Inflammation, Vol. 17, No. 3, 1993

CHEMILUMINESCENCE IN ACTIVATED HUMAN NEUTROPHILS: Role of Buffers and Scavengers

ISAAC GINSBURG,[1] RIVKA MISGAV,[1] DOUGLAS F. GIBBS,[2] JAMES VARANI,[3] and RON KOHEN[2]

[1]*Department of Oral Biology*
Hebrew University-Hadassah Faculty of Dental Medicine founded by the Alpha Omega Fraternity, Jerusalem, Israel

[2]*Department of Pharmacy, School of Pharmacy*
Hebrew University-Hadassah Faculty of Medicine, Jerusalem, Israel

[3]*Department of Pathology, University of Michigan*
Ann Arbor, Michigan 49109

Abstract—Human neutrophils (PMNs) suspended in Hanks' balanced salt solution (HBSS), which are stimulated either by polycation-opsonized streptococci or by phorbol myristate acetate (PMA), generate nonamplified (CL), luminol-dependent (LDCL), and lucigenin-dependent chemiluminescence (LUCDCL). Treatment of activated PMNs with azide yielded a very intense CL response, but only a small LDCL or LUCDCL responses, when horse radish peroxidase (HRP) was added. Both CL and LDCL depend on the generation of superoxide and on myeloperoxidase (MPO). Treatment of PMNs with azide followed either by dimethylthiourea (DMTU), deferoxamine, EDTA, or detapac generated very little CL upon addition of HRP, suggesting that CL is the result of the interaction among H_2O_2, a peroxidase, and trace metals. In a cell-free system practically no CL was generated when H_2O_2 was mixed with HRP in distilled water (DW). On the other hand significant CL was generated when either HBSS or RPMI media was employed. In both cases CL was markedly depressed either by deferoxamine or by EDTA, suggesting that these media might be contaminated by trace metals, which catalyzed a Fenton-driven reaction. Both HEPES and Tris buffers, when added to DW, failed to support significant HRP-induced CL. Nitrilotriacetate (NTA) chelates of Mn^{2+}, Fe^{2+}, Cu^{2+}, and Co^{2+} very markedly enhanced CL induced by mixtures of H_2O_2 and HRP when distilled water was the supporting medium. Both HEPES and Tris buffer when added to DW strongly quenced NTA-metal-catalyzed CL. None of the NTA-metal chelates could boost CL generation by activated PMNs, because the salts in HBSS and RPMI interfered with the activity of the added metals. CL and LDCL of activated PMNs was enhanced by aminotriazole, but strongly inhibited by diphenylene iodonium (an inhibitor of NADPH oxidase) by azide, sodium cyanide (CN), cimetidine, histidine, benzoate, DMTU and moderately by superoxide dismutase (SOD) and by deferoxamine.

0360-3997/93/0600-0227$07.00/0

LUCDCL was markedly inhibited only by SOD but was boosted by CN. Taken together, it is suggested that CL generated by stimulated PMNs might be the result of the interactions among, NADPH oxidase, (inhibitable by diphenylene iodonium), MPO (inhibitable by sodium azide), H_2O_2 probably of intracellular origin (inhibitable by DMTU but not by catalase), and trace metals that contaminate salt solutions. The nature of the salt solutions employed to measure CL in activated PMNs is critical.

INTRODUCTION

It is well established that neutrophils (PMNs), which are stimulated by a variety of both soluble and particulate agonists generate a series of oxygen-derived species (O_2, H_2O_2, $OH^{\cdot}$ and $^1O_2$) that can be measured by a variety of techniques (1–6). Concomitant with the generation of oxygen-derived species, stimulated PMNs also emit light (chemiluminescence, CL), which can be monitored either by scintillation counters or by a variety of luminometers (7–14). Light emission can be markedly amplified either by luminol (luminol-dependent chemiluminescence, LDCL) (7–13) or by lucigenin (lucigenin-dependent chemiluminescence LUCDCL) (12). While LDCL probably measures a mixture of oxygen-derived species, LUCDCL is believed to specifically monitor the generation of superoxide. This vast field of research has been reviewed elsewhere (13, 15). In addition to the standard stimulators of superoxide and CL generation by PMNs and macrophages regularly employed (antibody-opsonized particles, chemotactic peptides, phorbole esters), we have recently introduced a variety of cationic polyelectrolytes (poly-L-arginine, poly-L-histidine, histone) as potent stimulators of the respiratory burst in mammalian PMNs (16–22).

A strong relationship between the respiratory burst in neutrophils and the phenomenon of chemiluminescence has been established (13). The findings that either azide-treated normal PMNs or PMNs from patients with MPO deficiency failed to generate luminol-dependent chemiluminescence suggested a link between the respiratory burst, the phenomenon of LDCL, and the MPO–H_2O_2–halide system (10). In a more recent study, a critical analysis of the controversial issues connected with the employment of chemiluminescence to evaluate the role of discrete oxygen-derived species in light emission by activated PMNs has been reported (15). These authors came to the conclusion that "luminol-dependent chemiluminescence gives, at present, very little ability to discriminate between individual oxygen species." Furthermore, "luminol-dependent chemiluminescence used in biological systems is extremely prone to many interferences, which are very difficult to control." Thus, although measurement of chemiluminescence generation by PMNs is very simple to perform, many investigators of neutrophil activation are reluctant to employ chemiluminescence techniques to evaluate the nature of the oxygen-derived species that are responsible for light emission.

The availability of potent stimulators of the respiratory burst (PMA, polycation-opsonized streptococci) (16–22) and sensitive monitors of chemiluminescence prompted us to reevaluate the interrelationships among nonamplified (CL), luminol (LDCL), and lucigenin-amplified chemiluminescence (LUCDCL) generation by activated human neutrophils and under the effects of a large series of scavengers of putative oxygen-derived species. The present communication suggests that: (1) the initial generation of O_2^- and the subsequent accumulation of H_2O_2 are absolutely necessary for light emission; (2) that there is a strong parallelism between the nonamplified and luminol-amplified system; and (3) that a marked enhancement of nonamplified CL can be initiated by the addition of horseradish peroxidase (HRP) to azide-treated PMNs in the presence of an agonist, provided that trace elements (probably Fe^{2+}, Cu^{2+}, and Co^{2+}) capable of being chelated by deferoxamine (DESF) and EDTA, are present in the buffers employed. The possible nature of the oxygen-derived species responsible for light emission in the three chemiluminescence systems will be discussed.

