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The *Bacteroides* By-Product Succinic Acid Inhibits Neutrophil Respiratory Burst by Reducing Intracellular pH

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Short-chain fatty acids, particularly succinic acid, are major metabolic by-products of *Bacteroides* species. To determine their role as potential virulence factors in infections containing *Bacteroides* species, short-chain fatty acids were examined for their effect on the neutrophil respiratory burst. Succinate (30 to 50 mM) irreversibly impaired superoxide and hydrogen peroxide production in response to opsonized zymosan and phorbol myristate acetate when neutrophils were treated at pH 5.5 but not pH 6.0 or greater. Several other short-chain fatty acids tested produced similar inhibition. Reversible inhibition of oxygen consumption was found when neutrophils were incubated in succinate-containing medium (pH 6.0) as well as control medium (pH 6.0 and 5.5). Neutrophil cytoplasmic pH was measured by fluorimetric techniques to determine whether the inhibition was mediated via a reduction in intracellular pH. The intracellular pH of cells in control medium (pH 6.5 or less) was significantly reduced compared with pH 7.4. The addition of succinate (30 mM) to these media caused a further significant reduction in cytoplasmic pH at each pH level. The reduction in intracellular pH was sufficient to account for both the irreversible and reversible impairment of the neutrophil respiratory burst. Thus, short-chain fatty acids appear to exert their inhibition, at least in part, by reducing intracellular pH. These data also demonstrate the potential for interactions between *Bacteroides* species and their microenvironment to increase the virulence of an infection.

Anaerobic bacteria, particularly *Bacteroides* species, are frequently encountered in clinical infections as part of a polymicrobial flora (8). Their contribution to the pathogenicity of mixed infections has been well demonstrated, particularly in animal models of intra-abdominal and soft tissue infections (3, 14, 24), but the mechanism of this effect is not well defined. Whereas many investigators have focused on the pathogenic role of bacterial surface components such as the *Bacteroides* capsule (5, 20), others have identified a low-molecular-weight, soluble factor(s) present in the filtrate of *Bacteroides* culture broth which can inhibit several neutrophil (PMN) functions including chemotactic migration and phagocytic killing (17, 25). Short-chain fatty acids (SCFA), which are by-products of anaerobic bacteria, may be good candidates for this factor (11). They have a low molecular weight and are heat-stable products that are present in high concentrations both in vivo in infections containing *Bacteroides* species (9) and in vitro in *Bacteroides* culture filtrate (16). One SCFA, succinate, has been measured in particularly high concentrations in these studies (up to 31 mM in vivo [9] and up to 35 mM in vitro [16]). It has also been demonstrated that several SCFA can inhibit PMN chemotaxis, phagocytic killing of *Escherichia coli*, and chemiluminescence (2, 23). The inhibitory effect of succinate on PMN function was shown to be markedly pH dependent, i.e., it was inhibitory at pH 5.5 but not at neutral pH (23). The acidic pH at which succinate was inhibitory approximates levels measured in aspirated samples of human pus (pH 5.7) (4). The pH dependence suggested that the fatty acid in its undissociated state was responsible for these effects (pK_as of succinate are 5.57 and 4.19; D. E. Gueffroy [ed.], *Buffers*, p. 10, Calbiochem-Behring, La Jolla, Calif., 1978).

The purpose of the present studies was to further define the pH-dependent inhibition of the neutrophil respiratory burst by succinate and to examine the mechanism of this phenomenon. Specifically, we hypothesized that succinate effected this impairment by reducing intracellular pH.

MATERIALS AND METHODS

Preparation of human PMNs. PMNs were separated from the heparinized blood of healthy donors by centrifugation (400 × *g* for 25 min) over Ficoll-Hypaque (7). The PMNs were washed twice after hypotonic lysis of residual erythrocytes and suspended in Hanks balanced salt solution (HBSS; GIBCO Laboratories, Grand Island, N.Y.) The purity of the PMNs recovered was >95% as monitored by Wright staining of a Cytospin preparation.

SCFA. All fatty acids (succinic, propionic, butyric, isobutyric and adipic) were obtained in a highly purified form from Sigma Chemical Co., St. Louis, Mo. Solutions of concentrations from 0 to 50 mM were made up in HBSS and were titrated with sodium hydroxide (1.0 and 10.0 M) to pH values ranging from pH 5.5 to 7.4 as indicated below. This pH range encompasses those levels measured in pus obtained from clinical abscesses (4). Because pH 5.5 approximated the pH level measured in vivo and the pH level of an in vitro 20-h *Bacteroides fragilis* culture, it was chosen as the lower end of the pH range examined. For the studies examining PMN oxygen consumption and cytoplasmic pH, the control medium contained 140 mM NaCl, 5 mM KCl, 10 mM glucose, 2 mM $CaCl_2$, and 10 mM Tris-MES [Tris-2-(*N*-morpholine)ethanesulfonic acid; Sigma]. The succinate solution contained 30 mM sodium succinate (Fisher Scientific Co., Fairlawn, N.J.) replacing equimolar quantities of NaCl.

