EVIDENCE FOR THE GENERATION OF AN ELECTRONIC EXCITATION STATE(S) IN HUMAN POLYMORPHONUCLEAR LEUKOCYTES AND ITS PARTICIPATION IN BACTERICIDAL ACTIVITY[a)]

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SUMMARY: Heat-killed opsonized Propionibacterium shermanii (P. shermanii) and opsonized live Staphylococcus aureus (S. aureus) are shown to elicit a chemiluminescence (CL) from human polymorphonuclear leukocytes (PMN) but not from lymphocytes upon phagocytosis. The CL is correlated with hexose monophosphate (HMP) shunt activity, number of PMN, and bacteria. CL was evoked from PMN by opsonized latex particles. No CL was obtained from PMN exposed to serum alone, nor unopsonized bacteria. It is proposed that the CL of PMN reflects the generation of singlet oxygen, $^1O_2$, which acts per se, as the bactericidal and/or CL species.

Howes and Steele (1) described a CL evoked from rat liver microsomes by NADPH, and O_2 in the absence of an oxidizable substrate, and showed subsequently (2) that CL and lipid peroxidation were suppressed with the initiation of hydroxylation upon the addition of appropriate substrates to the system. They proposed that substrate hydroxylations, lipid peroxidations, and CL are mediated by $^1O_2$, and are mutually competitive for it. These observations led one of us (RCA) to reason that the bactericidal activity of PMN, which is thought to be mediated by myeloperoxidase (MPO), may be due, in part, to generated $^1O_2$, in which case PMN should exhibit a CL with the onset of phagocytosis. This communication describes the CL elicited from human PMN with the onset of phagocytosis.

EXPERIMENTAL: The reaction medium was phosphate-buffered-saline (PBS), pH 7.4, containing 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, and 0.8 g glucose per liter.

For opsonization 1 ml of heat-killed P. shermanii or live S. aureus (30 mg ml^{-1} dry wt. suspended in PBS) or 1 ml of Difco latex particles (0.8 μ diameter, suspended in water) was mixed with 3 ml serum from the subject donating the PMN, and the mixture was incubated at 37°C for 30 minutes. Non-opsonized bacteria were prepared by adding 1 ml bacteria to 3 ml PBS.

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PMN were harvested from human blood as described by Stjernholm et al. (3). Glycolysis and HMP shunt activity were assayed as described by Stjernholm and Manak (4). Human lymphocytes were isolated by the glass bead column technique of Rabinowitz (5).

CL, measured at 21°C and ambient pO_2, was recorded as counts per minute (cpm) vs time as described by Howes and Steele (1).

RESULTS AND DISCUSSION: Temporal traces of CL obtained with PMN are shown in Fig. 1. Both phagocytosis of opsonized heat-killed P. shermanii (curve-a), and phagocytosis of opsonized latex particles (curve-b) resulted in the production of CL. No CL was obtained from PMN plus non-opsonized P. shermanii (curve-c), or PMN plus serum alone (curve-d). PMN plus live opsonized S. aureus gave a CL response as for P. shermanii (curve-a). Lymphocytes plus opsonized P. shermanii gave no CL (response as for curves - c & d). The maximum CL (ordinates at the curve maxima at which points the accelerations are zero, and the systems are in transient steady state conditions) elicited from PMN upon the phagocytosis of bacteria and latex particles correlated directly with the HMP shunt activity induced in these systems (Table 1).

Glycolysis and HMP shunt activity observed with PMN during resting and phagocytizing conditions are recorded in Table 1. There is a small increment in glycolysis during phagocytosis and a dramatic increase in HMP shunt activity. We interpret the marked burst in CL which PMN display with the initiation of phagocytosis as reflecting an increased generation of NADPH via the activated HMP shunt. NADPH