MATERIALS AND METHODS

Human neutrophils (PMNs) were isolated from freshly drawn human blood in ACD employing a Ficol-Hypaque gradient and dextran sedimentation as described in detail (18, 19). Preparations containing greater than 95% viable PMNs (trypan blue exclusion test) were suspended either in Hanks' balanced salt solution (HBSS), pH 7.4, buffered with 3 mM HEPES, pH 7.35, or in HBSS alone, and kept on crushed ice for up to 4 h without losing their CL and superoxide generating capacities.

Generation and Measurement of Chemiluminescence. Chemiluminescence was measured in activated PMNs at 37°C in a LUMAC/3M Biocounter M2010 connected to a linear recorder. PMNs ($2–5 \times 10^5$–$2–5 \times 10^6$/ml) were pipetted into plastic cuvettes (supplied by the manufacturer), which contained 1 ml HBSS–HEPES buffer or HBSS. Either buffer (nonamplified system, CL) luminol (5×10^{-5} M) (LDCL), or lucigenin (3×10^{-5} M) (LUCDCL) were added followed by a variety of activators of the respiratory burst (see below). The cuvettes were mixed over a mechanical vortex, and chemiluminescence was monitored immediately and then until peak CL was reached. The following agents were employed to activate PMNs for the generation of chemiluminescence: (1) PMA, 1 μg/ml; and (2) cationized streptococci, 20 μl/ml. Ten milliliters of a saline-washed suspension of group A streptococci (type 3 strain C203S), of an optical density of 5.0 at 550 nm were treated for 15 min at 37°C either with 100 μg/ml of nuclear histone (type II-A) or with 100 μg/ml of poly-L-histidine (PHSTD) HCl (mol wt 23,000). The cationized suspensions were then washed in normal saline and resuspended to the original volume. Such cationized bacteria were employed to stimulate PMNs. These agonists were previously shown to activate human PMNs for the generation of LDCL, superoxide, and hydrogen peroxide (16–22). When the chemiluminescence responses were stabilized, additional modulators were added. In some experiments HRP was also added to the systems (see below) and the chemiluminescence responses were read immediately.

Modulation of Chemiluminescence Responses. The following agents were employed to modulate the chemiluminescence responses in the various systems: superoxide dismutase (SOD), catalase (CAT), sodium azide (AZ), sodium cyanide (CN), sodium benzoate (BENZ), histidine (HISTD), taurine (TAUR), dimethyl thiourea (DMTU), 3-amino 1,2,4-triazole (ATAZ), deferoxamine mesy-

late (DESF), cimetidine (TAG), methionine (METH), diphenylene-iodonium (DPI), and horse radish peroxidase (HRP). All other chemicals were purchased from Sigma Chemical Co., St. Louis, Missouri.

Chemiluminescence Induced by Hydrogen Peroxide–HRP Mixtures. Either reagent H_2O_2 or a mixture of glucose (1 mg/ml) and glucose oxidase (0.197 units/ml) were incubated with a variety of scavengers of oxygen-derived species in HBSS–HEPES, HBSS, or in distilled water. HRP (type I, 9 units/ml) was then added, and the chemiluminescence signals that emerged were monitored. In some experiments, a mixture of glucose and glucose oxidase was allowed to interact at 37°C for 2.5 min. This was followed by the addition of scavengers. Thirty seconds later, HRP was added, and the CL signals were monitored.

Measurement of Superoxide (O_2^-). Superoxide generation following stimulation by the various agonists was performed by measuring the reduction of cytochrome *c* (type III) at 550 nm, as described in detail (1, 20). Results were expressed as nanomoles per number of cells per 10 min.

Measurement of Hydrogen Peroxide. H_2O_2 was measured by the method of Thurman et al. [23] employing ferrous ammonium sulfate and sodium thiocyanate. Results were expressed as nanomoles per number of cells per 10 min.

Preparation of Metal Chelates. Nitrilotriacetate (NTA, 0.1 M) was mixed with 1 ml of an 0.1 M solution of $MnCl_2 \cdot 4H_2O$, $CuSO_4$, $CoCl_2 \cdot 6H_2O$; 0.2 M NTA was also mixed either with 0.1 M solutions of $FeSO_4$, or an 0.1 M solution of $FeCl_3$. The pH of the solutions was adjusted to 7.4 with powdered $NaHCO_3$. Such metal chelates (coined NTA-metals) were added to different supporting media (double-distilled water, water + 3 mM HEPES, HBSS, HBSS + HEPES, RPMI 1640 tissue culture medium) and the generation of CL was measured by adding hydrogen peroxide and HRP.

Lactic Dehydrogenase Release. The toxicity of the various agonists and modulators for PMNs was measured by the release of the cytosolic enzyme lactate dehydrogenase as described [20].

RESULTS

Generation of Chemiluminescence by Activated Neutrophils (PMNs)

It is accepted that while the luminol-dependent chemiluminescence (LDCL) that is generated by activated PMNs measures a variety of oxygen-derived species, including superoxide (10, 11, 13–15), lucigenin-dependent chemiluminescence (LUCDCL) is thought to monitor the generation of superoxide exclusively (12). Very few studies, however, have tried to define the nature of the oxygen-derived species that are emitted in a nonamplified chemiluminescence (CL) system (also see DeChatelet et al.) (10). To address this question we first verified that all the agonists we were employing were capable of inducing the generation of both superoxide, H_2O_2 and CL. In a typical experiment 10^6/ml of PMNs, stimulated by PHSTD-opsonized streptococci, generated approximately 32 nmol superoxide and about 15 nmol hydrogen peroxide. We found that the generation of O_2^-, H_2O_2, and chemiluminescence was totally inhibited

by as little as 5 μM of diphenyleneiodonium, a specific inhibitor of NADPH oxidase. We then compared the patterns of CL, LDCL, and LUCDCL that were generated from PMNs, stimulated either by polycation-opsonized streptococci or by PMA and under the sequential effects of azide, SOD, and HRP. The rationale for performing such experiments was that any superoxide that might be generated by activated PMNs will be dismutated by SOD to H_2O_2. Furthermore, the presence of azide during the activation of the respiratory burst might further facilitate the accumulation of hydrogen peroxide, because azide, which is a strong inhibitor of both catalase and MPO, will prevent the consumption of hydrogen peroxide by the MPO–halide system [24]. The hydrogen peroxide, which is expected to accumulate, may then be monitored as chemiluminescence by the addition of HRP [25].