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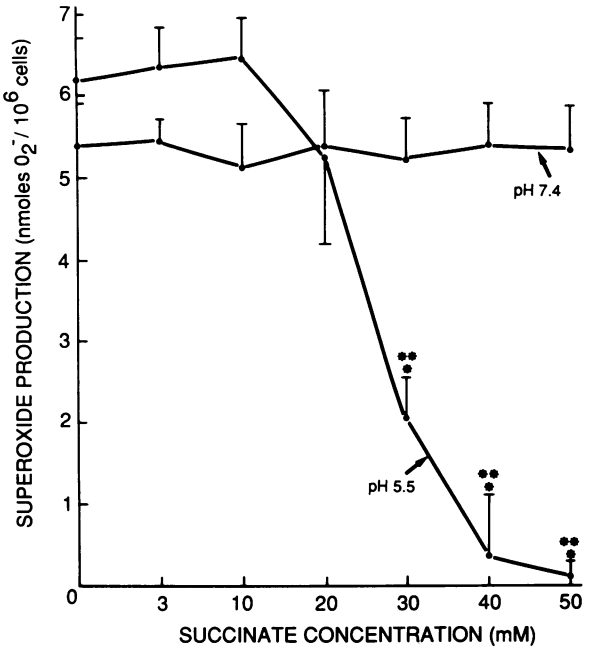

FIG. 1. Effect of succinate on superoxide production by neutrophils in response to OZ. Cells were incubated for 20 min at 37°C in solutions containing succinate (0 to 50 mM) adjusted to pH 5.5 or 7.4. Cells were then washed twice and suspended in HBSS at pH 7.4 before testing. Results are expressed as nanomoles of O_2^- per 10^6 cells per 20 min. Means ± SEM of at least four experiments, each performed in duplicate, are shown. Symbols: *, $P < 0.01$ versus control with corresponding succinate concentration at pH 7.4; **, $P < 0.001$ versus pH 5.5 control.

The sodium-free K^+ solution contained 140 mM KCl instead of NaCl. The pH of these media was adjusted to the values indicated below.

Incubation of PMNs in SCFA. For studies examining superoxide and hydrogen peroxide production, PMNs (20×10^6/ml) were incubated in the indicated medium for 20 min at 37°C, washed twice, and then suspended in HBSS (pH 7.4) before testing. Intracellular pH and oxygen consumption were determined while cells were incubating in the test medium.

Superoxide assay. The generation of superoxide anion (SO) was measured as the superoxide dismutase-inhibitable reduction of cytochrome *c* by measuring absorbance at 550 nm as described by Babior et al. (1). The standard assay mixture (1.0 ml) consisted of HBSS containing 2×10^6 PMNs and ferricytochrome *c* (BDH Chemical, Toronto, Canada). Superoxide release was stimulated by the addition of either opsonized zymosan (OZ; final concentration, 1.25 mg/ml) or phorbol myristate acetate (PMA; Sigma; final concentration, 0.5 μg/ml). The reaction mixture was incubated at 37°C for 20 min and then centrifuged for 5 min at 800 × *g* at 4°C. Absorbance of the supernatant at 550 nm was read on a Beckman DU-8 spectrophotometer. The blank contained all the components listed above plus 60 μg of superoxide dismutase (Sigma) per ml to correct for ferricytochrome *c* reduction by agents other than superoxide. The amount of reduced cytochrome *c* present was calculated by using an extinction coefficient of 21.1/mM/cm at 550 nm. Results are expressed as nanomoles of O_2^- per 10^6 cells per 20 min. Studies with OZ as the stimulus contained 26.3 mM ferricytochrome *c*, whereas those with PMA contained 52.3 mM ferricytochrome *c*. At each of these concentrations, the total amount of ferricytochrome *c* available for reduction, as determined with sodium dithionite, exceeded the amount reduced by either of these stimuli.

Hydrogen peroxide assay. Hydrogen peroxide release was measured by the oxidation of phenol red as described by Pick and Keisari (21). Briefly, the reaction mixture (1.0 ml) consisted of HBSS with 2×10^6 PMNs, 0.28 mM phenol red (Sigma), 8.5 U of horseradish peroxidase (Worthington Diagnostics, Freehold, N.J.), and either OZ or PMA as the stimulus. After a 20-min incubation at 37°C, 0.010 ml of 1 M NaOH was added, and the tubes were centrifuged at 800 × *g* for 5 min at 4°C. Absorbance of the supernatant at 610 nm was determined spectrophotometrically. Results were expressed as the percentage of H_2O_2 released by PMNs incubated in a similarly pH-adjusted HBSS control. Control cells incubated in HBSS at both pH 5.5 and 7.4 produced equivalent amounts of H_2O_2 (data not shown).

