LOW LEVEL CHEMILUMINESCENCE OF INTACT POLYMORPHONUCLEAR LEUKOCYTES

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1 Introduction

The ingestion of different phagocytizable materials such as bacteria, zymosan or latex particles by polymorphonuclear leukocytes (PMN) is associated with a cyanide-insensitive respiratory burst and the production of oxygen intermediates [1–4] Although the microbicidal effect of PMN has been attributed to several oxygen reduction intermediates, superoxide anion [3–7], hydrogen peroxide [2–6] and hydroxyl radical [4,7,8] and also to singlet oxygen [5,9,10], no clear evidence is yet available to indicate a predominant role of one of the proposed species

The occurrence of low level chemiluminescence, so termed according to [11], in PMN upon phagocytosis was first observed [9] and subsequently confirmed [5,10,12] The production of chemiluminescent singlet oxygen has been implicated in the phagocytic process that accompanies the initial oxidative burst of PMN [5,9,10] and in the activity of the isolated enzyme myeloperoxidase [12,13]

Here we present direct evidence that chemiluminescence is related to the oxygen uptake of the respiratory burst and to superoxide anion production and is apparently independent of the participation of myeloperoxidase activity and of singlet oxygen production

2 Materials and methods

2 1. *Cell preparation*

Guinea pig PMN were isolated from the peritoneal cavity by the method in [1] 16 h after injection of sterilized caseinate in 0 9% NaCl as in [14]. Leukocytes from the peritoneal exudate were washed once with Ca^{2+}-free Krebs Ringer phosphate buffer (pH 7 4), at 120 × *g* for 5 min at 4°C and resuspended in the same buffer kept cool in an ice bath Protein concentration in the PMN cell suspensions was assayed by the Lowry method [15] slightly modified [16], 10^7 cells/ml were equivalent to 1 mg protein/ml

2 2 *Photon counting and oxygen uptake*

Chemiluminescence was measured in a photon counting apparatus as in [17] in either a 35 × 35 × 5 mm (12 5 cm^2 surface) cuvette or a 35 × 51 × 10 mm cuvette holding an oxygen electrode in order to record chemiluminescence level and oxygen uptake simultaneously The cell suspension in the cuvette was bubbled with a gas mixture containing oxygen and nitrogen at a variable ratio through a gas proportioner until the desired initial concentration of oxygen was reached All assays were carried out at 37°C

2 3 *Chemicals*

Superoxide dismutase, catalase, casein and myristic acid were purchased from Sigma Chemical Co (St Louis, MO), *N,N'*-dimethylpiperazine, *tert*-butylhydroperoxide and DABCO (1,4-diazabicyclo-[2,2,2]-octane) were obtained from Aldrich Chemical Co (Milwaukee, WI), other reagents were of analytical grade

3. Results

3.1 *Chemiluminescence and oxygen uptake*

PMN produce low level chemiluminescence simul-

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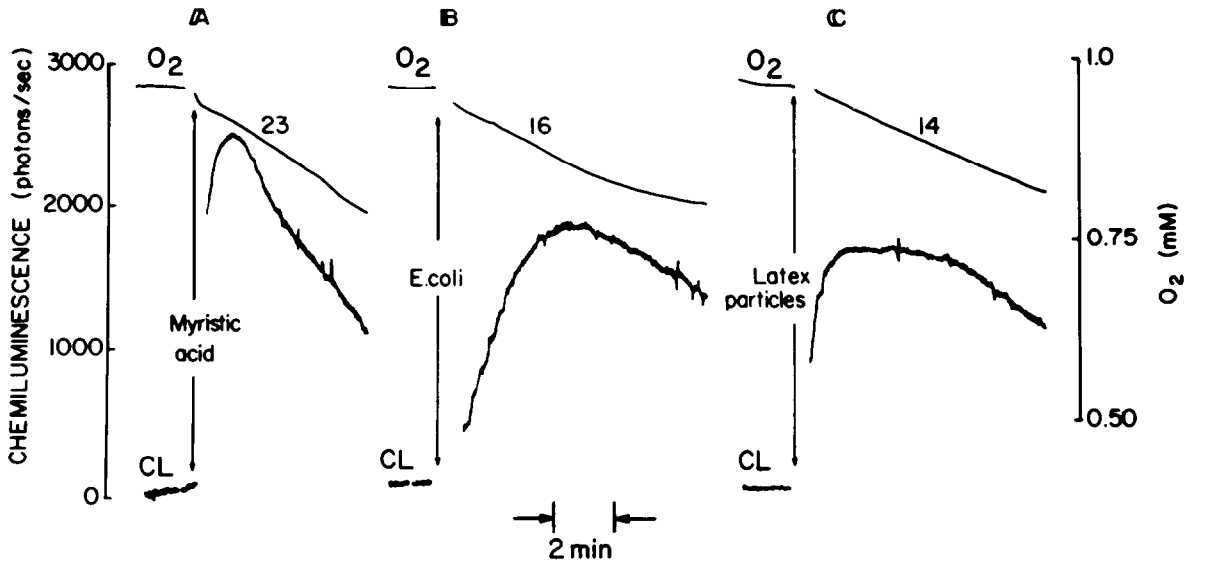

