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Enterococcus faecalis produces extracellular superoxide and hydrogen peroxide that damages colonic epithelial cell DNA

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Enterococcus faecalis **is a commensal microorganism of the human intestinal tract that produces substantial extracellular superoxide (O_2^-), and derivative reactive oxygen species such as H_2O_2 and hydroxyl radical, through autoxidation of membrane-associated demethylmenaquinone. Because these oxidants may be important as a cause of chromosomal instability (CIN) associated with sporadic adenomatous polyps and colorectal cancer, the ability of *E.faecalis* to damage eukaryotic cell DNA was examined using the alkaline lysis single cell gel electrophoresis (comet) assay. Both Chinese hamster ovary and HT-29 intestinal epithelial cells showed increased DNA damage after co-incubation with wild-type *E. faecalis* strain OG1RF, but not a transposon-inactivated mutant with attenuated extracellular O_2^- production. *E. faecalis*-mediated DNA damage was prevented by catalase, but not manganese superoxide dismutase, indicating H_2O_2 arising from O_2^- was the genotoxin. In a rat model of intestinal colonization, OG1RF resulted in significantly higher stool concentrations of H_2O_2 and 5,5-dimethyl-1-pyrroline *N*-oxide adducts of hydroxyl and thiyl radicals, as identified by electron spin resonance–spin trapping, compared with rats colonized with a mutant strain having attenuated O_2^- production. Using the comet assay, luminal cells from the colon of rats colonized with O_2^--producing *E. faecalis* showed significantly increased DNA damage compared with control rats colonized with the mutant. These findings suggest a potentially profound role for extracellular free radical production by *E. faecalis* in promoting CIN associated with sporadic adenomatous polyps and colorectal cancer.**

Introduction

Genomic instability is a characteristic feature of colorectal cancer that potentially contributes to the multi-step acquisition of mutations found in these tumors (1,2). Chromosomal instability (CIN) is the most common form of somatic genomic instability and is typified by genetic rearrangements and losses or gains of large DNA fragments including, on occasion, entire chromosomes (3,4). The usual features of CIN include aneuploidy and loss of heterozygosity. This form of instability is found in >80% of sporadic colon cancers and nearly all intestinal tumors due to familial adenomatous polyposis. Microsatellite instability (MIN) is another distinct form of genomic instability caused by defective DNA mismatch repair (3–5). MIN tumor cells show accelerated accumulation of mutations in repetitive nucleotide sequences located throughout the genome. Although <20% of sporadic colon cancers are classified as MIN, this form of genomic instability is highly associated with hereditary non-polyposis colorectal cancer (3–5).

Genomic instability affects not only colorectal cancer cells but also benign hyperplastic and pre-cancerous adenomatous polyp cells (1,6). The number of genomic alterations in polyps has been estimated by an inter (simple sequence repeat) PCR technique at >11 000 mutations/cell (1). A similar high frequency of chromosomal gains and losses has been reported for colorectal tumors using arbitrarily primed PCR methodology (7,8). Because CIN has been found in cells from very small adenomas, and hence at the earliest stage of tumorigenesis, genomic instability seems more likely a cause, rather than consequence, of cancer formation (1,6). Aneuploidy, cell-cycle checkpoint loss, break-induced replication, defects in double-strand repair-recombination machinery and mitotic non-disjunction have all been considered potential mechanisms for CIN (9,10), but none have proven entirely satisfactory. Therefore, the nature of this fundamental process in colorectal tumorigenesis remains obscure.

One less well-investigated mechanism for CIN proposes oxidative stress on the colonic epithelium from free radicals produced by intraluminal bacteria (11,12). This theory suggests that biologically derived radicals from commensal flora are directly mutagenic and may also indirectly promote genomic instability by forming carcinogens from dietary procarcinogens (13,14). This hypothesis was proposed by Babbs based on *ex vivo* observations of hydroxyl radical production by normal stool (12). Erhardt *et al*. extended these findings by describing a 13-fold increase in hydroxyl radical production by stool from human volunteers fed a high fat/meat and low fiber diet (15). An oxidative mechanism for CIN also potentially links the substantial epidemiological evidence for dietary risk factors with colorectal cancer (16,17).