Oxygen consumption. PMN oxygen consumption in response to PMA was measured with a model 53 biological oxygen monitor (Yellow Springs Instruments, Yellow Springs, Ohio), which uses a Clark type polarographic electrode. Cells (4×10^6) were suspended in 2 ml of the indicated medium at 37°C and stirred magnetically. PMA was added 10 min after the cells to permit correlation with the cytoplasmic pH measurements. Oxygen uptake was monitored continuously by using a *Y* versus time chart recorder. The O_2 electrode was titrated with dithionite in the various media to ensure that its performance was not affected by pH. O_2 consumption was calculated by using a solubility coefficient of 0.024 ml of O_2 per ml of medium at 37°C.

Cytoplasmic pH determination. Cytoplasmic pH (pH_i) was determined fluorimetrically using the probe 2,7-biscarboxyethyl-5(6)-carboxyfluorescein (10). Suspensions of 10^7 cells per ml in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Sigma)–RPMI (RPMI 1640 without HCO_3; GIBCO) were loaded with 2,7-biscarboxyethyl-5(6)-carboxyfluorescein by incubation with the parent acetoxymethylester (1 μg/ml; Molecular Probes, Junction City, Ore.) for 30 min at 37°C. The cells were then washed and suspended in HEPES-RPMI. Before each determination, 2×10^6 cells were sedimented and suspended in 1 ml of the indicated medium. Fluorescence was measured at 37°C with stirring using a Perkin-Elmer 650-40 fluorimeter with excitation at 485 nm and emission at 540 nm and 5- and 10-nm slits, respectively. Cell lysis with Triton X-100 (0.05%, vol/vol) was used for calibration. The K^+-nigericin method of Thomas et al. (28) was used for estimation of the correction factor required due to the blue shift induced by the detergent (22). K^+-nigericin was also used for manipulating and fixing the pH_i. This was performed by suspending PMNs in a K^+-containing, Na^+-free solution in the presence of the K^+-H^+ exchanger ionophore nigericin (Calbiochem-Behring). This procedure is based on the premise that, in the presence of high nigericin concentrations, pH_i will reach a steady state when $[K^+]_i/[K^+]_o = [H^+]_i/[H^+]_o$, where brackets indicate concentrations and subscript *i* and *o* indicate intracellular and extracellular, respectively. Because the intracellular concentration of K^+ is similar to that in the incubation medium pH_i will approximate pH_o. Thus, where indicated, pH_i clamping was achieved by suspending cells in K^+ media of various pH_o values and adding 15.0 μM nigericin.

OZ. Stock zymosan (1 ml; Sigma) was incubated with 3 ml of serum at 37°C for 30 min. After centrifugation at 800 × *g* for 5 min, the pellet was washed twice with Dulbecco

phosphate-buffered saline (Irvine Scientific, Santa Ana, Calif.) and suspended in 3.8 ml of phosphate-buffered saline to a final concentration of 12.5 mg/ml.

Statistics. Data were analyzed by a one-way analysis of variance, and differences between groups were tested by using Student's *t* test.

RESULTS

Effect of pH and SCFA on viability of human PMNs. A 20-min incubation at 37°C in various concentrations of SCFA (3 to 50 mM) at pH between 5.5 and 7.4 had no effect of the viability of human PMNs, which maintained >90% trypan blue exclusion.

Effect of succinate on superoxide and hydrogen peroxide production by PMNs. Figure 1 illustrates the effect of various concentrations of succinate at pH 5.5 and pH 7.4 on SO production by PMNs with OZ as a stimulus. Incubation of PMNs in 30 to 50 mM succinate at pH 5.5 significantly impaired their ability to produce SO when compared with both HBSS pH 5.5 or succinate-containing solutions (30 to 50 mM) at pH 7.4. After incubation and suspension in HBSS (pH 7.4), PMNs did not recover (for up to 3 h) their ability to produce SO (data not shown), thereby demonstrating the irreversible nature of the inhibition. Low doses of succinate (3 to 10 mM) at pH 5.5 caused a small but insignificant enhancement of SO production. Incubation in succinate at pH 7.4 at concentrations up to 50 mM did not affect SO production.

