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Chemiluminescent Response to Pathogenic Organisms: Normal Human Polymorphonuclear Leukocytes

PAUL ROBINSON,* DENIS WAKEFIELD, SAMUEL N. BREIT, JOAN F. EASTER, AND RONALD PENNY

Department of Immunology, St. Vincent's Hospital, and the Department of Medicine, University of New South Wales, Sydney, Australia

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Chemiluminescence (CL) is a sensitive indicator of phagocytosis and intracellular killing; however, little is known of the normal CL response by human polymorphonuclear leukocytes to different pathogenic microorganisms. We investigated the luminol-enhanced CL response of normal polymorphonuclear leukocytes to a number of common bacterial pathogens and two yeasts. We analyzed the CL response to viable and heat-killed microorganisms at 25 and 37°C. The CL response to all microorganisms was greater and more rapid at 37°C. Variable responses were observed with viable and heat-killed microorganisms; some were unaffected, whereas others demonstrated reduced CL. Each microorganism caused a reproducible response pattern, which could be placed into two general categories. In the first category were those which caused a rapid exponential rise and decay in CL: *Enterobacter cloacae*, *Salmonella typhimurium*, *Shigella flexneri*, *Staphylococcus aureus*, *Candida albicans*, and zymosan. In the second category were those which rose slowly over a longer time course to a poorly defined peak: *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Streptococcus pyogenes*. The CL response also reflected serum opsonic activity. The effects of inactivated complement, factor B, and removal of specific antibody were investigated. Increasing the concentration of zymosan gave a proportional rise in peak CL; however, a strain of *E. coli* caused a variation in peak time rather than peak height. Different CL kinetics were shown for three strains of *K. pneumoniae*, possibly a result of each having different membrane or cell wall characteristics. This study defines the nature and factors affecting the normal CL response to a variety of common pathogenic microorganisms.

The interaction between particles and phagocytic cells induces activation of a membrane oxidase (5, 16, 24, 26), which triggers a metabolic response, the so-called "respiratory burst." During the process of phagocytosis and subsequent killing, a number of electronically excited molecules such as singlet oxygen and oxygen radicals including superoxide and hydroxyl radicals are formed (6, 17). Light is produced as a result of the relaxation of electronically excited carbonyl chromophores generated as products of oxygenation (2). Phagocytosis of polymorphonuclear leukocytes (PMN) can be monitored using chemiluminescence (CL) (4), and with the addition of chemilumigenic probes such as luminol, light production is substantially enhanced (3).

Neutrophil function is now frequently assessed by CL induced by zymosan or by *Staphylococcus aureus*. This study examines the CL responses to a variety of common, clinically important human pathogens. We demonstrated different reproducible response patterns for each microorganism. This variation may further our understanding of the differences in the interaction of the infective agents with PMN and may be important in determining their pathogenicity.

MATERIALS AND METHODS

Phagocytic cells. Human PMN were obtained from 30 ml of heparinized blood by density gradient centrifugation (8) followed by dextran sedimentation. Briefly, 30 ml of blood was diluted 1:2 with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). This was layered onto 10 ml of Ficoll-Paque (Pharmacia Fine Chemicals, Upsala, Sweden) in 50-ml centrifuge tubes (Corning Glass Works, Corning, N.Y.) and centrifuged for 30 min at 600 × *g*. The upper interface of the gradient and excess Ficoll-Paque were discarded.

PBS (10 ml) and 3 ml of 3% dextran T500 in saline (Pharmacia) were added to the bottom PMN-containing layer. These tubes were mixed and placed in a 37°C water bath for 40 min. The buffy coat was removed and centrifuged at 200 × *g* for 10 min, after which the remaining erythrocytes were lysed with 10% PBS in distilled water for 30 s. PMN were washed three times in Hanks balanced salt solution (Ca^{2+}- and Mg^{2+}-free) containing 15% fetal calf serum. After the final wash, the PMN were counted and suspended in Hanks balanced salt solution at a concentration of 10^6 PMN per ml. The preparations of PMN demonstrated 95 to 97% viability as measured by trypan blue exclusion.

Opsonizing sera. Serum was collected from 15 healthy adults who were not on any drug therapy. Samples were pooled and portioned into 5-ml volumes which were immediately stored at −85°C until required. This was termed pooled normal human serum (NHS). Serum was also taken from each control subject for use in experiments where autologous serum was required. Inactivation of complement components was achieved by heating at 56°C for 30 min (total complement inactivation) and heating at 50°C for 30 min (inactivation of factor B and, therefore, alternative pathway activation). Blockage of the classical pathway was achieved by incubating serum at 37°C in the presence of 10 mM Mg-ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid (EGTA) (12). For removal of specific antibody, NHS was incubated at 37°C for 60 min with individual strains of bacteria followed by centrifugation at 1,000 × *g* for 20 min. Samples were filtered through 0.2-μm filters (Millipore Corp., Bedford, Mass.).

Microorganisms. All microorganisms used were clinical isolates (wound swabs or urine or blood cultures) and were

* Corresponding author.

inoculated into brain-heart infusion broth and incubated at 37°C for 24 h. *Streptococcus* sp. required serum (4% horse serum) to stimulate growth. Gram-positive microorganisms used included *S. aureus*, *Streptococcus pyogenes*, *Listeria monocytogenes*. Gram-negative microorganisms used included *Klebsiella pneumoniae* (including strains K43, STV1, AF2, AF3, and AF4), *Escherichia coli*, *Enterobacter cloacae*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Pseudomonas aeruginosa*, and *Proteus mirabilis*. Also included were *Candida albicans* and zymosan. After overnight culture, microorganisms were washed twice in PBS before suspension in PBS. The drop counting technique (22) was used for all bacterial counts. All microorganisms were diluted with PBS to a concentration of 10^8 microorganisms per ml. Heat killing was achieved by incubating dilutions of each microorganism at 60°C for 90 min.

