HALIDE DEPENDENCE OF THE MYELOPEROXIDASE-MEDIATED ANTIMICROBIAL SYSTEM OF THE POLYMORPHONUCLEAR LEUKOCYTE IN THE PHENOMENON OF ELECTRONIC EXCITATION

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SUMMARY: The chemiluminescent (CL) response from intact phagocytizing polymorphonuclear (PMN) leukocytes was found to be halide-dependent and halide-specific, and this suggested the participation of the myeloperoxidase antimicrobial system in the electronic excitation phenomenon. Myeloperoxidase extracted from these leukocytes also yielded a chemiluminescence if HOOH and a halide cofactor were available to the system. Each halide was unique with respect to the magnitude and kinetic character of the CL response for a given concentration. Bromide and chloride gave best results for both cellular and enzyme systems. Iodide gave a weak response, but fluoride gave no response. The CL response was labile to heat denaturation, 90°C for 10 minutes.

I. INTRODUCTION

Evidence for the generation of electronically excited molecules during the microbicidal metabolic activity of the human polymorphonuclear (PMN) leukocyte has been presented previously (1,2). Several lines of evidence suggest that the microbicidal enzyme myeloperoxidase (MPO) is at least partially responsible for electronic excitation. The chemiluminescence (CL) response, which was taken as an index of excited state generation from intact phagocytizing PMN leukocytes demonstrates a definite halide dependency, and inhibitors of MPO, such as sodium azide and sodium cyanide, also successfully inhibit CL activity from intact PMN (3).

Abbreviations are: MPO, myeloperoxidase; PMN, polymorphonuclear; CL, chemiluminescence; $^1O_2$, excited singlet multiplicity molecular oxygen; X^-, either chloride, bromide, or iodide.

Klebanoff has reported that MPO, obtained from leukocytes by weak acid extraction, is capable of microbicidal activity in the presence of HOOH and either I^-, Br^-, or Cl^-. The degree of microbicidal activity was dependent upon the halide used and upon its concentration (4).

In order to investigate the role of electronic excitation in microbicidal activity at the enzymatic level, intact human PMN leukocytes were obtained and extracted for MPO as described by Klebanoff (4), and the enzyme preparation was monitored for CL under varying conditions of halide concentrations. This report describes the CL response of the MPO-X^--HOOH system.

II. MATERIALS AND METHODS

Human PMN leukocytes were obtained from peripheral blood by venipuncture. The whole blood was collected and sedimented. The leukocytes obtained were further purified by a modification of the glass bead column method described by Rabinowitz (3,5). The purified PMN leukocytes were then washed two additional times with the halide-free 0.1 M phosphate buffer, pH 7.2, in order to remove any residual halides. These leukocytes were extracted for MPO as previously described, and the supernatant obtained was assayed for MPO activity using the o-dianisidine method described by Klebanoff (4).

Chemiluminescence (CL) was monitored by photomultiplication methods using a Packard Scintillation Spectrometer Model 3320, operated in the out-of-coincidence summation mode (3,6). For continuous recording, the summation signal was fed into a Packard Ratemeter, Model 280 A, and then to a Honeywell Elektronik Strip Chart Recorder. Numerical integrations of the CL response were obtained from a Packard-adapted Monroe Digital Printer. It should be stressed that the measurement of CL by this method does

NOT involve the use of radioactive materials or phosphors.

The reactions were carried out in glass scintillation counting vials. Each vial used was scrupulously cleaned and tested for background CL prior to use. The allowed range for background noise of a dark-adapted vial was 5,000 ± 1,000 cpm. The desired concentration of halide as its sodium salt was pre-added to the vials and dessicated to dryness. The desired quantity of enzyme was then added and after allowing for mixing 10-15 minutes, the reaction was initiated by addition of HOOH with a microliter syringe.

All chemicals were of reagent grade and H_2O was glass-distilled. Centrifugation requiring forces greater than 1,000 g was done with a Beckman Model L Centrifuge, using a No. 30 fixed head rotor. A Beckman Zeromatic pH meter was used for pH determinations.