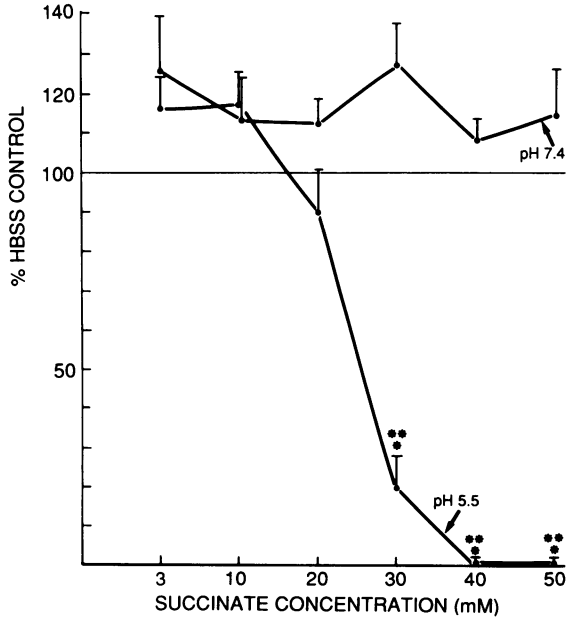

FIG. 2. Effect of succinate on hydrogen peroxide production by neutrophils in response to OZ. Cells were incubated for 20 min at 37°C in solutions containing various concentrations of succinate (0 to 50 mM) adjusted to pH 5.5 or 7.4. Cells were then washed twice and suspended in HBSS at pH 7.4 before testing. Results are expressed as the percentage of hydrogen peroxide produced by cells incubated in HBSS (i.e., zero succinate level) adjusted to the corresponding pH. Absolute H_2O_2 production by PMNs after incubation in HBSS at pH 5.5 and 7.4 was 15.1 ± 2.0 and 10.8 ± 1.1 nmol per 20 min, respectively. These values were not significantly different. Means ± SEM of four experiments, performed in duplicate, are shown. Symbols: *, $P < 0.001$ versus control with corresponding succinate concentration at 7.4; **, $P < 0.001$ versus pH 5.5 control.

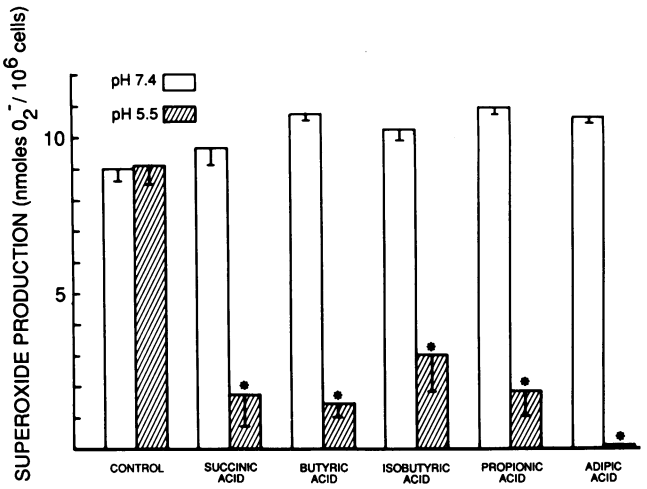

FIG. 3. Effect of different SCFA on superoxide production by PMNs in response to OZ. Cells were incubated in 30 mM solutions of various SCFAs at either pH 5.5 or 7.4 for 20 min at 37°C. Cells were then washed twice and suspended in HBSS at pH 7.4 before testing. Results are expressed as nanomoles of O_2^- per 10^6 cells per 20 min. Means ± SEM of at least two experiments, performed in duplicate, are shown. Symbol: *, $P < 0.01$ versus control with same SCFA at pH 7.4.

A similar phenomenon was demonstrated for H_2O_2 generation after incubation in succinate (Fig. 2). At pH 5.5, concentrations of 30 mM or greater caused a significant reduction in H_2O_2 production in response to OZ. However, at pH 7.4 no inhibition was found. In fact, there was slight enhancement of H_2O_2, although this did not reach statistical significance. Succinate at pH 5.5 also caused a dose-dependent inhibition of SO and H_2O_2 production when PMA was used as a stimulus (data not shown). The pattern of inhibition was markedly similar to that demonstrated for OZ,

