Succinic Acid, a Metabolic By-Product of *Bacteroides* Species, Inhibits Polymorphonuclear Leukocyte Function

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Anaerobes, in particular *Bacteroides* spp., are the predominant bacteria present in mixed intra-abdominal infections, yet their critical importance in the pathogenicity of these infections is not clearly defined. Succinic acid, a major fatty acid by-product of *Bacteroides* metabolism, was tested for its effect on neutrophil function to determine whether it might play a role in enhancing the virulence of *Bacteroides*-containing infections. At pH 5.5 but not pH 7.0, succinic acid at concentrations commonly found in clinical abscesses profoundly inhibits in vitro neutrophil function. It virtually obliterates phagocytic killing of *Escherichia coli* and reduces neutrophil random migration and chemotactic response to formyl-methionyl-leucyl-phenylalanine and C5a. These effects occur in conjunction with a reduced chemiluminescent peak and delayed time to the peak. The effect on neutrophils is only partially reversible by multiple washings. These findings suggest that succinic acid may be an important *Bacteroides* virulence factor when present in the microenvironment of a mixed intra-abdominal infection in which concentrations are high and the pH of the medium is reduced.

Bacteroides spp. are the predominant anaerobic microorganisms of the mixed flora present in intra-abdominal infections (10, 23, 43). Many investigators, including ourselves, have shown that under appropriate clinical or experimental conditions, these bacteria can act as synergistic copathogens with other virulent bacteria (3, 7, 39a; O. D. Rotstein, T. L. Pruett, and R. L. Simmons, Rev. Infect. Dis., in press). The mechanisms have not been thoroughly studied, and most studies of *Bacteroides* virulence factors have focused on the polysaccharide capsule (2, 4, 17, 31, 32, 40, 44). The capsule appears to be of critical importance in the production of experimental abscesses in both subcutaneous and intraperitoneal infection models (2, 4, 31, 44). The proposed mechanisms include inhibition of phagocytosis of encapsulated *Bacteroides* spp. strains of polymorphonuclear leukocytes (PMNs) (40) and increased peritoneal adherence of encapsulated *Bacteroides* spp. strains (32).

Other lines of evidence suggest that factors other than *Bacteroides* capsular polysaccharide may be important virulence factors (47, 48). Unencapsulated *B. distasonis* is an effective abscess former (48) and inhibits phagocytic killing of target aerobes (*Proteus mirabilis*) by PMNs as well as encapsulated *Bacteroides* spp. strains in vitro (47). Furthermore, Namavar et al. (26) demonstrated that a *Bacteroides* culture supernatant contained a low-molecular-weight heat-stable factor that could reproduce this inhibition of killing. We have found that both encapsulated and unencapsulated *Bacteroides* spp. strains can act in synergy with *Escherichia coli* in the production of lethal intra-abdominal infections in rats (O. D. Rotstein, T. L. Pruett, and R. L. Simmons. Abstr. Assoc. Acad. Surg., October 1984). The apparent incongruity of the results with respect to the role of the polysaccharide capsule may also be partially attributed to differences in experimental models which may be measuring somewhat different parameters.

Short-chain fatty acids (SCFAs) are a major by-product of anaerobic metabolism and are used for rapid clinical identification (9, 19, 34, 42). Gorbach et al. (9) performed quanti-

tative analyses of SCFAs present in clinical specimens and found a good correlation between the recovery of anaerobic gram-negative bacilli and the presence of isobutyric, butyric, and succinic acids. Succinic acid, a virtually ubiquitous SCFA among *Bacteroides* spp. (13), was generated in particularly high concentrations (up to 31 mM). The purpose of these studies was to determine whether succinic acid, at concentrations found in clinical specimens and at differing pH conditions, was able to alter PMN function and thereby play some role in the pathogenesis of mixed intra-abdominal infections.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A clinical isolate of *E. coli* (serotype O18 ab;K56/K7;-) has been used repeatedly in this laboratory (11, 12, 20). It was maintained at $-70°C$ in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) in 10% glycerol. Daily preparations of *E. coli* were made by inoculating 50 ml of brain heart infusion broth with a loop sample from a stock plate. After incubation at 37°C for 18 h in a shaker bath, the bacteria were washed three times by centrifugation at 1,600 × *g* for 10 min and suspended in sterile isotonic saline. Approximation of the number of organisms was performed by densitometry with a Klett-Summerson colorimeter, and viable numbers were precisely confirmed by pour plate enumeration.

