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Correlation Between Measurements of the Luminol-Dependent Chemiluminescence Response and Bacterial Susceptibility to Phagocytosis

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The generation of chemiluminescence by phagocytosing leukocytes has been suggested to reflect concomitant microbicidal activity. Correlation between measurements of the chemiluminescence response and susceptibility of bacteria to phagocytosis, however, has not been studied. To examine and compare a range of responses in the two assays, four *Escherichia coli* serotypes were chosen as test organisms with degrees of susceptibilities to phagocytosis ranging from 0 to 100% bacteria killed. No complete correlation between peak, slope, or curve area integral measurements of the chemiluminescence response and bacterial susceptibility to phagoocytosis were found, although a correlation between the two assays could be made after using specific opsonization procedures like the addition of antiserum to selected serotypes. Intrinsic differences present among the bacterial serotypes may be responsible for the observed lack of correlation between the two assays.

Chemiluminescence (CL), or the emission of light by phagocytosing polymorphonuclear leukocytes (PMNL), is thought to result indirectly from the generation of electronically excited oxidative species. The generated oxidative species postulated to be involved in CL reactions include O_2^-, $\cdot OH$, H_2O_2, and $^1O_2$ (2, 3). Addition of chemical and enzymatic inhibitors of these metabolites and the enzymes leading to their generation in PMNL-bacterial phagocytosis assays causes a decrease in PMNL bactericidal activity (8), suggesting their importance in microbicidal function. Several investigators have used the CL method to evaluate various serum opsonic capabilities (1, 6, 7, 10, 11) and to compare and correlate the resulting CL with PMNL bacterial killing (12, 14). Quantitation of the CL response in these studies was determined either graphically, by peak CL, initial slope, or curve area integration. Because no previous study has explored the interrelationship between such measurements of CL and bacterial killing, these parameters of PMNL-bacteria interactions were compared and analyzed by using a luminol-dependent CL (LDCL) and standard pour plate bactericidal assay.

MATERIALS AND METHODS

Bacteria. *Escherichia coli* serotype O73:K92:H1 was obtained from John C. Feeley of the special pathogens laboratory, Center for Disease Control, Atlanta, Ga. *E. coli* serotypes O1:K1:NM, O16:K1:NM, and O101:K nontypable were blood culture isolates from patients at UCLA Medical Center and were serotyped by Bertil Kaijser, Institute of Microbiology, University of Goteborg, Sweden.

Preparation of human PMNL and pooled human serum. Human PMNL were sedimented from whole blood by dextran sedimentation according to the method of Boyum (4) and processed as previously described (12). For preparation of pooled human serum, equal volumes of serum obtained from six normal healthy donors were pooled, divided into equal portions, and kept at −80°C until needed.

PMNL bactericidal assay. The in vitro bactericidal assay used has been previously described and found to be a sensitive indicator of opsonic activity and bacterial killing potential of human PMNL (13). The 1-ml reaction mixture consisted of 5×10^6 to 1.2×10^6 bacterial colony-forming units (CFU) adjusted by a MacFarland standard, 0.1 ml of pooled human serum, and 5×10^6 PMNL. The desired bacteria were grown to log phase in nutrient broth and then washed twice with normal saline before use. The percentage of bacteria killed was determined by dividing the difference in CFU from tubes with PMNL between 0 and 60 min by the base-line CFU at 0 min with PMNL and multiplying times 100. Serum sensitivity of bacterial isolates was determined similarly in tubes without PMNL.

LDCL. LDCL was performed as recently described (11). Briefly, 0.5 ml of 2×10^5 PMNL per ml in Hanks buffer was added to scintillation vials containing 1.5 ml of buffer and luminol (Aldrich Chemical Co., Milwaukee, Wis.) at a final concentration in the reaction vial of 1.13×10^{-7} M. LDCL was measured with a Beckman 230 liquid scintillation spectrophotometer at ambient temperature with a coincident circuit switched to the rear photomultiplier tube. The reaction was done in the dark in previously dark-adapted polypropylene vials.

Preopsonization of bacteria. A total of 10^9 CFU of the desired bacterial serotype were preopsonized with 50% pooled human serum in a total volume of 0.5 ml for 30 min at 37°C. Preopsonization of bacteria with antiserum was performed as previously described (14). LDCL was initiated by the addition of 20 μl of preopsonized bacteria to the reaction mixture for a final bacteria/PMNL ratio of 200:1.

Preparation of rabbit antisera. Four- to 6-kg New Zealand rabbits were immunized with live bacteria, using a minimum of two rabbits per bacterial serotype as previously described (14).