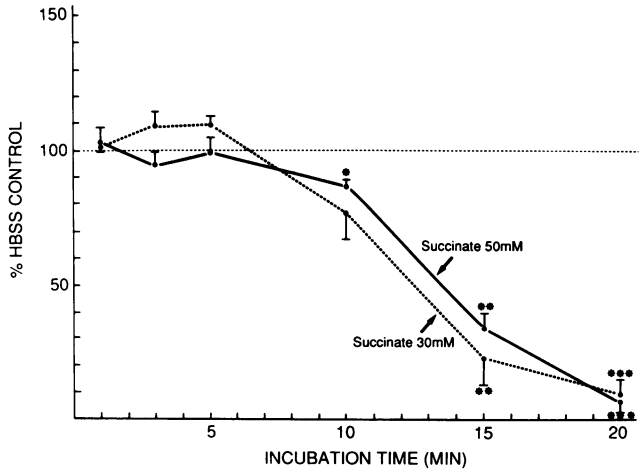

FIG. 4. Effect of incubation time in succinate pH 5.5 on inhibition of superoxide production by PMNs in response to OZ. Cells were incubated in succinate (30 or 50 mM) for 1 to 20 min at 37°C and then washed twice and suspended in HBSS at pH 7.4 before testing. Results are expressed as the percentage of superoxide produced by cells incubated for the corresponding period of time in HBSS at pH 5.5. Means ± SEM of at least three experiments, performed in duplicate, are shown. Symbols: *, $P < 0.01$ versus control; **, $P < 0.05$ versus control; ***, $P < 0.001$ versus control.

TABLE 1. Effect of incubation medium pH on the inhibition of superoxide production by succinate

Incubation medium	Superoxide production[a] at the following pH:			
	7.4	6.5	6.0	5.5
Control	6.28 ± 0.27	6.71 ± 0.21	6.26 ± 0.56	5.93 ± 0.63
Succinate (30 mM)	6.05 ± 0.25	6.59 ± 0.43	6.50 ± 0.45	1.69 ± 0.67[b]

[a] Cells were incubated in the indicated medium for 20 min at 37°C, washed, and suspended in HBSS at pH 7.4 before testing. Results are expressed as nanomoles of O_2^- per 10^6 PMN per 20 min. Shown are the means ± SEM of four experiments, each performed in duplicate.
[b] $P < 0.01$ versus the pH 5.5 control.

except that (i) inhibition of SO production after exposure to 30 mM succinate was more variable, and (ii) significant impairment in H_2O_2 production was effected at 20 mM concentration.

Since *Bacteroides* species produce several SCFA (9) in addition to succinic acid, the effect of butyric, isobutyric, and propionic acids on SO production was tested. Adipic acid, which is not a *Bacteroides* by-product, was also examined due to its structural similarity to succinic acid. All of the SCFA tested (30 mM) caused a pH-dependent, irreversible inhibition of PMN SO production in response to OZ (Fig. 3), similar to that produced by succinate.

The time course of inhibition of SO production by succinate is shown in Fig. 4. At least a 10-min exposure to succinate (30 or 50 mM) was necessary before inhibition became evident. Furthermore, inhibition increased progressively with prolonged incubation.

To further define the pH dependence of the inhibition of SO generation, PMNs were incubated in media containing succinate (30 mM) which were adjusted to pHs ranging from 5.5 to 7.4 before testing (Table 1). Incubation in succinate at pH 5.5 (but not the other pH levels tested) produced irreversible inhibition of SO production. As previously shown, incubation of PMNs in a medium of reduced pH in the absence of succinate was not inhibitory.

Effect of succinate on PMN intracellular pH. Because of the pH-dependent inhibition of the respiratory burst, we hypothesized that succinate in its undissociated form traversed the plasma membrane and reduced pH_i by dissociating in the cytoplasmic compartment. Therefore, the pH_i of PMNs suspended in media containing succinate was determined. The pH_i after a 10-min incubation in succinate (30 mM) at various external pH levels (pH_o) is shown in Table 2. The

TABLE 2. PMN intracellular pH in succinate (30 mM) at various medium pH values

Medium pH	Intracellular pH[a]	
	Control medium[b]	Succinate medium
7.4	7.16 ± 0.08	7.09 ± 0.08
6.5	6.83 ± 0.05[c]	6.60 ± 0.06[d,e]
6.0	6.66 ± 0.05[d]	6.11 ± 0.05[d,f]
5.5	6.32 ± 0.05[d]	5.52 ± 0.02[d,f]

[a] Cells loaded with 2,7-biscarboxyethyl-5(6)-carboxyfluorescein were suspended at 2 × 10^6/ml in the indicated medium, and pH_i was determined after a 10-min incubation. Shown are the means ± SEM of four or five experiments.
[b] Composition of medium for control and succinate groups as noted in Materials and Methods.
[c] $P < 0.01$ versus similar medium at pH 7.4.
[d] $P < 0.001$ versus similar medium at pH 7.4.
[e] $P < 0.05$ versus same pH control.
[f] $P < 0.001$ versus same pH control.

