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Evaluation of Serum Opsonic Capacity by Quantitating the Initial Chemiluminescent Response from Phagocytizing Polymorphonuclear Leukocytes

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Phagocytically activated polymorphonuclear leukocytes produced a chemiluminescence that could be correlated metabolically with the stimulated oxidation of glucose via the hexose monophosphate shunt. The chemiluminescence observed was considered to originate from the relaxation of electronically excited carbonyl groups produced during singlet molecular oxygen-mediated microbicidal oxidation of the ingested microbe. With adequate adjustment of leukocyte and bacterial concentrations, the rate of chemiluminescence increase was nearly constant for the first minutes after initiation of phagocytosis. This rate was dependent on the quantity of bacteria phagocytized by the leukocytes. If both leukocytes and bacterial concentrations were held constant, this initial rate of chemiluminescence reflected the opsonic capacity of the sera used for opsonization. The prior absorption of opsonins from serum resulted in a decreased rate of chemiluminescence related to the quantity of bacteria used for absorption. Heating of sera to 56°C for 30 min resulted in a great decrease in the chemiluminescent responses and may reflect the role of complement in the opsonization process.

The primary function of neutrophilic polymorphonuclear (PMN) leukocytes is the ingestion and killing of microorganisms. This function is associated with morphological and metabolic changes directed toward mobilization of enzymatic machinery against the ingested microbe. Metabolically there is a 100-fold increase in the oxidation of glucose via the hexose monophosphate shunt and an increase in non-mitochondrial oxygen consumption (16, 18, 21). These activities are thought to reflect the activation of nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH) oxidase, and it has been proposed that this flavoprotein univalently reduces oxygen to the superoxide anion (3, 15, 16). Enzymatic evidence for the production of superoxide has been presented, and evidence for the generation of singlet molecular oxygen by the phagocytically activated PMN leukocyte, and by the isolated myeloperoxidase-halide-peroxide microbicidal system, has been reported (H. Rosen and S. J. Klebanoff, Fed. Proc. 35:1391, 1976; 1, 2, 4, 6, 12).

PMN leukocytes generate a chemiluminescence subsequent to phagocytosis. This chemiluminescent response is considered to result from relaxation of electronically excited carbonyl groups generated during the microbicidal oxidation of the ingested organism by singlet molecular oxygen (1, 2, 4). The integral of chemiluinescence, that is, the total counts recorded over a given interval of time, can be closely correlated with oxidation of glucose via the hexose monophosphate shunt (4). Thus, the measurement of chemiluminescent response affords a sensitive, continuous, and nondestructive means of quantitating microbicidal metabolic activity. Recently, leukocyte chemiluminescence has been used to evaluate the humoral-phagocyte axis in host defense. In particular, it has been used in evaluating opsonophagocytic defects to *Escherichia coli* and in evaluating nonspecific opsonic activities (P. Stevens and L. S. Young, Fed. Proc. 35:738, 1976; R. J. Weinstein et al., Clin. Res. XXIV 3:354A, 1976; H. R. Hill et al., Clin. Res. XXIV 2:180A, 1976.

This report demonstrates the use of the initial rate of chemiluminescence as a technique for evaluating opsonic function. The prior absorption of opsonins from serum using various concentrations of the challenging organism is studied, and the effect of heating serum to 56°C for 30 min is also investigated. The latter method is known to inactivate both classical and alternate pathways of the complement sys-

tem (22). The effect of the post-venipuncture age of leukocytes with regard to chemiluminescent response is also presented.

MATERIALS AND METHODS

Human whole blood was obtained by venipuncture from normal male and female volunteers ranging in age from 22 to 40 years. The leukocytic fraction was isolated by a dextran sedimentation technique, with great care taken to minimize erythrocyte contamination (20). The leukocytes were washed three times with normal saline to remove any cellular absorbed globulins and were then resuspended in RPMI 1640 reagent without phenol red. Total cell counts were done by a hemocytometer technique, and differential counts were used to quantitate PMN leukocyte concentration. The monocyte contribution to the differential count was never greater thant 5%, and in most experiments it was below 3%. The mean PMN leukocyte count was 80% of the differential count. Both neutrophils and eosinophils were considered as PMN leukocytes. However, the mean eosinophil count was 3% of the total differential count.