Quantitation of LDCL. The initial LDCL slope from 20 to 50 min after addition of preopsonized bacteria was calculated by the linear regression least-squares method and expressed as counts per minute per minute. Curve area integration was determined by tracing the area under each LDCL curve on standard weight paper which was then cut and weighed. The weights are given in units of counts per 170 min. Statistical differences between peak LDCL, slope, and curve area integrals were determined by the unpaired Student *t* test, using a Monroe 1860 calculator.

RESULTS

Stimulation of LDCL by four *E. coli* serotypes. The LDCL responses of PMNL after addition of the four *E. coli* serotypes, O101:K nontypable, O73:K92:H1, O16:K1:NM, and O1:K1:NM, are shown graphically in Fig. 1. *E. coli* serotypes O101:K nontypable, O73:K92:H1, and O16:K1:NM all produced significant increases over background LDCL, but each showed a different response pattern. In contrast, *E. coli* serotype O1:K1:NM displayed a negligible increase in LDCL over background LDCL.

Correlation between measurements of LDCL and susceptibility to phagocytosis. To determine whether measurements of the LDCL response after addition of the four *E. coli* serotypes correlated with susceptibility to phagocytosis, three parameters of the LDCL response were compared with phagocytosis susceptibility as determined by percentage of bacteria killed and remaining CFU at the conclusion of bactericidal experiments. *E. coli* O101:K nontypable and O16:K1:NM demonstrated 97.3 and 56.6% bacteria killed, respectively, in contrast to serotypes O73:K92:H1 and O1:K1:NM, which showed 0% bacterial killed (Table 1). Examination of the difference in CFU from the initial inoculum and the CFU present in the reaction mixture 60 min later showed serotype O1:K1:NM with the highest remaining CFU, followed by O73:K92:H1, O16:K1:NM, and O101:K nontypable. There were approximately 7×10^6 more CFU in the O1:K1:NM reaction mixtures than O73:K92:H1 after incubation with PMNL. The CFU values between these serotypes had a *P* value of less than 0.02.

LDCL peak, slope, and curve area integration were then examined for correlation with these degrees of susceptibilities to killing. Peak LDCL values were not found to be greatly different between serotypes O101:K nontypable and O16:K1:NM ($P > 0.05$), although O16:K1:NM peaked approximately 25 min earlier than O101:K nontypable. Serotype O73:K92:H1, 0% killed, but with fewer remaining CFU than O1:K1:NM, showed a peak LDCL intermediate between the

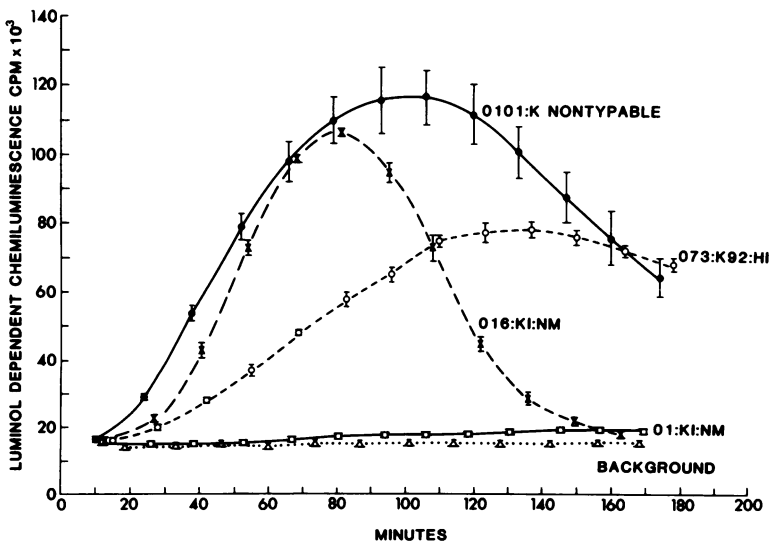

FIG. 1. *Temporal LDCL responses of PMNL after addition of four E. coli serotypes opsonized with pooled human serum. Points shown represent the mean of three experiments ± standard deviation.*

TABLE 1. *Comparison of the LDCL response by peak, slope, and curve area integration with susceptibility to in vitro phagocytosis, using four E. coli serotypes*[a]