Figure 1A shows that employing histone-opsonized streptococci as a stimulus of the oxidative burst, although much smaller CL signals were generated in the nonamplified system as compared with the amplified ones (B, C), the sequential addition of azide SOD and HRP to the activated cells resulted in a large peak of nonamplified light. On the other hand, no enhanced light was found either in the luminol- or in the lucigenin-amplified systems. Figure 2A further shows that even a larger nonamplified peak of light was obtained if the PMNs were first stimulated by the agonist in the presence of azide, and subsequently treated by SOD and HRP. Again, no significant enhanced light peaks were monitored in the two amplified systems (Figure 2B,C). Identical results were also obtained when PMA or PHSTD (20) were employed as stimulators of PMN luminescence (not shown). Table 1 further examines the effects of AZ, SOD, and HRP on CL, when these agents were added to PMNs at various stages of cell activation. The data in Table 1 show that activated PMNs treated with azide or with SOD generated only 20% and 50% of CL, respectively, as

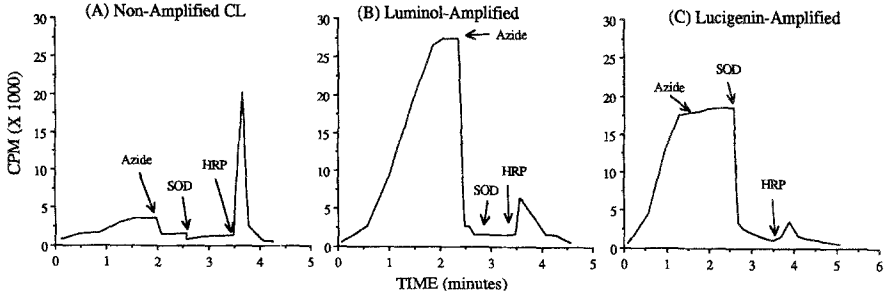

Fig. 1. Effects of azide, SOD, and HRP on CL, LDCL, and LUCDCL by PMNs stimulated by histone-opsonized streptococci. PMNs (10^6/ml) were stimulated by the cationized streptococci, 2 min later azide (1 mM) was added, followed 40 sec later by SOD (36 units/ml) followed one additional minute later by HRP (0.9 units/ml). Note the different patterns of luminescence generated by the three systems.

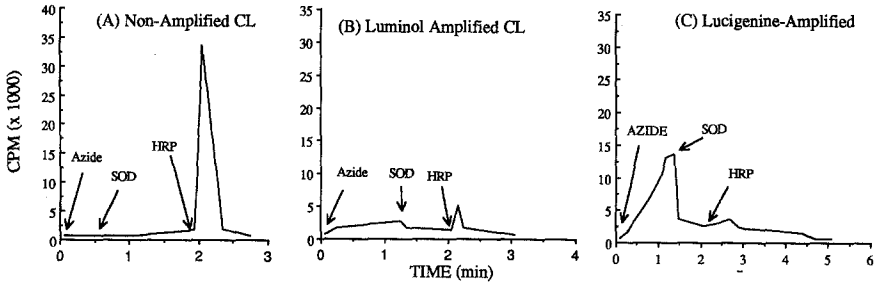

Fig. 2. Effects of azide, SOD, and HRP on chemiluminescence generated by PMNs stimulated by histone-opsonized streptococci. PMNs (10^6/ml) were stimulated by cationized streptococci in the presence of azide (1 mM) (arrow). SOD (36 units/ml) was added, followed 1 min later by the addition of HRP (0.9 units/ml). Note that a much larger luminescence peak was obtained when azide, followed by SOD and HRP, was employed in the nonamplified system.

Table 1. Effect of Azide and SOD on Nonamplified Chemiluminescence (CL) Induced in PMNs by Polyhistidine-Opsonized Streptococci[a]

Activated PMNs		Chemiluminescence generated					
Treated by	cpm	Followed by	cpm	Followed by	cpm	Followed by	cpm
None	300	SOD (units/ml)	96			HRP (9 units/ml)	2400
SOD (36 units)	150	AZ	70			HRP	300
AZ (1 mM)	60	SOD	66			HRP	4675
SOD + AZ	60					HRP	175
None	300	AZ	70	SOD	66	HRP	3800
None	300	SOD	140	Azide	70	HRP	3280
None	300	SOD + AZ	70			HRP	2280

[a]PMNs (1×10^6/ml) in HBSS + HEPES were simultaneously treated with the various agents and with PHSTD-opsonized streptococci. When peak CL was reached (about 4 min) a variety of agents were added in sequence at 1-min intervals. In the final stage, HRP was added, and the CL signals were measured immediately. Untreated PMNs yielded approximately 30 cpm of CL. The data are the aveage of five experiments.

compared with controls. These data suggest that the generation of CL was mainly myeloperoxidase-dependent and, to a lesser extent, superoxide-dependent. On the other hand, when PMNs were activated in the presence of azide and later on challenged by HRP, a very large CL peak was obtained, suggesting that azide, by inhibiting MPO, prevented the consumption of hydrogen peroxide by the MPO–halide reaction (24) and thus allowed HRP to interact with larger amounts of hydrogen peroxide. Since PMNs activated in the presence of SOD produced very little CL upon treatment with HRP, one can assume that the early generation of superoxide, by the activated PMNs, was absolutely necessary to allow the accumulation of hydrogen peroxide. The strongest inhibition of the

HRP CL was obtained when activated PMNs were simultaneously incubated with SOD + azide, further confirming the need for an early generation of superoxide to secure maximal HRP-induced CL.