Five million PMN leukocytes were suspended in 4.5 ml of RPMI 1640 reagent and placed in siliconized counting vials. Phagocytosis was initiated by the addition of 20 μl of a heat-killed suspension of *E coli* type 014 incubated in 0.5 ml of autologous serum for 15 to 20 min before challenge. Twenty microliters of *E. coli* added to 5.0 ml of saline yielded an absorbance of 0.40 at 525 nm. It is especially important that an optimal concentration of phagocytizable substrate is used in experiments studying the initial rate of phagocytosis. The rate will be increased with increased concentrations and decreased with decreased concentrations. Two vials containing leukocytes were monitored simultaneously, with each vial automatically counted for 1 min at intervals of approximately every 2 min. Equidistant spacing of the vials in the automated counting system insured that the vials were uniformly agitated before each counting. For experiments investigating in vitro aging, the cells were suspended in RPMI media at 23°C for various lengths of time before challenge.

The studies of prior absorption of opsonins from serum were carried out by allowing either 20 or 40 μl of the *E. coli* suspension per 0.5 ml of autologous serum to incubate for 15 to 20 min. The sera were then centrifuged at a relative centrifugal force of approximately 2,000 for 30 min to remove particulate bacteria. The supernatant sera obtained were then used for opsonization of 20 μl of fresh *E. coli* suspension. Bacterial challenge and counting were as previously decribed.

Heating of serum to 56°C for 30 min was used to inactivate the complement system (22). This serum was subsequently used to opsonize 20 μl of *E. coli* as previously described. Finally, as a control study, leukocytes were also challenged with serum in the absence of bacteria.

Chemiluminescence was monitored with a Packard model 3320 scintillation spectrometer operated in the out-of-coincidence mode at an ambient temperature of 23°C. Integral chemiluminescence for 1 min of counting was recorded by a Packard-adapted Monroe calculator, and a Packard model 280 A ratemeter attached to a Honeywell Electronik strip chart recorder was used for continuous recording of chemiluminescent intensity.

The *E. coli* type O14 were grown for 24 h and then harvested and washed three times with saline. RPMI 1640 reagent without phenol red was purchased from Associated Biomedics. All chemicals were of reagent grade, and sterile plastic and siliconized glassware was used in all procedures. Calculations of least-square slopes, standard deviations, and correlation coefficients were carried out on a Wang model 154 calculator. A Beckman DU spectrometer was used for absorbancy measurements, and an International model PR-2 centrifuge was used for centrifugation. No radioactive isotopes or phosphors were used in any of the experiments.

RESULTS

During the first minutes after the addition of serum-opsonized bacteria to incubating PMN leukocytes, the rate of increase in chemiluminescence is relatively constant. This rate is dependent on the quantity of bacteria and the quantity of the phagocytes used. When both leukocytes and bacteria are held constant, the rate of chemiluminescence reflects the opsonic capacity of the serum. The chemiluminescent response observed is considered to reflect the quantity of bacteria phagocytized via the increase in leukocyte microbicidal metabolism. Therefore, measurement of the initial rate of chemiluminescence is equivalent to quantitation of the rate of phagocytosis of bacteria.

Figure 1 presents the temporal traces of chemiluminescence elicited from leukocyte suspensions after the initiation of phagocytosis at time zero by the addition of *E. coli* suspended in autologous serum. Both leukocyte and bacterial concentrations were held constant, and the variable was the post-venipuncture age of the leukocyte preparations. The numerical slope given in these figures was calculated from all of the data points of the individual experiments taken collectively. With increase in the post-venipuncture age of the suspensions, up to 5.5 h, there was an increase in slope and y-intercept. However, there was also an increase in the standard deviation of the slope.

The effects of absorption of opsonins from serum are graphically demonstrated in Fig. 2. The sera used in these experiments were pretreated with various concentrations of *E. coli*. After removal of bacteria, the sera were used to opsonize fresh *E. coli*, and the resulting suspensions were used to initiate phagocytosis. A decrease in slope was observed that was relative to the quantity of bacteria used for absorption.