Zymosan preparation. Zymosan was prepared by boiling 500 mg of zymosan A (Sigma Chemical Co., St. Louis, Mo.) in 50 ml of PBS for 60 min and then washing and resuspending it to 50 mg/ml in PBS. This stock solution was found to remain stable at 4°C for several weeks. A 10-fold dilution was used for assays.

CL assay procedure. All CL measurements were performed with a Packard 2660 liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.) in the out of coincidence mode by using previously described methods (23). Minor modifications to this method included a reduction of the total reaction vial volume to 850 μl. To each vial was added 400 μl of PBS, 200 μl of PMN (2×10^5 cells), 50 μl of luminol (Sigma; 10^{-4} M), 100 μl of serum (treated as described above), and 100 μl of the microorganism suspension. The cells, buffer, serum, and luminol were preincubated at 30°C for 10 min before the addition of the relevant microorganism suspension.

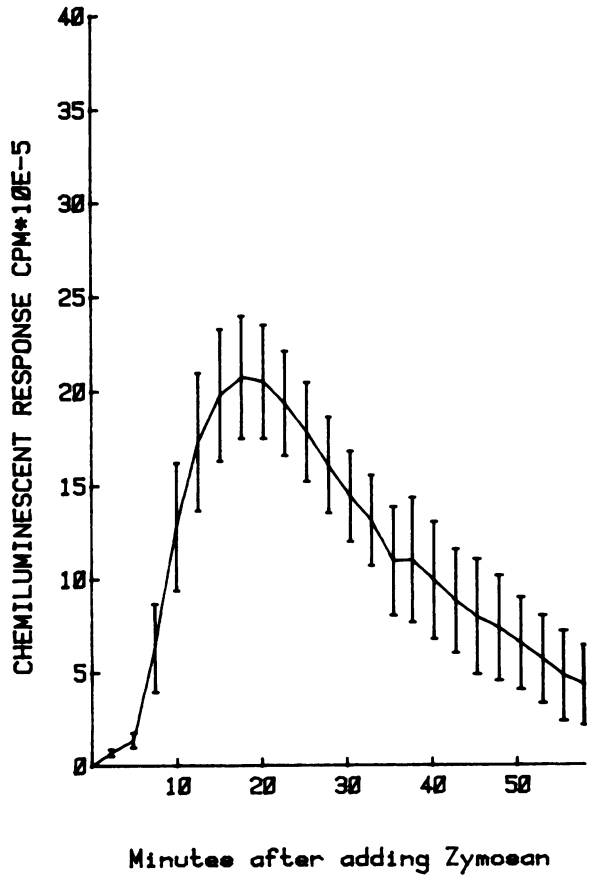

FIG. 1. CL response of PMN to zymosan represented by the mean curves of 44 individual controls over a 12-month period. Bars indicate mean ± 2 standard errors of the mean.

TABLE 1. Analysis of 44 individual PMN CL response curves to zymosan[a]

Parameter	Mean ± SE
Peak height (cpm × 10^5)	22.7 ± 1.60
Peak time (min)	19.1 ± 0.82
Integral to peak (cpm × 10^5)	561.0 ± 46.00
Integral to 60 min (cpm × 10^5)	1,211.0 ± 69.50
Initial slope	0.55 ± 0.06
Maximum slope	3.4 ± 0.39

[a] The curve represented by this data is shown in Fig 1.

CL measurements were made automatically for 6-s intervals on each of 10 vials over a 90-min period with a constant amount of agitation between measurements. The temperature was maintained at a constant 30 ± 0.2°C by a thermostatically controlled device installed inside the liquid scintillation counter (AST Electronics, Sydney, Australia). Assay vials (standard 20-mm scintillation vials) were preheated to 30°C. Vials were not dark adapted nor were assays carried out in a darkened room (except for the removal of fluorescent lights) because negligible differences were seen under these conditions when using luminol-assisted CL (unpublished observations).

Data analysis. The CL responses, measured in counts per minute, were recorded on line to a Hewlett-Packard 85 computer, which provided a visual curve and a six-parameter analysis based on peak height, time to peak, initial slope, maximum slope, integral to peak height, and total integral counts. Statistical analysis was performed using the Student's *t* test.

RESULTS

Response of PMN to zymosan. Figure 1 illustrates the response of normal PMN to zymosan at 30°C. The curve was derived from 44 individual cell preparations over a 12-month period. Table 1 shows the analysis of the standard curve in terms of average initial slope (first four points), maximum slope, time to peak, peak height, integral to peak, and total integral counts. The response to zymosan was characterized by the rapid rise and decay of the CL. The time taken to achieve peak height was normally between 20 and 30 min. The response fell to preactivation levels by approximately 90 min.

Comparison of CL responses to heat-killed and live organisms. When the CL responses to heat-killed (60°C, 90 min) microorganisms were compared to responses to the corresponding viable microorganisms, no distinct patterns emerged. *P. mirabilis* caused a slight increase in CL response (not significant), whereas response to *E. coli* remained unchanged. *S. aureus*, *S. typhimurium*, and *S. dysenteriae* all caused a significant reduction ($P < 0.001$) in peak CL response (Table 2). Although *P. mirabilis* caused no significant change in peak CL response, the time to peak was significantly increased ($P < 0.01$). Of the microorganisms which showed reduced CL, only *S. typhimurium* caused a prolonged response with a doubling of time to peak.