Several years ago our laboratory noted that *Enterococcus faecalis*, a human intestinal commensal, produced extracellular superoxide (O_2^-) (18). We subsequently demonstrated that the formation of this anionic free radical depended on the presence of membrane-associated demethylmenaquinone and was inhibited by exogenous fumarate or hematin (19). Similar to observations made by Babbs, colonic contents from rats colonized with O_2^--producing *E. faecalis* produced hydroxyl radical as measured by electron spin resonance (ESR)–spin trapping (19). These findings led to consideration of extracellular O_2^- produced by *E. faecalis* near the oxygenated luminal surface of colonocytes as a potential source of genomic instability. Oxidative stress would presumably occur through secondary reactive oxygen species such as H_2O_2 and hydroxyl

Abbreviations: CIN, chromosomal instability; CHO, Chinese hamster ovary; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; ESR, electron spin resonance; MnSOD, manganese superoxide dismutase.

radical (20). In the mildly acidic environment of the large intestine O_2^- would spontaneously disproportionate to H_2O_2:

$$E.\ faecalis \rightarrow O_2^- \quad (1)$$
$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

H_2O_2 generated near the intestinal epithelial surface could passively diffuse into colonocytes and form hydroxyl radical at DNA sites through iron-mediated Fenton reactions. This would lead to DNA–protein crosslinks, DNA breaks and base modifications that could cause nucleotide transitions and transversions (21,22). Thus, chronic oxidative stress from intestinal enterococci might be an important source of ongoing DNA damage, leading to genomic instability and cumulative mutations found in adenomatous polyps and colorectal cancer (23). Here we show that extracellular O_2^- generated by *E. faecalis* does indeed form H_2O_2 and hydroxyl radical, and that these bacterially derived oxidants can damage colonic epithelial cell DNA both *in vitro* and *in vivo*. Our findings suggest a potentially profound role for this common intestinal commensal in generating CIN.

Materials and methods

Bacteria and media

E. faecalis strain OG1RF is plasmid-free and has been shown to produce extracellular O_2^- using whole bacteria in a ferricytochrome *c* assay (18). TM1 is a Tn*917* derivative of OG1RF with inactivation of *aroC*, a gene encoding chorismate synthase, by transposon insertion (19). PW18, another OG1RF derivative, was prepared by allelic inactivation of *menB* using the plasmid p3erm. This gene encodes 1,4-dihydroxy-2-naphthoic acid synthase and is essential for biosynthesis of membrane-associated demethylmenaquinone (19). Both mutants are attenuated in production of extracellular O_2^- through loss of membrane-associated demethylmenaquinones. These mutants grow well in brain–heart infusion (BD Biosciences, Franklin Lakes, NJ) with doubling times no different than for OG1RF. Unless specified otherwise, bacteria were grown in closed tubes in brain–heart infusion at 37°C overnight.

Glutathione peroxidase assay for H_2O_2

A glutathione peroxidase assay was used to measure H_2O_2 produced by live *E. faecalis* and in intestinal contents (24). Bacteria were washed and resuspended to 10^9 c.f.u./ml in 25 mM Tris (pH 7.4) with 7 mM glucose or McCoy's medium with 10% fetal calf serum. After timed incubations 1 vol of 0.6 N perchloric acid was added to 2 vol of bacteria and samples assayed immediately. Intestinal contents were similarly acidified and stored at –20°C for testing later. Supernatants from bacteria or stool were neutralized with 0.8 M $KHCO_3$ and after 15 min 1 vol of a reaction buffer (4 mM EDTA, 3 mM sodium azide, 0.6 mM NADPH, 5 mM glutathione and 3 U/ml glutathione reductase) was added to 2 vol of clear supernatant. The mixture was incubated at 37°C and the change in absorbance at 340 nm followed before and after addition of glutathione peroxidase (final concentration 0.28 U/ml). H_2O_2 concentrations were calculated from differences in absorbance using an extinction coefficient for NADPH of 6.27 mM/cm.

Electron spin resonance–spin trapping

Cultures of bacteria were washed with phosphate-buffered saline (PBS) and resuspended to a density of 10^9 c.f.u./ml in 25 mM Tris buffer (pH 7.4) with 7 mM glucose. The spin trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) (Oxis, Portland, OR) was added to a final concentration of 20 mM (19,25). Intestinal contents were suspended in 200 µl of 25 mM Tris (pH 7.4), clarified by centrifugation and the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) (Aldrich, Milwaukee, WI) added to a final concentration of 40 mM. Spectra were immediately recorded following the addition of a spin trap at ambient temperature (22°C) in a quartz flat cell using an X-band ER300E spectrometer (Bruker, Rheinstetten, Germany) with the following parameters: 100 kHz field modulation, microwave power 20 mW, modulation amplitude 1.0 Gauss, sweep width 100 Gauss/84 s and time constant 164 ms. DMPO radical adducts were quantified using the stable nitroxide 4-hydroxy TEMPO (Sigma, St Louis, MO) as a spin standard and identical instrument settings. Xanthine, xanthine oxidase, catalase, Tris, EDTA, sodium azide, glucose, manganese superoxide dismutase (MnSOD), NADPH and glutathione peroxidase were purchased from Sigma.