Fig.1. Chemiluminescence and oxygen uptake of polymorphonuclear leukocytes upon addition of different activators. 1.5 × 10^7 cells/ml in Ca^{2+}-free Krebs Ringer phosphate buffer, 5 mM glucose (pH 7.4) were added of: (A) myristic acid (0.2 mM); (B) *E. coli* (0.2 mg protein/ml); (C) latex particles (2.2 × 10^9 particles/ml). Numbers near oxygen traces indicate oxygen uptake (nmol/min per 10^7 cells).

taneously with the burst in oxygen uptake upon addition of myristic acid, *E. coli* or latex particles (fig.1). Chemiluminescence level and the rate of oxygen uptake produced by myristic acid were slightly higher than that observed after addition of *Escherichia coli* or latex particles. Myristic acid is the most effective, among several fatty acids, in inducing drastic changes of the leukocyte oxidative metabolism [16]. The differences between myristic acid, bacteria and latex particles in chemiluminescence production parallels, though in lower magnitude, those reported for superoxide anion generation [16]. Oxygen uptake appears to correlate well (r = 0.99) with the maximal chemiluminescence level observed with the different activators. The increase of chemiluminescence was observed immediately after the addition of the activators; the maximal chemiluminescence signal was achieved within 1–2 min. After that, chemiluminescence decreased slowly until reaching the initial baseline about 5–15 min later; some kinetic details such as half-time for maximal chemiluminescence or the rate of decay were different with the different activators.

Chemiluminescence and the rate of oxygen uptake upon the respiratory burst depended on the oxygen concentration in the reaction medium (fig.2). Nearly maximal chemiluminescence was observed with

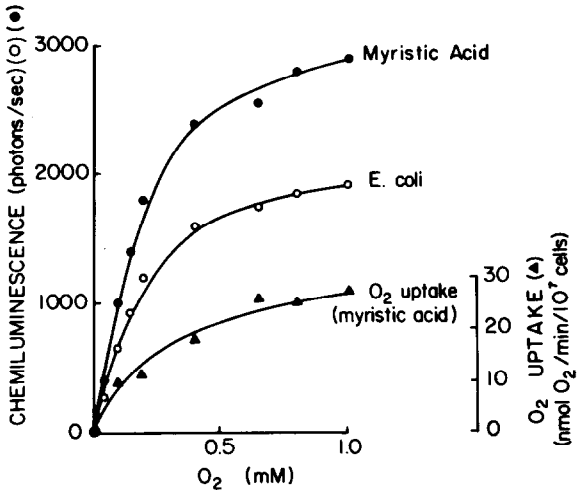

Fig.2. Dependence of chemiluminescence and oxygen uptake on oxygen concentration. Experimental conditions as in fig.1, 1.3 × 10^7 cells/ml. The reaction was started with either myristic acid (0.2 mM) or *E. coli* (0.18 mg protein/ml).

~1 mM oxygen, both in the presence of myristic acid and *E coli* The oxygen uptake upon myristic acid stimulation showed a similar oxygen dependence (fig 2) Double reciprocal plots gave an app K_m for oxygen of ~0 38 mM and 0 36 mM when myristic acid or *E. coli* were used as activators, respectively, the app K_m for oxygen uptake in the presence of myristic acid was 0 41 mM As long as the K_m for oxygen uptake and chemiluminescence are similar, it is sensible to consider that both activities belong to a single system

3 2 *Effect of inhibitors and enhancers of chemiluminescence*

Chemiluminescence produced by the treatment of PMN with myristic acid was decreased ~20% upon addition of catalase, ~38% upon addition of superoxide dismutase and ~77% when both, catalase and dismutase, were added (fig 3)