Succinic acid. Succinic acid was obtained in a highly purified crystalline form from Sigma Chemical Co., St. Louis, Mo. Concentrations ranging from 0.3 to 30 mM were made up in Hanks balanced salt solution (HBSS; GIBCO Laboratories, Grand Island, N.Y.) and were titrated with sodium hydroxide (1.0 and 10 M) to either pH 5.5 or 7.0 by using a Beckman model 3500 digital pH meter.

Preparation of human PMNs. PMNs were separated from the heparinized blood of healthy donors by centrifugation (400 × *g* for 25 min) over Ficoll-Hypaque (8). The PMNs were washed twice after hypotonic lysis of residual erythrocytes and resuspended in HBSS. The purity of the PMNs recovered was >95% as monitored by Wright staining of a Cytospin preparation.

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Incubation of PMNs with succinic acid. PMNs (20 × 10⁶ PMNs per ml) were incubated in concentrations of succinic acid ranging from 0.3 to 30 mM at either pH 5.5 or 7.0 or in appropriate control HBSS (pH 5.5 and 7.0) at 37°C for 20 min and then chilled on ice. If dictated by the particular experiment being performed, the PMNs were washed twice and resuspended in HBSS. The cells were counted by Coulter Counter (Sysmex, P-110, Toa; Medical Electronics), and cell viability was tested by trypan blue exclusion.

Assays of chemotaxis. Random migration and chemotaxis assays were performed by a modified under-agarose technique as described by Nelson et al. (29). Briefly, agarose plates with a 6-ml volume and containing a final concentration of 1% agarose, 0.5% gelatin, 1× Eagle minimal essential medium (MEM; GIBCO Laboratories), and 0.025 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) at pH 7.0 were made in 60-by-15-mm plastic petri plates (A/S Nunc, Kamstrop, Denmark). After the agarose had hardened, wells were cut as previously described (29). Chemotaxis toward 1.1 × 10⁻⁷ M formyl-methionyl-leucyl-phenylalanine (FMLP; Calbiochem-Behring, La Jolla, Calif.) and zymosan-activated serum (ZAS) was performed. The reaction was stopped after 2 h, and the cells were fixed with 2.5% glutaraldehyde. The following day the agarose was lifted off, and the cells were stained with Wright stain.

The migration patterns were magnified 40 times and projected onto a light background with a projecting microscope. Quantification of the migration was measured by the linear distance from the lip of the well to the outer margin of the cells migrating towards the chemotactic well. Random migration of PMNs was determined by measuring the distances covered when HBSS was placed in the outer well.

The effect of succinic acid on PMN chemotactic response was tested in three ways: (i) cells were placed in the wells on the agarose plates while still being bathed in succinic acid (i.e., unwashed cells); (ii) cells were twice washed with HBSS at pH 5.5 or 7.0 and then resuspended in HBSS (pH 5.5 or 7.0) before being placed in the wells on the agarose plate (i.e., washed cells); and (iii) washed cells were placed on agarose plates titrated to pH 7.0 (as above) or 5.5 by adjusting the pH of the minimal essential medium-buffer mixture just before addition to the agarose.

Studies in which cells were treated by the first two methods (i.e., chemotaxis on a pH 7.0 agarose plate) were expressed as a percentage of the migration of cells incubated in HBSS at their corresponding pH (e.g., chemotactic migration after incubation in 10 mM succinic acid at pH 5.5 was expressed as a percentage of the chemotactic response of cells incubated in HBSS at pH 5.5). The chemotactic response of cells after incubation in HBSS at pH 5.5 did not differ from that of cells incubated in HBSS at pH 7.0 (Table 1). Expression of cell migration on pH-adjusted plates was an absolute measurement of projected distance of migration onto a light background.

Assay of phagocytic killing of *E. coli* by PMNs. Incubated PMNs were twice washed in HBSS (pH 7.0) and resuspended in HBSS (pH 7.0). A 1-ml mixture of PMNs (5 × 10⁶), *E. coli* cells (1 × 10⁷), and human serum (final concentration, 15%) was made in a 12-by-75-mm polystyrene tube and rotated slowly at 37°C for 1 h. At the end of the incubation period, the PMNs were lysed with distilled water, and exact enumeration was performed by the agar pour plate technique after serial dilution in isotonic saline (15). Tubes with *E. coli* but without PMNs were similarly treated to serve as controls. Results are expressed as a killing index (KI), i.e., the percentage of bacteria killed during the incubation period;

KI = [(number of viable bacteria without PMNs − number of viable bacteria with PMNs)/number of viable bacteria without PMNs] × 100%.

