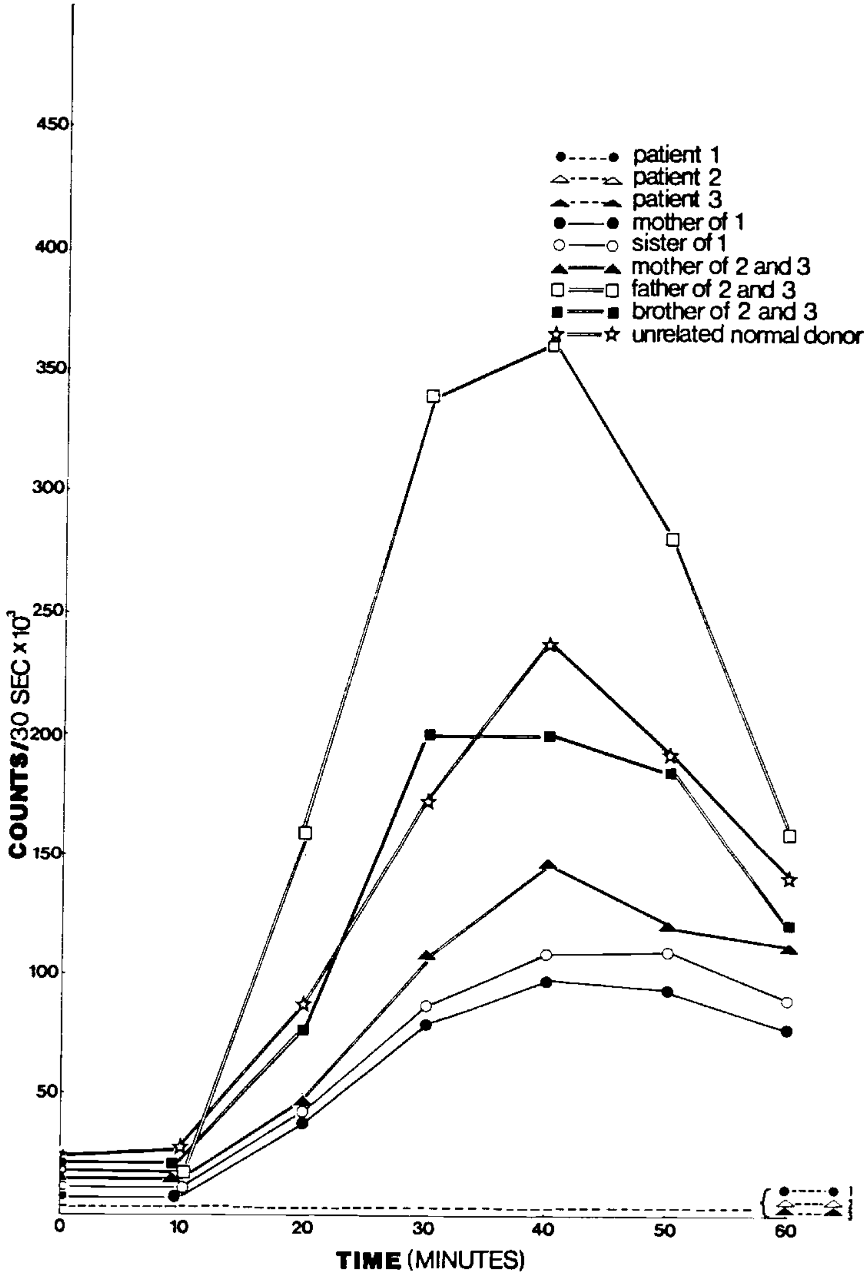


Figure 2. Chemiluminescence response of chronic granulomatous disease patients and relatives with ZAP.