Tissue culture co-incubation assays

For co-incubation experiments, bacteria at 10^8 or 10^9 c.f.u./ml, or other reagents, were resuspended in McCoy's with fetal calf serum and added to a confluent growth of tissue culture cells. Flasks were incubated at 5% CO_2 and 37°C. At the end of timed experiments, tissue culture medium was aspirated and cells harvested by scraping in a small amount of mincing solution [Hank's balanced salt solution (Ca^{2+} and Mg^{2+} free, Gibco, Rockville, MD), 20 mM EDTA (Sigma), 10% dimethylsulfoxide (Fisher, Pittsburgh, PA)]. Chinese hamster ovary (CHO) cells were provided by Sheila Galloway at Merck Research Laboratories (West Point, PA) (26). HT-29 cells are a colonic epithelial line that was obtained from the American Type Culture Collection (Bethesda, MD, USA) (27). Cells were routinely maintained in McCoy's medium containing fetal calf serum at 5% CO_2 and 37°C.

Rat intestinal colonization model

Intestinal tracts of male Wistar rats were colonized with spontaneous streptomycin- and spectinomycin-resistant derivatives of OG1RF or PW18 (OG1RF-SS and PW18-SS, respectively) as described previously (19). In brief, rats were individually caged and fed a conventional diet *ad libitum*. After 2 weeks streptomycin- and spectinomycin-containing water (each at 500 mg/l) was provided to eliminate endogenous enterococcal flora and promote exogenous colonization with OG1RF-SS or PW18-SS. Three days later, rats were orogastrically administered 10^9 c.f.u. of *E. faecalis* in 0.2 ml of PBS. Five days post-inoculation, rats were anesthetized using inhaled isoflurane and ileal and colonic segments surgically excised. Samples of luminal contents were obtained and segments flushed with 60–100 ml of mincing solution. Segments were longitudinally opened and epithelial cells gently scraped from the luminal surface using a sharp scalpel. Cells were diluted in mincing solution for comet assay slide preparation (see below). Hematoxylin and eosin stained preparations from intestinal scrapings showed >90% of cells to be of epithelial origin. Enterococci in stool were enumerated using bile-esculin-azide agar plates (Difco, Detroit, MI).

Comet assay

The alkaline lysis single cell gel electrophoresis (comet) assay was used to measure single-strand breaks, alkali-labile sites and crosslinking of DNA from tissue culture and intestinal epithelial cells. Comet assays were performed as described previously (28). In brief, ~10^4 tissue culture cells, or material from 3–4 cm of scraped luminal intestine, in 5–10 µl of mincing solution were mixed in 75 µl of low-melting point agar at 37°C and layered onto microscope slides coated with 1% agarose. Tissue culture cells were assessed by fluorescent microscopy (Olympus BM-40X, Melville, NY) using 5,6-carboxyfluorescein diacetate (Molecular Probes, Portland, OR) and ethidium bromide (Sigma). Greater than 90% cell viability was considered adequate (29).

A third layer of agarose was applied to the embedded tissue culture or epithelial cells and slides refrigerated for >60 min in a lysing solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10), 1% Triton X-100, 10% dimethylsulfoxide]. Slides were electrophoresed for 20 min in 10 N NaOH and 200 mM EDTA (pH >13) at 25 V and 300 mAmp. Slides were neutralized with 400 mM Tris (pH 7.4) and fixed using 100% cold ethanol. After drying slides were stained with ethidium bromide and 50–100 randomly selected cells/slide scored by fluorescent microscopy (Figure 1). Slides were prepared and coded by one of us (V.A.) and read by another person (M.M.H.) masked to treatments.

Statistical analysis

The distribution of comet scores between tests and controls were compared using ridit analysis (30). This method assumes discrete measures represent intervals in an underlying continuous distribution. No other assumptions about the distribution are made using this technique. Ridits range from 0 to 1, and by definition the ridit for all control distributions is set at 0.50. If the mean ridit for a test distribution were >0.50, then more than half the time randomly selected measures from this distribution would have a value greater than randomly selected measures from a control distribution. For the comet assay a mean ridit >0.50 would indicate greater DNA damage compared with a control, whereas a value <0.50 would indicate lesser damage. Group comparisons were performed using JMP (Version 3.1, SAS Institute, Cary, NC). P values ≤ 0.05 were considered significant.

Results

E. faecalis produces superoxide and H_2O_2

As reported previously, *E. faecalis* strain OG1RF produced (mean ± SD) 17.2 ± 0.3 nmol of extracellular O_2^-/min/10^9 c.f.u. at 37°C (19). Under similar conditions the strain also produced 23 nmol H_2O_2/min/10^9 c.f.u. For the mutant strains