Assay of chemiluminescence. The method used was one devised in this laboratory (27). Briefly, 2 × 10⁶ washed PMNs and 1.25 mg of opsonized zymosan in a total volume of 2.5 ml of phosphate-buffered saline were placed within a scintillation counter (Beckman L5330), and measurements were taken every 5 min until the peak response was past. Both the peak chemiluminescence and time to peak response were measured. The luminol-enhanced (2 × 10⁻⁸ M) chemiluminescent response to FMLP (10⁻⁷ M) was also tested (1).

Opsonized zymosan. Stock zymosan (1 ml) (Sigma) was incubated with 3 ml of serum at 37°C for 30 min. After centrifugation at 2,000 rpm for 5 min, the pellet was washed twice with Dulbecco phosphate-buffered saline (Irvine Scientific, Santa Ana, Calif.) and resuspended in 3.8 ml of phosphate-buffered saline to a final concentration of 12.5 mg/ml.

Statistics. Data were evaluated by using an unpaired Student *t* test and standard tables.

RESULTS

Effect of pH and succinic acid on viability of human PMNs. A 20-min incubation at 37°C in various concentrations of succinic acid (0 to 30 mM) at either pH 5.5 or 7.0 had no effect on the viability of human PMNs, maintaining >95% trypan blue exclusion.

Effect of succinic acid on chemotaxis and random migration. The effect of various concentrations of succinic acid at pH 5.5 and 7.0 on PMN response to FMLP and ZAS and PMN random migration is shown in Fig. 1 and 2. The cells were not washed before the chemotactic assay was performed. Neutrophils preincubated in various concentrations of succinic acid at pH 5.5 demonstrated (i) a significant reduction in random migration at concentrations of 20 ($P < 0.05$) and 30 mM ($P < 0.01$) when compared with control cells incubated in HBSS at pH 5.5; (ii) a depressed chemotactic response to ZAS at concentrations of 10 ($P < 0.02$), 20 ($P < 0.02$), and 30 mM ($P < 0.01$); and (iii) a reduced chemotactic response to FMLP at concentrations of 20 ($P < 0.05$) and 30 mM ($P < 0.01$). At pH 7.0, the response to ZAS at 30 mM was significantly reduced (Fig. 2), but to a significantly lesser degree when compared with the effect at pH 5.5 (78.9% of control at pH 7.0 versus 10.1% of control at pH 5.5 [$P < 0.001$]). At 30 mM succinic acid, random migration was also slightly but significantly reduced but again not nearly as markedly as at pH 5.5 (91.7% at pH 7.0 versus 16.0% at pH 5.5, $P < 0.01$). At pH 7.0, there was no reduction in chemotaxis or random migration at succinic acid concentrations less than 30 mM. In fact, at 3.0 mM and pH 7.0, there

TABLE 1. Neutrophil migration after exposure to HBSS at pH 5.5 and 7.0

| pH | Chemotactic response[a] to: | | Random migration (cm) |
	FMLP	ZAS	
5.5	6.57 ± 0.68	4.60 ± 0.70	2.20 ± 0.35
7.0	5.69 ± 0.60	3.80 ± 0.45	1.88 ± 0.27

[a] Projected distance of migration in centimeters. Values represent the mean and standard error derived from four separate experiments, with each experiment performed in triplicate. PMNs were incubated with HBSS (pH 7.0 or 5.5) for 20 min at 37°C before evaluation of chemotaxis by the standard under-agarose technique with plates at pH 7.0. There was no significant difference between cells incubated at pH 7.0 or 5.5 when tested for chemotactic response and random migration.

was a significant stimulation of random migration ($P < 0.05$) and chemotaxis to FMLP ($P < 0.01$).

To determine whether the inhibitory effect on PMN motility induced by succinic acid at pH 5.5 was reversible, PMNs were tested after two washes with HBSS (pH 5.5) for their response to chemotaxins and for random migration (Table 2). In paired studies, in which PMNs were tested either before or after washing, the washed PMNs responded slightly better to the chemotaxins but were still reduced from control values at 20 and 30 mM concentrations for FMLP and at 10 to 30 mM for ZAS. Random migration was significantly reduced only at 30 mM ($P < 0.05$). In general, these responses mimic those seen for the unwashed cells (Fig. 1). All subsequent experiments described were performed with doubly washed PMNs.