CL responses of PMN to gram-positive organisms. The CL

TABLE 2. Effect of microorganism viability on CL response[a]

Microorganism	No. of assays	Viable microorganisms		Heat-killed microorganisms	
		Mean peak height (cpm × 10^5) ± SE	Mean time (min) ± SE	Mean peak height (cpm × 10^5) ± SE	Mean time (min) ± SE
P. mirabilis	6	3.42 ± 0.9	44.0 ± 1.2	3.9 ± 0.7	76.4 ± 5.9[b]
E. coli	6	19.0 ± 4.3	42.0 ± 8.2	16.0 ± 2.5	30.8 ± 0.8[c]
S. aureus	9	11.8 ± 3.1	31.5 ± 6.7	5.4 ± 0.8[b]	31.8 ± 0.9
S. typhimurium	7	26.5 ± 5.6	27.8 ± 6.0	5.7 ± 1.6[b]	54.2 ± 7.9[b]
S. dysenteriae	5	15.4 ± 2.5	30.4 ± 0.5	4.0 ± 0.7[b]	31.2 ± 0.8

[a] The responses to *P. mirabilis* and *E. coli* remained unchanged when heat-killed microorganisms were substituted for live microorganisms under the same conditions for CL measurement. The CL responses to *S. aureus*, *S. typhimurium*, and *S. dysenteriae* showed significantly reduced peak heights ($P < 0.001$). The kinetics of the responses did not, however, follow the same pattern, since *P. mirabilis* almost doubled the time taken to achieve peak height ($P < 0.001$), whereas the other two microorganisms had unchanged times to peak height. *E. coli* actually reduced the time taken to achieve peak height ($P < 0.05$). All experiments were carried out with pooled normal serum.

[b] Significant at $P < 0.001$.

[c] Significant at $P < 0.05$.

responses of PMN to *S. pyogenes*, *S. aureus*, and *C. albicans* were examined at 30°C. The shapes of the mean response curves are shown in Fig. 2. Peak heights were lower and response times were slower as compared with zymosan. *S. aureus* and *C. albicans* response curves were similar in shape to those of zymosan, with a less dramatic CL decay. *Streptococcus* sp. showed less-well-defined peaks, with little decay of the CL response during the time period measured.

CL responses of PMN to gram-negative bacteria. Shown in Fig. 3 are the response curves caused by several members of the family *Enterobacteriaciae*. Two *Klebsiella* species caused extremely slow and gradual increases in CL response, often extending to over 100 min before a decay was observed. *P. aeruginosa* caused a rapid PMN CL response; however, the CL response failed to show significant decay over the 60 min measured. The other microorganisms tested caused a rapid rise and a discernible peak followed by a rapid decay in CL. This group included *E. coli*, *E. cloacae*, *P. mirabilis*, and *S. dysenteriae*.

CL variation due to cell wall differences. When three different *K. pneumoniae* strains were compared, it appeared that surface characteristics contributed significantly to the CL response. Figure 4 shows the variation in CL response of PMN to encapsulated (AF3), slime-producing (AF2), and nonencapsulated, non-slime-producing strains (AF1). The encapsulated strain caused a much slower CL response to occur; however, the slime producer actually caused a greater CL response than did the non-slime producer.

Change in concentration of activator. Increasing the concentration of zymosan increased the peak height of the CL response without significantly altering the time to peak (Fig. 5a). However, when the concentration of *E. coli* was increased, peak height remained relatively constant, whereas time to peak decreased proportionally with increased concentration (Fig. 5b). The total reactivity of each concentration as measured by the integral to 50 min showed no great variation over the concentration range. The measurements were made on the same PMN population on the same day to exclude any variation due to PMN from different donors.

Relative CL response at 37 and 25°C. When several different microorganisms were tested for their CL responses at 37 and 25°C, their responses in all cases were more rapid and resulted in higher peaks at 37°C (Fig. 6a). Most pronounced was a 440% increase in slope with zymosan when the temperature was increased from 25 to 37°C. Greater variations were seen in the change in slope than were seen for other parameters (Fig. 6b). Time to peak height showed a

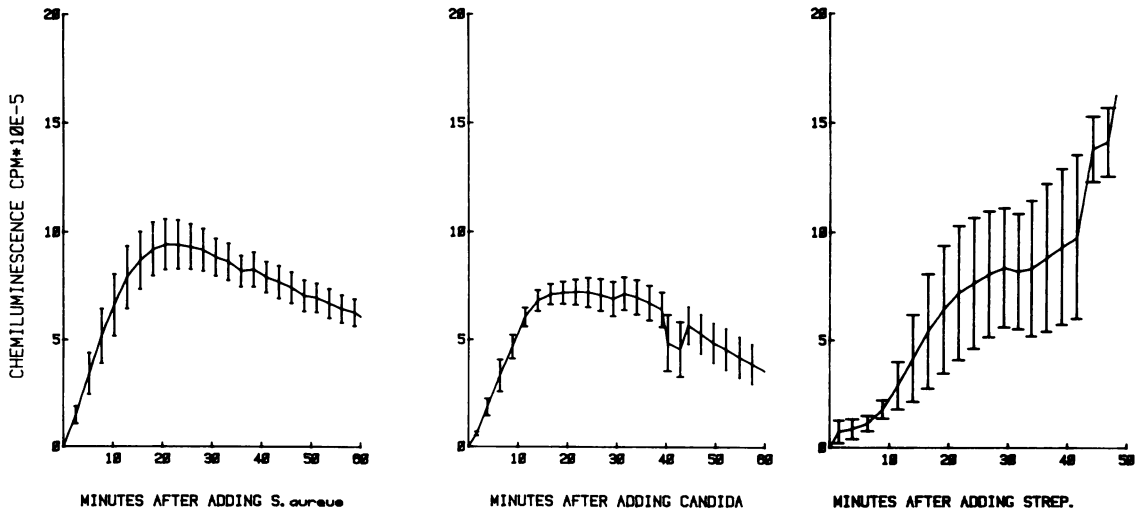

FIG. 2. Mean CL responses of PMN to two gram-positive bacteria and *C. albicans*. The number of individual controls for each microorganism was: *S. aureus*, 22; *Streptococcus* group A, 7; *Candida*, 14. Bars represent the means ± 2 standard errors of the mean. (The aberration in the *Candida* CL curve is artifactual.)

