INFECTION AND IMMUNITY, June 1986, p. 657–663
0019-9567/86/060657-07$02.00/0

Vol. 52, No. 3

Effects of Human Serum on Bacterial Competition with Neutrophils for Molecular Oxygen

BRADLEY E. BRITIGAN[1] AND MYRON S. COHEN[1,2]*

Department of Medicine[1] and Department of Microbiology and Immunology,[2] University of North Carolina, Chapel Hill, North Carolina 27514

Received 16 December 1985/Accepted 21 February 1986

A dialyzable factor(s) in human serum is known to stimulate gonococcal oxygen consumption. Its effect on other human pathogens was investigated. A 10% serum solution increased peak O_2 consumption for *Escherichia coli* and *Staphylococcus aureus* to 157% ($P < 0.05$) and 199% ($P < 0.02$), respectively, of their O_2 consumption when suspended in Hanks balanced salt solution, compared with a 356% increase for *Neisseria gonorrhoeae* with serum. Dialyzed serum lacked stimulatory capacity. Bacteria, serum, and neutrophils are often incubated to evaluate neutrophil bactericidal activity. Samples of 10^8 *N. gonorrhoeae*, *S. aureus*, and *E. coli* turned resazurin colorless (anaerobic conditions, $E_h < -42$ mV) after 7.4, 13.3, and 15.1 min, respectively. Because neutrophil formation of reactive oxygen intermediates requires ambient O_2, the effect of live bacteria and serum on this process was explored. After 5 min of incubation of 10^8 *N. gonorrhoeae* or *S. aureus* in 10% normal or dialyzed serum, 10^5 neutrophils were added. Phorbol myristate acetate was then added to assure neutrophil stimulation, and luminol-dependent luminescence was measured. *N. gonorrhoeae* and *S. aureus* incubation in normal serum decreased peak LDL 91.7 and 88.6%, respectively, relative to incubation in dialyzed serum. A sample of 10^8 *E. coli* totally eliminated LDL. A sample of 10^8 *E. coli* incubated in Hanks balanced salt solution for 5 min also eliminated phorbol myristate acetate induced neutrophil H_2O_2 production. LDL inhibition increased in proportion to bacterial concentration and time of incubation and was prevented by inclusion of KCN. Increasing the concentration of neutrophils to 10^8 (1:1 particle-to-cell ratio) only partially reversed LDL inhibition. Re-aeration of the system allowed brief LDL which persisted only if KCN was added. Addition of KCN after bacterial incubation also permitted LDL, arguing against depletion of other factors from the media or accumulation of bacterially derived inhibitory substances. A dynamic competition for O_2 occurs between bacteria and neutrophils. Serum stimulation of bacterial O_2 utilization may contribute to virulence by increasing bacterial capacity to inhibit neutrophil function.

After membrane stimulation, neutrophils consume large quantities of ambient O_2 and subsequently generate reactive oxygen intermediates (2, 24). These products are critical for neutrophil-mediated killing of some bacterial species (17, 19). Previous investigators have reported a decrease in neutrophil formation of reactive oxygen intermediates under anaerobic conditions (12–14) that are similar to conditions described for experimental abscesses (16). Depletion of local oxygen by aerobic bacteria has been proposed as contributing to the pathogenesis of abscess formation both by interfering with neutrophil oxygen-dependent microbicidal activity and by creating a microenvironment capable of supporting the growth of obligate anaerobes (reviewed in reference 23).

Neisseria gonorrhoeae undergoes a marked increase in metabolic rate, including a fourfold increase in O_2 consumption, after exposure to a heat-stable dialyzable component(s) of human serum (6). Vaginal mucosal secretions and ascitic fluid exhibit a similar stimulatory capacity (5). This stimulatory factor(s) may allow gonococci to rapidly consume sufficient oxygen to compromise local neutrophil function. The abilities of other pathogens to increase their metabolic rates in response to this stimulatory factor(s) could have broader implications for understanding bacterial pathogenesis.

We therefore examined the effect of serum on the oxygen metabolism of two common human pathogens, *Staphylococcus aureus* and *Escherichia coli*, and compared the results with those seen with *N. gonorrhoeae*. By use of luminol-dependent luminescence (LDL) as a measure of neutrophil oxygen metabolism and formation of reactive oxygen intermediates, the effect of bacterial metabolism on this process was explored. Our results demonstrate that bacteria compete with neutrophils for O_2 in vitro. Exposure to serum increased bacterial metabolism sufficiently to lower the bacterial concentration required to interfere with the neutrophil functions examined.

(Part of this research was presented previously [B. E. Britigan and M. S. Cohen, Program Abstr. 25th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 12, 1985].)

MATERIALS AND METHODS

Preparation of bacteria. *N. gonorrhoeae* FA19, *S. aureus* ATCC 502A, and *E. coli* 026622 (a blood culture isolate provided by the Clinical Microbiology Laboratory, North Carolina Memorial Hospital, Chapel Hill), were maintained by daily subculture on GCB agar (GC Medium Base; Difco Laboratories, Detroit, Mich.) containing 1 and 0.5% Kellogg defined supplements 1 and 2, respectively, as described previously (18). FA19 is a well-characterized laboratory strain of *N. gonorrhoeae*, originally derived from a clinical isolate (8, 26). A nonpiliated opaque isolate (28) of FA19 was used in all experiments with *N. gonorrhoeae*. All three bacterial species were serum resistant (25).