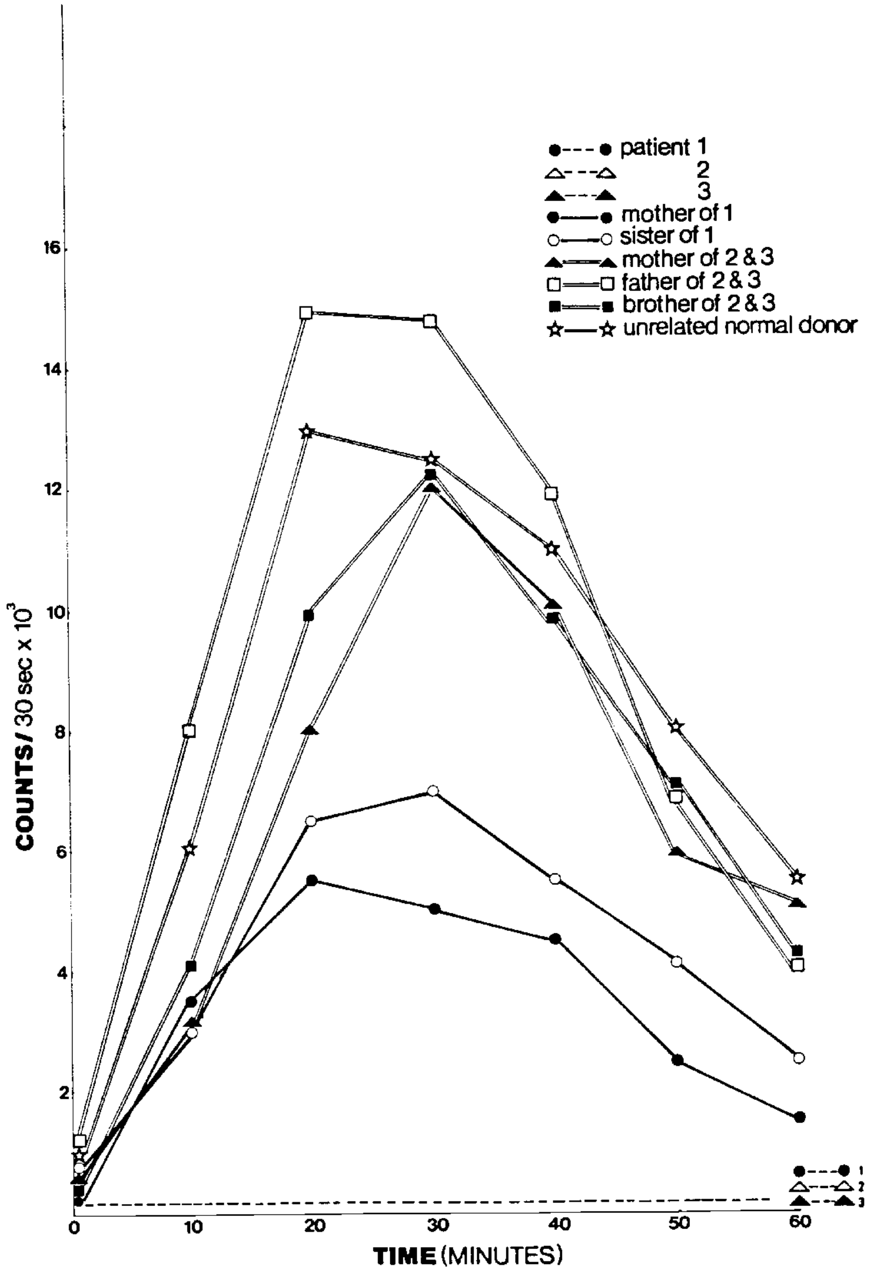


Figure 3. Chemiluminescence response of chronic granulomatous disease patient and relatives with Biobeads.

