PHAGOCYTIC ACTIVATION OF A LUMINOL-DEPENDENT CHEMILUMINESCENCE IN RABBIT ALVEOLAR AND PERITONEAL MACROPHAGES

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SUMMARY: No chemiluminescent response above background was obtained from rabbit alveolar or peritoneal macrophages either in the resting state or subsequent to phagocytosis. If luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was added to the cell suspension, a chemiluminescence was observed following phagocytosis of bacteria by both alveolar and peritoneal macrophages. The chemiluminescent response obtained was of high intensity, and could be easily monitored using the standard in-coincidence scintillation spectrophotometry equipment employed in routine isotope counting. Both rabbit and human polymorphonuclear leukocytes were also tested in the presence of luminol, and the results of the various cell systems are compared. The chemiluminescent response was further investigated by the use of superoxide dismutase and sodium benzoate, and the inhibition of chemiluminescence by the agents is discussed.

I. INTRODUCTION

Polymorphonuclear leukocytes (PMN) yield a chemiluminescence (CL) following phagocytosis that can be correlated with metabolic activation of the hexose monophosphate (HMP) shunt, and is also oxygen dependent (1,2). However, when alveolar or peritoneal macrophages are monitored for CL either prior to or subsequent to bacterial phagocytosis, no chemiluminescent response is obtained.

Cyclic hydrazides, such as luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) yield a 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

Abbreviations are: PMN, polymorphonuclear leukocytes; CL, chemiluminescence; luminol-FCS, luminol saturated fetal calf serum; $^1O_2{}^*$, excited singlet molecular oxygen; S.O.D., superoxide dismutase; $\cdot O_2^-$, superoxide anion.

state by photon emission (3,4). Utilizing this reaction, luminol has been used in the study of enzymes (5) and subcellular fractions (6) involved in the generation of activated oxidizing species.

In an attempt to follow the metabolic events involved in the reduction of 0_2 by phagocytically stimulated macrophages, luminol was introduced into the cell suspension medium as luminol-saturated fetal calf serum. This report describes the luminol-mediated chemiluminescent response obtained from the various macrophage systems and from PMN.

II. MATERIALS AND METHODS

Alveolar and peritoneal macrophages were obtained from female New Zealand white rabbits (2-3 kg.) by tracheal lavage (7) and by peritoneal lavage (4 days after an intraperitoneal injection of 30 ml of 12% Na caseinate) respectively (8). Macrophages were collected from their respective lavage fluids by centrifugation at 300 g for 10 minutes. The cells were resuspended in RPMI-1640 culture solution. PMN were obtained from the New Zealand rabbits by ventricle puncture and from human peripheral blood by venipuncture. The leukocytes were separated from erythrocytes by dextran sedimentation, and following centrifugation, were also resuspended in RPMI-1640 solution (9).

Total cell counts were obtained using hemocytometer technique, and differential counts were used as an index of preparation purity. Phagocytosis was initiated by the addition of 0.5 ml of autologous serum containing 20 ul of heat killed Escherichia coli (Type 014) suspension (20 µl diluted to 5.0 ml in saline yields an absorbance at 525 nm of 0.40).

Fetal calf serum was incubated with luminol to saturation for one hour, and then centrifuged at 700 g for 30 min to remove any residual particulate luminol, and the supernatant, designated

as luminol-saturated FCS (Luminol-FCS) was added to the cell suspension to a final concentration of 20% by volume. Although this approach has a serious drawback with respect to quantitation, it was found to give relatively reproducible results, and effectively avoided the necessity of using organic solutions.

Chemiluminescence was monitored using a Packard Scintillation Spectrometer Model 3320, operated in the in-coincidence summation mode. Note, previous studies on PMN utilized the more sensitive out-of-coincidence summation mode; however, this mode was found to be too sensitive for the high chemiluminescent yields obtained from the luminol systems. The summation signal was fed into a Packard Ratemeter Model 280A, and recorded on a Honeywell Electronik Strip Chart Recorder (2,10).

Superoxide dismutase was purchased from Sigma Chemical Co. (2,900 units/mg), and luminol was purchased from Eastman Organic Chemicals. RPMI-1640 reagent (no phenyl red) and fetal calf serum were purchased from Associated Biomedic Systems and Grand Island Biological Co. respectively. Other chemicals were of reagent grade. Sterile plastic and siliconized glassware were used in all procedures.

III. RESULTS

Figure 1 describes the CL obtained from rabbit peritoneal macrophage, alveolar macrophage, and PMN suspensions incubated in RPMI-1640 containing 20% by volume luminol-FCS. After 20-30 minutes of incubation, E. coli in 0.5 ml of autologous serum was added to the suspension. Chemiluminescent intensity was monitored intermittently and plotted against time. No CL above that described for the resting cell system was observed when bacteria in serum was added to cell-free RPMI-1640 containing luminol-FCS.