To further elucidate the nature of the enhanced light obtained by HRP in the nonamplified system, we also examined the possibility that trace metals, which are known to contaminate the salts that constitute HBSS, might have been responsible for initiating a Fenton-driven reaction and for the generation of light (26). To address this question, we first stimulated PMNs by opsonized streptococci in the presence of azide (to inhibit MPO). We then added SOD to further allow the accumulation of hydrogen peroxide, and then added either DESF, EDTA, DETAPAC (all chelators of metals), or catalase prior to the addition of HRP. Table 2 shows that employing 5×10^6 PMN/ml, a very strong inhibition of CL occurred in the presence of all the chelators and of catalase, suggesting that both trace metals and hydrogen peroxide are involved in the initiation of CL following the addition of HRP (see below).

Experiments with Glucose Oxidase and with Reagent H_2O_2

Glucose + Glucose Oxidase (GO). Since chelating agents were found to be inhibitory to the HRP reaction in PMNs stimulated by cationized bacteria in the presence of azide and SOD (Table 2), it was also of interest to examine the effects of chelating agents and catalase on nonamplified CL emitted when hydrogen peroxide interacted with HRP. Tubes containing either distilled water, 1

Table 2. Effect of Chelating Agents and Catalase on Nonamplified Chemiluminescence Induced in PMNs by PHSTD-Opsonized Streptococci[a]

Activated PMN +	Followed by	Followed by	Followed by	cpm	Inhibition (%)
Azide (AZ)	SOD	None	HRP	11000	
AZ	SOD	DESF	HRP	600	94.0
AZ	SOD	EDTA	HRP	3000	93.0
AZ	SOD	DETAPAC	HRP	918	92.0
AZ	SOD	Catalase (CAT)	HRP	492	95.0
AZ	SOD	CAT + DESF	HRP	100	99.0

[a]PMNs (5×10^6) in HBSS + HEPES (containing 1 mg/ml glucose) were activated by PHSTD-opsonized streptococci in the presence of 1 mM AZ; 2.5 min later SOD (36 units/ml) was added followed 1 min. later by the addition either of DESF (2.5 mM), EDTA (2.5 mM), DETAPAC (diethylentriamine pentaacetic acid) (2.5 mM), CAT (110 units/ml). One additional minute later HRP (9 units/ml) was added, and the CL responses were monitored. PMNs stimulated by the PHSTD-opsonized streptococci yielded 1200 cpm of luminescence. Data are the average of three separate experiments.

mg/ml of glucose and GO (0.197 units/ml) or HBSS-HEPES + GO were incubated for 2.5 min at 37°C to allow generation of hydrogen peroxide. HRP (9 units/ml) was then added, and the nonamplified CL signals that emerged were immediately monitored. Table 3 shows that, as in the case of activated PMNs (Table 2), the nonamplified CL signals that were monitored in the glucose oxidase system can be inhibited either by catalase or by deferoxamine, again suggesting that the interaction of H_2O_2 with HRP involved additional agents (trace metals?) present in HBSS–HEPES buffer, but not in distilled water. Addition of HBSS at 15% v/v to distilled water + glucose, restored the near-full CL responses upon addition of HRP.

Reagent H_2O_2. To further study the nature of the metals that might be involved in the generation of CL, when HRP interacted with hydrogen peroxide, we first added various concentrations of manganese, iron, copper, and cobalt (250–1000 μM) to cuvettes containing distilled water H_2O_2 and HRP. No significant enhancement of CL above baseline was observed. Since, however, certain of the divalent metals employed might not be stable under these conditions, we also prepared nitrilotriacetate (NTA) chelates with the four metals and tested their effect on CL generated by mixtures of HRP and H_2O_2. Figure 3A shows that when double-distilled water was the supporting medium, NTA-Co^{2+} and NTA-Cu^{2+} were more efficient than either NTA-Mn^{2+} or NTA-Fe^{2+} in the stimulation of HRP-induced CL. Deferoxamine at 2.5 mM caused 97% inhibition of CL induced either by Co^{2+}, Mn^{2+}, or Cu^{2+} (not shown). Deferoxamine, however, formed a brown complex with Fe^{2+}, which interfered with the CL readings. Figure 3B shows that when HEPES (3 mM) was added to distilled

Table 3. Effect of Deferoxamine and Catalase on Nonamplified CL Induced by Glucose–Glucose Oxidase

Glucose oxidase added to Media[a]	Followed by	Followed by	Luminescence (cpm)
HBSS + HEPES (containing glucose)	None	HRP	3000
HBSS + HEPES	DESF	HRP	350
HBSS + HEPES	CT	HRP	280
Distilled water (DW) + Glucose (1 mg/ml)	None	HRP	300
DW + glucose (1 mg/ml)	DESF	HRP	100
DW + glucose	CAT	HRP	88
DW + HBSS + HEPES (15% v/v)	None	HRP	2800
DW + HEPES	None	HRP	800
DW + glucose + HBSS (15% v/v)	DESF	HRP	600

[a]Glucose oxidase (0.197 units/ml) was added to the various media and incubated for 2.5 min at 37°C to allow the generation of H_2O_2. This was followed by the addition of either deferoxamine (DESF) (2.5 mM) or catalase (CAT) (110 units/ml) and finally by HRP (9 units/ml). The changes in CL were monitored immediately. The data are the average of three experiments.