For all experiments, 16- to 24-h-old GCB agar colonies were scraped into Hanks balanced salt solution (HBSS) to a final concentration of 10^9 organisms per ml as determined by turbidity in a Klett-Summerson colorimeter (Klett Manufac-

* Corresponding author.

TABLE 1. Effect of serum on bacterial oxygen consumption

Organism	Mean peak oxygen consumption ± SEM[a] (nmol/min) in buffer system[b]		
	HBSS	HBSS + NS[c]	HBSS + DS
E. coli	53.9 ± 3.8	85.0 ± 7.9	56.0 ± 1.6
S. aureus	18.0 ± 1.5	36.0 ± 4.2	17.7 ± 1.6
N. gonorrhoeae	16.6 ± 2.4	57.0 ± 6.7	18.3 ± 3.1

[a] $n = 3$ or 4.
[b] NS, 10% normal serum; DS, 10% dialyzed serum.
[c] Significant increases relative to the controls: *E. coli*, $P < 0.05$; *S. aureus*, $P < 0.02$; *N. gonorrhoeae*, $P < 0.01$.

turing Inc., New York, N.Y.); turbidity had been previously correlated with direct enumeration or CFUs (4).

Serum preparation. Serum from five to eight normal donors lacking histories of *N. gonorrhoeae*, *S. aureus*, or *E. coli* infection was pooled, filtered, and frozen at −70°C. Some serum was thawed and dialyzed overnight (dialyzed serum) in 3,500-molecular-weight membrane exclusion tubing (Spectra/Por 6; Spectrum Medical Industries, Los Angeles, Calif.) against 2 liters of HBSS (6).

Bacterial oxygen consumption. O_2 consumption was measured in a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) with a 1-ml volume containing 10^8 bacteria (6). Results were expressed as the maximal O_2 consumption rate observed (nanomoles per minute).

Determination of anaerobic conditions. Samples of 10^8 bacteria were added to glass tubes (12 by 75 mm) containing 10% normal or dialyzed serum and 185 ng of resazurin (Sigma Chemical Co., St. Louis, Mo.) in 1 ml of HBSS. Tubes were stoppered, placed vertically in a 37°C water bath (72 strokes per min), and examined at 1-min intervals. Experiments were performed in triplicate, and results were expressed as the mean time necessary for resazurin to become colorless, indicating an oxidation-reduction potential (E_h) of < -42 mV. Falcon tubes (12 by 75 mm, polypropylene; Becton Dickinson Labware, Oxnard, Calif.) could not be used because of their interference with the sensitivity of the resazurin indicator.

Neutrophil separation. Whole blood from normal human donors was obtained in heparinized syringes. Neutrophils were separated from other cellular components by using Plasmagel (Roger Bellon, Neuilley, France) and Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, N.J.) sedimentation with subsequent osmotic lysis of contaminating erythrocytes (6). Neutrophils were suspended in HBSS to a concentration of 10^7/ml as determined by a model D2N automated blood cell counter (Coulter Electronics, Inc., Hialeah, Fla.). Giemsa stain revealed that >98% of the cells were neutrophils and viability was >95% by trypan blue dye exclusion.

Neutrophil LDL. Bacteria were added to Eppendorf tubes (1.5-ml capacity) containing 10% normal pooled or dialyzed serum, 10^{-4} M luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma), and 0.5% bovine serum albumin (Sigma) in HBSS to a final concentration of 10^6 to 10^8 organisms per ml (volume, 1.5 ml). Tops were closed, and LDL was determined in an integrating photometer model 3000 (SAI Technology Co., San Diego, Calif.) at 31°C (ambient temperature of the photometer [15]). After defined time periods, tubes were removed and 15 μl of neutrophils (10^7/ml of HBSS) and 15 μl of phorbol myristate acetate (PMA) (10 μg/ml of dimethyl sulfoxide; Midland Chemical, Brewster, N.Y.) added. Only neutrophils were added to tubes containing *S. aureus*. Tubes were returned to the photometer, and LDL was measured. Controls experiments were performed in the absence of serum or bacteria or both. Because of the presence of bovine serum albumin, serum, and bacteria, the chemiluminescence detection system used in these experiments was more complex than systems used to characterize the chemistry of neutrophil light emission (1, 9, 11, 15, 27, 30); the system used here appears to indirectly reflect formation of superoxide, hypochlorous acid, and perhaps other reactive oxygen intermediates as well (unpublished observation). KCN (Sigma) was added to some tubes (final concentration, 1 mM) to inhibit bacterial O_2 consumption. By use of this chemiluminescence detection system in the absence of bacteria, it was observed that 1 mM KCN inhibited PMA-stimulated neutrophil LDL by 30% (unpublished observation). Results were expressed as the maximal LDL measured and always occurred in 10 min or less.

Measurement of neutrophil hydrogen peroxide release. The rate of neutrophil hydrogen peroxide (H_2O_2) release was measured as previously described (21). Briefly, 4 μM scopoletin (Sigma) and 22 nM horseradish peroxidase (Sigma) in 2.5 ml of HBSS was placed in a Perkin-Elmer fluorescence spectrophotometer (model MPF-3L; Perkin-Elmer Corp., Mountain View, Calif.) and maintained at 37°C by a circulating water-bath apparatus. After a 5-min incubation, 25 μl of neutrophils (2.5×10^8/ml) and 25 μl of PMA (10 μg/ml of dimethyl sulfoxide) were added. H_2O_2 production was measured as the reduction in emission fluorescence at 460 nm (recorded continuously) with a 350-nm activating light source.