PMNs incubated in HBSS at pH 7.0 or 5.5 had equivalent absolute random migration and response to chemotaxins when tested in our standard under-agarose system (which was performed at pH 7.0) (Table 1). To examine the effect of pH and the interaction of pH with succinic acid on cell migration, cells were incubated with various concentrations of succinic acid at pH 5.5 or 7.0, doubly washed with HBSS of the corresponding pH, and then tested for chemotactic response and random migration on the appropriately pH-adjusted agarose plates. Figure 3 shows the projected distance of migration of PMNs at various succinic acid concentrations and pHs. PMNs incubated in HBSS at pH 5.5 and
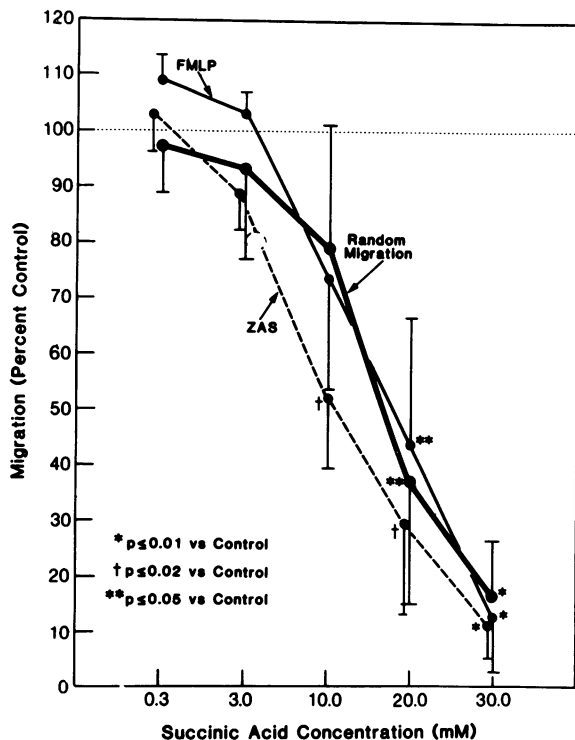
FIG. 2. Effect of succinic acid on PMN chemotactic response to FMLP and ZAS and on PMN random migration after incubation at pH 7.0. Cells were incubated at 37°C for 20 min in various concentrations of succinic acid (0 to 30 mM) titrated to pH 7.0. The PMNs were then added unwashed to the wells in the agarose plate for testing. Results are expressed as percent control migration (cells incubated in HBSS at pH 7.0). Mean and standard error of four separate experiments each performed in triplicate are shown.

tested for chemotaxis and random migration at pH 5.5 agarose system migrate significantly less than at pH 7.0 ($P < 0.01$). At pH 7.0, succinic acid had no effect on cell migration. At pH 5.5, there was virtually complete obliteration of chemotaxis and random migration at succinic acid concentrations of 20 and 30 mM. At a concentration of 10 mM, PMN chemotactic response and random migration began to return toward control values but were still significantly depressed.

Effect of succinic acid on phagocytic killing of *E. coli* by PMNs. The effect of preincubation of PMNs in various

FIG. 1. Effect of succinic acid on PMN chemotactic response to FMLP and ZAS and on PMN random migration after incubation at pH 5.5. Cells were incubated at 37°C for 20 min in various concentrations of succinic acid (0 to 30 mM) titrated to pH 5.5. The PMNs were then added unwashed to the wells in the agarose plate for testing. Results are expressed as percent control migration (cells incubated in HBSS at pH 5.5). Mean and standard error of four separate experiments each performed in triplicate are shown. At succinic acid concentrations of 20 and 30 mM there was marked reduction in chemotactic response and random migration.
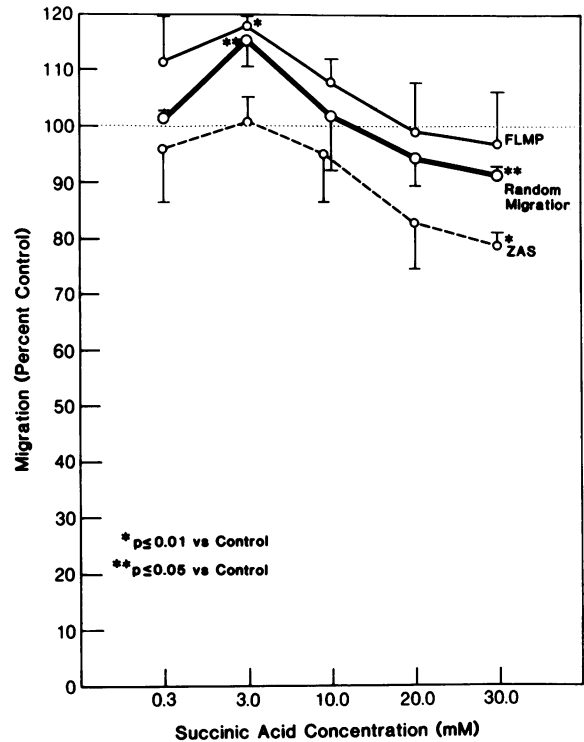
TABLE 2. Reversibility of the effect of succinic acid at pH 5.5 on neutrophil locomotion

