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Butyrate and Propionate: Important Components of Toxic Dental Plaque Extracts

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Extracts of in vitro-cultured human dental plaque contain factors toxic to mammalian cells. Previous studies demonstrated that those toxic factors most readily released from cultured plaque had very low molecular weights and were heat stable. Studies reported here demonstrate that metabolic end products including short-chain fatty acids were present in fractions containing the low-molecular-weight, heat-stable factors. The salts of two of these acids, butyrate and propionate, inhibited proliferation of both mouse L929 cells and human gingival fibroblasts. Furthermore, when tested at concentrations present in plaque extracts, the inhibitory effects of butyrate and propionate accounted for essentially all the inhibitory potential of the extracts. These findings, taken together with those of other groups, suggest that butyrate and propionate, end products of dental plaque metabolism, may have an etiological role in periodontal disease.

It is well established that the complex bacterial plaque that accumulates on teeth along the gingival margin prompts development of gingival inflammation in humans (11). Considerable evidence indicates that these bacteria typically do not infiltrate into the gingival tissues (20). Rather, it appears likely that the bacteria of dental plaque produce substances that penetrate periodontal tissues and in turn initiate the etiology of periodontitis (21). One hypothesis is that plaque bacteria produce water-soluble cytotoxic molecules that diffuse into and through the gingival epithelium to facilitate development of gingival inflammation (10). Clearly, it is of interest to identify toxic factors elaborated by dental plaque and determine whether such molecules have a role in the disease pathology.

The bacteria of dental plaque contain many substances that are potentially toxic to mammalian cells (9, 10, 12). However, definitive proportions of the total activity produced by dental plaque have not been ascribed to specific molecules. Investigators have partially characterized toxic factors after complete disruption of the plaque bacteria (10, 12). However, studies to date have not identified that fraction of toxic molecules that account for the water-soluble toxic activity readily released from viable human dental plaque.

To address this need, we sought to identify those toxic molecules readily released from a heterogeneous human dental plaque which had been cultured in vitro. Thus, it was reported previously (16) that, during culture, dental plaque elaborated into culture media substances toxic to mammalian cells. Such factors had very low molecular weights and were heat stable. Similar toxic factors were found in water extracts of cultured plaque. In contrast, homogenates of cultured plaque were found to contain high-molecular-weight toxins, some of which were heat inactivated.

The purpose of experiments reported here was to determine whether bacterial metabolites might contribute to those toxic products readily released from cultured dental plaque. These experiments demonstrate that certain end products of bacterial metabolism, butyric and propionic acids, contribute a large proportion of the total toxic activity elaborated by human dental plaque in vitro.

MATERIALS AND METHODS

Culture of mammalian cells in vitro. All tissue culture media and supplementary materials were purchased from GIBCO Laboratories, Grand Island, N.Y. Tissue culture media were prepared as described previously (16).

Human gingival fibroblasts were derived from connective tissue dissected from the roots of freshly extracted human teeth, which were obtained from a local dental surgeon. After extraction, the teeth were immediately immersed in sterile tissue culture medium. To develop primary cell cultures, the tissue was held in place on the bottom of a sterile petri dish with a human fibrin clot containing chicken embryo extract (GIBCO; no. 620-5115). After 2 weeks in McCoy 5a medium (GIBCO; no. 320-6610), the cells were subcultured and carried in RPMI 1640 (GIBCO; no. 430-1800) as a stock culture, using standard tissue culture techniques as described by Kruse and Patterson (7).

Mouse L929 fibroblasts (American Type Culture Collection CCLI) were also cultured by standard tech-

niques. Minimal essential medium (GIBCO; no. 140-1600) supplemented with 10% fetal bovine serum (GIBCO; no. 200-6140) and 1% antibiotic antimycotic (GIBCO; no. 600-5240) was used to culture mouse L929 fibroblasts. Human fibroblasts were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% antibiotic/antimycotic, and 5 μg of amphotericin B per ml (GIBCO; no. 600-5295). Cell proliferation was determined by counting with a hemacytometer the trypan blue-excluding cells recovered from individual culture dishes as previously described (16). Each experimental point was the mean of counts from at least duplicate cultures and, more typically, triplicate cultures of cells.