III. RESULTS

Klebanoff has previously demonstrated that halides, with the exception of F^-, were necessary as cofactors in the MPO-X^--HOOH microbicidal system, and that the degree of microbicidal activity was dependent on the halide used (4). If MPO is involved in electronic excitation, and if halides are necessary cofactors for MPO-mediated microbicidal activity, then the CL response from intact PMN leukocytes should demonstrate a halide dependency. Figure 1 shows the temporal traces of CL from intact PMN leukocytes resuspended in four different PBS media. Each medium was the same with the exception that the halide was exclusively F^-, Cl^-, Br^-, or I^-. The PMN were challenged with serum opsonized bacteria. A good response was obtained with either Br^- or Cl^- as cofactor. The I^- containing system gave a poor, but detectable response, but no CL activity

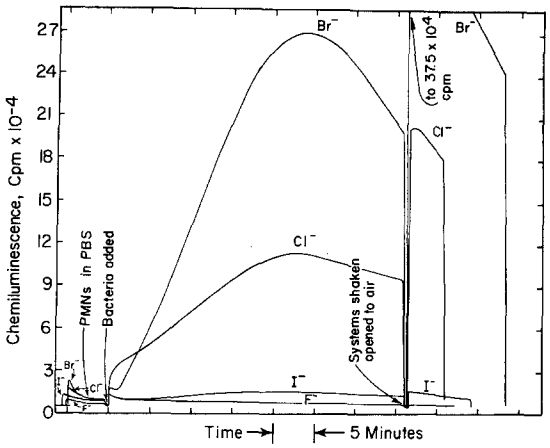


Figure 1. Continuous temporal traces of chemiluminescent intensity elicited from 1 x 10^7 PMN leukocytes upon addition of 0.5 mg (dry weight) Propionibacterium shermanii (heat killed) in 0.25 ml autologous serum. The cells were suspended in 5 ml phosphate buffered saline containing 80 mg % glucose and either F^-, Cl^-, Br^-, or I^- exclusively.

was detectable from the F^- system. With the exception of I^-, these results correlate with the microbicidal observations of Klebanoff (4) and Paul et al. (9).

Klebanoff has also reported direct iodination as a microbicidal mechanism when a sufficient concentration of I^- is available as the oxidizable cofactor in the MPO-X^--HOOH system (7,8). There is, therefore, the possibility that electronic excitation is unnecessary in the I^--mediated killing. Furthermore, I^- is an effective quencher of electronically excited molecules, and the decreased CL response might reflect this activity (10).

In order to investigate more directly the role of MPO in the electronic excitation phenomenon, purified intact human PMN were obtained and extracted for MPO. Figure 2 shows the temporal traces of CL obtained from the MPO system when the pH, the concentration of MPO, and HOOH are held constant and the concentration of Cl^- is varied. Figures 3 and 4 represent the CL