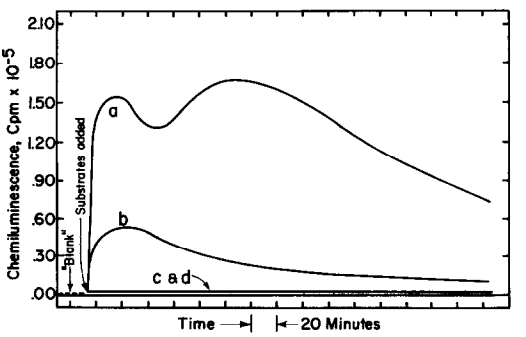

Fig. 1. Temporal traces of CL (cpm) elicited from PMN. Systems: 2.5×10^7 PMN in 7 ml PBS. Curve-a, 0.5 ml opsonized P. shermanii (7.5 mg dry wt. ml^{-1}); curve-b, 0.5 ml opsonized latex particles; curve-c, 0.5 ml non-opsonized P. shermanii; and curve-d, 0.5 ml serum, with all 0.5 ml additions being made at zero time.

supplies reducing equivalents to the PMN redox system which is activated upon degranulation (Selvaraj and Sbarra, 7; Zatti and Rossi, 8). It is proposed that these reducing equivalents then react via oxygen intermediates to generate $^1O_2$.

TABLE 1

GLUCOSE UTILIZATION BY PMN

Condition	Glycolysis μmoles glucose hr^{-1} per 10^8 PMN	HMP-shunt μmoles glucose hr^{-1} per 10^8 PMN
Resting Cells	6.2	0.02
Phagocytosis (latex)	6.6	0.62
Phagocytosis (bacteria)	8.5	2.10

5 ml of Eagle's minimal essential medium containing 10^7 PMN ml^{-1} and 1 ml of autologous serum were incubated for 1 hr at 37°C in a metabolic shaker. Phagocytosis was induced with latex particles or heat-killed P. shermanii. Calculations were made as described by Wood et al. (6).

Fig. 2 illustrates temporal traces of CL elicited by increasing numbers of PMN phagocytizing P. shermanii in systems with the PMN/bacteria ratio held constant. It is evident that the CL rates increase, and the times to attain transient steady state conditions (rates at curve maxima) decrease, as the PMN-bacterial populations in the system are increased.

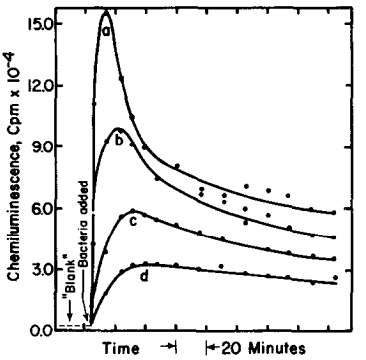

Fig. 2. Temporal traces of CL (cpm) elicited for different numbers of PMN at a constant PMN/bacteria ratio. System: 1.3 ml P. shermanii (30 mg dry wt.ml^{-1}) were added to 4 ml serum. 1 ml aliquots of serial dilutions of this suspension were added to 7 ml of serial dilutions of PMN: curve-a, 23.1 x 10^6 PMN + 7.4 mg bacteria; curve-b, 11.5 x 10^6 PMN + 3.7 mg bacteria; curve-c, 5.75 x 10^6 PMN + 1.85 mg bacteria, curve-d, 2.875 x 10^6 PMN + 0.925 mg bacteria. Bacteria were added at zero time.

Fig. 3 illustrates the temporal traces of CL elicited from phagocytizing PMN systems in which the PMN population was held constant while the number of bacteria was varied. It is seen again that the CL rates increase, and the times to reach transient steady state conditions decrease as the number of bacteria added is increased. Since Hirsch and Cohen (9) demonstrated that the degree of degranulation within the PMN, which should determine the amount of MPO released from the granules (Schultz et al., 10), correlated with the number of bacteria per infected PMN then these results might be anticipated if the MPO is involved in the light reaction. We consider the changes in the kinetic parameters of Figs. 2 and 3 to be due to changes in the absolute concentrations of the PMN-bacterial populations which, as increased, provide for more rapid cellular contact, enhanced phagocytosis, HMP shunt activity, PMN degranulation, and bactericidal activity.