E. coli serotype	% of organisms killed in 1 h	Change in CFU[b] ($\times 10^7$)	Peak LDCL (cpm $\times 10^3$)	Initial LDCL slope[c] (cpm^2 $\times 10^3$)	Integral value of area under LDCL curve (counts/170 min)
O101:K nontypable	98.4 ± 0.4	−1.10 ± 0.12	117 ± 8	1.69 + 0.85	1.99 ± 0.17 × 10^7
O16:K1:NM	56.6 ± 5.13	−0.53 ± 0.11	107 ± 3[d]	1.86 ± 0.22[e]	1.11 ± 0.06 × 10^{7f}
O73:K92:H1	00.0	+1.56 ± −0.13[g]	78 ± 3	0.67 ± −0.06	1.17 ± 0.05 × 10^{7h}
O1:K1:NM	00.0	+2.29 ± 0.32	19 ± 0.5	−0.02 ± 0.01	5.59 ± 0.01 × 10^5

[a] Results are mean ± standard deviation of three experiments.
[b] Change from initial inoculum after a 60-min incubation.
[c] From 20 to 50 min.
[d] $P > 0.05$ compared with O101:K nontypable.
[e] $P > 0.3$ compared with O101:K nontypable.
[f] $P < 0.005$ compared with O101:K nontypable.
[g] $P < 0.02$ compared with change in CFU of O1:K1:NM.
[h] $P < 0.005$ compared with O101:K nontypable, $P > 0.1$ compared with O16:K1:NM.

phagocytosis-susceptible serotype O101:K nontypable (greater than 50% killed) and serotype O1:K1:NM (0% killed).

Comparing LDCL slopes, the phagocytosis-susceptible serotypes O101:K nontypable and O16:K1:NM had different mean values, although not significantly different ($P > 0.30$), with the latter serotype possessing a slightly higher slope value, 1.86 versus 1.69. Serotype O73:K92:H1 again demonstrated an intermediate value between the phagocytosis-susceptible serotypes and the serotype O1:K1:NM (0% killed).

A third measurement of the LDCL response, curve area integration, was also compared with the susceptibility to phagocytosis. *E. coli* serotype O101:K nontypable had the greatest area, followed by O73:K92:H1 and O16:K1:NM. Although these latter two curve area integrals were both less than that of O101:K nontypable ($P < 0.005$), they were not statistically different from each other, with a P value of greater than 0.1. The curve area integral of O1:K1:NM was approximately 1.5 orders of magnitude less than those of serotypes O101:K nontypable, O16:K1:NM, and O73:K92:H1.

Increased LDCL response after opsonization of O73:K92:H1 with capsular antiserum over pooled human serum. To determine whether the intermediate LDCL response of serotype O73:K92:H1 could be increased to a level comparable to that of the phagocytosis-susceptible serotypes O101:K nontypable and O16:K1:NM, O73:K92:H1 was opsonized with capsular-directed antiserum (Fig. 2). The addition of capsular-directed antiserum to PMNL bactericidal experiments has been previously shown to effectively opsonize the respective bacterial strain (14) (>90% bacteria killed). The resulting LDCL response demonstrated a significant increase over pooled human serum-opsonized O73:K92:H1, with peak, slope, and integral values of 141 ± 4 × 10^3, 3.69 ± 0.69, and 1.35 ± 0.10, respectively.

DISCUSSION

PMNL killing of bacteria is thought to depend in part and perhaps primarily on the generation of highly reactive oxidative species by the PMNL (9). CL by phagocytosing PMNL is also believed to result from the generation of similar oxidative species, i.e, O_2^-, $^1O_2$, · OH, and H_2O_2 (2, 3). Although correlation between CL and susceptibility to phagocytosis has been reported (12, 14), no study has yet analyzed or compared various means of quantitating the CL response with bacterial susceptibility to phagocytosis.

Of the four serum-resistant *E. coli* serotypes examined in this study, all demonstrated different temporal LDCL responses. Analysis of various measurements of the LDCL responses, such as slope, peak, and curve area integration, and comparison with susceptibility to phagocytosis of the serotypes studied, however, did not reveal a complete correlation. Surprisingly, two of the serotypes, O73:K92:H1 and O1:K1:NM, had equal percentages of bacteria killed (0%), but O73:K92:H1 gave an LDCL response intermediate between the two serotypes. Although no bacteria were killed in the bactericidal assay with either serotype O73:K92:NM or O1:K1:NM, i.e., the final CFU were greater than the starting CFU, bactericidal assay experiments with O73:K92:H1 were found to have significantly fewer CFU remaining than in experiments with O1:K1:NM, suggesting that serotype O73:K92:H1 is more susceptible than O1:K1:NM to phagocytosis. Variation in replication time may exist among *E. coli* serotypes but probably could not account for the observed large difference in CFU