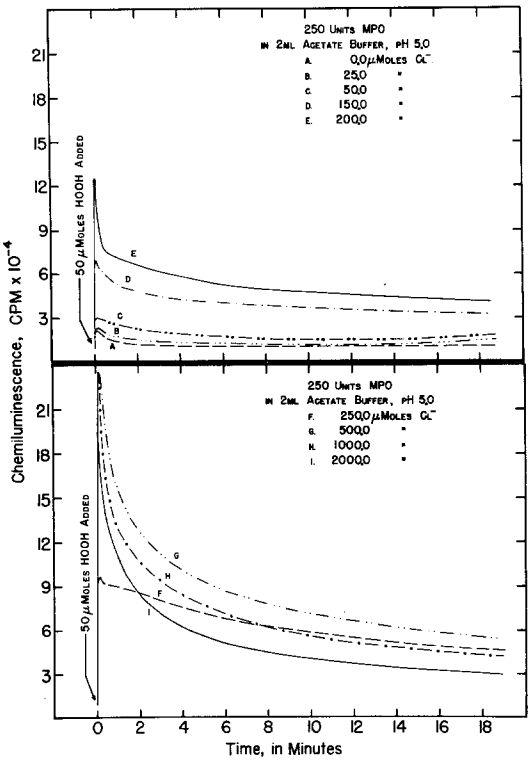
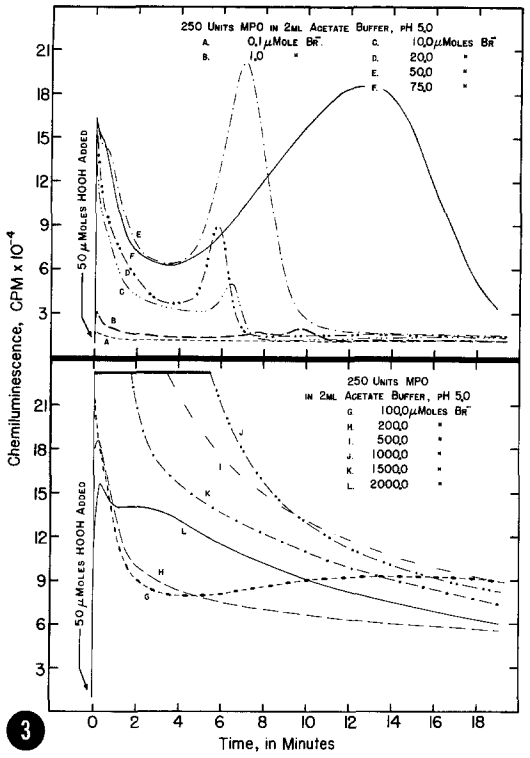


Figure 2. Comparison of continuous temporal traces of chemiluminescent intensity from the myeloperoxidase microbicidal system over a range of chloride concentrations.

responses from similar systems when the concentration of Br^- and I^- were varied respectively.

As in Figure 1, these curves of CL are velocity curves (change in intensity with time), and reflect the various kinetic differences obtained with varying concentrations of the different halides. Figure 5 is a composite plot of the integrals of CL against the log of concentration for the various halides. Note that F^- was ineffective as a cofactor, but a CL response was obtained with I^-, Br^-, and Cl^-. The response also demonstrated the optimal concentration for the particular halide. As in Figure 1, using intact PMN leukocytes, the order of decreasing CL response was $Br^- > Cl^- > I^- > F^-$. Bromide was more effective



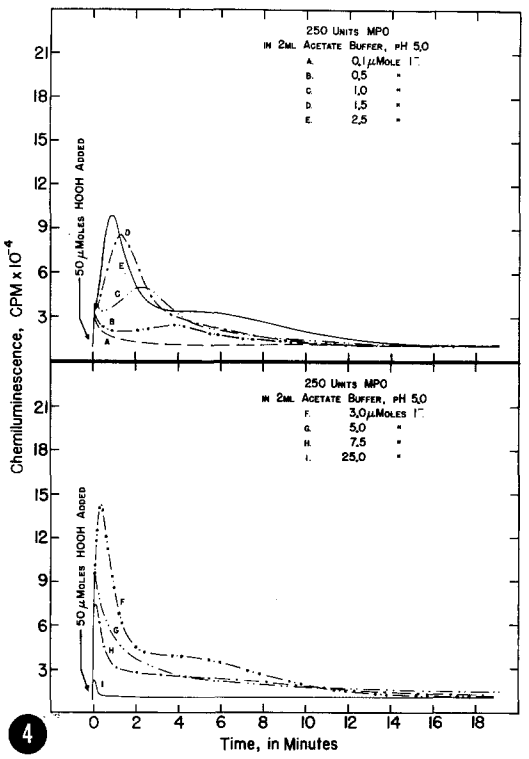


Figure 3. Comparison of continuous temporal traces of chemiluminescent intensity from the myeloperoxidase microbicidal system over a range of bromide concentrations.