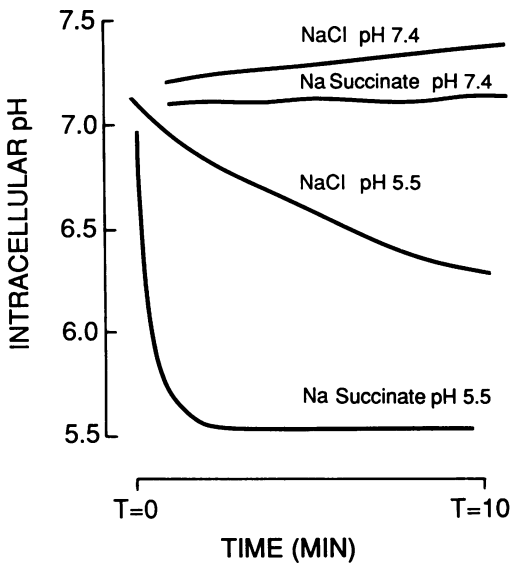

FIG. 5. Effect of succinate on the cytoplasmic pH (pH_i) of neutrophils. Cells (2 × 10^6/ml) loaded with 2,7-biscarboxyethyl-5(6)-carboxyfluorescein were suspended in NaCl medium adjusted to pH 5.5 to 7.4 with or without succinate (30 mM). The pH_i was measured fluorimetrically. The traces demonstrate pH 5.5 and 7.4 levels only and are representative of four or five experiments.

pH_i at time $t = 0$ was 7.00 ± 0.05 (mean ± standard error of the mean [SEM] of four experiments). At pH 7.4, the pH_i did not differ between control and succinate-treated cells. However, at all other external pH values tested there was a significant reduction in pH_i in succinate-treated cells compared with controls. This was particularly marked at pH_o 5.5. The extremely rapid and profound reduction of pH_i effected in the presence of succinate at pH 5.5 is illustrated in Fig. 5. Of further note is that, even in the absence of succinate, media of lowered pH_o caused a significant reduction in pH_i compared with that at pH_o 7.4 (Table 2).

One possible explanation for the irreversible inhibition of

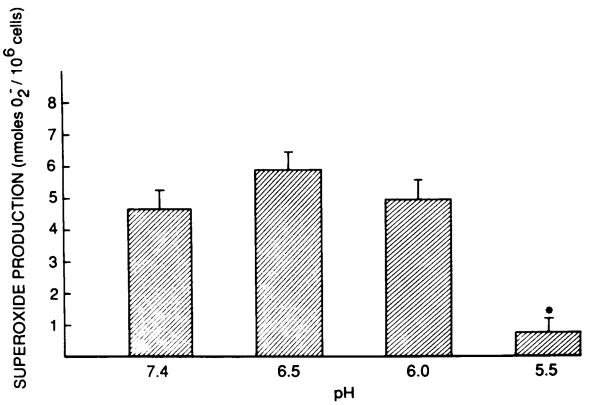

FIG. 6. Effect of clamping cytoplasmic pH of neutrophils at different pH levels on subsequent superoxide production in response to OZ. Cells (20 × 10^6/ml) were incubated in sodium-free K^+ medium of varied pH_o in the presence of 15 μM nigericin for 20 min at 37°C. Cells were then washed twice and suspended in HBSS at pH 7.4 before testing. Results are expressed as nanomoles of O_2^- per 10^6 cells per 20 min and represent the means ± SEM of five experiments, each performed in duplicate. Symbol: *, $P < 0.01$ versus pH 7.4 control.

the respiratory burst was that the pH_i remained low after the cells were washed and suspended in control pH 7.4 medium. To test this hypothesis, after incubation for 10 min in succinate-containing pH 5.5 medium, PMNs were sedimented and suspended in control pH 7.4 medium while monitoring pH_i. The pH_i returned to 7.24 ± 0.04 (mean ± SEM of four experiments) within 20 min.

To determine whether pH_i reduction alone was sufficient to explain the irreversible impairment of SO production, the pH_i of the PMNs was clamped at various pHs ranging from 5.5 to 7.4 by using nigericin and K^+ as described in Materials and Methods. The cells were then incubated at 37°C for 20 min, washed, and suspended in HBSS at pH 7.4 before testing. SO production in response to OZ in these cells is shown in Fig. 6. Clamping the pH_i at 5.5 but not 6.0 or 6.5 during the 20-min incubation caused inhibition of SO production in response to OZ as well as to PMA (data not shown).

Effect of succinate on PMN O_2 consumption. Whereas these data suggested that pH_i reduction to pH 5.5 was responsible for the irreversible inhibition of SO production in PMNs, others have shown that less profound pH_i reductions could cause reversible impairment of the PMN respiratory burst (10, 26). To determine whether succinate-containing media could produce similar reversible inhibition, PMN oxygen consumption was determined in various pH-adjusted media with and without succinate (30 mM, Table 3). A typical experiment is shown in Fig. 7. SO production was not assessable with the ferricytochrome *c* assay, because high concentrations of succinate in the incubation mixture interfered with the assay. At pH 5.5 and 6.0, there was significant reduction in O_2 consumption in both control and succinate-containing media compared with their corresponding pH 7.4 controls. At each of these pH levels, O_2 consumption was significantly less in the succinate-containing media than in the control solutions. Reversibility was assessed by adding 10 μl of 1 M Tris base to the incubation mixture. The O_2 consumption rate by control groups at pH 6.0 and 5.5 and the succinate group at pH 6.0 returned to normal after alkalinization to pH neutrality. However, the reversal did not occur in the succinate-containing pH 5.5 group, consistent with the irreversible inhibition of the respiratory burst produced by these conditions (see above).