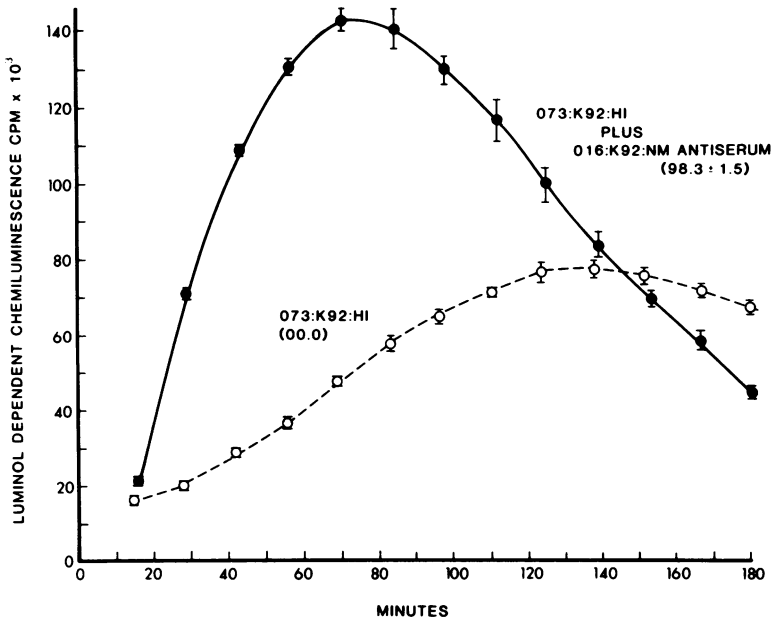

FIG. 2. *Increase in LDCL after addition of the capsular-directed antiserum (anti-O16:K92:NM)-opsonized E. coli O73:K92:H1 over pooled human serum-opsonized O73:K92:H1. Numbers in parentheses represent mean percentage of bacteria killed in 1 h ± standard deviation of three experiments. Points on curves are mean ± standard deviation of three experiments.*

after a 60-min incubation period. A greater susceptibility to phagocytosis of serotype O73:K92:H1 over O1:K1:NM may then explain the respective increase in LDCL seen with O73:K92:H1 over O1:K1:NM.

CL elicited by PMNL stimulated with opsonized bacteria is most likely the result of bacteria-PMNL membrane interactions leading to the production of microbicidal oxidative species. The nature of this membrane interaction may be due to specific perturbations of the PMNL membrane via Fc, C3b, or C5a receptors or from direct contact with bacterial outer membrane components. Previous reports have documented the increased production of O_2^- by human PMNL after exposure to heat-aggregated immunoglobulin G (IgG), a C3b receptor stimulus, C5a (5), and generation of LDCL with aggregated IgG (N. J. Doll et al., Fed. Proc. **39**:673, 1980) and IgG-coated bacteria in the absence of heat-labile serum factors (14). Serotypes O73:K92:H1, O101:K nontypable, and O16:K1:NM may thus be more prone to successful PMNL membrane interaction than O1:K1:NM because (i) more antibody may be bound and available for appropriate PMNL membrane contact, (ii) greater amounts of C3b may be attached, or (iii) the outer membrane structure of serotypes O73:K92:H1, O101:K nontypable, and O16:K1:NM may be more conducive to interaction with the PMNL membrane, possibly due to their large degree of hydrophobicity compared with the O1:K1:NM outer membrane.

Although O73:K92:H1 showed an LDCL response greater than that of O1:K1:NM and background LDCL levels, the LDCL response as measured by peak, slope, and curve area integration was significantly less than that of the phagocytosis-susceptible serotype O101:K nontypable (>90% bacteria killed). However, addition of specific capsular antiserum resulted in a greatly increased LDCL response (Fig. 2), even higher than the LDCL response seen with O101:K nontypable, when comparing slope and peak LDCL measurements. The use of this capsular antiserum has previously been shown to generate increased LDCL over pooled human serum-opsonized bacteria and to promote effective killing of serotype O73:K92:H1 by human PMNL (>90% bacteria killed) (14). Thus, the effect of specific opsonic procedures on certain bacterial strains can be correlated with bacterial susceptibility to phagocytosis and with slope, peak, and curve area integration measurements of the resulting LDCL response.

In conclusion, the results do not show a complete correlation between the slope, peak, and curve area integration measurements of the LDCL response and bacterial susceptibility to phagocytosis. Inherent differences among the

four *E. coli* serotypes studied may account for this lack of correlation. However, greater bacterial susceptibility to phagocytosis as achieved by opsonization with antiserum (or other methods) may be correlated with respective increases in slope, peak, and curve area integration measurements of the LDCL response.

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