Succinic acid concn (mM)	Chemotactic response[a] (% control) to:		Random migration (% control)
	FMLP	ZAS	
30	50.2 ± 2.6[b]	24.4 ± 0.6[c]	35.5 ± 10.0[b]
20	80.7 ± 4.2[b]	59.8 ± 1.6[b]	87.5 ± 18.0
10	94.3 ± 2.8	65.2 ± 0.3[c]	104.3 ± 27.9
3	94.4 ± 4.5	90.5 ± 5.4	97.0 ± 10.5

[a] Values represent the mean and standard error of two experiments, each performed in triplicate. Neutrophils were incubated with various concentrations of succinic acid at pH 5.5 for 20 min at 37°C and then doubly washed with HBSS (pH 5.5) before migration was tested in the under-agarose system. There was still marked inhibition of chemotactic response and random migration despite washing, albeit not as pronouced as in unwashed cells.
[b] $P < 0.05$ versus control.
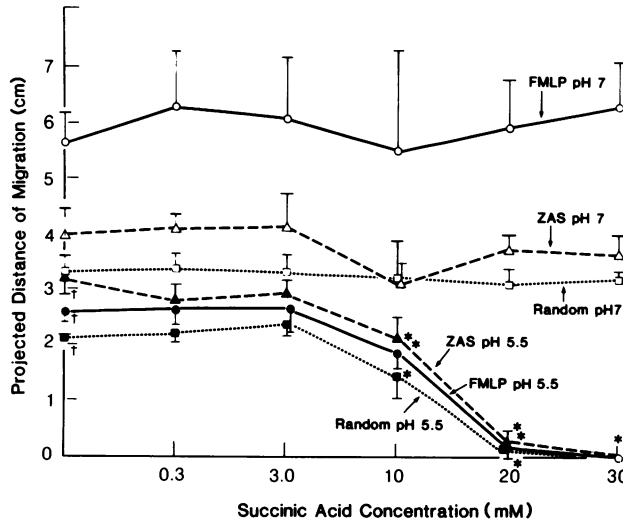[c] $P < 0.01$ versus control.

FIG. 3. Effect of succinic acid and its interaction with pH on neutrophil migration. Cells were incubated at pH 5.5 or 7.0 with varying concentrations of succinic acid (0 to 30 mM) for 20 min at 37°C. Cells were doubly washed with HBSS of the corresponding pH and added to the wells of the appropriately pH-adjusted agarose plate. Results are expressed as projected distance of migration (centimeters) measuring from the lip of the well to the leading front of the cell migration. Mean and standard deviation of two experiments, each performed in triplicate, are shown. At pH 5.5, there was significant reduction in migration at succinic acid concentrations of 10 to 30 mM, whereas at pH 7.0, migration was unaffected. Reduction in pH also significantly reduced both chemotactic and random migration in the absence of succinic acid. Symbols: *, $P \leq$ 0.01 versus pH 5.5 control; †, $P \leq 0.01$ versus pH 7.0 control.

DISCUSSION

Intra-abdominal infections are almost always polymicrobial, but the critical role played by the various components of the mixed flora is still being defined. A biphasic response to a polymicrobial infection has been shown in the rat peritonitis-gelatin capsule model (33, 49). With this model, all mortality occurs during an early phase, the peritonitis phase, which lasts 3 to 4 days. The mortality rate (approximately 40%) appears to correlate most closely with the size of the initial inoculum of *E. coli* (30). The survivors of this phase enter the second or abscess phase. Abscess formation appears to depend on the presence of an encapsulated *Bacteroides fragilis* strain in the inoculum. Subsequent studies have pointed out that the *B. fragilis* capsular polysaccharide is an important virulence factor (17, 31, 32, 40). Its presence has been related to increased resistance to phagocytosis by neutrophils (40) and increased adherence to the peritoneal surface (32), thus predisposing it to abscess formation. The rat intraperitoneal fibrin clot model has been used to show that *B. fragilis* can also enhance *E. coli* lethality (7, 39a). This effect appears to be mediated through a heat-stable extracellular product of *B. fragilis* (39a). Brook et al. (3) have used a subcutaneous inoculation model in mice to demonstrate that several anaerobes, including *B. fragilis*, are capable of producing synergistic lethality with *E. coli* (and other aerobes). Furthermore, several in vitro