In experiments containing *E. coli*, suspensions were inoculated with sufficient organisms to achieve a final concentration of 10^8 bacteria per ml. *E. coli* by themselves had no effect on recorded fluorescence. As previously reported (21), serum inhibits scopoletin oxidation precluding evaluation of serum stimulation of bacterial metabolism on neutrophil H_2O_2 generation.

Statistical analysis. Paired or unpaired Student's *t* tests were used for all statistical analysis. Results were considered significant at $P < 0.05$. Although for the purpose of the presentation, data may be expressed as a percentage of appropriate control, only original (raw) data were used for statistical comparisons.

RESULTS

Serum effect on *N. gonorrhoeae*, *S. aureus*, and *E. coli* oxygen consumption. The ability of a low-molecular-weight (dialyzable) factor(s) in human serum and other body fluids to stimulate bacterial metabolism has been previously demonstrated for *N. gonorrhoeae* (3, 5, 6). Because the capacity of this factor(s) to stimulate metabolism of other bacterial pathogens was unknown, we examined O_2 consumption rates of *S. aureus* and *E. coli* following exposure to human serum and compared them with O_2 consumption rates for *N. gonorrhoeae*. Samples of 10^8 plate-scraped organisms were placed in the 1-ml chamber of a Clark O_2 electrode, and peak O_2 consumption rates were measured while the samples were suspended in HBSS, HBSS plus 10% pooled serum, or HBSS plus 10% dialyzed serum (Table 1 and Fig. 1A to C). The O_2 consumption rate in HBSS was much greater for *E. coli* than it was for *S. aureus* or *N. gonorrhoeae* (Table 1 and Fig. 1A to C). A 10% serum solution increased peak O_2 consumption rates of *E. coli*, *S. aureus*, and *N. gonorrhoeae* to 157 ± 10, 199 ± 11, and 356 ± 90%, respectively, of the peak oxygen consumption rates of these organisms in HBSS

(Table 1 and Fig. 1A to C). Dialyzed serum showed no stimulatory effect for any of the three organisms (Table 1 and Fig. 1A to C). The increase in bacterial oxygen consumption required the continued presence of serum; organisms which had been incubated in serum for 30 min, washed, and resuspended in HBSS (i.e., opsonization) exhibited oxygen consumption rates identical to those of the controls (data not shown). Opsonized bacteria remained capable of enhanced metabolism when reexposed to a fresh serum source (data not shown). The three serum-stimulated organisms appeared to consume nearly all O_2 available in the 10% serum-buffer solution over 5 to 10 min (Fig. 1A to C).

Bacterial production of an anaerobic environment. Resazurin has been used in microbiological systems as an anaerobic indicator (19); it becomes colorless when the E_h is decreased to <-42 mV, suggesting low oxygen availability. Using this indicator, we examined the effect of serum-stimulated *N. gonorrhoeae*, *S. aureus*, and *E. coli* on the E_h of buffer in glass tubes (12 by 75 mm) (4-ml column of air above) when incubated vertically in a 37°C shaker water bath.

In the presence of 10% normal serum, *N. gonorrhoeae*, *S. aureus*, and *E. coli* at concentrations of 10^8/ml turned the resazurin colorless after 7.4 ± 0.5, 13.3 ± 1.9, and 15.1 ± 1.3 min (mean ± standard error of the mean, $n = 3$), respectively (Table 2). A small rim of color remained at the air-liquid interface. Neither *N. gonorrhoeae* nor *S. aureus* in 10% dialyzed serum turned resazurin colorless (Table 2). Suspension of *E. coli* in 10% dialyzed serum led to a disappearance of color after 17.2 ± 1.2 min (Table 2). The pH at the completion of incubation varied from 7.07 to 7.16 (dialyzed serum) and 7.28 to 7.44 (normal serum).