DISCUSSION

Several mechanisms by which *Bacteroides* species may interact with other bacteria to enhance the virulence of mixed infections have been postulated (15). The most avidly investigated mechanism has been the ability of these bacteria to impair host defense mechanisms, particularly PMN phagocytic killing (12, 13, 17, 29) and migration (18, 25). A

TABLE 3. Effect of succinate (30 mM) at various pH levels on PMN oxygen consumption

Medium pH	Oxygen consumption[a]	
	Control medium	Succinate medium
7.4	4.4 ± 0.5	4.5 ± 0.8
6.5	4.2 ± 0.2	4.9 ± 0.3
6.0	2.2 ± 0.3[b]	0.6 ± 0.3[b,c]
5.5	1.0 ± 0.3[b]	0.1 ± 0.1[b,c]

[a] Cells (4×10^6) were suspended in 2 ml of the specified medium for 10 min before stimulation with PMA. Results are expressed as nanomoles of O_2 per 10^6 cells per minute. Shown are means ± SEM of two to four experiments.
[b] $P < 0.01$ versus similar medium at pH 7.4.
[c] $P < 0.05$ versus same pH control.

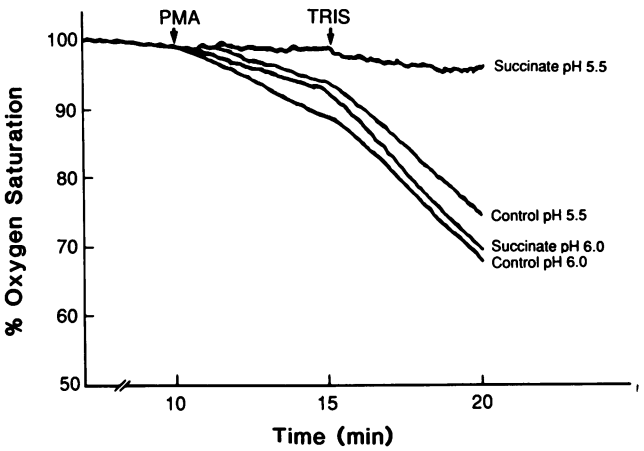

FIG. 7. Oxygen consumption by PMNs (4×10^6) incubated in 2 ml of NaCl medium with or without succinate (30 mM) and adjusted to various pHs. PMA was added as indicated after a 10-min incubation at 37°C; 5 min later, 10 μl of 1 M Tris base was added to return the pH of the medium to >7.0. Representative traces of PMN oxygen consumption in pH 5.5 and 6.0 media are shown.

soluble factor present in the culture filtrate of several *Bacteroides* species has recently been shown to be capable of producing this inhibition (17, 25). Namavar et al. (17) demonstrated that a low-molecular-weight (<3,500), heat-stable material present in *Bacteroides gingivalis* supernatant impaired phagocytic killing of *Proteus mirabilis* by PMNs. A factor with similar characteristics present in *B. fragilis* supernatant has also been shown to inhibit PMN chemotactic and random migration (25). SCFA, which are major by-products of *Bacteroides* metabolism, are low-molecular-weight, heat-stable molecules and have been noted to inhibit PMN chemotaxis, impair phagocytic killing of *E. coli*, and reduce the chemiluminescence response to both particulate and soluble stimuli (2, 23). Two further lines of evidence support the concept that SCFA may be the toxic soluble product present in the *Bacteroides* supernatant. First, the inhibition of PMN migration caused by both succinate and the *B. fragilis* supernatant was markedly pH dependent (25), i.e., inhibition occurred at pH 5.5 but not pH 7.4. Second, the succinate levels measured in the *B. fragilis* supernatant were sufficient to account for the inhibition when the culture filtrate was found to be inhibitory to PMN migration (25a).

The present studies demonstrate that incubation of PMNs in succinate can result in both an irreversible and reversible impairment of the neutrophil respiratory burst, depending upon the conditions of incubation. We examined succinic acid in greatest detail because it is produced in large quantity by *Bacteroides* species in vitro and in vivo (9, 16). However, it is clear that several different SCFA demonstrate a similar pH-dependent inhibition of the respiratory burst, albeit to a somewhat variable degree.