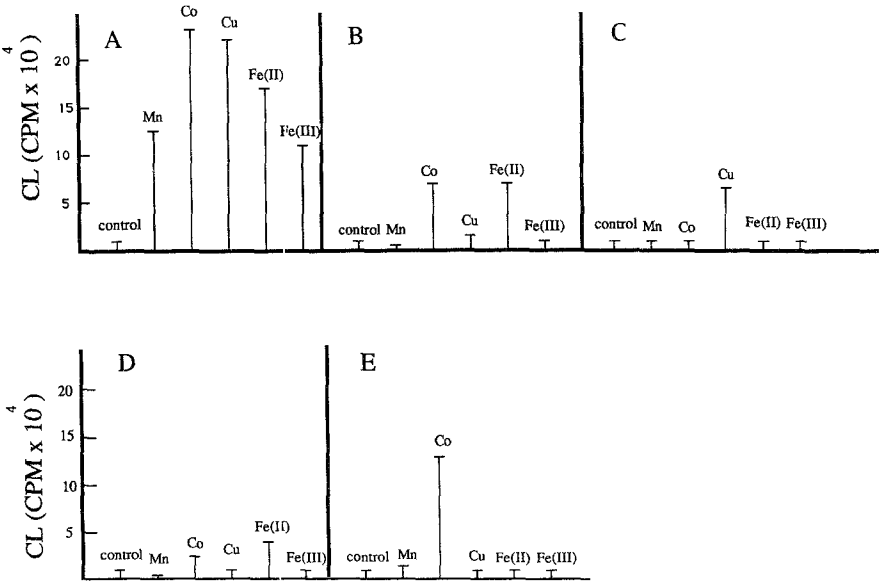

Fig. 3. Effect of NTA-metal chelates on HRP CL. To cuvettes containing either 1 ml of distilled water (A), distilled water plus 3 mM HEPES buffer (B), HBSS medium (C), HBSS + HEPES (D) or RPMI 1640 medium (E), 2 mM of hydrogen peroxide and 1 mM of metal chelates (NTA-Mn^{2+}, NTA-Co^{2+}, NTA-Cu^{2+}, NTA-Fe^{2+}, NTA-Fe^{3+}) were added, followed by the addition of HRP (18 units/ml) and the CL generated was measured immediately. Note the inhibitory effects of HBSS, HBSS + HEPES and RPMI medium on HRP CL. The results represent a typical experiment performed on the same day.

water, there was a marked decrease in the ability of NTA-Mn^{2+} and NTA-Cu^{2+} to stimulate CL. HEPES had a lesser inhibitory effect on CL induced either by NTA-Co^{2+}, NTA-Fe^{2+}, or NTA-Fe^{3+}.

When HBSS was the supporting medium (Figure 3C) only NTA-Cu^{2+} had some stimulatory effect on HRP-CL but, unlike in the case of distilled water (Figure 3A), deferoxamine totally failed to quench HRP CL (not shown). The addition of HEPES to HBSS medium (Figure 3D) also shows a marked depression in metal-enhanced HRP CL by practically all the metal chelates employed. When RPMI 1640 was the supporting medium (Figure 3E), only NTA-Co^{2+} was stimulatory for HRP CL. Its effect was totally inhibited by deferoxamine (not shown). The data in Figure 3A–E clearly demonstrate that the choice of supporting medium is very important in experiments involving metal chelates.

The ability of metal chelates to markedly boost HRP CL led us to determine the smallest amount of hydrogen peroxide that could be detected when distilled water was the supporting medium. Figure 4 shows that as little as 10 μM of hydrogen peroxide can be detected when 1 mM NTA-Co^{2+} was added to mix-

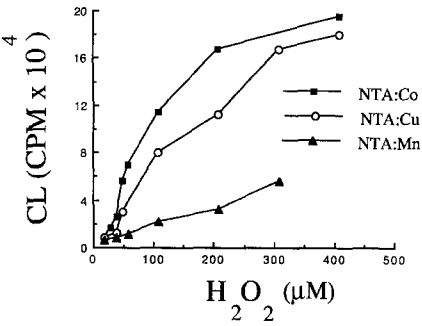

Fig. 4. Effect of NTA-metal chelates on HRP CL generated by increasing concentrations of hydrogen peroxide. To cuvettes containing 1 ml of distilled water, increasing concentrations of reagent hydrogen peroxide were added, 1 mM of NTA-Mn^{2+}, NTA-Co^{2+}, and NTA-Cu^{2+} were added followed by the addition of HRP (18 units/ml). Note that NTA-Co^{2+} was the most effective metal chelate capable of detecting small amounts of hydrogen peroxide. The results represent a typical experiment performed on the same day.

tures of hydrogen peroxide and HRP. NTA-Cu^{2+} and NTA-Mn^{2+} were inferior to NTA-Co^{2+} as stimulators of HRP CL. We also determined the smallest amounts of NTA-metals that are capable of stimulating HRP CL. Figure 5 shows that NTA-Co^{2+} is by far the most efficient metal chelate capable of boosting HRP CL.

Effect of NTA-Metal Chelates on CL Induced in PMNs

Since NTA-metals were involved in HRP-induced CL in cell-free systems (see above), we also tested the effects of such complexes on luminol-dependent CL induced in PMNs by histone-opsonized streptococci (substituted for PHSTD streptococci). PMNs (10^6/ml) suspended in HBSS were stimulated in the absence and presence of NTA-metals, and the luminol-dependent chemiluminescence was measured for several minutes. Of the four NTA-metal chelates tested (NTA-Fe^{2+}, NTA-Cu^{2+}, NTA-Mn^{2+}, NTA-Co^{2+}), only NTA-Co^{2+} at 1 mM markedly boosted PMN-induced LDCL either when added at peak CL or when mixed with PMNs prior to stimulation by histone-opsonized streptococci. In both cases, the addition of deferoxamine at the peak LDCL very markedly depressed light emission. Deferoxamine also markedly inhibited LDCL when added together with NTA-Co^{2+}, PMNs, and histone-opsonized streptococci. NTA-Mn^{2+} and NTA-Fe^{2+} failed to enhance CL, while NTA-Cu^{2+} even at 0.01 mM was strongly inhibitory. To test the nature of the enhancement of PMN-LDCL by NTA-Co^{2+}, we examined the possibility that NTA-Co^{2+} might interact with luminol, resulting in light emission. Indeed, we found that a very steep peak of

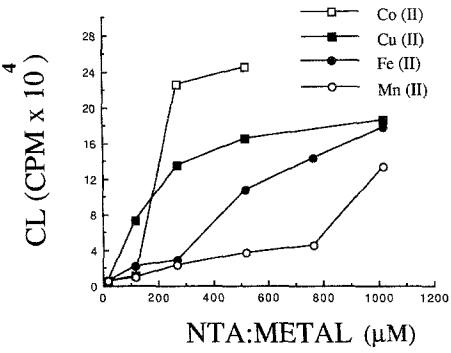

Fig. 5. Effect of increasing concentrations of NTA-metal chelates on HRP CL. To cuvettes containing 1 ml distilled water were added increasing concentrations of NTA-metal chelates followed by the addition of 2 mM of H_2O_2 and 18 units/ml of HRP. The results represent a typical experiment performed on the same day.

light was emitted by such mixtures in the absence of added activated PMNs. Since PMNs stimulated by cationized streptococci also generated nonamplified CL, we added NTA-Co^{2+} to such mixtures and found no enhancement of CL, suggesting, therefore, that the effect of NTA-Co^{2+} on LDCL induced by activated PMNs was not due to the effect on the oxidase but due to an artifact caused by complexing luminol with NTA-Co^{2+}.