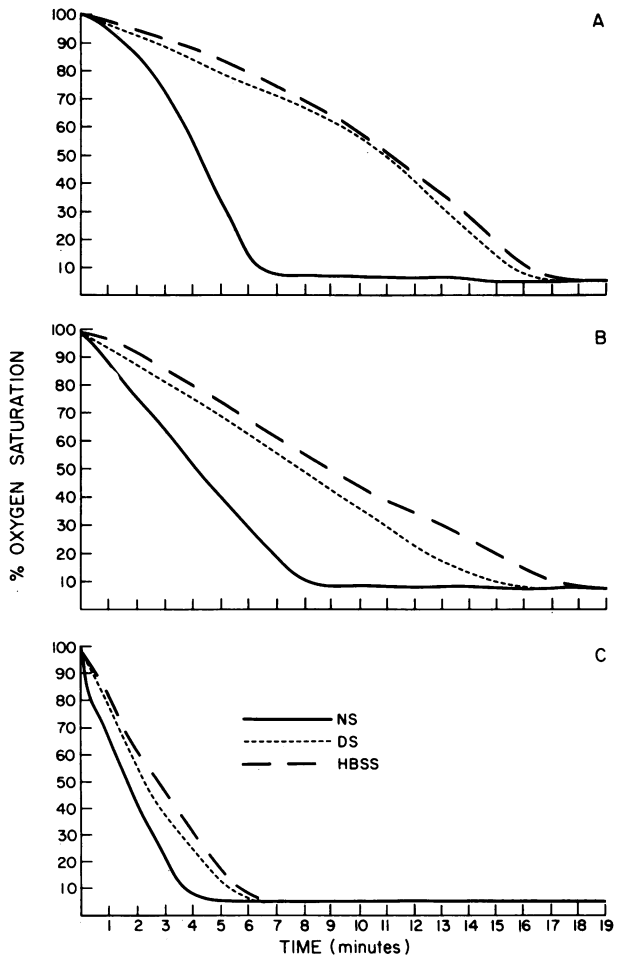

FIG. 1. Representative Clark oxygen electrode tracings from three to four determinations of oxygen consumption rates for *N. gonorrhoeae* (A), *S. aureus* (B), and *E. coli* (C) suspended in HBSS, HBSS plus 10% dialyzed serum (DS), or HBSS plus 10% normal serum (NS).

TABLE 2. Ability of bacteria to create an anaerobic environment

Organism	Mean time (min) ± SEM[a] until resazurin colorless in 10% serum:	
	Normal	Dialyzed
N. gonorrhoeae	7.4 ± 0.5	No change[b]
S. aureus	13.3 ± 1.9	No change[b]
E. coli	15.1 ± 1.3	17.2 ± 1.2

[a] $n = 3$.
[b] Neither *N. gonorrhoeae* nor *S. aureus* turned the resazurin colorless during 30 min of observation.

The conditions of incubation determined the buffer E_h at the completion of bacterial incubation. After use of a system in which bacteria and serum were tumbled end-over-end or shaken at a 30° angle with 240 strokes per min, the indicator remained colored ($E_h > -42$ mV). However, when the tubes were shaken vertically at 240 strokes per min or at 30° with 72 strokes per min E_h approached -42 mV.

Bacterial inhibition of neutrophil chemiluminescence. In view of the above observations, we explored the possibility that serum stimulation of microbial metabolism might produce an environment which would interfere with formation of reactive oxygen intermediates by neutrophils. In the presence of luminol, membrane stimulation of these cells by soluble or particulate stimuli resulting in O_2 consumption leads to generation of visible light (1, 9, 27). *N. gonorrhoeae*, *S. aureus*, or *E. coli* (10^8/ml) was incubated in either 10% normal or dialyzed serum; neutrophils (10^5/ml) and PMA were then added, and neutrophil LDL was measured. PMA was added to assure neutrophil stimulation (10, 20, 30) beyond that mediated by interaction with bacteria. Because of the inclusion in the reaction mixture of bovine serum albumin, serum, and bacteria, the light emission observed is more difficult to ascribe to a particular reactive oxygen intermediate(s) than LDL measured by others (1, 9, 11–13, 15, 22, 27, 30). This system is less dependent on neutrophil myeloperoxidase activity for luminescence than luminol-dependent systems are (9; unpublished observations), because luminol-independent luminescence occurs in the presence of protein substrates (30). Stimulation in response to *S. aureus* was equivalent to that of PMA, and therefore PMA was not added in experiments in which these bacteria were used.

LDL was not detected in the absence of neutrophils (data not shown). Peak neutrophil LDL was reduced 91.7 ± 2.4% in *N. gonorrhoeae* and 88.6 ± 2.0% in *S. aureus* ($n = 4$, $P < 0.05$) following a 5-min incubation of 10^8 organisms in 10% normal serum as compared with the LDL of the same organisms in 10% dialyzed serum (Fig. 2). *E. coli* (10^8) totally suppressed LDL whether preincubated in 10% normal or dialyzed serum, while 10^7 *E. coli* in 10% normal serum reduced LDL 52.7 ± 4.0% ($n = 3$, $P < 0.05$) relative to the same concentration of organisms in 10% dialyzed serum. Decrease in LDL was proportional to the concentration of

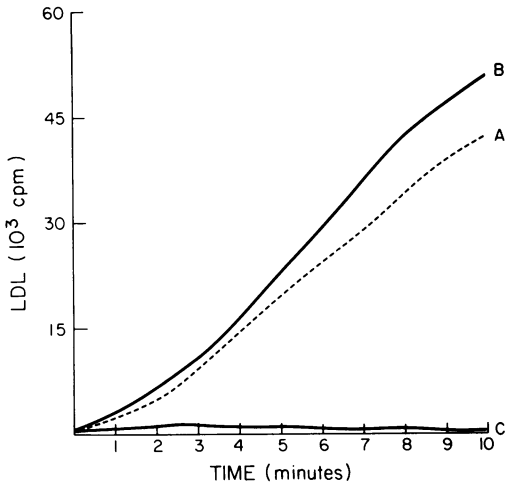

FIG. 2. Representative tracings of LDL taken directly from the strip chart recorder attached to the luminometer (n = 3) after stimulation of neutrophils (10^5/ml) by PMA while suspended in HBSS (A), HBSS plus 10% dialyzed serum containing preincubated *N. gonorrhoeae* (10^8/ml) and PMA (B), and HBSS plus 10% normal serum containing preincubated *N. gonorrhoeae* (10^8/ml) and PMA (C). Time zero is the time of neutrophil and PMA addition. Similar results were obtained with *S. aureus* (10^8/ml, n = 3).

bacteria present and independent of the particle-to-cell ratio or number of neutrophils added. Inhibition of LDL was consistently seen at bacterial concentrations of 10^7 or greater (Fig. 3). Increasing the concentration of neutrophils to 10^8/ml (1:1 bacteria-to-neutrophil ratio) in the presence of 10^8 *N. gonorrhoeae* or *E. coli* did not prevent bacteria-mediated inhibition of LDL (data not shown).