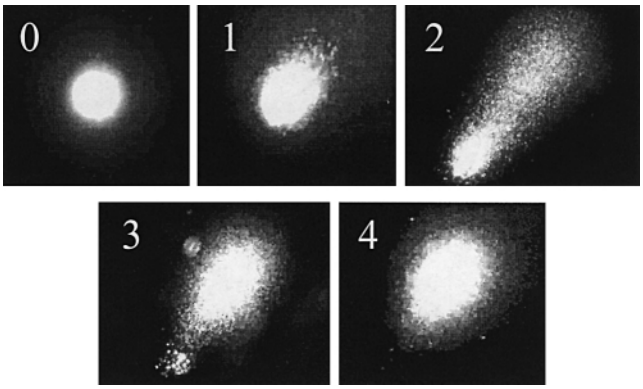

Fig. 1. The alkaline lysis single cell gel electrophoresis (comet) assay for measuring single-strand breaks, alkali-labile sites and crosslinking of DNA in tissue culture cells co-incubated with *E. faecalis*. Randomly selected cells were scored by fluorescent microscopy using a five point scale: 0, no visible DNA migration from the nucleus; 1, minimal DNA migration with an intact nucleus; 2, moderate DNA migration with reduction in the size of the nucleus; 3, extensive DNA migration with only a pinpoint nucleus remaining; 4, complete migration of DNA into a comet tail with no visible nucleus remaining.

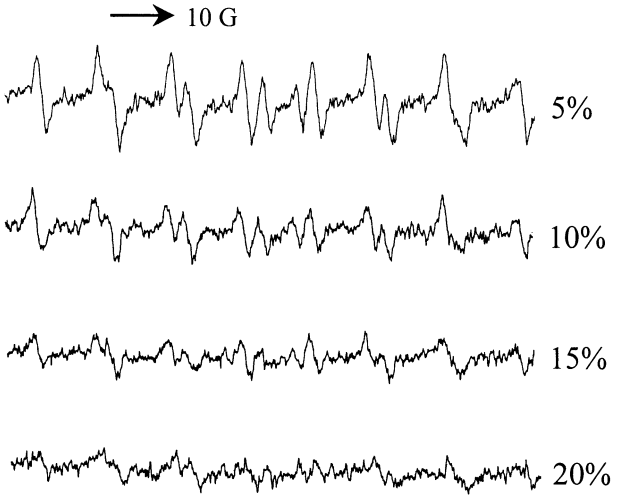

Fig. 2. ESR–spin trapping spectra for OG1RF in McCoy's with varying percentages of fetal calf serum. Bacteria grown in brain–heart infusion were washed and resuspended in McCoy's to 10^9 c.f.u./ml for 30 min. DEPMPO was added to 20 mM. Arrow points up field and length indicates 10 Gauss.

TM1 (*aroC*::Tn*917*) and PW18 (*menB*::p3erm) extracellular O_2^- production was <1.0 nmol/min/10^9 c.f.u. (19), and no H_2O_2 was detectable, implying that H_2O_2 was entirely generated by disproportionation of O_2^-.

H_2O_2 damages eukaryotic cell DNA

The effect of fetal calf serum on extracellular O_2^- production by OG1RF was investigated prior to tissue culture studies. OG1RF was resuspended in McCoy's with varying concentrations of fetal calf serum, incubated for 30 min and O_2^- and hydroxyl radical measured using ESR–spin trapping. Decreasing signal intensities for DEPMPO–hydroxyl radical and DEPMPO–superoxide adducts were found as the percentage of fetal calf serum increased (Figure 2). This effect was probably due to contamination of fetal calf serum with small amounts of MnSOD as reported previously (31), or from non-enzymatic scavenging of O_2^- by other serum constituents. As CHO and HT-29 cells grew well in McCoy's containing 10% fetal calf serum, and McCoy's supplemented with 5 or 10% fetal calf serum gave the strongest ESR–spin trapping signals, 10% fetal calf serum was selected for all subsequent experiments.

CHO and HT-29 cells were incubated for 30 min with 0, 25, 50, 100 or 200 μM H_2O_2. All mean ridits for all H_2O_2-treated cells were significantly >0.50 using unexposed cells as the control distribution (Table I). These results confirmed the comet assay and ridit analyses as sensitive measures of H_2O_2-mediated damage to genomic DNA. In addition, increasing mean ridits for greater concentrations of H_2O_2 were consistent with a dose response. Ridits at each H_2O_2 concentration were greater for CHO than HT-29 cells, possibly due to an enhanced resistance of HT-29 cells to H_2O_2-mediated DNA damage. The addition of catalase to CHO and HT-29 cells led to a modest, but statistically significant, decrease in mean ridits (Table II). Mean ridits, however, were unchanged for cells exposed to both 200 μM H_2O_2 and catalase. No treatment led to cell losses $>10\%$.

Under neutral conditions xanthine oxidase and xanthine produce O_2^- and H_2O_2 (32). To test whether enzymatically generated reactive oxygen species damaged HT-29 cell DNA, combinations of xanthine, xanthine oxidase and MnSOD were incubated with these cells for 30 min (Table III). Compared with untreated controls, xanthine modestly increased the mean ridit to 0.57 ($P = 0.01$), whereas xanthine oxidase alone had no significant effect (ridit = 0.48, $P = 0.35$). The combination of xanthine and xanthine oxidase, however, increased the mean ridit to 0.82 ($P < 0.001$), indicating substantial genomic DNA damage. As MnSOD rapidly converts O_2^- to O_2 and H_2O_2, MnSOD alone afforded presumably no protection to cells if DNA damage was due to H_2O_2 (mean ridit = 0.83, $P < 0.001$).