Chemiluminescence was found to be stimulated by 50–70% when leukocytes were supplemented with cyanide and azide (fig 4) Cyanide and azide showed half-maximal effect at ~0 23 mM and 0 42 mM, respectively Addition of *tert*-butyl hydroperoxide to leukocytes produced a 53% increase in the chemiluminescence produced upon addition of myristic acid The tertiary amines, DABCO and dimethylpiperazine, inhibited the chemiluminescence by 85% and 79%, respectively, with a half-maximal effect at ~1 7 mM for DABCO and 3 0 mM for dimethylpiperazine Dimethylfuran was found without effect at 10–30 mM

4 Discussion

The participation of superoxide anion and hydrogen peroxide in the killing mechanism of PMN has been reported [1–4] Both superoxide anion and hydrogen peroxide production are markedly increased upon phagocytosis in the intact cells [1–4] and in the isolated subcellular granules [6,16] In the present experiments the use of superoxide dismutase and catalase have provided evidence that superoxide anion and hydrogen peroxide are also involved in the chemiluminescence process

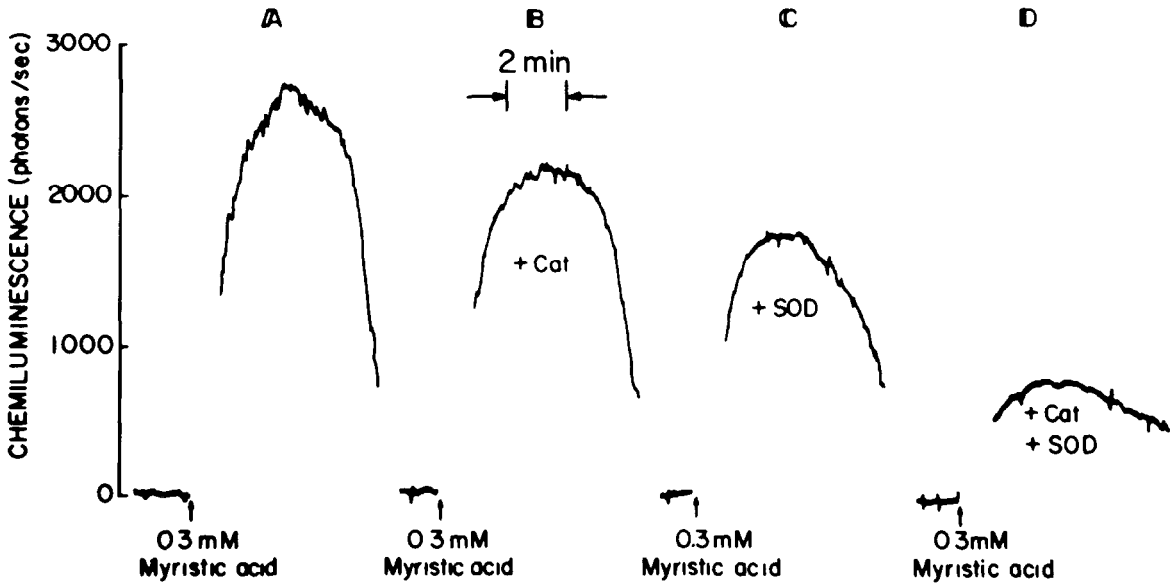

Fig 3 Effects of catalase and superoxide dimutase on the chemiluminescence of polymorphonuclear leukocytes Assay mixture contained 2 2 × 10^7 cells/ml and 5 mM glucose in air-saturated Ca^{2+}-free Krebs Ringer phosphate buffer (pH 7 4) Myristic acid was added to start the reaction (A) control, (B) +25 μg catalase/ml (cat), (C) +25 μg superoxide dismutase/ml (SOD), (D) +25 μg catalase/ml + 25 μg superoxide dismutase/ml