Time of bacterial incubation in serum also affected subsequent neutrophil LDL. Increase in bacterial incubation time from 0 to 5 min resulted in progressively greater inhibition of LDL (Fig. 4). When neutrophils, bacteria, and PMA were added simultaneously, LDL increased for 3 min but reached a premature plateau (Fig. 4).

Although these data suggested a decrease in neutrophil LDL secondary to bacterial utilization of O_2, other possible explanations were examined. Addition of 10% serum to HBSS in the absence of bacteria reduced neutrophil LDL in response to PMA to 39.1 ± 6.9% of the control (mean ± standard error of the mean, n = 5). Slightly less reduction was seen when dialyzed serum was substituted (42.9 ± 9% of control, n = 5), but this was insufficient to account for our results. Bacterial metabolism of luminol to a form incapable of luminescence could also lead to similar results if this phenomenon were greater in normal than in dialyzed serum. However, addition of 10^{-3} M luminol (10 times the required concentration) at the same time as neutrophils and PMA failed to alter bacterial inhibition of LDL (Fig. 5). Under the conditions in these experiments, buffer pH decreased to 6.8 after bacterial incubation in normal serum (data not shown). However, neutrophils suspended in HBSS at this pH exhibited an LDL of 87.3 ± 5.4% of the control (mean ± standard error of the mean, n = 3, $P > 0.05$).

Addition of KCN to the mixture markedly interfered with the capacity of bacteria to inhibit LDL (Fig. 5), indicating the importance of ongoing bacterial metabolism to this process. Vigorous buffer agitation immediately before neutrophil addition resulted in a brief burst of LDL (Fig. 5), but this could only be maintained in the presence of KCN (Fig. 5). Addition of KCN to the system after bacterial preincubation allowed LDL to occur at a rate equivalent to that during reaeration in the presence of KCN (Fig. 5).

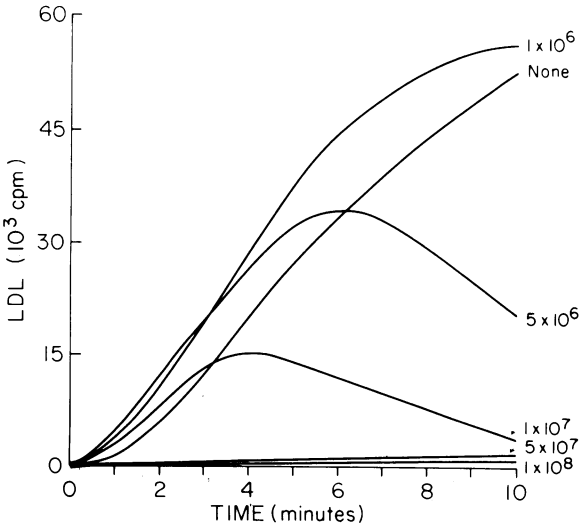

FIG. 3. Tracings demonstrating the effect of *N. gonorrhoeae* concentration on LDL inhibition. Tracings represent LDL after PMA stimulation of 10^5 neutrophils suspended in HBSS plus 10% normal serum in which various concentrations of *N. gonorrhoeae* (denoted to the right of each tracing) had incubated at 37°C for 5 min before the addition of neutrophils (n = 2). LDL in the same buffer but in the absence of bacteria (designated none) served as a control. Similar results were obtained with *E. coli* (n = 3), although approximately 0.5 log fewer organisms were required for a similar degree of LDL inhibition.

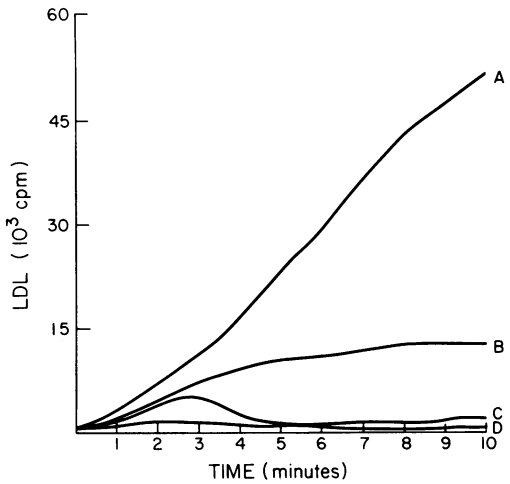

FIG. 4. Tracings demonstrating the effect of the duration of bacterial incubation in 10% normal serum (before neutrophil addition) on subsequent inhibition of LDL generation (n = 5). These tracings show LDL after PMA stimulation of neutrophils (10^5/ml) suspended in HBSS plus 10% dialyzed serum containing *N. gonorrhoeae* (10^8/ml) which had incubated for 5 min (A), or HBSS plus 10% normal serum containing *N. gonorrhoeae* (10^8/ml) which had incubated for 0 (B), 1 (C), or 5 (D) min before addition of neutrophils. Time zero is the time of addition of neutrophils and PMA. Data were similar regardless of bacterial strain employed.

However, LDL failed to reach the level seen when KCN was present throughout the period of bacterial preincubation (Fig. 5).