E. faecalis damages eukaryotic cell DNA

To determine whether extracellular O_2^- from *E. faecalis* damaged eukaryotic cell DNA, OG1RF and TM1 at 10^8 and 10^9 c.f.u./ml were co-incubated with CHO and HT-29 cells for 30 min (Table IV). Mean ridits were calculated using OG1RF as the test distribution and TM1 as the control. For all experiments significantly increased mean ridits, or increased genomic DNA damage, were observed for cells exposed to OG1RF compared with TM1. A dosing effect was apparent with increased mean ridits at the higher concentration of bacteria. Similar levels of genomic DNA damage were found when the incubation time was extended to 60 min (data not shown). Finally, the mean ridit for TM1 at 10^9 c.f.u./ml was not significantly different from control cells not exposed to any bacteria.

Catalase protects against E. faecalis-induced damage of eukaryotic cell DNA

To determine which reactive oxygen species generated by *E. faecalis* damaged tissue culture cell DNA, co-incubations were performed using CHO cells and OG1RF at 10^9 c.f.u./ml. Beforehand, MnSOD, catalase or MnSOD and catalase were added to bacteria in McCoy's medium. After a 30 min co-incubation, comet assays were performed and supernatants studied by ESR–spin trapping (Figure 3). For MnSOD-treated cells no decrease occurred in the mean ridit. This indicated that MnSOD conferred no protection against genomic DNA damage. In contrast, catalase alone, or in combination with MnSOD, significantly reduced the mean ridits suggesting substantial protection against DNA damage.

ESR–spin trapping of tissue culture supernatants containing MnSOD showed no DEPMPO adduct signals for O_2^- and hydroxyl radical (Figure 3), indicating hydroxyl radical arose

Table I. Effect of H_2O_2 on CHO and HT-29 cell DNA using the comet assay

Comet scores	Frequency of scores (%)[a]									
	CHO cells, H_2O_2 (μM)					HT-29 cells, H_2O_2 (μM)				
	0	25	50	100	200	0	25	50	100	200
0	38	12	50	1	0	54	35	21	30	9
1	38	32	17	10	0	36	45	53	35	26
2	12	20	23	15	6	9	16	24	20	28
3	8	23	39	44	27	1	3	2	15	20
4	4	12	12	30	67	0	1	0	0	17
Ridit	0.50	0.70	0.77	0.88	0.95	0.50	0.61	0.68	0.67	0.84
P value	–	<0.001	<0.001	<0.001	<0.001	–	0.004	<0.001	<0.001	<0.001

[a]Thirty minute incubations; assays performed twice with data combined.

Table II. Effect of catalase and H_2O_2 on CHO and HT-29 cell DNA using the comet assay

Comet scores	Frequency of scores (%)[a]							
	CHO cells				HT-29 cells			
	Control	Catalase	H_2O_2	Catalase and H_2O_2	Control	Catalase	H_2O_2	Catalase and H_2O_2
0	65	76	11	62	30	44	11	40
1	28	18	19	31	41	36	21	38
2	6	4	27	7	25	12	29	18
3	1	2	30	0	3	5	25	4
4	0	0	13	0	1	3	14	0
Ridit	0.50	0.44	0.74	0.52	0.50	0.43	0.75	0.44
P value	–	0.006	<0.001	0.41	–	<0.001	<0.001	0.003

[a]Thirty minute incubations; catalase, 100 U/ml; H_2O_2, 200 μM; assays performed twice with data combined.

Table III. Effect of xanthine and/or xanthine oxidase on HT-29 cell DNA using the comet assay

Comet scores	Frequency of scores (%)[a]				
	Control	Xanthine	Xanthine oxidase	Xanthine and xanthine oxidase	Xanthine, xanthine oxidase and MnSOD
0	42	19	45	8	5
1	34	60	38	21	20
2	16	18	8	11	11
3	6	2	9	34	39
4	2	1	0	26	25
Ridit	0.50	0.57	0.48	0.82	0.83
P value	–	0.01	0.35	<0.001	<0.001

[a]Thirty minute incubations; xanthine, 2 mM; xanthine oxidase, 100 U/ml; MnSOD, 100 U/ml.

entirely from O_2^- by iron-catalyzed Fenton reactions. In the presence of catalase, O_2^- and hydroxyl radical–DEPMPO adducts were still detectable. Loss of ESR signals occurred only when MnSOD had been added. The lack of effect of catalase on hydroxyl radical production from O_2^- is consistent with previous reports and represents either an accelerated decay of the O_2^-–DEPMPO adduct to a hydroxyl adduct by the tissue culture cells (33), or incomplete scavenging of H_2O_2 by catalase at the concentration used in this experiment. In aggregate, these data show that H_2O_2 arises from extracellular O_2^- produced by *E. faecalis*, and that H_2O_2 damages CHO cell DNA following short-term exposure.