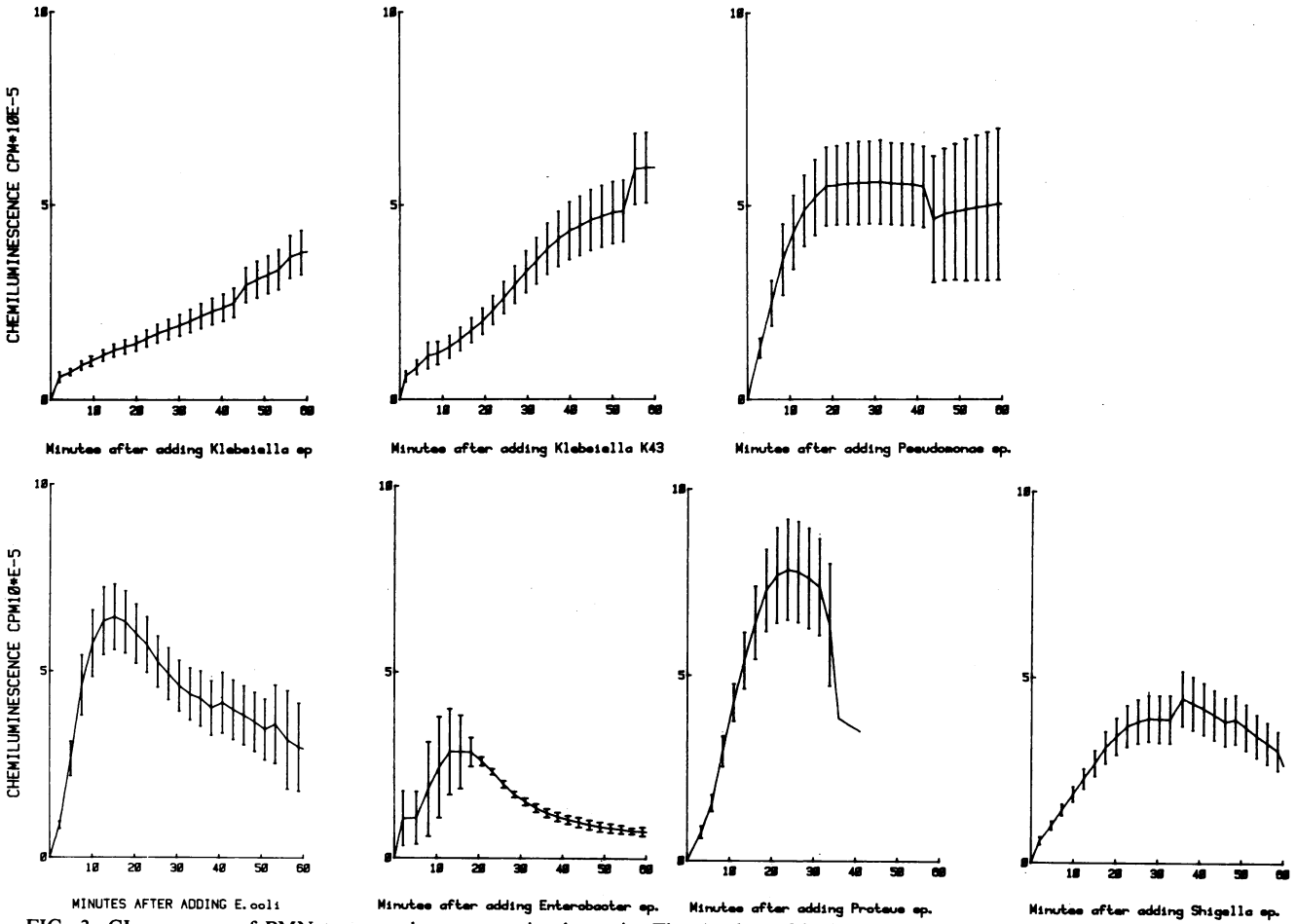

FIG. 3. CL response of PMN to several gram-negative bacteria. The number of individual controls represented in each graph is: *K. pneumoniae* STV1, 32; *K. pneumoniae* K43, 32; *Pseudomonas* sp., 12; *E. coli*, 14; *Enterobacter* sp., 5; *Proteus* sp., 8; *Shigella* sp., 32.