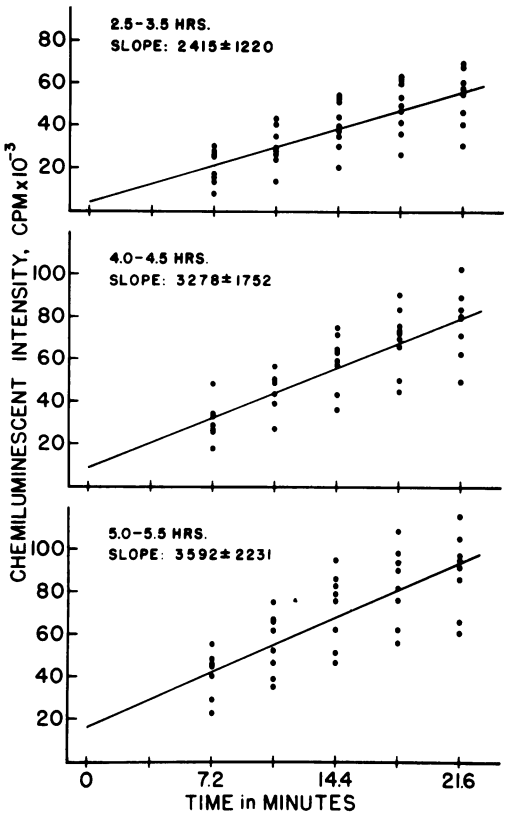

FIG. 1. *Temporal traces of chemiluminescence elicited from 5 × 10⁶ PMN leukocytes after various periods of in vitro aging. The collective slope for all data points within a group is plotted. Slope 2,415 represents the activity 2.5 to 3.5 h post-venipuncture. Slopes 3,278 and 3,592 represent the activity after 4.0 to 4.5 and 5.0 to 5.5 h, respectively. Time zero is the time of addition of E. coli in autologous serum to the leukocytes.*

The role of heat-labile components of the opsonin system in phagocytosis was investigated (Fig. 3). The sera used in these experiments were heated to 56°C for 30 min and then allowed to cool to ambient temperature (22°C) before use for opsonization. Heating of the sera markedly decreased the initial rate of chemiluminescence and suggested that the heat-labile components of the complement system play a significant role in the phenomenon of opsonization. The bottom graph of Fig. 3 is a control study in which sera without bacteria were added to the leukocyte preparations.

Table 1 provides numerical data for quantitative comparison of the various systems studied. The slope, y-intercept, and correlation coefficients reflect mean values obtained from the individual experiments and also allow for com-

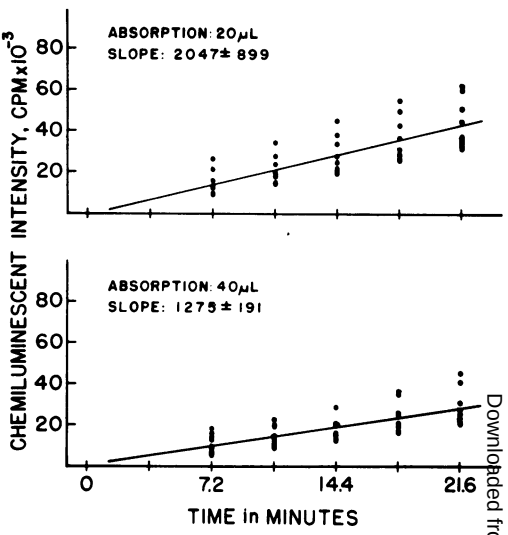

FIG. 2. *Temporal traces of chemiluminescence from 5 × 10⁶ PMN leukocytes after addition of E. coli in autologous serum previously treated with varying concentrations of antigen to remove opsonins by absorption. The collective slopes 2,048 and 1,275 represent the activity after absorption with 20 and 40 μl of E. coli suspensions, respectively. Twenty microliters of fresh E. coli opsonized with the previously absorbed serum was added at time zero.*