E. faecalis damages colonic epithelial cell DNA in vitro

A rat intestinal colonization model was used to evaluate the *in vivo* production of reactive oxygen species by *E. faecalis*. Colon contents from rats that had been colonized with OG1RF-SS or PW18-SS for 5 days had similar mean concentrations of enterococci (Table V). Four-fold greater mean concentrations of H_2O_2 were found in colon contents for OG1RF-SS compared with PW18-SS colonized rats ($P = 0.05$). Ileal contents, however, had lower concentrations of both enterococci and H_2O_2, and no significant differences were noted in these measures between the two groups of colonized rats. ESR–spin trapping of ileal and colon contents for the OG1RF-SS

Table IV. Damage to CHO and H-29 cell DNA by *E. faecalis* using the comet assay

Comet scores	Frequency of scores (%)[a]									
	CHO cells					HT-29 cells				
	Control	10^8 c.f.u./ml		10^9 c.f.u./ml		Control	10^8 c.f.u./ml		10^9 c.f.u./ml	
		TM1	OG1RF	TM1	OG1RF		TM1	OG1RF	TM1	OG1RF
0	16	24	11	13	1	38	54	30	41	3
1	41	43	46	50	27	44	17	33	35	33
2	12	14	16	17	30	14	5	19	17	35
3	19	14	20	14	21	4	15	13	4	17
4	11	5	7	6	21	0	9	5	3	12
Ridit		0.50	0.58	0.50	0.71		0.50	0.59	0.50	0.78
P value		–	0.004	–	<0.001		–	0.01	–	<0.001

[a]Thirty minute incubations; assays performed twice with data combined.

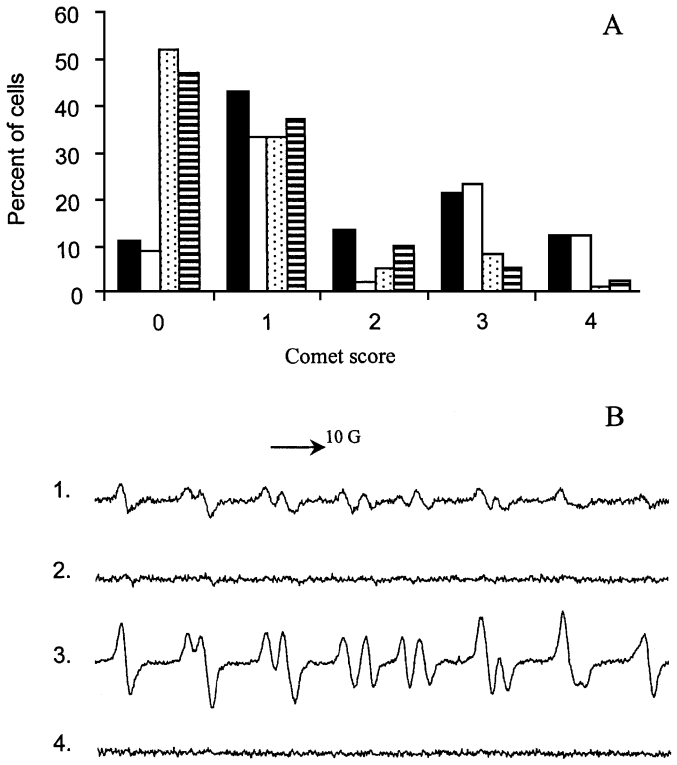

Fig. 3. Effect of catalase (1000 U/ml) and/or MnSOD (100 U/ml) on CHO cell DNA co-incubated with OG1RF at 10^9 c.f.u./ml for 30 min by the comet assay. (**A**) No addition (black bars); MnSOD (white bars, ridit = 0.54, $P = 0.13$); catalase (spotted bars, ridit = 0.24, $P < 0.001$); MnSOD and catalase (striped bars, ridit = 0.25, $P < 0.001$). Ridits were calculated using cells co-incubated only with OG1RF as the control distribution. (**B**) ESR–spin trapping of aliquots of supernatants from (A) following the 30 min incubation with DEPMPO at 20 mM: 1, no additions; 2, MnSOD; 3, catalase; 4, MnSOD and catalase. Arrow points up field and length indicates 10 Gauss.

colonized rats demonstrated DMPO adducts for both hydroxyl and thiyl radical (Figure 4). The characteristic hyperfine splitting constants for thiyl radical–DMPO adducts presumably arose from partial oxidation by *E. faecalis* of thiol compounds in stool (34,35). Colonic concentrations of hydroxyl and thiyl–DMPO adducts were 3-fold greater for rats colonized with OG1RF-SS compared with PW18-SS ($P = 0.04$).