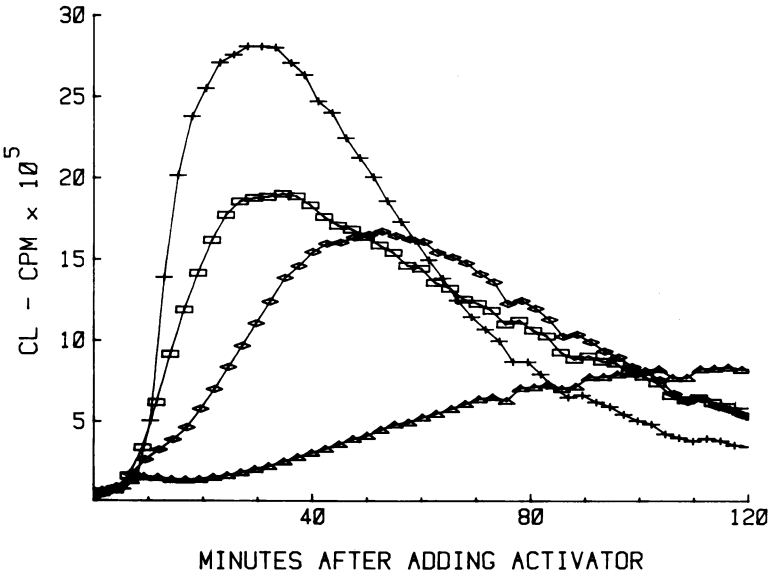

FIG. 4. Effect of the type of bacterial membrane on the CL response of PMN. Shown are the CL responses to three species of *K. pneumoniae* whose characteristics include: □, slime but no capsule (AF2); ◇, capsule but no slime (AF3); ◇, neither slime nor capsule (AF1). Also shown is the CL response to zymosan under identical conditions (+–+). Curves represent three experiments performed on each microorganism.

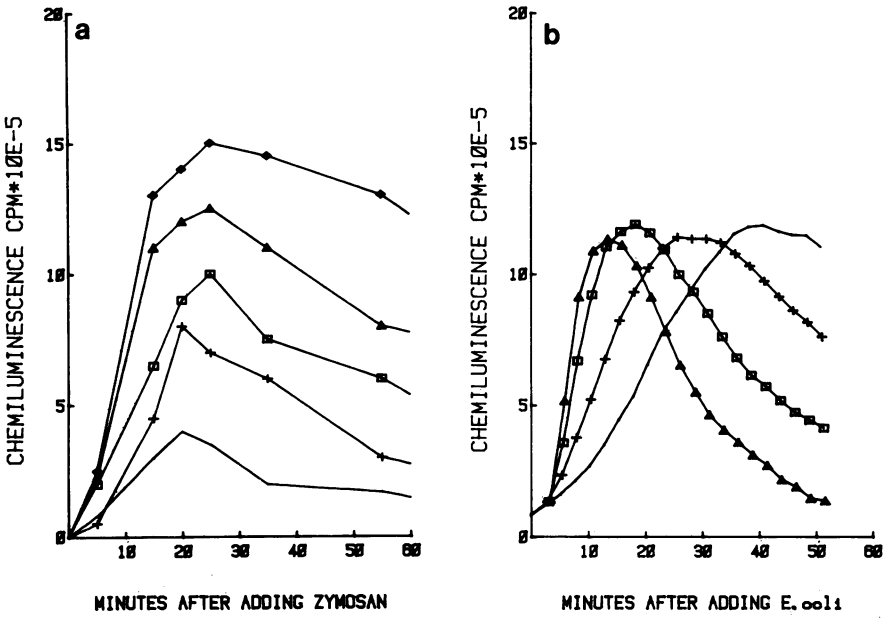

FIG. 5. (a) Change in CL response to increasing concentrations of zymosan: — , 0.2 mg; +, 1.0 mg; □, 5.0 mg; △, 10.0 mg; ◇, 15.0 mg. (b) Change in CL response to increasing concentrations of *E. coli*: △, 2×10^4/ml; □, 2×10^5/ml; +, 5×10^5/ml; —, 1×10^6/ml.

mean decrease of 50% for the microorganisms tested at 25°C compared with those tested at 37°C (Fig. 6c). The increase in peak height at 37°C ranged from 40 to 140%.

Effects of serum adsorption. A total of 14 microorganisms were compared for their abilities to cause PMN CL responses in the presence of NHS and portions of serum which had been adsorbed against the specific microorganisms. Unopsonized microorganisms were added to PMN at the same microorganism-to-PMN ratios. Adsorption was undertaken immediately before the measurements, and NHS was incubated under the same conditions as adsorbed serum but without microorganisms. The CL responses were placed into three categories based on changes in peak CL: (i) no change, (ii) moderate reduction of CL to 30% of the NHS response, and (iii) highly reduced CL responses (>30%). Table 3 details the responses to each microorganism tested, showing both peak heights and time to achieve peak height.

Effects of complement inactivation. Table 4 tabulates the PMN CL responses (peak and time to peak) to four microorganisms measured in the presence of either NHS or adsorbed serum which had been inactivated (56°C, 30 min), inactivated factor B (50°C, 30 min), or untreated serum. Two microorganisms (*C. albicans* and *L. monocytogenes*) demonstrated a complement dependence which was independent of factor B. Both peak CL response and time to peak were affected. *L. monocytogenes* showed an absolute dependence upon the classical complement components and was not affected by adsorption of specific antibody or inactivation of factor B or both. Neither *E. coli* nor *S. aureus* showed significant alterations in peak CL response; however, *S. aureus* caused a significant increase in time to peak ($P < 0.05$) for each of the serum treatments.