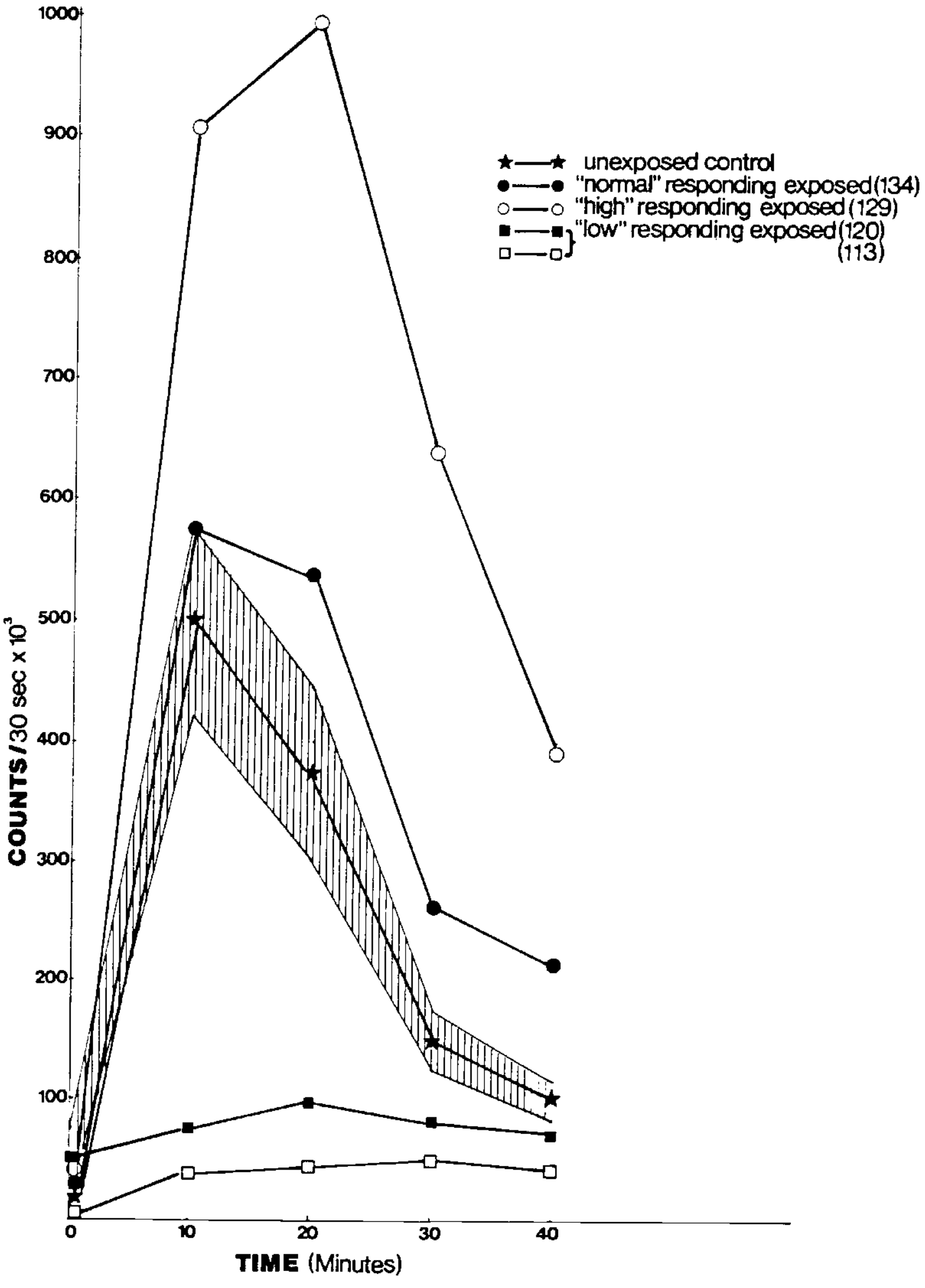


Figure 4. Representative neutrophil curves from a cohort of patients exposed to dioxin with ZAP reagent.

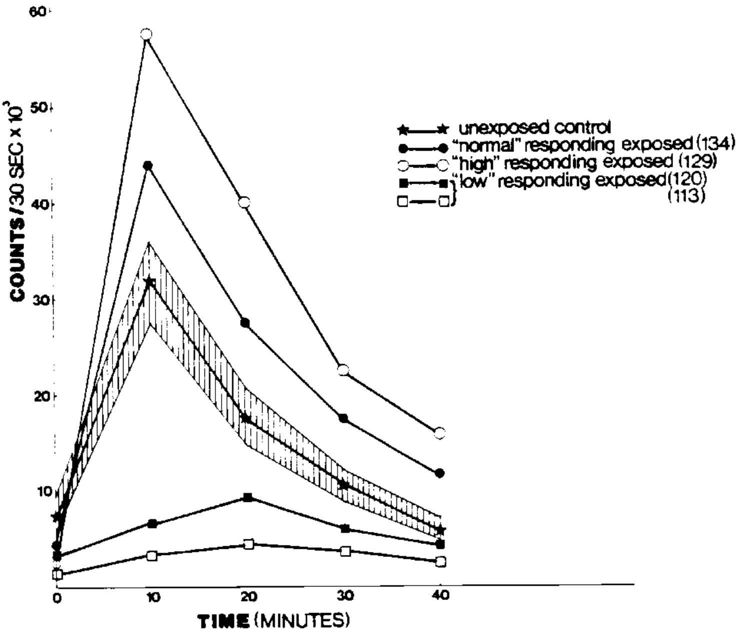


Figure 5. Representative isolated neutrophil curves from a cohort exposed to dioxin. Biobead reagent.

the ZAP and Biobead preparation.