Short-chain fatty acids used in these experiments were obtained from J. T. Baker Chemical Co., Phillipsburgh, N.J. (sodium acetate); Matheson Coleman and Bell, Cincinnati, Ohio (sodium propionate and sodium butyrate); Eastman Kodak Co., Rochester, N.Y. (isocaproic acid); and Sigma Chemical Co., St. Louis, Mo. (sodium lactate; valeric, isovaleric, isobutyric, and caproic acids).

Culture of human dental plaque in vitro. The plaque culture system used in these studies was that of J. J. Kirkland, of Procter & Gamble Co., and has been described in detail by Singer and Buckner (16). Briefly, the system used an inoculum of human plaque scrapings from which a complex microflora would develop during culture in a programed sequence of media changes. This microbial plaque accumulated on extracted teeth mounted on metal supports in a growth chamber and qualitatively resembled in vivo plaque in microbial composition (16).

Preparation of plaque-exposed medium and plaque extracts. To obtain factors elaborated by plaque during culture, tissue culture medium (minimal essential medium) was incubated for 4 h with cultured human dental plaque in vitro (16). After exposure to plaque, supplementary tissue culture medium nutrients were added to the medium to compensate for any components depleted by bacterial metabolism. Water extracts of cultured dental plaque were prepared as previously described by dislodging plaque bacteria from surfaces of the growth chamber and then centrifuging the bacteria out of a water suspension.

Gas chromatographic analysis of plaque-exposed media and plaque extracts. Plaque-exposed media and water extracts of human plaque cultured in vitro were analyzed for short-chain fatty acid content by a modification of the method described by Holdeman and co-workers (6). The principal modification involved the use of a Supelco no. 1-2144 column (Supelco, Inc., Bellefonte, Pa.) and butyric and propionic acid standard solutions. A Perkin-Elmer 900 gas chromatograph with flame ionization detector and Infotronics CRS-104 peak integrator was used to quantitate levels of butyric and propionic acids in plaque extracts and parallel standard solutions.

RESULTS

Inhibition of cell proliferation by end products of bacterial metabolism. The finding that the water-soluble toxic activity elaborated by cultured plaque had a very low molecular weight (16) suggested that metabolic end products might contribute to such activity. Thus, in preliminary experiments, qualitative gas chromatographic analyses were performed to determine whether significant levels of volatile, nonvolatile, or long-chain fatty acids could be detected in various extracts of cultured human plaque. Indeed, such extracts were found to contain significant levels of acetic, lactic, propionic, and butyric acids. Only trace levels of long-chain fatty acids or other nonvolatile metabolic acids (e.g., succinic and oxalic acids) were detected.

To determine whether these common metabolic end products could affect mammalian cell proliferation, mouse L929 fibroblasts were cultured with various concentrations of the salts of the four principal acids. After 6 days of culture, numbers of viable cells were determined. Butyrate and propionate markedly reduced mammalian cell proliferation at much lower concentrations than either acetate or lactate (Fig. 1).

Quantitative determination of metabolic acids in plaque extracts. Since millimolar levels of the salts of butyric and propionic acids inhibited mammalian cell proliferation, it was of interest to quantitate amounts of these metabolites both in culture media exposed to in vitro-cultured plaque and in water extracts of that same plaque. Concentrations of both butyric and propionic acids released into culture media (Table 1) during exposure to cultured plaque were within ranges inhibitory to fibroblasts (Fig. 1). Similarly, levels of these metabolic end products in water extracts of in vitro plaque (Table 1)