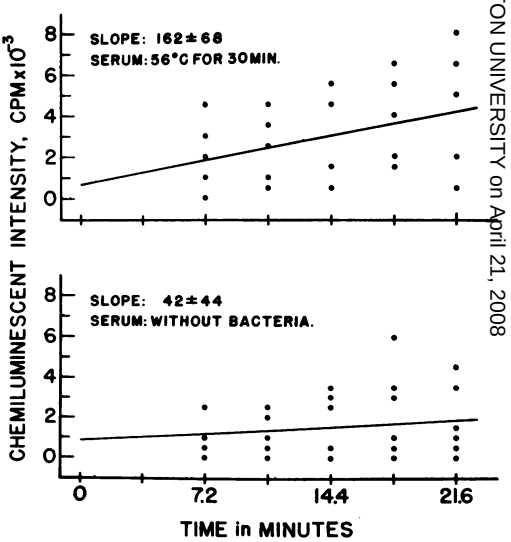

FIG. 3. *Temporal traces of chemiluminescence from 5 × 10⁶ PMN leukocytes. The serum used for opsonization was previously heated to 56°C for 30 min. Time zero is the time of addition of heat-inactivated autologous serum containing E. coli to the PMN leukocytes. Bottom graph shows the temporal traces of chemiluminescence from 5 × 10⁶ PMN leukocytes when autologous serum without bacteria is added at time zero.*

TABLE 1. *Mean least-squares estimates of slope and y-intercept with mean correlation coefficients for the velocity curves of chemiluminescence from 5 × 10⁶ PMN leukocytes after initiation of phagocytosis*

Conditions[a]	Post-venipuncture age of leukocytes (h)	Mean slope ± SD[b]	Mean y-intercept ± SD	Mean correlation coefficient	No. of expts
A	2.5–3.5	2,415 ± 555	6,678 ± 3,387	0.9952 ± 0.0000	9
	4.0–4.5	3,278 ± 800	8,856 ± 7,687	0.9912 ± 0.0000	9
	5.0–5.5	3,592 ± 713	15,875 ± 10,375	0.9833 ± 0.0000	8
	2.5–5.5	3,076 ± 838	10,296 ± 8,239	0.9901 ± 0.0000	26
B	4.0–4.5	1,998 ± 510	4,038 ± 2,137	0.9980 ± 0.0000	8
	4.5–5.0	2,472	2,500	0.9993	1
	4.0–5.0	2,051 ± 502	3,867 ± 2,064	0.9981 ± 0.0000	9
C	3.5–4.0	1,091 ± 150	529 ± 4,308	0.9948 ± 0.0000	7
	4.5–5.0	1,917 ± 412	1,350 ± 6,010	0.9980 ± 0.0000	2
	3.5–5.0	1,275 ± 413	711 ± 4,309	0.9955 ± 0.0000	9
D	4.0–4.5	250	2,200	0.9594	1
	4.5–5.0	153 ± 78	600 ± 424	0.9443 ± 0.0574	2
	5.0–5.5	139 ± 96	867 ± 513	0.9636 ± 0.0265	3
	4.0–5.5	162 ± 83	1,000 ± 710	0.9565 ± 0.0316	6
E	2.5–3.0	43 ± 57	422 ± 988	0.4151 ± 0.5627	6
	3.5–4.0	97	800	0.4244	1
	2.5–4.0	50 ± 56	476 ± 913	0.4164 ± 0.5136	7

[a] Conditions were as follows. (A) Phagocytosis was initiated with 20 μl of *E. coli* suspension opsonized with 0.5 ml of autologous serum. (B) Autologous sera were preincubated with 20 μl of *E. coli* suspension for 20 min and then centrifuged to remove bacteria. The resultant sera were then used to opsonize 20 μl of fresh *E. coli* suspension as described in A. (C) Autologous sera were preincubated with 40 μl of *E. coli* suspension for 20 min and then centrifuged to remove bacteria. The resultant sera were then used to opsonize 20 μl of fresh *E. coli* suspension as in A. (D) Autologous sera were heated to 56°C for 30 min. The heated sera were then used to opsonize 20 μl of *E. coli* as described in A. (E) 0.5 ml of autologous sera containing no bacteria was added to the leukocyte suspension.