Results of other possible drugs upon the PMN cell phagocytic response demonstrated an increase in phagocytic response above the normal range in a brief kidney transplant study. Figure 6 shows the CL results from a patient who received a kidney transplant and was tested before treatment and preoperatively and postoperatively following a dose of antilymphocyte serum and 6-aminopurine (Imuran®)*.

A comparison of CL, bactericidal ability and NBT dye reduction (Table 1) in normal and one CGD family demonstrates that CL correlates with the other parameters. Similarly a comparison of the NBT data shown in Table II with CL data are given in Figures 1-6 shows that there is reasonably good correlation.

*Burroughs Wellcome

TABLE 1. Chemiluminescence, Bactericidal Ability and Nitroblue Tetrazolium Dye Reduction

Clinical Condition	Chemiluminescence (Peak Counts/30 sec) ZAP[a]	BB[b]	Bactericidal Ability *S. aureus* Killed (% of Inoculum)	Viable Intracellular (% of Incolum)	NBT[c] CFN[d]	PFN[e]	AFN[f]
Normal Control-1	800,000	53,000	80	0.8	95	4	1
Normal Control-2	600,000	30,000	70	2.8	80	5	15
CGD-4	1,600	1,000	0	44	0	99	2
Mother (carrier)	250,000	10,000	52	12.1	45	40	15

[a] ZAP = zymosan A preparation
[b] BB = polyacrylamide bead to which IgG and luminol were chemically bonded
[c] NBT = nitroblue tetrazolium
[d] CFN = completely functional neutrophil; ingests beads and reduces dye
[e] PFN = partially functional neutrophil; ingests beads but does not reduce dye
[f] AFN = afunctional neutrophil; neither ingests beads nor reduces dyes