DISCUSSION

Since the first report by Allen et al. on the use of native CL to measure phagocyte activity (4), there have been a number of publications demonstrating the variables involved

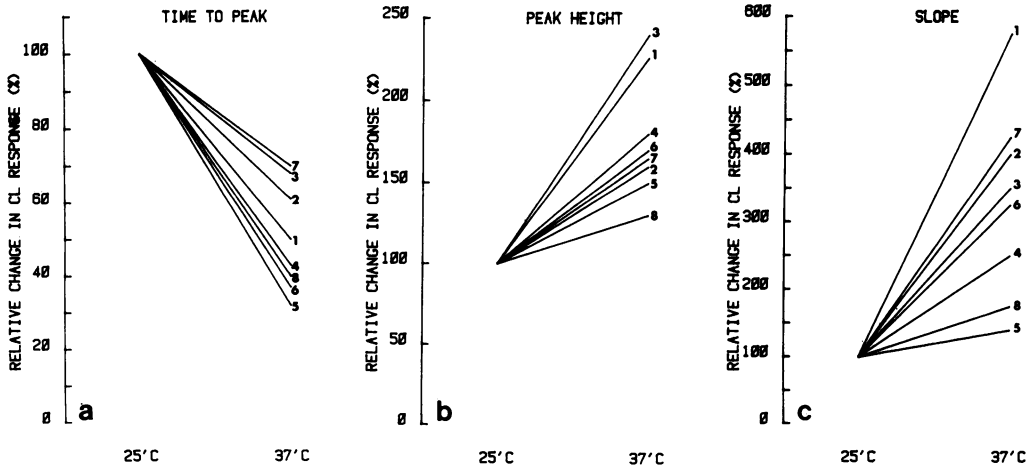

FIG. 6. PMN CL response to changes in temperature. Variations in time to peak (a), peak height (b), and slope (c) with a change in temperature from 25 to 37°C. Organisms or stimulus: 1, zymosan; 2, *C. albicans*; 3, *Streptococcus dysgalactiae*; 4, *S. pyogenes*; 5, *S. aureus*; 6, *E. coli*; 7, *P. aeruginosa*; 8, *P. mirabilis*.

TABLE 3. Effect of specific antibody on CL responses[a]

Group[b]	Stimulus	No. of assays	Normal pooled serum		Adsorbed serum	
			Mean peak height (cpm × 10^5) ± SE	Mean time (min) ± SE	Mean peak height (cpm × 10^5) ± SE	Mean time (min) ± SE
A	*S. dysenteriae*	8	15.4 ± 4.2	30.4 ± 0.54	16.3 ± 4.6	30.6 ± 0.5
	E. coli	14	13.2 ± 3.1	34.0 ± 12.7	13.5 ± 4.3	30.4 ± 8.2
	K. pneumoniae AF1	9	10.2 ± 3.8	24.9 ± 8.7	10.8 ± 4.1	36.9 ± 5.9
	L. monocytogenes	8	3.2 ± 0.8	28.0 ± 13.0	3.1 ± 0.89	37.0 ± 14.5
	K. pneumoniae AF3	9	16.9 ± 4.2	31.5 ± 0.5	16.8 ± 4.2	31.8 ± 0.8
B	*S. aureus* (Oxford)	10	15.3 ± 3.8	26.4 ± 4.9	11.1 ± 3.2	53.8 ± 6.4
	S. aureus (Cowan)	10	11.8 ± 2.1	31.5 ± 6.7	8.4 ± 1.3	65.6 ± 16.3
	P. aeruginosa	8	17.9 ± 4.2	49.8 ± 7.0	13.2 ± 3.4	102.0 ± 10.4
C	*K. pneumoniae* AF2	8	7.8 ± 1.9	105.0 ± 6.4	2.9 ± 0.8	166.0 ± 10.4
	S. typhimurium	10	26.5 ± 5.6	27.8 ± 6.0	14.5 ± 3.1	70.4 ± 18.0
	P. mirabilis	10	3.4 ± 1.0	44.0 ± 1.0	0.9 ± 0.25	90.6 ± 0.8
	K. pneumoniae K43	12	16.7 ± 4.2	40.1 ± 8.9	2.1 ± 0.68	84.0 ± 12.7
	C. albicans	6	14.1 ± 3.1	21.3 ± 4.05	5.0 ± 1.1	10.7 ± 4.2
	Zymosan	13	33.3 ± 4.6	23.6 ± 4.5	15.4 ± 3.3	82.4 ± 11.2

[a] Each microorganism was unopsonized and was added to reaction vials containing either normal pooled serum or serum from the same pool which had been adsorbed against the specific microorganism immediately before measuring CL. Each microorganism was tested with PMN isolated from the same individual when comparing NHS and adsorbed sera.

[b] Group A, Microorganisms which caused no change in CL response in the presence of adsorbed sera; group B, a moderate reduction in CL response with adsorbed sera; group C, a highly reduced CL response.

in measurements of CL, especially the measurement of luminol-enhanced CL (3, 9, 11).

The exact interpretation of the oxidative events during phagocytosis, particularly with respect to the clinical interpretation of CL assays, remains unclear. Nevertheless, several authors have attempted to correlate CL response with phagocytic and bactericidal activity (14, 15, 21, 29). Generally, it is thought that luminol is dioxygenated to yield aminophthalate anions and subsequent electronically excited carbonyl chromophores, which upon relaxation to ground state produce light (2). The process is subject to complex interactions between activating particles and cellular receptors. We have taken one aspect of this complex series of events to analyze the role of different organisms in the CL response.

Many reports on CL activity have utilized zymosan for activation. We studied the responses of normal PMN to a variety of organisms as well as to zymosan. Zymosan, a cell wall component of *S. cerevisiae*, has always proved to be an excellent activator of CL in PMN. As shown in Fig. 1, the initial slope and maximum slope rose exponentially over the first 20 min of the reaction followed by a well-defined peak and rapid decay of the response. The curve fell to preactivation levels after approximately 90 min.