Irreversible inhibition of SO and H_2O_2 production occurred when the PMNs were incubated in succinate at pH 5.5, but not pH 6.0 or greater, for longer than 10 min. The similar patterns of inhibition in response to both OZ and PMA suggest that the fatty acids may affect protein kinase *c* activation or a subsequent step rather than an early transduction mechanism. In addition, the inhibition in response to PMA suggests that chelation of calcium by succinate is an unlikely mechanism, since stimulation of protein kinase *c* by PMA can occur in the virtual absence of calcium (6).

We hypothesized that at low pH, undissociated succinate entered the cells and, in effect, acted as a shuttle for proton movement. The rapid and profound reduction in intracellular pH and the rapid intracellular accumulation of ^{14}C-labeled succinate during incubation of PMNs in succinate-containing medium at pH 5.5 (data not shown) supported this contention. Furthermore, although lowering the pH_o of the succinate-free solutions resulted in a progressive reduction of the intracellular pH, the presence of succinate in the incubation medium caused a further significant reduction in pH_i at each pH_o tested below pH 7.4. To determine whether the reduced pH_i along could account for the irreversible inhibition of the respiratory burst, the pH_i was clamped at pHs ranging from 5.5 to 7.4 in the absence of succinate. SO production by cells incubated at pH_i 5.5 was impaired, whereas cells exposed to pH_i 6.0 or greater exhibited a normal response. Thus the pH_i attained by the PMNs in the succinate pH 5.5 group (pH_i 5.52 ± 0.02) was sufficiently low to explain the inhibition of SO production. Furthermore, the pH_i levels attained during incubation by the PMNs in all other groups (minimum pH_i was 6.11 ± 0.05) were consistent with their ability to demonstrate normal SO production upon their return to a pH 7.4 control medium.

The respiratory burst, as measured by O_2 consumption, was impaired in cells incubated in both succinate-containing and control media at pH 6.0 or less. The mean pH_i levels measured in cells incubated in the control media under similar conditons were 6.66 and 6.32 for pH_o 6.0 and 5.5, respectively. Using the pH_i clamping technique, Nasmith and Grinstein recently demonstrated comparable reductions in O_2 consumption at similar pH_i levels (10). Furthermore, as in our study, inhibition of O_2 consumption was reversed when the pH_o returned to neutrality. Simchowitz (26), using the ammonium chloride prepulse method to produce cytoplasmic acidification, also noted reversible impairment of SO production. The virtually complete obliteration of O_2 consumption by cells incubating in succinate media at pH_o 6.0 could also be explained by the reduction in the pH_i alone. The pH_i level achieved (6.11 ± 0.05) was low enough, based on the data of Nasmith and Grinstein (19), to effect complete inhibition of the respiratory burst, and the effect was reversible with return of the pH_i to neutrality. However, although this explanation is likely and sufficient, one cannot entirely rule out the possibility that succinate (or the other SCFA tested) may have directly contributed to the inhibition.

In further support of the hypothesis that the inhibition of O_2 consumption was mediated via pH_i reduction was the rough correlation between measured pH_i and O_2 consumption in the PMNs incubated (with and without succinate) at pH 6.0 or less (compare Tables 2 and 3). A discrepancy arises when one compares the O_2 consumption in the succinate-containing pH_o 6.5 group and the succinate free pH_o 6.0 group. The pH_i measurements were almost identical, yet the O_2 consumption in the former was approximately twice that in the latter. This discrepancy may have been due to a particularly steep relationship between O_2 consumption and pH_i in this pH_i range or, alternatively, due to a slight stimulatory effect of succinate on the respiratory burst under these conditions.

The mechanism of inhibition in response to a reduction in the pH_i was not examined in detail in these studies. Because the response to PMA was inhibited, it can be concluded that either the stimulation of protein kinase *c* or the activation of the effector mechanism was affected. In this regard, it has been reported that the NADPH oxidase system displays a pH optimum in the 6.8 to 7.9 range in vitro (27). Thus, inhibition of this enzyme system alone could account for the observed results.

The role of SCFA as potential virulence factors produced by *Bacteroides* species in vivo is not well defined. SCFA, particularly succinic acid, are present in infections containing *Bacteroides* species in high concentration (9). Although the present studies did not examine mixtures of SCFA, which are found both in abscesses and in culture broth, the ability of several different SCFA to effect inhibition, presumably via reduction in pH_i, suggests that the effect of combinations of SCFA may be additive. Gross measurements of the pH of aspirates of human pus yield values as low as pH 5.7 (4). No studies have been performed to examine whether low pH and high SCFA concentrations coexist in either clinical or experimental abscesses. However, if these conditions do occur, the present studies suggest that at a given reduced extracellular pH, SCFA further reduce the intracellular pH of PMNs, thereby exaggerating the deleterious effect on PMN function. These data thus demonstrate a potential mechanism by which *Bacteroides* species and their microenvironment might interact to contribute to the virulence of a polymicrobial infection.

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