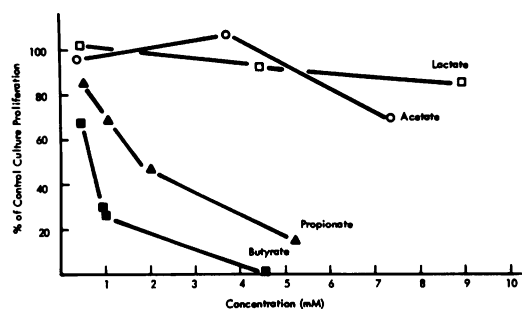

FIG. 1. *Inhibition of L929 fibroblast proliferation by organic acid salts. Mouse L929 fibroblasts were cultured in minimal essential medium containing the indicated concentration of sodium acetate, sodium lactate, sodium propionate, or sodium butyrate. The cells were incubated for 6 days in these media at 37°C in a 5% CO_2 atmosphere, with the media being changed on the third day. Cell proliferation was determined as described in Materials and Methods. In control cultures, L929 cells grew to a density of 6.11 × 10^6 cells per plate.*

were also within those ranges inhibitory to cultured fibroblasts. These extracts also contained quantities of isobutyric, isovaleric, and isocaproic acids. Valeric, caproic, and heptanoic acids were not found. Precise determinations of levels of isobutyric, isovaleric, and isocaproic acids were not made; however, approximate comparisons to butyric acid levels were possible. Within the extracts, typical concentrations of isobutyric, isovaleric, and isocaproic acids were one-tenth, one-half, and two-thirds, respectively, the concentration of butyric acid.

Inhibition potential of plaque metabolites. To determine relative toxicities of these metabolites and related compounds, the sodium salt of each was assayed at a concentration of 1 mM for the ability to inhibit proliferation of mouse fibroblasts in vitro (Fig. 2). The data indicate that butyrate had a greater inhibitory potential than any of the other compounds. In particular, salts of acids present in plaque extracts at concentrations lower than that of butyric acid (e.g., isovaleric, isobutyric, and isocaproic acids) were less inhibitory than sodium butyrate. Although these latter compounds might have had some effect upon the total toxicity of plaque extracts, this contribution would appear to have been small relative to that of butyrate and propionate. If true, levels of butyrate and propionate in plaque extracts would account for a large proportion of the total inhibitory potential associated with such extracts.

To test this hypothesis, the inhibitory potential of medium formulated from a plaque extract containing known levels of butyrate and propionate was compared with that of media formulated with salts of the two acids at the same concentrations. The combined inhibitory potential of butyrate and propionate was statistically equivalent to that of a corresponding water extract of in vitro plaque (Table 2).

Toxicity toward human gingival fibroblasts. To determine whether effects of butyrate and propionate upon mouse fibroblast proliferation were indicative of effects upon human gingival cells, the toxicities of these compounds toward both L929 fibroblasts and human gingival fibroblasts were compared (Table 3). Both human and mouse fibroblasts were similarly sensitive to inhibitory effects of butyrate and propionate.

TABLE 1. *Propionic and butyric acid concentrations in extracts of plaque cultured in vitro*[a]

Plaque extract	Period of plaque culture (days)	Metabolite levels (mM)	
		Propionic acid	Butyric acid
Culture medium	4	4.44 (±7%)	0.30 (±36%)
Culture medium	8	5.37 (±24%)	0.64 (±12%)
Water extract	8	2.00 (±12%)	0.71 (±17%)

[a] Tissue culture medium (minimal essential medium) was exposed (see Materials and Methods) for 4 h to human dental plaque cultured in vitro for either 4 or 8 days. The water extract of plaque was prepared (see Materials and Methods) from human dental plaque which had been cultured in vitro for 8 days. The gas chromatographic analyses for determination of propionic and butyric acids levels are described in Materials and Methods.