Modulation of PMN-Chemiluminescence by Scavengers

The nature of the oxygen-derived species that are responsible for the CL phenomenon might involve superoxide, hydrogen peroxide, OH·HClO and still undefined oxygen-derived species (13, 15). It is accepted, however, that while at least in the case of the luminol-enhanced luminescence (LDCL) of stimulated PMNs, myeloperoxidase plays a central role in light emission (10, 11, 14), the lucigenin CL (LUCDCL) might specifically monitor the generation of superoxide (12). No clear picture is, however, available on the oxygen-derived species that are generated in a nonamplified (natural) luminescence of stimulated PMNs. We have addressed this point by comparing the three luminescence assays of activated PMNs under the effect of a series of scavengers of well-defined oxygen-derived species. Two systems have been analyzed. In system A, PMNs were incubated with the agonist in the presence of the modulating agent (scavenger) and the nonamplified (CL), luminol-amplified (LDCL), and lucigenin-amplified luminescence (LUCDCL) were monitored. In system B, we first allowed PMNs to interact with the agonist. When peak luminescence was reached, we added the various scavengers and immediately monitored the changes

in light emission. While the first system might measure both the antimetabolic and scavenging effects of the agents, the second system probably measures their scavenging effects, exclusively. Table 4 shows that when system A (scavenger added during cell activation) was employed, a significant inhibition of both CL and LDCL took place with diphenyleneiodonium, SOD, AZ, CN, TAG, HISTD, BENZ, DMTU, and, to a lesser extent, with DESF and CAT. On the other hand, a marked enhancement of both CL and LDCL occurred with ATAZ. LUCDCL was significantly inhibited only by SOD and to a much smaller extent by ATAZ, but very markedly enhanced by CN.

Employing system B (scavenger added at peak luminescence) revealed a strong parallelism with system A. A very marked inhibition of peak CL and LDCL occurred with diphenyleneiodonium, AZ, CN, TAG, HISTD, BENZ, DMTDU, but little inhibition was caused by SOD, CAT, or DESF. Both CL and LDCL were markedly enhanced by ATAZ. As in the case of system A, LUCDCL was inhibited to a significant degree only by SOD, but was again markedly boosted by CN. The data in Table 4 suggest strong parallelism between

Table 4. Effect of Various Agents on Nonamplified, Luminol- and Lucigenin-Amplified Chemiluminescence Responses of PMNs Induced by Poly-Histidine-Opsonized Streptococci

Scavenger luminescence	Inhibition (%) of Chemiluminescence					
	System A, scavenger added with activator[a]			System B, scavenger added at peak[b]		
	CL	LDCL	LUCDCL	CL	LDCL	LUCDCL
Diphenylene iodonium (5 μM)	100.0	100.0	100.0	100.0	100.0	100.0
SOD (110 units)	53.0	61.0	81.0	38.8	33.8	72.0
Catalase (110 units)	5.0	27.0	0	7.0	15.0	12.0
Azide (1 mM)	76.0	93.0	21.0	83.0	91.0	0
Cyanide (1 mM)	69.8	69.0	+ 195.0[c]	64.0	0.0	+228.0[c]
Aminotriazole (3 mM)	+180.0[c]	+34.0[c]	57.0	+188.0[c]	+169.0[c]	29.0
Cimetidine (8 mM)	44.0	76.0	5.0	53.0	61.0	15.0
Desferal (2.5 mM)	6.0	51.0	4.0	20.0	24.0	0
Histidine (10 mM)	64.0	70	0	71.0	67.0	0
Benzoate (10 mM)	61.0	61.0	0	55.0	45.0	0
DMTU (10 mM)	59.6	58.0	0	68.0	76.0	5.0

[a] Neutrophils (4×10^6) in the case of nonamplified CL and 1×10^6 cells in the cases of luminol- or lucigenin-enhanced CL were stimulated by PHSTD-opsonized streptococci in the presence of a variety of scavengers, and the peak CL was recorded 4 min later.

[b] Neutrophils were first stimulated by opsonized streptococci. When peak CL was reached (3–4 min) the various scavengers were added, and the immediate change in CL was monitored.

[c] + indicates enhanced luminescence; CL yielded 1100 cpm, LDCL 12,000 cpm, and LUCDCL 6000 cpm. The data are the average of five experiments.

the luminol and nonamplified CL systems and that the various agents employed probably acted as scavengers rather than as antimetabolic agents.

To establish whether or not the results with the various scavengers were specific for the particulate agonist employed (opsonized streptococci), we have also tested their effects on the chemiluminescence-induced by PMA. The patterns of inhibition of CL, LDCL, and LUCDCL generated by PMA-activated PMNs, (employing system A scavenger added together with the agonist) were very similar to those obtained with the opsonized streptococci (not shown).

DISCUSSION

The evaluation of chemiluminescence to assess the generation of oxygen-derived species generated by activated neutrophils is controversial due to the lack of knowledge on the exact nature of the reactants, which are involved in this reaction. The employment of luminol and lucigenin to enhance light emission is fraught with many pitfalls (28), as it might involve secondary reactions between the amplifier and the oxygen-derived species (15).

The present communication compared both the enhanced and nonenhanced chemiluminescence responses initiated in activated human PMNs under the effects of scavengers of known oxygen-derived species.

A major difference in the luminescence patterns between the nonamplified and the luminol or lucigenin systems was found when activated PMNs were treated with AZ and SOD followed by HRP (Figures 1 and 2), where a very intense light is measured in the nonamplified system, as compared with the amplified one. The reason for the inability to measure an intense light response, when luminol is employed as an amplifier, is not known. Since, however, PMNs stimulated with streptococci in the presence of azide and SOD (in the absence of an amplifier) and treated with luminol + HRP also generated intense light (not shown), we may assume that luminol did not inhibit the interaction of HRP with hydrogen peroxide, presumably generated by the activated PMNs.