Table I gives the numerical integrals of CL obtained from

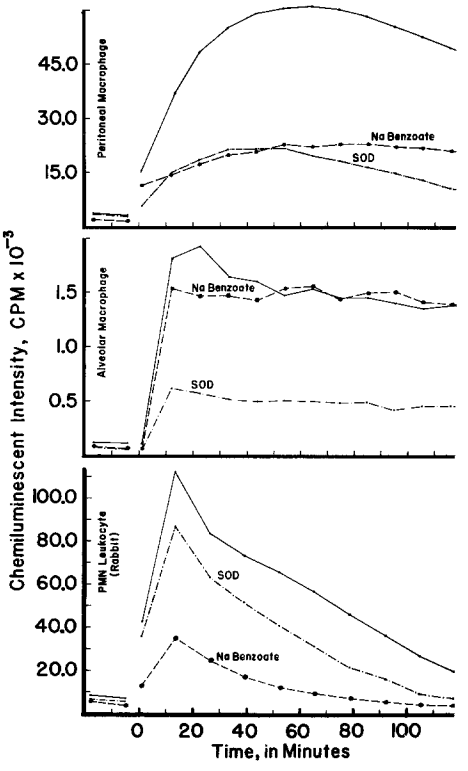

Figure 1. Temporal traces of CL from $5x10^6$ peritoneal macrophages, $5x10^6$ alveolar macrophages, and $1x10^6$ PMN in 4.5 ml of RPMI-1640 culture reagent containing 1 ml of luminol-FCS. Phagocytosis was initiated at Time 0 by the addition of 0.5 ml of autologous serum containing E. coli. Superoxide dismutase was added to a concentration of 50 µg/vial. Na benzoate was added to a concentration of 0.0083 M. Both inhibitors were added 20-30 min before bacterial challenge.

the various cell systems as well as the percent inhibition of CL when superoxide dismutase (S.O.D.) or Na benzoate were added. The individual counts/30 min/$1x10^6$ cells are given such that the activity over the 2 hours following bacterial challenge can be compared.

Worthy of note is the magnitude of luminol-mediated CL obtained from the human PMN. These leukocytes gave a response that was approximately 2 orders of magnitude greater than that achieved with the rabbit PMN, and approximately 3 and 5 orders

TABLE I.

COMPARISON OF LUMINOL-MEDIATED CHEMILUMINESCENCE FROM PERITONEAL MACROPHAGES, ALVEOLAR MACROPHAGES AND PMN LEUKOCYTES, AND THE INHIBITORY EFFECTS OF Na BENZOATE AND SUPEROXIDE DISMUTASE ON THIS CHEMILUMINESCENCE

CELL TYPE [1]	DIFF. COUNT PMN	LYMPH	MACRO	COUNTS/30 MIN. INTERVAL		% INHIBITION SOD [3]	NaBenzoate [5]
#1 1x10^6 Peritoneal Macrophage	--	50	50	1st 30 min.	0.25x10^5	85	-
				2nd	0.22	84	-
				3rd	0.14	80	-
				4th	0.09	78	-
#2 1x10^6 Peritoneal Macrophage	13	--	87	1st	5.10x10^5	74	50
				2nd	7.80	79	52
				3rd	5.70	80	49
				4th	3.60	82	45
#3 1x10^6 Peritoneal Macrophage	20	20	60	1st	2.20x10^5	60 [4]	59
				2nd	3.50	64	64
				3rd	3.50	70	62
				4th	3.10	75	57
#4 1x10^6 Peritoneal Macrophage	8	9	83	1st	0.10x10^5	38 [4]	42
				2nd	0.17	56	58
				3rd	0.12	63	62
				4th	0.08	66	64
#1 1x10^6 Alveolar Macrophage	--	--	100	1st	1.02x10^3	62	-
				2nd	1.14	65	-
				3rd	1.06	67	-
				4th	0.86	61	-
#2 1x10^6 Alveolar Macrophage	3	--	97	1st	8.30x10^3	71	3
				2nd	7.60	75	16
				3rd	3.80	75	5
				4th	2.70	80	1
#3 1x10^6 Alveolar Macrophage	13	--	87	1st	8.70x10^3	68 [4]	18
				2nd	9.40	68	6
				3rd	8.80	67	-2
				4th	8.30	68	-6
#4 1x10^6 Alveolar Macrophage	--	--	100	1st	3.90x10^3	44 [4]	38
				2nd	8.70	45	40
				3rd	11.20	49	41
				4th	11.60	56	43
#2 1x10^6 PMN [2] (Rabbit Blood)	25	75	--	1st	0.95x10^6	33	-
				2nd	0.61	23	-
				3rd	0.25	24	-
				4th	0.10	27	-
#4 1x10^6 PMN [2] (Rabbit Blood)	23	77	--	1st	2.57x10^6	23 [4]	70
				2nd	2.09	33	78
				3rd	1.46	49	85
				4th	0.82	60	85
#5 1x10^6 PMN [2] (Human Blood)	47	50	--	1st	65.0x10^6	20 [4]	32
				2nd	116.0	-5	24
				3rd	134.0	4	23
				4th	134.0	6	21