To determine whether the increased production of reactive oxygen species by OG1RF-SS *in vivo* damaged epithelial cell DNA, scrapings from ileal and colon segments were assessed using the comet assay. The mean ridit for cells from ileal segments were not different for rats colonized with OG1RF-SS compared with PW18-SS (Table VI). For colon cells, however, the mean ridit was significantly higher for OG1RF-SS compared with PW18-SS colonized rats (ridit = 0.65, $P < 0.001$). The results suggest epithelial cell DNA in the colon was damaged through the production of extracellular O_2^- by *E. faecalis*.

Discussion

No single model has yet been accepted that can account for CIN found in sporadic adenomatous polyps and colorectal cancer (1–4,16,17,23). One novel hypothesis, however, proposes reactive oxygen species from colonic bacteria as direct initiators of genetic mutability (12). A corollary to this theory is potential modulation of bacterial free radical production and alteration of cellular antioxidant defenses by diet, linking genomic instability with existing epidemiological evidence on nutritional factors known to affect the prevalence of colorectal cancer (11,12,15,36).

Production of reactive oxygen species by colonic bacteria necessarily assumes the presence of O_2 in the colon. This would be expected at the epithelial–luminal interface where O_2 passively diffuses from cells into luminal contents. Leaching of O_2 from the epithelium is detectable in flatus where a mean pO_2 of 30 torr (range 0–119 torr) has been measured for healthy persons (37). Any O_2^- produced by colonic bacteria would disproportionate to H_2O_2 in the mildly acidic conditions of the colon, and be available to diffuse back into colonic epithelial cells to cause genomic DNA damage (38).

The discovery of extracellular O_2^- production by *E. faecalis*, a normal intestinal commensal, suggests a bacterial source for colonic reactive oxygen species. Enterococci have robust reducing properties (39,40) and were initially reported to produce extracellular O_2^- in 1981 (41). We subsequently found this phenotype typical for *E. faecalis*, but not other enterococci or facultative bacteria that colonize the colon (18,42). Genetic studies of *E. faecalis* strain OG1RF show demethylmenaquinone is essential for extracellular O_2^- production, and that O_2^- is only produced upon the conditional loss of terminal quinol oxidases that redox cycle this membrane-associated charge carrier (19).

Table V. Concentrations of enterococci, H_2O_2 and DMPO adducts of hydroxyl and thiyl radicals in rat intestinal contents[a]

Concentration	Ileum			Colon		
	PW18-SS	OG1RF-SS	*P* value	PW18-SS	OG1RF-SS	*P* value
Enterococci (log_{10} c.f.u./g stool ± SD)	5.3 ± 0.8	8.6 ± 1.7	0.25[b]	9.1 ± 0.4	9.1 ± 0.6	0.91[b]
H_2O_2 (μM/g stool ± SD)	0.8 ± 1.2	1.3 ± 1.6	0.13[c]	3.1 ± 2.7	11.7 ± 14	0.05[c]
DMPO-radical adducts (μM/g stool ± SD)	0.8 ± 0.9	0.7 ± 1.6	0.84[c]	0.6 ± 0.3	2.0 ± 2.2	0.04[c]

[a]Twenty-eight rats were colonized, 14 per group.
[b]Student's *t*-test.
[c]Wilcoxon signed rank test.

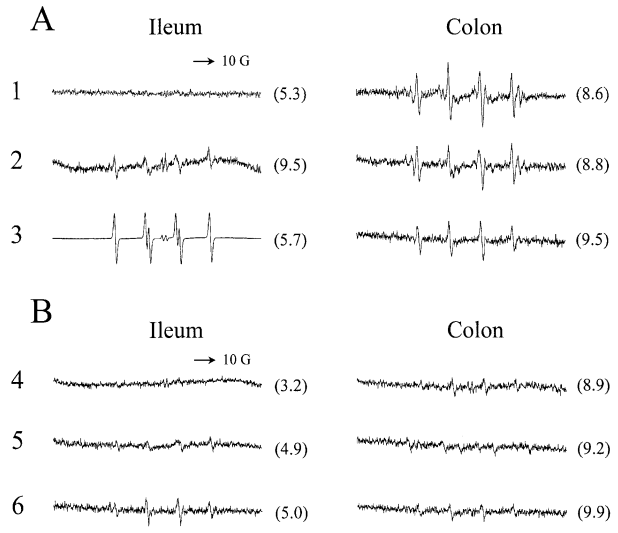

Fig. 4. Consecutive ESR spectra of intestinal contents for representative rats colonized with *E. faecalis* strain OG1RF-SS (**A**) or PW18-SS (**B**) using DMPO as a spin trap. Hyperfine coupling constants for DMPO adducts showed: 1, hydroxyl radical (a_N = 14.9 G and a_H = 14.9 G) for ileal (6) and colon (3 and 6) contents; 2, thiyl radical (a_N = 14.3 G and a_H = 16 G) for ileal contents (3); 3, composites of hydroxyl and thiyl radical adducts for ileal (2) and colon (1 and 2) contents. Small signals for ascorbyl radical were detected in ileal contents (2 and 3). All spectra were recorded identically except with the ileal contents for 3 were recorded at 10-fold lower sensitivity. Concentrations of enterococci are shown in parentheses as log_{10} (c.f.u./g stool). Arrows point up field and length indicates 10 Gauss.