Figure 4. Comparison of continuous temporal traces of chemiluminescent intensity from the myeloperoxidase microbicidal system over a range of iodide concentrations.

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than Cl^- at all concentrations tested. However, in considering these data it should be noted that in the physiological milieu of the blood, only Cl^- is available in adequate concentration. The actual serum concentrations for each halide as reported in "FASEB Biological Handbook: Blood and Other Body Fluids" are: Cl^- 104.0 mM, Br^- 0.00012 mM, and I^- 0.00055 mM. The *in vivo* contribution of Br^- and I^- to the CL and microbicidal activity of the PMN is therefore possible, but physiologically improbable.

Chemiluminescence was totally inhibited by heating the enzyme system (MPO-Cl^--HOOH) for 10 minutes at 90°C. Heat

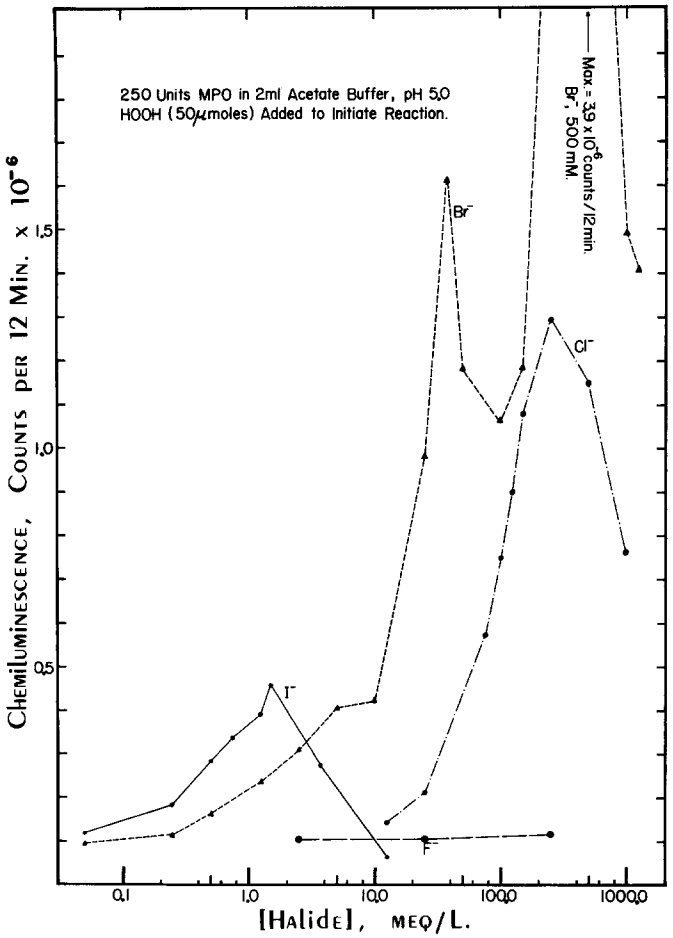

Figure 5. Semilog plot of halide concentration against integral of chemiluminescence obtained from the various myeloperoxidase-halide systems.

denaturation has also been reported to inhibit the antimicrobial activity of this system (4).

IV. DISCUSSION

The PMN leukocyte is characterized by its high concentration of myeloperoxidase (11), and this heme enzyme is capable of microbicidal activity in the presence of HOOH and a halide co-factor (4). The functionality of electronic excitation in the microbicidal activity of this MPO-X^--HOOH system can be deduced by comparison of the microbicidal data reported by Klebanoff with the CL data here present.

Certain conditions of pH and halide concentration must be satisfied in order to obtain both microbicidal activity and CL response from the MPO-X^--HOOH system. These conditions are specific for the halide used as cofactor. The more electronegative the halide, the greater the concentration of halide required to obtain a CL response. It has been suggested that the microbicidal activity of the MPO-X^--HOOH system resides in its capacity to mediate halide oxidation (12,13,14). In fact, when I^- is the halide oxidized, electrophilic iodination of bacteria is accomplished (7). However, no chlorination of bacteria occurs when Cl^- serves as the halide cofactor. It has been postulated that HOCl is the mediator of killing (13,14), but attempts at isolation have been unsuccessful (15).