1. Cells were suspended in 4.5 ml of RPMI - 1640 Media and 1 ml of luminol-saturated fetal calf serum. Phagocytosis was initiated by the addition of 0.5 ml autologous serum containing E. coli. (See Methods)
2. Both rabbit and human PMN leukocytes were obtained from peripheral blood by sedimentation. (See Methods)
3. Superoxide dismutase (S.O.D.) at a concentration of 40 µg/vial.
4. Superoxide dismutase (S.O.D.) at a concentration of 50 µg/vial.
5. Na Benzoate at a final concentration/vial of 0.0083 M.

of magnitude greater than that achieved with peritoneal and alveolar macrophages respectively. The luminol-mediated CL from human PMN surpassed the capacity of the scintillation counter to count accurately even in the in-coincidence summation mode. This relative difference in cellular activity might well explain the absence of a detectable non-luminol chemiluminescent response from these macrophage systems. It further points out the gain

in sensitivity achieved by the use of luminol as an oxidizable substrate because of its high quantum efficiency.

The greater response obtained with the PMN system also raises the possibility that the CL from the macrophage systems might be accounted for by the presence of PMN contaminant. However, the various systems show differences with respect to the kinetics of CL, and there are also differences with respect to inhibition of the different systems by S.O.D. and Na benzoate.

IV. DISCUSSION

Previous investigations have centered on the generation of electronically excited molecules by the PMN and the myeloperoxidase enzyme systems (11,12,13). In these investigations bacteria served as the physiological substrate oxidized and the chemiluminescent response obtained is proposed to have originated from relaxation of electronically excited carbonyl groups generated in oxidative microbicidal activity.

Metabolic activation has also been reported subsequent to phagocytosis by macrophages, and the type of metabolic stimulation appears to be similar to that observed in the PMN (14). However, no CL response is observed from the macrophage system following phagocytosis. This might reflect the lower concentration or absence of peroxidase in these macrophage systems (15). However, the type of metabolic activation suggests that the $\cdot O_2^-$ is generated. Furthermore, Gee et al. have reported an increase in HOOH production in alveolar macrophages during phagocytosis (15). In an attempt to increase the quantum efficiency in monitoring the macrophage oxidizing system, luminol was added to the incubating suspension of cells, and the cells were then metabolically activated by allowing phagocytosis to occur. Oxidation of the luminol was then monitored as chemiluminescence as described in Figure 2.

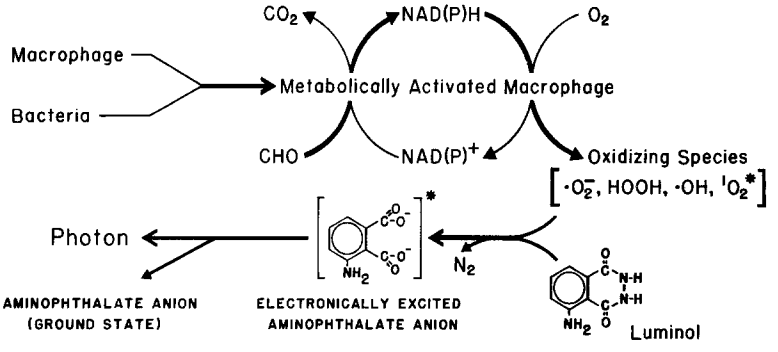

Figure 2. Schematic description of the proposed mechanism to describe luminol-mediated chemiluminescence from the various phagocytes.

Luminol, when dissolved in dimethyl sulfoxide in the presence of solid potassium superoxide, yields an intense greenish CL lasting for hours, and luminol chemiluminescence has also been used in the investigation of xanthine oxidase, a $\cdot O_2^-$ generating enzyme (5). The inhibition of CL by S.O.D. might reflect the removal of $\cdot O_2^-$ resulting in a decrease in luminol oxidation.

Na benzoate has been demonstrated to be effective in trapping hydroxyl free radicals ($\cdot$OH) generated in aqueous solutions with the formation of salicylates (16,17). Na benzoate is effective in inhibiting luminol-mediated CL especially from the peritoneal macrophage system. However, little information is available concerning the reaction of $\cdot$OH with luminol to produce light. Arneson's proposal that $\cdot O_2^-$ and $\cdot$OH might react to give OH^- + $^1O_2^*$ is of interest (18). Trapping of $\cdot$OH would prevent generation of $^1O_2^*$ by this mechanism, and may thus decrease luminol oxidation by $^1O_2^*$. Although the role of Na benzoate is well established with respect to $\cdot$OH trapping, more information would be desirable regarding the interaction of $\cdot O_2^-$ and $^1O_2^*$ with this oxidizable trap.

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