Bacterial inhibition of neutrophil hydrogen peroxide production. Since LDL provides only indirect evidence of neutrophil formation of reactive oxygen intermediates (1, 9), we undertook direct measurement of the effect of bacterial metabolism on generation of O_2 reduction products. Superoxide release by neutrophils could not be tested, because all three species of bacteria reduced ferricytochrome *c* (7) by a superoxide-dismutase-resistant mechanism (data not shown). As an alternative, neutrophil H_2O_2 production was measured (21). Exogenous serum markedly reduces the sensitivity of the system we employed (21). Since 10^8 *E. coli* per ml inhibited LDL independent of the presence of the serum metabolic stimulating factor(s), the effect of this concentration of *E. coli* suspended in HBSS alone on neutrophil H_2O_2 production was determined (Fig. 6). In the absence of bacteria, neutrophil (2.5×10^6/ml) stimulation by PMA resulted in sustained secretion of H_2O_2 (Fig. 6). When neutrophils were added to a suspension of *E. coli* which had been allowed to preincubate for 5 min, no neutrophil H_2O_2 was detected (Fig. 6). Simultaneous addition of *E. coli*, neutrophils, and PMA led to initial H_2O_2 release equivalent to the control which then ceased after 2.5 min (Fig. 6).

DISCUSSION

N. gonorrhoeae develop a marked burst in oxygen consumption in vitro following exposure to a heat-stable dialyzable component(s) of human serum (6) and other body fluids (5). Our data indicate that oxygen consumption by *S. aureus* and *E. coli* is also stimulated in response to a similar serum factor(s), although to a lesser degree. This variation may reflect differences in the base-line O_2 consumption rate of these organisms. The identity and mechanism of action of the serum factor(s) responsible for this stimulation are currently being investigated (3).

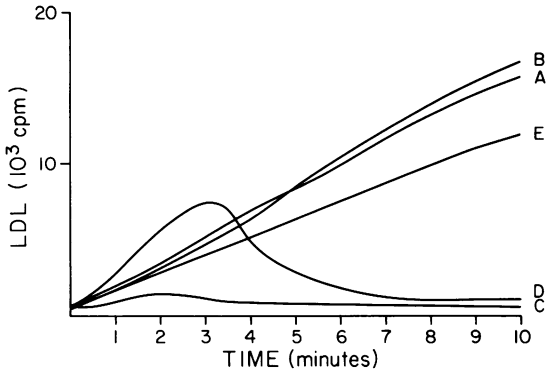

FIG. 5. Tracings illustrating the effect of KCN or reaeration of the system on bacterial inhibition of LDL ($n = 6$). Neutrophils (10^5/ml) and PMA were added to HBSS plus 10% normal serum (A) or HBSS plus 10% normal serum following 5 min preincubation of *E. coli* (10^8/ml) in the presence (B) or absence (C) of 1 mM KCN. Addition of 10^{-3} M luminol to the system immediately after the addition of neutrophils resulted in a curve identical to tracing C. Conditions for tracing D were identical to those for tracing C except the system was reaerated at the completion of bacterial preincubation. Tracing E was generated in a manner identical to tracing D except that 1 mM KCN was added at the time of reaeration. Addition of KCN after bacterial preincubation without reaerating the system led to a tracing identical to tracing E. In the absence of bacteria, 1 mM KCN led to a 30% decrease in LDL relative to that shown by tracing A, perhaps because of KCN-mediated inhibition of neutrophil myeloperoxidase activity. Time zero is the time of addition of neutrophils and PMA. Similar results occurred with addition of 10^8 *N. gonorrhoeae* or *S. aureus*.

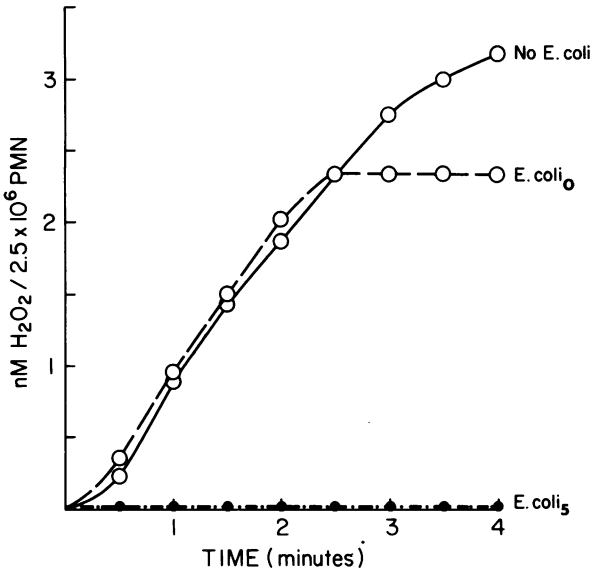

FIG. 6. Tracings ($n = 2$) of mean H_2O_2 production by 2.5×10^6 neutrophils after PMA (100 nM) stimulation while suspended in HBSS (no *E. coli*); HBSS containing *E. coli* (10^8/ml) which had been incubated for 5 min at 37°C before neutrophil and PMA addition (*E. coli*$_5$); and HBSS in which *E. coli* (10^8/ml) were added at the same time as neutrophils and PMA (*E. coli*$_0$).