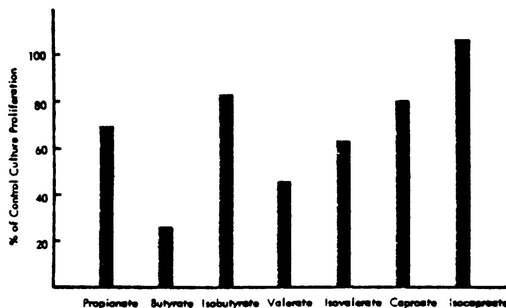

FIG. 2. *Relative inhibitory effects of organic acid salts versus mouse L929 fibroblasts. Mouse L929 fibroblasts were cultured in minimal essential medium containing the sodium salts of the indicated organic acids (1 mM). Cells were incubated for 6 days at 37°C in a 5% CO_2 atmosphere and the media were changed on the third day of culture. Cell proliferation was determined as described in Materials and Methods.*

TABLE 2. *Toxicity of a plaque extract and corresponding levels of butyrate and propionate*[a]

Test medium	Mean cells/plate ($\times 10^6$)	% Control
Control	2.15*[b]	
Propionate (2.27 mM)	1.06*	49
Butyrate (0.56 mM)	1.29*	60
Propionate + butyrate (2.27 mM + 0.56 mM)	0.47	22
Water extract of plaque	0.35	16

[a] A water extract was prepared (see Materials and Methods) from human dental plaque which had been cultured in vitro for 8 days. This extract was found by gas chromatographic analysis (see Materials and Methods) to contain 2.27 mM propionic acid and 0.56 mM butyric acid. Mouse L929 cells were cultured for 4 days at 37°C (5% CO_2) in minimal essential medium containing the indicated levels of butyrate and propionate. The water extract of plaque was used in place of water in preparation of tissue culture medium for that test system. Cell proliferation was determined as described in Materials and Methods.

[b] Means followed by an asterisk are significantly different at $\alpha = 0.05$.

Concentrations of butyric and propionic acids within cultured plaque. During the development of periodontitis, dental plaque accumulates in contact with the gingival tissues; therefore, it is important to determine attainable concentrations within plaque of possible pathogenic mediators. After 4, 6, and 8 days of culture, in vitro plaque was collected and extracted with water; these extracts were then analyzed for levels of propionic and butyric acids (Table 4). Concentrations of both acids within cultured plaque exceeded by one to two orders of magnitude levels of the respective salts that were inhibitory toward fibroblast proliferation in vitro.

TABLE 3. *Relative toxicity of the salts of plaque metabolites toward mouse L929 fibroblasts and human gingival fibroblasts*[a]

Salt of the metabolite	Concn (μg/ml)	% of control cell culture	
		L929	Human gingival
Acetate	500	107	78
	50	96	104
Lactate	500	93	100
	50	102	88
Propionate	500	15	30
	50	85	58
Butyrate	500	1	15
	50	68	46

[a] Mouse L929 fibroblasts were cultured in minimal essential medium, and human gingival fibroblasts were cultured in RPMI 1640 containing either 50- or 500-μg/ml concentrations of sodium salts of the indicated organic acids. Cells were incubated for 6 days at 37°C in 5% CO_2, with the media replenished on the third day. Cell proliferation was determined as described in Materials and Methods. Control cultures of L929 cells reached 7.23×10^6 cells per plate; control cultures of human gingival fibroblasts attained 1.005×10^6 cells per plate.

DISCUSSION

One mechanism proposed to be involved in the etiology of periodontal disease is the production of toxic water-soluble molecules that diffuse from the bacteria of dental plaque and interact with cells of the periodontium to further the disease etiology (10). To investigate this possibility, it is necessary to obtain preparations of water-soluble toxic molecules readily released or elaborated from plaque bacteria which have not been fully disrupted. Subsequently, it is desirable to chemically characterize such toxic molecules. To this end, in vitro systems of plaque and mammalian cell culture have been used in our laboratory to identify molecules that account for the predominant part of the toxic activity elaborated by cultured human dental plaque. Thus, it was found (16) that water extracts of cultured plaque contained low-molecular-weight, heat-stable factors toxic to mammalian cells. During culture of plaque in vitro, similar factors were released into the surrounding culture medium. Data presented here demonstrate that the levels of butyric and propionic acids found could account for toxic effects of water extracts of cultured human plaque. Furthermore, during culture similar levels of butyric and propionic acids were elaborated by plaque into the surrounding medium.