The organisms tested fell into two general categories: those organisms which caused a rapid CL response by PMN

TABLE 4. Effect of heat-treated serum on CL response[a]

Microorganism	Serum heated at (°C):	Pooled serum		Adsorbed serum	
		Mean peak height (cpm × 10^5) ± SE	Mean time (min) ± SE	Mean peak height (cpm × 10^5) ± SE	Mean time (min) ± SE
C. albicans	37	21.0 ± 4.7	28.8 ± 0.5	16.2 ± 4.0	40.5 ± 15.4
	50	18.3 ± 3.3	33.0 ± 6.6	16.4 ± 4.4	37.2 ± 8.3
	56	11.8 ± 3.0	53.7 ± 13.2	12.5 ± 2.9	62.4 ± 6.2
L. monocytogenes	37	16.4 ± 2.4	53.2 ± 13.0	16.4 ± 3.4	43.7 ± 1.2
	50	16.1 ± 3.8	50.2 ± 7.3	17.2 ± 3.3	55.1 ± 8.0
	56	3.6 ± 0.7	127.0 ± 30.0	2.9 ± 0.8	118.7 ± 31.5
E. coli	37	27.9 ± 5.8	15.0 ± 0.3	28.2 ± 4.6	15.7 ± 0.5
	50	27.4 ± 5.6	15.0 ± 0.2	29.4 ± 3.8	15.6 ± 0.6
	56	26.7 ± 4.8	15.0 ± 0.7	28.1 ± 4.8	16.0 ± 0.9
S. aureus (Oxford)	37	22.1 ± 3.5	28.6 ± 0.5	21.3 ± 3.4	62.4 ± 9.0
	50	21.5 ± 3.7	38.6 ± 7.5	22.2 ± 4.1	62.8 ± 6.4
	56	23.7 ± 4.9	61.6 ± 6.4	22.5 ± 5.0	78.3 ± 7.4

[a] NHS or adsorbed serum was heated at either 56 or 50°C. Measurements were made with the same PMNs when comparing NHS or adsorbed sera. Four individual PMN populations were measured against each microorganism and each of the six serum treatments. Each CL measurement was performed in duplicate. *C. albicans* and *L. monocytogenes* demonstrated a significant reduction in peak CL ($P < 0.05$) and increased time to peak height ($P < 0.05$) in the presence of heat-inactivated serum (56°C, 30 min). *E. coli* and *S. aureus* did not show the same effects except for slower CL kinetics for *S. aureus* with all serum treatments.

and those which caused a gradual increase with time. Since the ratios of organisms to phagocytes were held constant, it would appear that the membrane composition of the organisms and interactions with opsonic proteins were contributing factors to the differences. Two gram-positive organisms, *S. aureus* and *S. pyogenes*, demonstrated the different response patterns. *S. aureus* caused a response similar to that for zymosan, with a rapid but lower peak height, whereas *S. pyogenes* caused a less defined peak and a prolonged decay of CL.

When a range of gram-negative organisms was tested, their responses also fell into two groups. *Enterobacter*, *Salmonella*, *Proteus*, and *Shigella* species all caused responses similar to that for zymosan, whereas *Pseudomonas* species and the two *Klebsiella* species (K43 and STV1) caused a gradual rise over a period of 60 to 80 min before decay occurred. The reasons for the two response patterns are unknown. Gram staining separates bacteria based on chemical differences in that gram-positive organisms contain teichoic acids and many gram-negative organisms contain lipopolysaccharides; however, there appeared to be no relationship between the major surface antigens and the type of CL response. Therefore, a membrane-dependent mechanism may be responsible for the variations in the CL response such as the content of protein A or lipid A present in the cell wall.

Since one contributing factor in the CL response is the surface structure of the activating organism, CL responses were measured with heat-killed and live organisms. Each organism was tested against the same cell population by using NHS. The results shown in Table 3 suggest that some organisms are not affected by heat killing (e.g., *E. coli*), whereas others showed considerably less ability to stimulate the respiratory response of PMN (e.g., *S. aureus*). Previous studies have also demonstrated no change in CL response to a viable and Formalin-killed unopsonized strain of *E. coli* (IID953) (26). It was suggested that CL was increased only because of phagocytosis by macrophages of live rather than killed opsonized organisms. In the present study, organisms were not preopsonized, but were added to reaction vials containing serum. One interesting observation was that the kinetics of the response appeared to be independent of the actual peak CL obtained. This was evident with heat-killed *P. mirabilis*, which caused no change in peak height, but the time to achieve peak was doubled. On the other hand, peak CL response to heat-killed *S. dysenteriae* was significantly reduced, without a variation in time to peak. The removal of the capsular antigens by heat killing in the case of *S. dysenteriae* did not cause the expected increase in CL due to the exposure of the bacterial membrane to opsonins. These results suggest that any alteration of the cell surface by heat treatment was in part dependent upon the cell wall composition of the organism concerned, with the possibility of destruction of bacterial antigens such as hydrophilic proteins conductive to PMN-bacterial interaction. It is also evident that the kinetics of the response varied independently of the quantitative CL production by PMN.

This variation in CL kinetics was also observed when different strains of *K. pneumoniae* were used to stimulate PMN CL. These strains were different in their cell wall components in that one contained a capsule, one was a slime producer, and the other had neither capsule nor slime. The response curves (Fig. 4) demonstrated that the encapsulated organisms caused a slow rising CL curve without a defined peak. This has been previously demonstrated with *S. typhi* as being related to the presence of virulence factors (18). The unencapsulated strains both produced well-defined peaks (similar to zymosan). It is clear that the presence of a capsule in this organism caused a considerable increase in the time to peak. The presence of slime did not alter the shape of the response curve.