The findings that the presence of SOD in the early phases of PMN activation (Table 1) markedly depressed light emission upon addition of HRP suggest that the initial generation of superoxide is mandatory for light emission (see 25). On the other hand, addition of SOD to mixtures of activated PMNs and azide at a later stage had stimulatory effects on CL, suggesting that the early generation of SOD led to a metal-mediated Haber Weiss reaction. Indeed, data presented in Table 2 demonstrate that a chelator of iron (deferoxamine) and chelators of cations (EDTA and DETAPAC) strongly quenched light emission when PMNs were stimulated by cationized streptococci in the presence of AZ and SOD. Since light emission was also markedly depressed by catalase and DMTU (not

shown), we might also assume that the hydrogen peroxide generated interacted with a trace metal (present in HBSS-HEPES) and a peroxidase (HRP) to induce light. The inability to measure intense light in reaction mixtures containing phosphate-buffered saline probably stems from the lack, in such solutions, of trace metals.

Further support for the assumption that HRP-mediated CL, which was generated by PMNs stimulated by cationized streptococci in the presence of AZ and SOD, was linked to a metal-driven Haber Weiss reaction came from experiments described in Table 3, in which a cell-free system comprised of GO and HRP was used. The data strongly suggest that HBSS supplied agents (presumably trace metals) that boosted CL. The data presented in Tables 2 and 3 suggest, therefore, that the source of nonamplified CL, which is emitted when PMNs are stimulated in the presence of azide and SOD, might be the result of the generation of hydrogen-peroxide (which is destroyed by catalase and by DMTU) and which, in the presence of a trace metal (contaminants in HBSS), interacted with a peroxidase to generate light.

The nature of the trace metals that participated in the generation of light when H_2O_2 interacted with HRP was further studied by introducing exogenous metals. When distilled water was the supporting medium, all four metal chelates very markedly boosted CL generated by mixtures of H_2O_2 and HRP (Figure 3A). This effect was very markedly decreased by deferoxamine. Addition of HEPES buffer to distilled water very markedly depressed HRP CL (Figure 3B). HBSS, which is regularly employed as a suspending medium for the determination of PMN-induced CL, was very inhibitory to HRP CL and only NTA-Cu^{2+} induced some stimulation (Figure 3C). HBSS + HEPES was also very inhibitory to HRP CL (Figure 3D) while NTA-Co^{2+} was the only metal chelate capable of boosting CL when RPMI 1640 medium was employed (Figure 3E). This effect was totally quenched by deferoxamine. The ability to boost CL by the introduction of NTA-metal chelates allowed the determination of relatively very small amounts of hydrogen peroxide, provided that the test was conducted in distilled water (Figure 4). NTA-Co^{2+} was by far the most efficient chelate capable of boosting HRP CL (Figure 5) under the experimental conditions described. Attempts to boost CL generated by activated PMNs or by the introduction of exogenous metal chelates were not successful. While NTA-Co^{2+}, which apparently boosted PMN-induced CL, interacted with luminol to form a luminescent complex, which interfered with the CL measurements, NTA-Cu^{2+}, which was the only metal chelate capable of stimulating CL in HBSS (Figure 3C), was extremely toxic to the PMNs. Thus NTA-metal chelates, which are active when distilled water is the supporting medium, fail to exert their stimulatory activities in the presence of salts that constitute HBSS, the suspending medium of choice for neutrophils.

The assumption that LDCL generation by activated PMNs is the result of

the activation of NADPH oxidase is based on our findings that CL generation was totally inhibited by very low concentrations of diphenyleneiodonium, a specific inhibitor of NADPH oxidase. Our findings in Table 4, system A, further show that scavengers of known oxygen-derived species markedly inhibited CL, implicating hydrogen peroxide, the early generation of superoxide, and also the possible involvement of hydroxyl radicals.

Similar patterns are seen when the scavenger was added at peak CL (Table 4, system B). The patterns of modulation of the LUCDCL system differed from the two other systems. Here only SOD had a marked inhibitory effect, but CN markedly enhanced light emission. This might be linked with the ability of CN to boost NADPH production, which might enhance the activity of NADPH oxidase linked to the generation of superoxide.

The finding that ATAZ, a distinct inhibitor of catalase, markedly enhanced light emission both in the absence and presence of luminol is also of interest. ATAZ, by inhibiting catalase activity (25), might contribute to the accumulation of hydrogen peroxide, which then interacts with a trace metal and with a peroxidase (myeloperoxidase) to generate light.

Taken together, it is suggested that chemiluminescence, which is generated by activated PMNs, might be the result of the interactions among NADPH oxidase (inhibitable by diphenyleneiodonium chloride), hydrogen peroxide, probably of intracellular origin (inhibitable by dimethylthiourea but not by catalase), a peroxidase (myeloperoxidase inhibitable by both azide and cyanide), and trace metals, which catalyze the generation of hydroxyl radicals (inhibitable by benzoate, histidine and cimetidine). Our preliminary findings that both purified MPO and lactoperoxidase could replace HRP as enhancers of light emission in a cell-free system comprised of hydrogen peroxide and contaminating metals (which could be chelated by deferoxamine) further support the assumption that the nature of the supporting buffer employed to measure CL is of utmost importance for the evaluation of leukocyte activation.

Acknowledgments—This study was supported by a research grant from Dr. S. M. Robbins of Cleveland, Ohio, by grant IM-432 from the American Cancer Society, and by grants HL-31963 and GM 29507 from the National Institutes of Health, Bethesda, Maryland.

The authors acknowledge with thanks the help and suggestions of Dr. J. C. Fantone during the performance of this study.

REFERENCES

1. BABIOR, B. M. 1984. Oxidants from phagocytes: Agents of defence and destruction. *Blood* **64**:959–966.
2. KLEBANOFF, S. J. 1988. Phatocytic cells: Products of oxygen metabolism. *In* Inflammation

Basic Principles and Clinical Correlates. J. I. Gallin, I. M. Goldstein, and R. Snyderman, editors. Raven Press, New York. 391–444.