Inhibitory effects reported here of butyrate and propionate versus mammalian cell proliferation are consistent with findings of other investigators (4). Aside from cytostatic and cytotoxic effects, a variety of other effects of butyrate upon mammalian cell function also have been demonstrated and reviewed (13). For example,

TABLE 4. *Concentrations of propionic and butyric acids in in vitro plaque*[a]

Period of plaque culture (days)	Plaque mass[b]		Mass of acid present in plaque (mg/ml)[c]		Concn of acid (mM)[d] in plaque	
	Wet wt (g)	Dry wt (g/ml)[e]	Propionic	Butyric	Propionic	Butyric
4	3.13	0.40	23	2.2	112	9.1
6	4.73	0.74	26	2.4	87	6.8
8	7.46	1.06	42	7.6	88	13

[a] Human dental plaque was cultured for 4, 6, and 8 days in vitro and extracted with water (see Materials and Methods). Triplicate 1-ml portions of the intact plaque suspension were dried for 6 h at 95°C and weighed. Wet weights were taken on the material sedimented by centrifugation at $27{,}000 \times g$. Levels of propionic and butyric acids in the water extracts of the cultured plaque were determined by the gas chromatographic analysis described in Materials and Methods.

[b] Plaque taken from two growth chambers in a total of 200 ml of water.

[c] In 200 ml of water.

[d] An estimated value obtained by dividing the total acid (grams) by the amount (liters) of water in plaque (total wet weight minus total dry wieght) and dividing by the molecular weight of the acids (75 for propionic; 89 for butyric).

[e] Average dry weight in 200 ml of water.

butyrate has been shown to inhibit deoxyribonucleic acid synthesis (5), inhibit histone deacetylation (14), induce morphological differentiation (15, 23), and induce changes in the membrane receptor composition (20) of cultured mammalian cells. The effects of butyrate and propionate upon cells (i.e., growth inhibition, cytostasis, and cytotoxicity) vary as a function of concentration (1, 4). Ginsburg and co-workers (4) have reported that 2.5 mM butyrate reduces the viability of mammalian cells. We have seen similar effects. For example, in Table 3, 63% of the L929 cells treated with 500 μg of butyrate per ml were dead (as determined by trypan blue exclusion). D'Anna and co-workers (1) have reported that short-term exposure to 7.5 mM butyrate causes a subsequent decrease in mammalian cell viability.

A molecular component or end product of dental plaque proven to have a significant role in the etiology of periodontitis would fulfill a number of predictable criteria. Obviously, the substance would have the same biological activity as factors contained in or produced by human dental plaque in situ. The factor would be present in dental plaque in situ at concentrations sufficient to have a pathogenic effect upon gingival cells. In addition, levels of that molecule in plaque in situ would, to some degree, reflect the disease state of the site sampled. Finally, the molecule either would be readily released from plaque bacteria or would be freely available for pathogenic interaction with cells of the periodontium. The plaque metabolites butyric and propionic acids appear to fulfill a number of these characteristics.

Butyrate and propionate have molecular weights and effects upon mammalian cell proliferation consistent with those reported for toxic factors found in extracts of human plaque grown in situ. In this regard, Levine and co-workers (9, 10) demonstrated that saline extracts of homogenized human plaque scrapings contain very low-molecular-weight molecules that inhibit proliferation of HeLa cells. These inhibitory factors were not inactivated by heat. Using another approach, Mackler (12) demonstrated that human plaque sonic extracts contain a dialyzable factor capable of inhibiting lymphocyte blastogenesis. Kyner et al. (8) have demonstrated that butyrate can prevent lymphocyte transformation, a finding we have confirmed in experiments not shown here.