The serum-induced increase in the metabolism of several human pathogens has potential implications for in vitro evaluation of host defenses. Several experimental techniques employed to assess neutrophil bactericidal activity involve the simultaneous incubation of neutrophils, live bacteria, and serum. In the presence of normal serum, all three organisms that we examined established an anaerobic environment as reflected by the indicator resazurin ($E_h < -42$ mV). It is unclear why the oxygen consumption rates exhibited by these organisms did not correlate with the times required to turn resazurin colorless. However, since resazurin measures E_h and not O_2 concentration, it seems possible that bacterial factors other than those associated with oxygen depletion contributed to the low E_h observed. Although our experiments differed in some aspects of experimental design from systems used to investigate phagocyte bactericidal activity, these data emphasize the need to continuously aerate suspensions containing serum and live bacteria to assure the maintenance of aerobic conditions.

Measurement of LDL has been employed as an indirect means to detect formation of reactive oxygen intermediates by neutrophils (1, 9, 11–13, 15, 22, 27, 30). In the system we used, light emission by neutrophils was stimulated by either bacteria or PMA. The exact chemistry of the reaction leading to light formation was not explored. Nevertheless, when exposed to normal serum, all three microorganisms that we examined rapidly created an environment in which neutrophil LDL was suppressed. As is consistent with an earlier report (27), neither serum nor bacterial interference with luminol oxidation could be shown to account for these data. In addition, these observations were confirmed by demonstrating that metabolically active bacteria inhibited the ability of neutrophils to produce H_2O_2. Change in pH,

depletion of glucose, or accumulation of a bacterially derived inhibitory substance (22) could not be implicated. Instead, these data suggest that bacteria and neutrophils are in direct competition for available oxygen. When bacteria were in excess of neutrophils, bacteria exhibited a clear superiority in this contest. Even when the number of neutrophils and bacteria was the same (10^8/ml), bacterial oxygen consumption was still sufficient to markedly alter neutrophil LDL. Although the concentration of bacteria (10^7 to 10^8/ml) used in these experiments is large relative to that used in many in vitro systems, it is in keeping with conditions observed in many closed-space infections such as intra-abdominal abscesses.

Edwards et al. (12) have shown that neutrophil LDL begins to decrease at an O_2 concentration of <120 μM and is $<20\%$ of normal at an O_2 concentration of <10 μM. Although the O_2 concentration of our LDL system could not be measured, serum-stimulated bacterial metabolism led to an O_2 concentration in the Clark oxygen electrode of $<10\%$ (24 μM, Fig. 1). Thus the inhibition of LDL we observed would be predicted by this earlier study (12). However, Gabig et al. (14) reported that neutrophil superoxide production does not decrease until O_2 concentration is reduced to $<1\%$ ($\sim$2.4 μM). Although direct bacterial reduction of ferricytochrome *c* made evaluation of neutrophil superoxide production impossible, bacterially mediated inhibition of H_2O_2 generation was similar to inhibition of LDL. It does not seem likely that these reactive oxygen intermediates require different O_2 availability.

Formation of reactive oxygen intermediates by human neutrophils is critical to the microbicidal activity of these cells against many human pathogens as reflected by in vitro studies (17, 19) and the recurrent infections experienced by individuals whose cells lack this capacity (e.g., chronic granulomatous disease of childhood) (29). The ability of several different human pathogens to generate an environment which could negate oxygen-dependent neutrophil-killing mechanisms suggests the potential importance of this observation to bacterial pathogenesis, particularly in infections involving closed spaces or other areas of limited oxygen availability (16, 23). In addition, the factor(s) in serum and other body secretions which stimulates microbial metabolism may decrease the time and number of bacteria necessary to create such an environment, leading to an increase in bacterial virulence.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI15036-07 and 2T32AI07001-09 from the National Institutes of Health. B.E.B. is a recipient of the Burroughs Welcome Fellowship of the Infectious Disease Society of America.

We thank P. Frederick Sparling for his critical review of the manuscript and acknowledge the technical assistance of Martha French. Ronda Terrel and Belinda Wilson assisted in preparation of the manuscript.