[b] SD, Standard deviation.

parison of results with respect to post-venipuncture age of the leukocyte preparations.

DISCUSSION

Phagocytosis by PMN leukocytes is characterized by increased glucose oxidation via the hexose monophosphate shunt and increased O_2 consumption (16, 18, 21). This shift to microbicidal-directed metabolism is considered to reflect the phagocytic activation of NADPH oxidase, and this oxidase has been proposed to univalently reduce oxygen to superoxide at the expense of reducing equivalents from NADPH (3, 5, 15, 16). Evidence for the generation of superoxide has been presented, and the role of this agent in microbicidal activity has been considered (6, 7, 10). Acid disproportionation of superoxide produces peroxide and molecular oxygen. The generation of singlet molecular oxygen through this disproportionation has been proposed, but evidence opposed to this thesis has also been presented (8, 13, 17). Hydrogen peroxide has been isolated from phagocytically activated PMN leukocytes, and it serves as substrate for the myeloperoxidase microbicidal system (9, 14). Myeloperoxidase makes up approximately 5% of the dry weight of the PMN leukocyte, and, in the presence of an acid pH, an oxidizable halide cofactor, and peroxide, it has been shown to generate a potent microbicidal effect (9, 11, 19). This myeloperoxidase-halide-peroxide system has been demonstrated to produce a chemiluminescence that is both halide and pH dependent (1, 2). Myeloperoxidase is thought to catalyze the oxidation of the halide cofactor to a halogonium-type intermediate, OCl^- or Cl_2 by hydrogen peroxide (2, 23; K. Agner, 4th Proc. Int. Congr. Biochem 15:64, 1958). This intermediate can then react with a second molecule of hydrogen peroxide to generate singlet molecular oxygen (2, 8).

Chemiluminescence has been reported from intact phagocytizing PMN leukocytes, and the integral of light, that is, the total number of counts recorded over a given interval of time, correlates well with glucose oxidation via the hexose monophosphate shunt and is oxygen dependent (4). Singlet molecular oxygen has been proposed as a microbicidal agent generated. This electronically excited state of oxygen has a

wide spectrum of chemical reactivity, and it is proposed that chemiluminescent responses from both viable phagocytizing PMN leukocytes and the myeloperoxidase system reflect the relaxation of electronically excited carbonyl groups generated during singlet molecular oxygen-mediated oxidation of the ingested organism (2, 4, 5).

If the chemiluminescent response from PMN leukocytes reflects the oxidative destruction of the ingested microbe, then measurement of chemiluminescence allows for a sensitive, continuous, and nondestructive means for quantitating leukocyte microbicidal function. Phagocytosis requires not only a functional leukocyte, but also an intact humoral-phagocyte axis. Therefore, if the variable of leukocyte function is held constant, the technique of chemiluminescent measurement can be used to reflect opsonic capacity of serum.

Recently, the use of the chemiluminescent assay for evaluating humoral opsonic capacity has been reported for evaluating opsonophagocytic defects to *E. coli* (Stevens and Young, Fed. Proc. 35:738, 1976) and in evaluating nonspecific opsonic activity (Hill et al., Clin. Res. XXIV 2:180A, 1976). The present report demonstrates the value of measuring the initial rate of chemiluminescence in evaluating serum opsonic capacity. The rate of chemiluminescence is dependent upon the post-venipuncture age of the leukocyte preparation to some extent. However, the initial slope of chemiluminescence is found to be inversely related to the quantity of bacteria used to absorb opsonins from serum. The chemiluminescent method also demonstrates a heat-labile component of the opsonin system. This lability most probably reflects the role of complement in opsonization.

An alternative method of quantitating phagocytic activity would be quantitation of the integral of chemiluminescence, the total number of counts over a specified time interval. The use of peak height of chemiluminescence is not desirable, but may be sufficient if the relationship of peak chemiluminescence to time after initiation of phagocytosis is indicated.

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