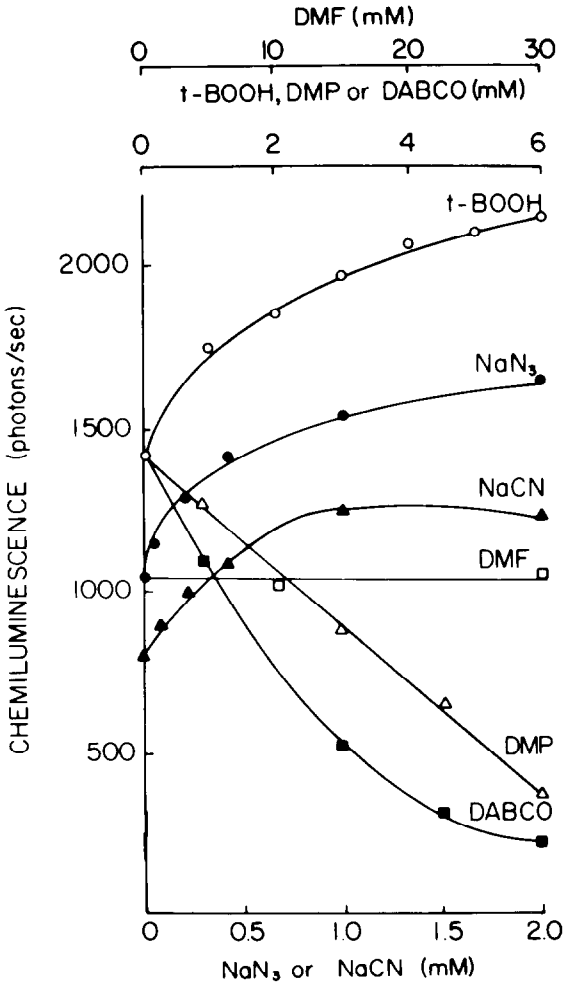

Fig.4. Effect of inhibitors and enhancers of chemiluminescence of polymorphonuclear leukocytes. Experimental conditions were as in fig.3 (○, △, □) 1.3×10^7 cells/ml (○, □) 1×10^7 cells/ml; (△) 0.8×10^7 cells/ml; 0.2 mM myristic acid was used to start the reaction.

Abbreviations: *t*-BOOH, *tert*-butyl hydroperoxide; DMF, dimethylfuran; DMP, dimethylpiperazine; DABCO, 1,4-diazabicyclo-[2,2,2]-octane

The small increase in chemiluminescence produced by both, azide and cyanide (fig.4), could be explained because of their inhibitory effect on the cytosolic forms of leukocyte catalase and dismutase [18,19]. Moreover, the cyanide- and azide-insensitive chemiluminescence in myristate-treated cells indicates a source of chemiluminescence other than the myeloperoxidase system, as long as cyanide and azide are effective inhibitors of myeloperoxidase [13,20]. The chemiluminescence signal (140–450 photons/s) observed in the myeloperoxidase systems at 0.1–1.0 μM myeloperoxidase and 25–50 mM H_2O_2 at pH 7.4 [21,22] seems to be much smaller (5–15%) than the one of phagocytosing PMN (1000–3000 photons/s with ~10^7 cells/ml (fig.1–4; [9]), considering that 10^7 cells contained 0.08 μM myeloperoxidase [20].

The stimulatory effect of *tert*-butyl hydroperoxide on chemiluminescence was reported [17]; this effect apparently indicates the existence of a reaction between *tert*-butyl hydroperoxide and superoxide anion [23] either (a) producing chemiluminescence singlet oxygen or (b) initiating a free radical process resulting in C–C bond rupture and the production of fragments with excited carbonyl groups [24].

The involvement of singlet oxygen in phagocytosis is still discussed on the ground of indirect evidence. The tertiary amines, DABCO and dimethylpiperazine, at 0.1–10 mM, have been shown [25] to enhance singlet oxygen dimol chemiluminescence in aqueous solutions. The effect of 10^{-5} M DABCO inhibiting by 90% the chemiluminescence from myeloperoxidase system was claimed to support the production of singlet oxygen [13]. However, at that concentration, DABCO had no effect on the chemiluminescence of intact PMN (fig.4) and, moreover, it has been shown that 10^{-5} M DABCO reacts with ClO^{-1} [21]. In our conditions, assuming that there is not interference in the myristic acid action due to charge interaction with the tertiary amines, the marked inhibition produced by both, DABCO and dimethylpiperazine, is inconsistent with an involvement of singlet oxygen, and quenchers, azide [25] and dimethylfuran [26] were found without effect on the chemiluminescence from myristate-treated cells (fig.4). Then, it could be concluded that the effect of tertiary amines, dimethylfuran and azide in the chemiluminescence of intact leukocytes did not support singlet oxygen production.

The low level chemiluminescence [11] measured from phagocytosing leukocytes ($1–3 \times 10^3$ photons/s per 10^7 cells/ml) corresponds, according to our calibrations (absolute quantum efficiency of ~0.1%), to a chemiluminescence species generation rate of ~0.1–0.3. fmol/min per 10^7 cells/ml.

Acknowledgements

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