concentrations of succinic acid on their ability to perform phagocytic killing of *E. coli* in vitro is illustrated in Fig. 4. Succinic acid at pH 7.0 had no effect on phagocytic killing at any concentration. However, at pH 5.5, killing was significantly reduced ($P < 0.0001$) at succinic acid concentrations of 20 and 30 mM compared with HBSS pH 5.5 control. At all succinic acid concentrations tested, the killing at pH 5.5 was significantly less than that at pH 7.0 ($P < 0.05$). Reduction of pH in the absence of succinic acid had no effect.

Chemiluminescent response of PMNs preexposed to succinic acid. Chemiluminescence was used to examine the effect of succinic acid on the respiratory burst. Table 3 shows the peak chemiluminescent response to opsonized zymosan of neutrophils preincubated in succinic acid. Neither peak chemiluminescent response nor the time of the peak was affected by any concentration of succinic acid when the preincubation occurred at pH 7.0. However, at pH 5.5, concentrations of 30 and 20 mM lowered the peak response and prolonged the time to peak response compared both with the control at pH 5.5 ($P < 0.02$) and with the corresponding concentration at pH 7.0 ($P < 0.001$ for 30 mM and $P < 0.05$ for 20 mM). At a succinic acid concentration of 3.0 mM, the peak response of the PMNs was significantly enhanced compared with control at pH 5.5 ($P < 0.02$) and the corresponding concentration at pH 7.0 ($P < 0.01$). Similar results for both pH values were obtained with FMLP as a stimulant, and a representative curve of the response for cells incubated at pH 5.5 is shown in Fig. 5. One difference, however, was that preincubation in succinic acid at 20 mM produced a late stimulation of the FMLP-induced chemiluminescent response.
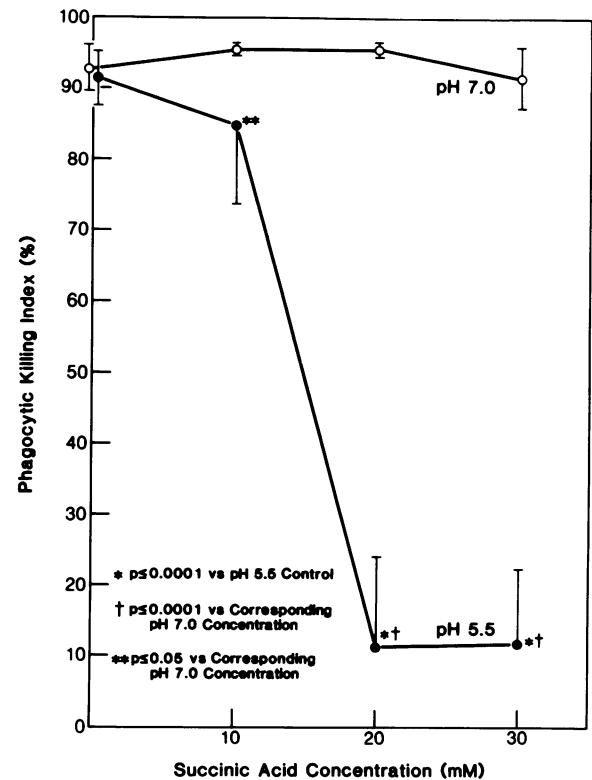

FIG. 4. Effect of succinic acid on phagocytic killing of *E. coli* by PMNs. PMNs were incubated in succinic acid (0 to 30 mM) at pH 5.5 or 7.0 for 20 min at 37°C and then doubly washed with HBSS (pH 7.0). A 60-min killing assay was performed. Results are expressed as a phagocytic killing index (see text). Each point was derived from at least six killing assays. Preincubation of PMNs at pH 5.5 significantly impaired killing capacity at all succinic acid concentrations when compared with corresponding concentrations at pH 7.0.