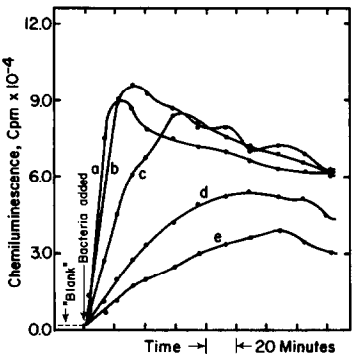

Fig. 3. Temporal traces of the CL (cpm) elicited from constant numbers of PMN (11.9×10^6) for different numbers of opsonized P. shermanii. Curves: a, b, c, d, e: 1 ml of bacterial suspensions containing 7.4, 3.7, 1.85, 0.925, and 0.462 mg (dry wt.) respectively, were added to 7 ml of PMN-PBS suspensions at zero time.

A schematic outline of the events giving rise to the light emission from the PMN with the onset of phagocytosis is given in Fig. 4. These processes appear to agree with the known metabolic parameters of PMN. It is possible that $^1O_2$ species, per se, are responsible for some of the light emission via:

$$2\ (^1\Delta_g)\ O_2 \longrightarrow 2(^3\Sigma_g^-)\ O_2 + h\nu\ (CL) \qquad (1)$$

(Khan and Kasha, 11, 12; Arnold et al., 13). The $^1O_2$ may react as an electrophile at certain sites of high electron density, π-systems (Fenical et al., 14; Kearns, 15; Foote, 16), within the cell and/or bacterium to form labile dioxetane structures. These structures may then cleave with the formation of electronically excited carbonyl groups which may then relax with light emission (McCapra, 17). McCapra

and Hann (18) have stressed the significance of this mechanism and describe how light with energy greater than would be expected from pooled ($^1\Delta_g$) 0_2 interactions (Eq. 1) can be obtained from the exothermicities of the dioxetane cleavage. White et al. (19) have described a novel mechanism which may be related to the bactericidal activity in which the photon released from the excited carbonyl group may itself participate in a photochemical event. High energy light intracellularly may conceivably be expected to affect that environment.

The proposed scheme for the intra-PMN environment represents, in essence, an analog of the well documented (Topley and Wilson, 20) dye-oxygen-photosensitized effects on a wide vareity of "substrates" ranging from chemicals, toxins and viruses to bacteria and paramecia. The mechanisms of these photodynamic effects are now recognized, retrospectively (Spikes, 21), as being mediated in many instances by $^1O_2$ as depicted in the last mechanism in Fig. 4 (Foote, 22). It is interesting to consider that the liver microsomal- and the PMN-systems, both of which are designed in part to detoxify and/or destroy foreign substrates, may do so by a common mechanism involving activated oxygen as $^1O_2$.

In subsequent communications details of our proposed mechanism for the generation of $^1O_2$ in the PMN system will be described,as well as studies of PMN from children with chronic granulomatous disease in which CL is absent during the phagocytosis of opsonized bacteria.

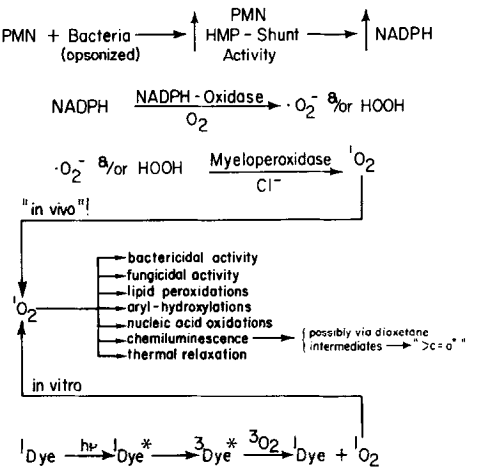

Fig. 4. Schematic of a proposed mechanism for the generation and utilization of electronic excitation states, as $^1O_2$, via the PMN redox system. It is depicted as an in vivo analogue of the in vitro $^1O_2$-generation by 0_2-dye-photosensitized reactions (see text).

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