3. BELLAVITE, P. 1988. The superoxide-forming enzymatic system of phagocytes. *Free Radical Biol. Med.* **4**:225–261.
4. BORREGARD, N. 1988. The respiratory burst; an overview. *In* The Respiratory Burst and Its Physiological Significance. A. J. Sbarra and R. Strauss, editors. Plenum Publishing, New York. 1–31.
5. BABIOR, B. M. 1988. Microbicidal oxidant production by phagocytes. *In* Oxyradicals in Molecular Biology and Pathology. P. A. Cerutti, editor. Allan R. Liss, New York. 39–51.
6. HALLIWELL, B. 1989. Current status review: Free radicals, reactive oxygen species and human disease: A critical evaluation with special references to artherosclerosis. *Br. J. Exp. Pathol.* **70**:737–757.
7. ALLEN, R. C., and LOOSE, L. D. 1976. Phagocytic activation of luminol-dependent chemi luminescence in rabbit alveolar and peritoneal macrophages. *Biochem. Biophys. Res. Commun.* **69**:245–252.
8. TRUSH, M. A., M. E. WILSON, and K. VAN DYKE. 1978. Generation of chemiluminescence (CL) by phagocytic cells. *Methods Enzymol.* **57**:462.
9. WESTRICK, M. A., P. SHIRLEY, and L. R. DECHATELET. 1980. Generation of chemiluminescence by neutrophils exposed to soluble stimuli of oxidative metabolism. *Infect. Immun.* **30**:385–390.
10. DECHATELET, L. R., G. D. LONG, P. S. SHIRLEY, D. A. BASS, M. J. THOMAS, F. W. HENDERSON, and M. S. COHEN. 1982. Mechanisms of the luminol dependent chemiluminescence of human neutrophils. *J. Immunol.* **129**:1589–1593.
11. DAHLGREN, C., and O. STENDAHL. 1983. Role of myeloperoxidase in luminol dependent chemiluminescence of polymorphonuclear leukocytes. *Infect. Immun.* **39**:736–741.
12. MULLER-PEDDINGHAUS, R. 1984. In vitro determination of phagocyte activity by luminol and lucigenin-amplified chemiluminescence. *Int. J. Immunopharmacol.* **6**:455–466.
13. VAN DYKE, K., and V. CASTRANOVA. 1987. Cellular chemiluminescence, Vols. I and II. CRC Press, Boca Raton, Florida.
14. DAHLGREN, C. 1989. Is lysosome fusion required for the granulocyte chemiluminescence reaction? *Free Radical Biol.* **6**:399–403.
15. VILIMM, V., and J. WILHELM. 1989. What do we measure by a luminol-dependent chemiluminescence of phagocytes? *Free Radical Biol.* **6**:623–629.
16. GINSBURG, I., R. BORINSKI, M. LAHAV, K. E. GILLERT, M. WINKLER, and S. MULLER. 1982. Bacteria and zymozan opsonized with histone and polyanethole sulfonate trigger intense chemiluminescence in human blood leukocytes, platelets and in mouse peritoneal macrophages: Modulation by metabolic inhibitors in relation to leukocyte-bacteria interactions in inflammatory sites. *Inflammation* **6**:343–364.
17. GINSBURG, I., R. BORINSKI, M. LAHAV, Y. MATZNER, I. ELIASSON, P. CHRISTENSEN, and D. MALLAMUD. 1984. Poly-L-arginine and *N*-formylated chemotactic peptide act synergistically with lectins and calcium ionophore to induce intense chemiluminescence and superoxide production in human blood leukocytes. Modulation by metabolic inhibitors, sugars and polyelectrolytes. *Inflammation* **8**:1–26.
18. GINSBURG, I., R. BORINSKI, D. MALAMUD, F. STRUCKMAYER, and V. KILMETZEK. 1985. Chemiluminescence and superoxide generation by leukocytes stimulated by polyelectrolyte opsonized streptococci: Role of polyargine, polylysine, polyhistidine, cytochalasins and inflammatory exudates as modulators of the oxygen burst. *Inflammation* **9**:245–271.
19. GINSBURG, I., and R. BORINSKI. 1987. "Cocktails" of soluble ligands and bacteria "opsonized" with cationic or anionic polyelectrolytes trigger intense chemiluminescence and superoxide production by leukocytes. *In* Cellular Chemiluminescence, Vol. II. K. van Dyke and V. Castranova, editors. CRC Press, Boca Raton, Florida. 121–156.

20. GINSBURG, I., R. BORINSKI, M. SADOVNIC, Y. EILAM, and K. RAINSORD. 1987. Poly-L-histidine, a potent stimulator of superoxide generation by human blood leukocytes. *Inflammation* **11**:253–277.
21. GINSBURG, I. 1987. Cationic polyelectrolytes: A new look at their possible role as opsonins as stimulators of the respiratory bursts in leukocytes in bacteriolysis and as modulators of immune complex disease (a review hypothesis). *Inflammation* **11**:489–515.
22. GINSBURG, I. 1989. Cationic polyelectrolytes: Potent opsonic agents which activate the respiratory burst in leukocytes. *Free Radical Res. Commun.* **8**:11–26.
23. THURMAN, R. G., H. G. LEYLAND, and R. SCHOLZ. 1972. Hepatic microsomal ethanol oxidation hydrogen peroxidase formation and the role of catalase. *Eur. J. Biochm.* **25**:420–430.
24. KLEBANOFF, S. J. 1968. Myeloperoxidase halide-hydrogen peroxide antibacterial system. *J. Bacteriol.* **95**:2131–2138.
25. MARGOLIASH, E., and A. NOVOGRODSKY. 1958. A study of the inhabit of catalase by 3-amino-1,2,4-triazole. *Biochem. J.* **68**:468.
26. LOCK, R., A. JOHANSSON, K. ORSELIUS, and C. DAHLGREN. 1988. Analysis of horseradish peroxidase-amplified chemiluminescence production by human neutrophils reveals a role for the superoxide anion in the light emitting reaction. *Anal. Biochem.* **173**:450–455.
27. TOTH, K. M., J. M. HARLAN, C. J. BEEHLER, L. M. BERGER, N. B. PARKER, S. L. LINAS, and J. E. REPINE. 1989. Dimethylthiourea prevents hydrogen peroxide and neutrophil mediated damage to lung endothelial cells *in vitro* and disappears in the process. *J. Clin. Invest.* **50**:2226–2229.
28. MCCORD, J. M., and E. D. J. DAY. 1989. Superoxide dependent production of hydroxyl radical catalyzed by iron-EDTA complex. *FEBS Lett.* **80**:130–136.