The slope of the CL response appears to be a significant measure of serum opsonic activity (1, 20). Therefore, since it is the slope that is the most significant variable with organisms such as *Klebsiella* and *Pseudomonas* species, the differences in CL responses may be a reflection of opsonic differences. Similarly, the longer time to peak height is indicative of slower kinetics, often as a result of variation in opsonization. The four organisms presented in Table 4 demonstrated considerable variation in the effects of different heat-treated sera on the CL response. PMN CL responses to unopsonized *E. coli* were unaffected by any of the serum treatments since both peak CL and time to peak remained constant. CL response to *S. aureus* was unchanged; however, the time to peak was increased with all serum treatments. This effect was smaller with alternative pathway inactivation than with total complement inactivation. This does not correlate with the findings of Verbrugh et al. (28), who concluded that optimal opsonization of staphylococci requires both immunoglobulin and intact classical pathway. The inactivation of complement components in this study did cause a significant slowing in the kinetics of the CL response, not in the magnitude of the total response (total integral counts). This is consistent with the findings of Verbrugh et al. (27), who showed that in the absence of the classical pathway of complement, maximal opsonization was only achieved after 30 to 60 min of incubation of the bacteria.

Both *C. albicans* and *L. monocytogenes* showed some dependence upon serum complement in promoting efficient oxidative metabolism in PMN. Only in the presence of inactivated serum were response kinetics altered, regardless of the presence or absence of specific antibody. The contribution of specific antibody to the CL responses of PMN differed according to the particular organism measured. Some organisms showed no antibody dependence at all (e.g., *S. dysenteriae*), whereas others showed a marked dependence upon antibody-specific opsonization (e.g., *S. typhimurium*). The organisms could be place into three groups related to the degree of reduction in peak CL response; however, no correlation was possible regarding morphology or other characteristics of pathogenicity. All experiments were performed with the same pool of serum, and only the specific antigens were absorbed from the pool for each organism. This allowed measurements to be made under similar conditions with identical serum components present except for the factors removed by adsorption. Since the adsorption process was carried out at 37°C, it is possible that other opsonic factors may have been removed from sera, affecting the CL response.

The activating agent is also of considerable importance when assessing the initial response. Indeed the rate of CL response, i.e., the slope, must depend upon several factors, one of which is the ratio of cells to organisms. Zymosan showed a concentration-dependent rise in peak height. The time to reach this peak remained relatively constant. With *E. coli*, although the peak height remained constant, the time to peak altered with increasing bacterial concentration. The reason for this must be in the nature of the activating particles which cause variable stimulation of the PMN membrane oxidase. Allen et al. (4) showed that increasing the number of *Propionibacterium shermanii* increased the peak height but reduced the time to peak. Experiments with

S. pneumoniae, *P. aeruginosa* (25), and the soluble stimulus phorbol myristate acetate (10) all demonstrated similar results. In each case, with increasing concentration of activator the curve shifted up and to the left, indicating faster, more intense CL production. Mangan and Snyder (19), using *E. coli* 19^+, demonstrated much less difference in time to peak, with only a 10-min variation over bacteria-to-cell ratios of 60:1 to 200:1. Our data with both zymosan and *E. coli* did not fit this pattern.

Zymosan is an excellent activator of the alternative complement pathway, which may account for the constant response patterns obtained and the single peak with first-order kinetic response. *E. coli*, on the other hand, showed a correlation between time to a peak and bacterial concentration. This may reflect differences in the rate of contact between particle and cell. Most previous reports have indicated that little or no agitation of reaction vials was done, whereas in the present study all vials were agitated in a constant manner throughout. Therefore, since all particles had equal opportunity to make contact with PMN, this would account for the longer time, but similar response height, with decreasing *E. coli* concentrations. It is also possible that the increase in number of particles causes a greater number of cell-particle interactions. The variability in cell wall composition of *E. coli* may contribute to the variable response patterns of different strains.

A second significant factor in the CL response is the effect of temperature. Many authors report the use of "ambient" temperature in CL methodology. This no doubt creates major differences in results, since ambient temperature will differ from laboratory to laboratory. It is usual for the temperature of most liquid scintillation counters to slowly rise during the assay period, especially under the conditions used in CL assays. We showed considerable differences in response over a temperature range of 25 to 37°C, with the increase in slope being the most dramatic effect. A similar effect has been previously shown for zymosan-stimulated PMN CL (13). Increasing the temperature from 25 to 37°C reduced the time to peak by half and increased the peak height by an average of 80% for the organisms tested. Thus, considerably greater phagocytosis and opsonization occurred at the higher temperature, as expected. The considerable differences in CL responses over this temperature range indicates the need to carefully regulate vial temperatures.

There are interesting similarities between our data and those of Barbour et al. (7), in which PMN from patients with active bacterial infection were shown to have elevated CL responses when activated by zymosan. The range of organisms isolated from patients used in that study was similar to that reported here. They also reported a wide range of peak CL responses. Although Barbour et al. only reported CL responses to zymosan, the differences were attributed to the presence of the infective organism, with the possibility that bacterial factors were significant in the alteration of the responsiveness of PMN. Our data indicate that each microorganism is capable of PMN activation and that the response pattern produced may be related to the characteristics of the microorganism. There may well be a relationship between the type of response pattern and the severity of infection.

It is clear that although the CL response to one species of bacteria is reproducible with different PMN, differences may also exist between serotypes. These differences may be due to organism membrane composition, subpopulations of PMN responding to specific bacterial components, variations in avidity binding of cell receptors, opsonic factors, and serum-dependent inhibition. Factors such as assay conditions, agitation, temperature, and particle/cell ratios must also be considered. By considering all of these factors, it may be possible to more easily identify more pathogenic strains of organisms by their CL response profiles, enabling an earlier resolution to severe bacterial infections.

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