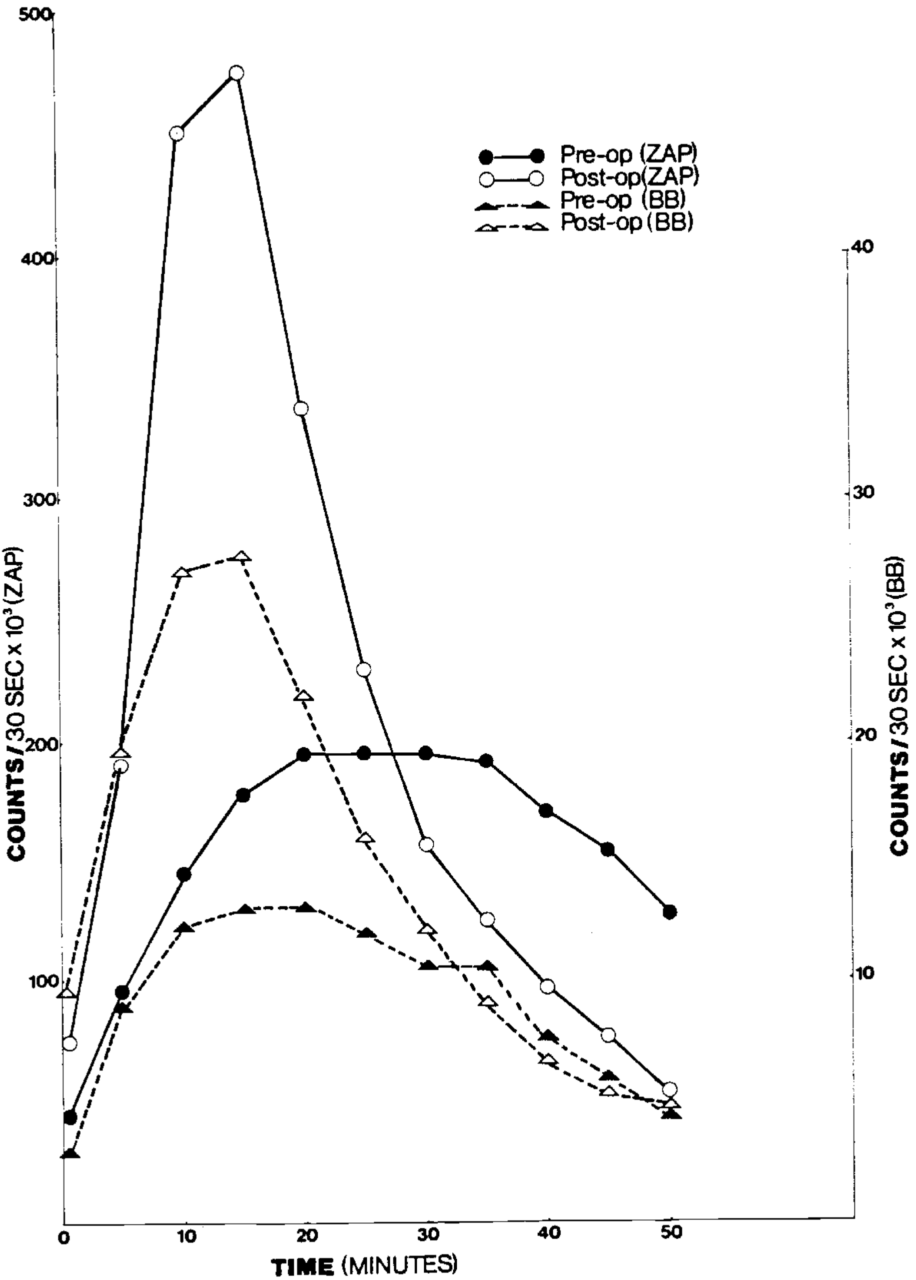


Figure 6. Pre- and post-operative chemiluminescence response of neutrophils from a kidney transplant patient.

TABLE II. Nitroblue Tetrazolium Dye Reduction by Neutrophils

Clinical Condition	Subject	CFN(%)[a]	PFN(%)[b]	AFN(%)[c]	Viability(%)[d]
Exposed to Dioxin	Normal Control	73	15	12	97
	134	83	8	9	100
	129	86	8	6	100
	120	27	59	14	75
	113	47	41	12	69
Chronic Granulomatous Disease (CGD) Families	CGD-1	0	99	1	99
	Sister	73	27	0	94
	Mother (carrier)	60	40	0	96
	CGD-2	1	99	1	100
	CGD-3	0	98	2	100
	Brother (normal)	96	3	1	97
	Father (normal)	98	2	0	98
	Mother (carrier)	83	15	2	98
Kidney Transplant	Preoperative	74	6	20	97
	Postoperative	90	5	5	94

a) CFN = Completely functional neutrophil; ingests beads and reduces dye

b) PFN = Partially functional neutrophil; ingests beads but does not reduce dye

c) AFN = Afunctional neutrophil; neither ingests beads nor reduces dyes

d) Viability measured by trypan blue exclusion

NBT dye reduction for functional neutrophils and trypan blue exclusion to determine cell viability are very time consuming microscopic measurements. We have found results of these measurements can be biased by premeasurement suggestion. Thus, the methodology is not only laborious, fatiguing, quite time consuming, but very subjective. Sample preparation is frequently a required technique in both microscopic and CL measurements. The CL may be performed automatically in newer instrumentation providing quantitative numbers in place of estimates.

CONCLUSIONS

The data presented are consistent with the interpretation that the products of cell metabolism, under certain conditions, result in chemiluminescence that is detectable with a luminometer and which may be of diagnostic significance. For example, when comparing CL with bacterial ability (Table I), reduced CL correlates with impaired killing ability. However, the bactericidal assay has several inherent disadvantages. It is semiquantitative at best and interpretation of the final killing "rate" is difficult because of the diverse processes involved. The test is time consuming and labor intensive. Finally, prolonged incubation times are required before the results are available. On the other hand, CL is simple, rapid and quantitative. In the case of whole blood, results are available in about forty minutes, and even when cells are first isolated and then tested, only a few hours are required.

When comparing luminol-amplified CL with the common biochemical method of assessing PMN function by the reduction of NBT, the correlation is excellent, particularly in patients with CGD. Again, the NBT test is labor intensive and semiquantitative.

The data presented are also consistent with the observations of other workers who used LSCs to detect CL. In general, LSCs require a larger number of cells per assay than luminometry. This presents a problem when dealing with infants or neutropenic patients. Luminometry also offers the advantage of a physiologic environment.

Finally, we have demonstrated that PMN from compromised patients exhibit altered CL responses.

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