TABLE 3. Effect of succinic acid on peak chemiluminescent response to opsonized zymosan

Succinic acid concn (mM)	Peak response (counts) at pH 7.0[a]	Time to peak (min) at pH 7.0[a]	Peak response (counts) at pH 5.5[a]	Time to peak (min) at pH 5.5[a]
Control	1,151.7 ± 77.3	41.7 ± 4.4	1,119.7 ± 91.3[b]	41.7 ± 1.7
30	1,025.3 ± 73.5[b]	38.3 ± 3.3[b]	102.8 ± 51.7[c,f]	87.5 ± 12.5[d,e]
20	957.3 ± 63.8[b]	41.7 ± 7.3[b]	372.0 ± 189.5[c,e]	88.3 ± 6.0[c,f]
10	999.0 ± 31.6[b]	41.7 ± 4.4[b]	1,424.0 ± 336.2	70.0 ± 7.6[c,e]
3	949.3 ± 119.8[b]	38.3 ± 6.0[b]	1,566.2 ± 43.6[c,f]	38.3 ± 4.4

[a] Mean ± standard error of the mean of two or three experiments.
[b] Not significant versus control at pH 7.0.
[c] P < 0.02 versus control at pH 5.5.
[d] P < 0.05 versus control at pH 5.5.
[e] P < 0.05 versus corresponding concentration at pH 7.0.
[f] P < 0.01 versus corresponding concentration at pH 7.0.

studies (14–16, 26, 45, 47) have demonstrated the ability of various anaerobes, especially *Bacteroides* spp., to inhibit uptake and phagocytic killing of an accompanying target aerobe by neutrophils, further supporting the concept that anaerobes contribute to the virulence of mixed infections. This phenomenon has been demonstrated with both encapsulated and unencapsulated strains of *Bacteroides*, suggesting that other factors in addition to the polysaccharide capsule may be important *Bacteroides* virulence factors. Further support for this concept is derived from work by Walker and Wilkins (48), in which an unencapsulated *B. distasonis* strain was an efficient abscess former in the mouse subcutaneous abscess model.
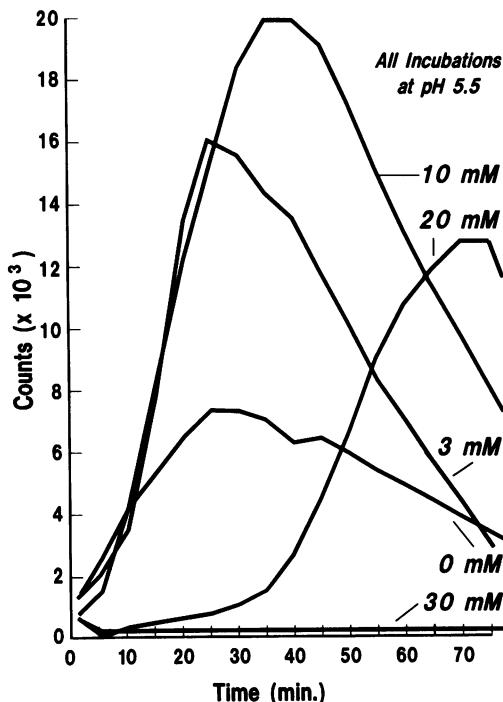

FIG. 5. Chemiluminescent response of PMNs to the soluble stimulant FMLP after incubation with various concentrations of succinic acid at pH 5.5. Cells were doubly washed with HBSS at pH 7.0 before being tested. At a succinic acid concentration of 30 mM, the response was virtually obliterated, whereas at 3 and 10 mM there was enhancement of response. Interestingly, at 20 mM there was initial blunting of response followed by later stimulation. This figure is representative of two experiments. Succinic acid at pH 7.0 produced no significant effect on FMLP-induced chemiluminescence (data not shown).

The SCFA products of anaerobic metabolism are clinically used as markers for the presence of anaerobes in infections (9, 19, 34, 42). Gorbach et al. (9) quantitated the concentrations of these fatty acids in various infected fluids and correlated them with the presence of various anaerobe species. Infections containing *Bacteroides* spp. (usually *B. fragilis*) produced particularly high concentrations of succinic acid (up to 31 mM).

The present studies demonstrate that, when incubated at high concentrations and low pH with neutrophils, succinic acid markedly impairs the phagocytic killing capacity of the cells, reduces the ability of the cell to respond to chemotactic stimuli, and impairs the generation of the respiratory burst. This effect was most evident at concentrations similar to those measured by Gorbach et al. (9) from clinical specimens. The inhibitory effect is also seen to be more pronounced at pH 5.5 than at 7.0. The lower pH value approximates that pH noted in some experimental abscesses (5, 6). Although high concentrations of succinic acid and low pH levels have been shown to occur independent of each other in abscess cavities, no studies have been done to examine whether they coexist either in clinical or in experimental settings.