Butyrate and propionate are present in plaque in situ at concentrations sufficient to inhibit mammalian cell proliferation. Geddes (2) has reported that millimolar levels of propionate are present in human plaque, whereas Gilmour and co-workers (M. N. Gilmour, G. C. Green, L. B. Zhan, J. Curzon, and C. D. Sharman, J. Dent. Res. **55**:441, 1976; M. N. Gilmour, G. C. Green, L. M. Zahn, C. D. Sparman, and J. Pearlman, J. Dent. Res. **54**:L-299, 1975) have reported that millimolar levels of butyrate and propionate are present in plaque grown in situ. For example, Gilmour and co-workers (3) found median butyrate and propionate concentrations in the plaque of children to be 3.7 and 7.2 mM, respectively. Although they reported considerable variability among the plaque samples analyzed ($n = 47$), the minimum levels of butyrate and propionate they found exceeded levels we found to be capable of inhibiting fibroblast proliferation (Fig. 1). Since Gilmour and co-workers (3) reported a maximum butyrate level of 75 mM, it is also clear that some in situ plaques contain butyrate at concentrations substantially greater than that which can be cytotoxic (1, 4).

Recent studies indicate that in situ levels of butyrate and propionate in the plaque of patients diagnosed as having periodontal disease are higher than levels found in plaque from control subjects. Bricknell and co-workers (K. Bricknell, V. Grinenko, D. Carlton, and M. G. Newman, J. Dent. Res. **57**:1106, 1978) reported that concentrations of butyrate and propionate in the subgingival plaque of patients with periodontitis were markedly elevated from those levels found in plaque of healthy subjects. Subsequently, Carlton and co-workers (D. Carlton, K. Bricknell, M. G. Newman, N. Yoon, S. Woolfe, and A. Horikoshi, J. Dent. Res. **58**:334, 1979) reported similar findings from studies of plaque associated with juvenile periodontitis. Such findings are not entirely unexpected in that butyric and propionic acids are common metabolites of gram-negative and anaerobic bacteria (6). A number of studies have associated elevated levels of gram-negative (11, 17–19, 22) and anaerobic (E. C. Moore, L. V. Holdeman, and K. G. Palcanis, J. Dent. Res. **59**:220, 1980; S. A. Syed and W. J. Loesche, J. Dent, Res. **59**:484, 1980) bacteria in plaque with experimental gingivitis and periodontitis. Thus, it seems reasonable to expect concomitant increases in plaque levels of the respective metabolic end products.

Being end products of bacterial metabolism, butyric and propionic acids are excreted by viable bacteria. We have shown both metabolites to be readily released by whole plaque in culture. Thus, it seems reasonable to assume that butyric and propionic acids produced by plaque in situ would be available to interact with gingival tissues.

The ability of any molecule to interact with the cells of the peridontium will be dependent upon the ability of that compound to penetrate gingival tissues. Siegel (I. A. Siegel, J. Dent. Res.

56:52, 1977) has reported that butyric and propionic acids can penetrate the oral mucosa. Since macromolecules (21) are reported to penetrate the gingival epithelium, it seems likely that butyrate and propionate could also.

In sum, the current data indicate that butyrate and propionate fulfill a number of predicted criteria for pathogenic components of dental plaque. Butyrate and propionate account for the water-soluble toxic activity of extracts of cultured human plaque, have characteristics similar to those of inhibitory factors found in plaque grown in situ, are produced by oral bacteria typically associated with gingivitis and periodontitis, are readily released by oral bacteria, are present in situ at concentrations sufficient to inhibit mammalian cell proliferation in vitro, are present at elevated levels in plaque associated with periodontitis, and can penetrate mucosal tissues. Of course, one must be cautious in extrapolating from in vitro systems to the in vivo disease pathology. On the other hand, we have found (R. E. Singer, B. A. Buckner, A. H. Meckel, and G. J. Leonard, J. Dent. Res. 59:670, 1980) that when solutions of reported plaque levels (3) of the sodium salts of these acids are occluded with the gingiva of beagle dogs, there is induced a significant increase in gingival inflammation. Taken together, these findings suggest that butyrate and propionate may have a role in the etiology of periodontitis.

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