If HOCl or some other oxidized halogonium-type species is generated by the MPO-mediated oxidation of Cl^- by HOOH, and if this species is subsequently destroyed through the reaction with a second molecule of HOOH, then it is quite apparent why no HOCl has been isolated. The reaction of HOOH with either OCl^- or Cl_2 is the classic chemical reaction for the generation of $^1O_2$ (16). This reaction also holds true for OBr^- or Br_2 (17).

The increased CL response obtained with Br^-, relative to Cl^-, most probably reflects the relatively greater oxidizability of Br^- in comparison with Cl^-. Iodide is more easily oxidized than either Br^- or Cl^-, and it does give a CL response at concentrations lower than those required for the other halides. However, I^-, as well as its oxidized products, are notorious as quenchers of electronically excited molecules (10). Furthermore, the ease of I^- oxidation indicates a difficulty in terms of its subsequent reduction by a second molecule of HOOH. The greater CL response with Br^- probably reflects its greater ease

in transition between its oxidized and reduced states:

Reaction 1 $HOOH + Br^- \xrightarrow{MPO} OBr^- + H_2O$

Reaction 2 $HOOH + OBr^- \longrightarrow Br^- + O_2$

Net Reaction: $2\ HOOH \xrightarrow[Br^-]{MPO} 2\ H_2O + {}^1O_2$ ($^1O_2$ Singlet Molecular Oxygen)

The $^1O_2$ generated by this system is thus available as a microbicidal agent.

BIBLIOGRAPHY

1. Allen, R.C., Stjernholm, R.L., Steele, R.H. Biochem. Biophys. Res. Comm. 47, 679-684 (1972).
2. Allen, R.C., Stjernholm, R.L., Benerito, R.R., Steele, R.H. Chemiluminescence and Bioluminescence (ed. M.J. Cormier, D.M. Hercules, and J. Lee), p. 498, Plenum Press, New York (1973).
3. Allen, R.C., Dissertation, Tulane Univ., University Microfilms, 74-291 (1973).
4. Klebanoff, S.J. J. Bacteriol. 95, 2131-2138 (1968).
5. Rabinowitz, Y. Blood 23, 811 (1964).
6. Van Dyke, K. Packard Technical Bulletin, Packard Instrument Co. 20, 1-13 (1974).
7. Klebanoff, S.J. J. Exp. Med. 126, 1063-1078 (1967).
8. Klebanoff, S.J. Biochemistry of the Phagocytic Process (ed. J. Schultz), pp. 89-110, North Holland Publishing Co., Amsterdam (1970).
9. Paul, B.B., Strauss, R.R., Jacobs, A.A., and Sbarra, A.J. Infect. Immunity 1, 338-344 (1970).
10. Szent-Györgi, A. Bioenergetics. Academic Press, New York (1957).
11. Schultz, J. and Kaminker, K. Arch. Biochem. Biophys. 96, 465-467 (1962).
12. Agner, K. Proc. Int. Congr. Biochem. 4th Vienna 15, 64 (1958).
13. Zgliezynski, J.M., Stelmaszynska, T., Domanski, J., and Ostrowski, W. Biochem. Biophys. Acta 235, 419-424 (1971).
14. Zgliezynski, J.M., Stelmaszynska, T., Ostrowski, W., Naskalski, J., and Sznajd, J. Eur. J. Biochem. 4, 540-547 (1968).
15. Sbarra, A.J., Paul, B.B., Jacobs, A.A., Strauss, R.R., and Mitchell, G.W., Jr. J. Reticuloendothel. Soc. 12, 109-126 (1972).
16. Kasha, M. and Khan, A.U. Ann. N.Y. Acad. Sci. 171, 5-23 (1970).
17. KcKeown, E. and Waters, W.A. J. Chem. Soc., B, 1040 (1966).