As noted, succinic acid at pH 5.5 also inhibits production of the respiratory burst as demonstrated by the reduced chemiluminescent peak and delayed response. Interestingly, although these neutrophil functions are markedly inhibited at high succinic acid concentrations, there appears to be some stimulation at lower concentrations. This is true for chemotaxis and chemiluminescence. Failure to demonstrate the phenomenon in the phagocytic killing assay is not surprising, as virtually all *E. coli* are killed in the control groups and it would be difficult to exceed this already high killing index.

The mechanism of the inhibitory effect of succinic acid on neutrophil function has not been examined in these studies. However, it is clear that the neutrophils must be exposed to succinic acid at low pH to be affected. The pK_a's of the two carboxyl groups of succinic acid are 4.19 and 5.57 (*In* O. E. Gueffroy (ed.), *Buffers*, p. 10, Calbiochem-Behring Corp., 1978). This suggests that succinic acid in its undissociated state may be the molecule responsible for these effects. The question of whether this moiety acts at the level of the surface receptor or in the intracellular compartment requires further study. The increased activity at pH 5.5 is compatible with activity at either of these two locations, presumably as a result of its greater lipid solubility in its undissociated state. Because the observed stimulation of neutrophil activity at low doses with inhibition at higher concentrations mimics the effect of soluble stimulators on neutrophil surface

receptors (24, 28), we speculate that a membrane effect might be present.

SCFAs have been shown in other model systems to be deleterious to mammalian cell function (18, 35–37, 41, 46) and inhibitory to bacterial cell growth (21, 25). The best studied is the four-carbon butyrate molecule. Butyrate is inhibitory or toxic to mammalian cells in culture (including Vero cells [46], human and mouse fibroblasts [37], and mouse neuroblastoma cells [36]) as well as inhibitory to lymphocyte activation by phytohemagglutinin, concanavalin A, and endotoxin (18). Butyrate also reduces the activity of several cellular enzymes, including lactate dehydrogenase and pyruvate kinase (35). Similar properties have been demonstrated for propionate and succinate, which inhibit acetyl glutamate synthesis in rat liver mitochondria, presumably by depleting the intracellular acetyl-coenzyme A (37). Touw et al. (46) have correlated butyrate production by a W83 strain of B. gingivalis with the ability of the culture supernatant to inhibit Vero cells in culture. Similarly, Singer and Buckner (41) demonstrated that dental plaque contained levels of butyrate and propionate sufficient to inhibit human and mouse fibroblasts. In their study bacteria in the plaque were not characterized, but presumably they were the usual mixed flora with a predominance of anaerobes including Bacteroides spp. (22). Namavar et al. (26) demonstrated that a heat-stable low-molecular-weight factor in the culture supernatant of several Bacteroides spp. strains inhibited phagocytic killing of Proteus mirabilis by neutrophils and also reduced chemiluminescence. They found butyrate levels to be normal but did not measure pH or succinate concentrations. Jones and Gemmell (16) also noted that a Bacteroides culture supernatant depressed the phagocytosis of [³H]thymidine-labeled bacteria. The presence of SCFAs in the supernatant was not evaluated. Several different SCFAs (including butyrate, propionic, isobutyric, acetic, and formic acids) have also been shown to inhibit growth of both aerobes and anaerobes in vitro, an effect which was more pronounced at low pH and low Eh (21, 25). As in our studies, the inhibition was postulated to be due to the presence of the undissociated molecule.

The inhibition of leukocyte chemotaxis at low pH has been previously demonstrated (38, 39). Our studies show that brief exposure (20 min) of neutrophils to low pH followed by return to pH 7.0 does not reduce PMN response to chemotaxins, whereas continued exposure to these low pH levels during the chemotactic assay does so. The acid environment appears to have a more adverse effect on responses to FMLP than to ZAS.

In conclusion, the results of the present study show that succinic acid when present at high concentrations and low pH (conditions present in mixed flora abscesses) profoundly reduces phagocytic killing of E. coli cells by neutrophils as well as neutrophil random migration and response to chemotaxins. This occurs in conjunction with an impairment of the respiratory burst while viability is maintained. Succinic acid may represent at least one leukotoxic virulence factor produced by Bacteroides spp. The presence of succinic acid in culture filtrates and clinical abscesses may help to explain some of the in vitro phenomena caused by this genus of bacteria as well as its predominance in the clinical setting.

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