LITERATURE CITED

1. **Allen, R. C., and L. D. Loose.** 1976. Phagocytic activation of a luminol-dependent chemiluminescence in rabbit alveolar and peritoneal macrophages. Biochem. Biophys. Res. Commun. **69:** 245–252.
2. **Baldridge, C. W., and R. W. Gerard.** 1933. The extra respiration of phagocytosis. Am. J. Physiol. **103:**235–236.
3. **Britigan, B. E., Y. Chai, and M. S. Cohen.** 1985. Effects of human serum on the growth and metabolism of *Neisseria gonorrhoeae*: an alternative view of serum. Infect. Immun. **50:** 738–744.
4. **Britigan, B. E., and M. S. Cohen.** 1985. Growth in human serum inhibits uptake of *Neisseria gonorrhoeae* by human neutrophils. J. Infect. Dis. **152:**330–338.
5. **Cohen, M. S., J. R. Black, R. A. Proctor, and P. F. Sparling.** 1985. Host defenses and the vaginal mucosa. Scand. J. Urol. Nephrol. **86**(Suppl.):13–22.
6. **Cohen, M. S., and M. H. Cooney.** 1984. A bacterial respiratory burst: stimulation of the metabolism of *Neisseria gonorrhoeae* by human serum. J. Infect. Dis. **150:**49–56.
7. **Cohen, M. S., J. A. Metcalf, and R. K. Root.** 1980. Regulation of oxygen metabolism in human granulocytes: relationship between stimulus binding and oxidative response using plant lectins as probes. Blood **55:**1003–1009.
8. **Daly, J. A., T. J. Lee, J. K. Spitznagel, and P. F. Sparling.** 1982. Gonococci with mutations to low-level penicillin resistance exhibit increased sensitivity to the oxygen-independent bactericidal activity of human polymorphonuclear leukocyte granule extracts. Infect. Immun. **35:**826–833.
9. **DeChatelet, L. R., G. D. Long, P. S. Shirley, D. A. Bass, M. J. Thomas, F. W. Henderson, and M. S. Cohen.** 1982. Mechanism of the luminol-dependent chemiluminescence of human neutrophils. J. Immunol. **129:**1589–1593.
10. **DeChatelet, L. R., P. S. Shirley, and R. B. Johnston, Jr.** 1976. Effect of phorbol myristate acetate on the oxidative metabolism of polymorphonuclear leukocytes. Blood **47:**545–554.
11. **Easmon, C. S. F., P. J. Cole, A. J. Williams, and M. Hastings.** 1980. The measurement of opsonic and phagocytic function by luminol-dependent chemiluminescence. Immunology **41:**67–74.
12. **Edwards, S. W., M. B. Hallett, and A. K. Campbell.** 1984. Oxygen-radical production during inflammation may be limited by oxygen concentration. Biochem. J. **217:**851–854.
13. **Fazeli, A., and L. Richards.** 1984. Chemiluminescence induced by phagocytosis of *Escherichia coli* by polymorphonuclear leucocytes. J. Gen. Microbiol. **130:**2267–2276.
14. **Gabig, T. G., S. I. Bearman, and B. M. Babior.** 1979. Effects of oxygen tension and pH on the respiratory burst of human neutrophils. Blood **53:**1133–1139.
15. **Hatch, G. E., D. E. Gardner, and D. B. Menzel.** 1978. Chemiluminescence of phagocytic cells caused by *N*-formylmethionyl peptides. J. Exp. Med. **147:**182–195.
16. **Hayes, R. C., and G. L. Mandell.** 1974. pO_2, pH, and redox potential of experimental abscesses. Proc. Soc. Exp. Biol. Med. **147:**29–30.
17. **Ingham, H. R., P. R. Sisson, R. L. Middleton, H. K. Narang, A. A. Codd, and J. B. Selkon.** 1981. Phagocytosis and killing of bacteria in aerobic and anaerobic conditions. J. Med. Microbiol. **14:**391–399.
18. **Kellogg, D. S., Jr., W. L. Peacock, Jr., W. E. Deacon, L. Brown, and C. I. Pirkle.** 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. J. Bacteriol. **85:**1274–1279.
19. **Mandell, G. L.** 1974. Bactericidal activity of aerobic and anaerobic polymorphonuclear neutrophils. Infect. Immun. **9:**337–341.
20. **Repine, J. E., J. G. White, C. C. Clawson, and B. M. Holmes.** 1974. The influence of phorbol myristate acetate on oxygen consumption by polymorphonuclear leukocytes. J. Lab. Clin. Med. **83:**911–920.
21. **Root, R. K., J. Metcalf, N. Oshino, and B. Chance.** 1975. H_2O_2 release from human granulocytes during phagocytosis. 1. Documentation, quantitation, and some regulating factors. J. Clin. Invest. **55:**945–955.
22. **Rotstein, O. D., T. L. Pruett, V. D. Fiegel, R. D. Nelson, and R. L. Simmons.** 1985. Succinic acid, a metabolic by-product of *Bacteroides* species, inhibits polymorphonuclear leukocyte function. Infect. Immun. **48:**402–408.
23. **Rotstein, O. D., T. L. Pruett, and R. L. Simmons.** 1985. Mechanisms of microbial synergy in polymicrobial surgical infections. Rev. Infect. Dis. **7:**151–170.
24. **Sbara, A. J., and M. L. Karnovsky.** 1959. The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. J. Biol. Chem. **234:**1355–1362.

25. **Shafer, W. M., K. Joiner, L. F. Guymon, M. S. Cohen, and P. F. Sparling.** 1984. Serum sensitivity of *Neisseria gonorrhoeae*: the role of lipopolysaccharide. J. Infect. Dis. **149**:175–183.
26. **Sparling, P. F., F. A. Sarubbi, Jr., and E. Blackman.** 1975. Inheritance of low-level resistance to penicillin, tetracycline, and chloramphenicol in *Neisseria gonorrhoeae*. J. Bacteriol. **124**:740–749.
27. **Stevens, P., D. J. Winston, and K. van Dyke.** 1978. In vitro evaluation of opsonic and cellular granulocyte function by luminol-dependent chemiluminescence: utility in patients with severe neutropenia and cellular deficiency states. Infect. Immun. **22**:41–51.
28. **Swanson, J.** 1978. Studies on the gonococcus infection. XII. Colony color and opacity variants of gonococci. Infect. Immun. **19**:320–331.
29. **Tauber, A. I., N. Borregaard, E. Simons, and J. Wright.** 1983. Chronic granulomatous disease: a syndrome of phagocyte oxidase deficiencies. Medicine (Baltimore) **62**:286–309.
30. **Westrick, M. A., P. S. Shirley, and L. R. DeChatalet.** 1980. Generation of chemiluminescence by human neutrophils exposed to soluble stimuli of oxidative metabolism. Infect. Immun. **30**:385–390.