Table VI. Damage to rat colonic epithelial cell DNA by intestinal colonization with *E. faecalis* using the comet assay

Comet scores	Frequency of scores (%)[a]			
	Ileum		Colon	
	PW18-SS	OG1RF-SS	PW18-SS	OG1RF-SS
0	10	4	10	1
1	26	23	24	18
2	27	36	43	33
3	33	27	15	31
4	6	10	8	17
Ridit	0.50	0.53	0.50	0.65
P value	–	0.08	–	<0.001

[a]Sixteen rats were colonized, eight per group; 50 cells scored per intestinal segment.

O_2^- is reactive under many *in vitro* and *in vivo* conditions and leads to H_2O_2 and hydroxyl radical (43). Our findings indicate that *E. faecalis* can readily generate O_2^- in the rat intestinal tract milieu. This was indirectly confirmed by ESR–spin trapping of ileal and colon contents where derivative hydroxyl and thiyl radicals were found. Sulfur-centered thiyl radicals most likely represent an oxidation of intestinal contents such as mucin which contains numerous cysteine-rich subdomains (44), glutathione from shed intestinal epithelial cells (45) or cysteine-rich compounds (e.g. mycothiol) synthesized by other intestinal microorganisms (46).

In this study we developed a modified glutathione peroxidase assay to measure H_2O_2 in intestinal contents. This technique, along with the ESR–spin trapping to quantify aqueous free radicals, showed significant differences in the concentrations of reactive oxygen species for rats colonized with the wild-type compared with mutant *E. faecalis* strains. Of note were lower, but still detectable, concentrations of H_2O_2 and DMPO adducts in intestinal samples from PW18-SS colonized rats. This may have been due to residual O_2^- production by PW18-SS through partial complementation of the biosynthetic block in the bacterial 1,4-dihydroxy-2-naphthoic acid synthase by endogenous naphthoate compounds in the intestine (19). Alternatively, a low level of reactive oxygen species might have arisen spontaneously from other intestinal constituents such as vitamin K_1, a phylloquinone produced by bacteria that readily forms reactive semiquinone radicals when mixed with bile salts and iron (47). Finally, anaerobic intestinal flora not suppressed by streptomycin or spectinomycin could have been another potential source of H_2O_2. Anaerobic bacteria are ordinarily present in high concentrations in the intestine and can generate H_2O_2 through poorly defined mechanisms upon exposure to oxygen (48). What effect, if any, basal levels of oxidative stress from these sources might have on epithelial cell DNA, however, was not addressed in this model.

We primarily undertook this investigation to determine whether *E. faecalis* colonizing the colon could damage epithelial cell DNA. The comet assay was selected to measure genomic DNA damage because: (i) it is among the most sensitive assays for detecting single or double-strand breaks and crosslinking in oxidatively damaged eukaryotic DNA; (ii) demonstrates a dose–response relationship for levels of DNA breakage at the lower end of detection for most other methodologies and (iii) has proven reliability in multiple studies (28,49). *In vitro* results show that H_2O_2 arising from O_2^- produced by *E. faecalis* rapidly damages tissue culture cell DNA. Similar results were observed *in vivo* for luminal cells from the colon of rats colonized with *E. faecalis*. Evidence for DNA damage, however, was not observed for cells from the ileum, perhaps because H_2O_2 and DMPO adduct concentrations in this portion of the intestine were less than in the colon where *E. faecalis* colonization was 50–10 000-fold more dense. Although con-

centrations of enterococci in tissue culture assays and colonizing the rat colon were 10–100-fold higher than what is normally recovered from human stool, these values still fall within the upper range for what has been reported previously for healthy persons (50).

Any plausible theory of colorectal carcinogenesis should consider why certain dietary factors are positively and negatively associated with these tumors (16,17). Oxidative stress from colonic flora is attractive in this respect. For example, high-risk diets—such as those rich in meat—could promote colon cancer through increased ingestion of iron. Iron is largely unabsorbed by the intestine and higher concentrations in the colon could accelerate catalysis of O_2^- to hydroxyl radical (12,51,52), increasing oxidation of polyunsaturated fatty acids in epithelial cell membranes. Peroxidation products of polyunsaturated fatty acids include the long-lived and freely diffusable clastogens 4-hydroxy-2-nonenal and 4-oxo-2-nonenal (53,54). In addition, dietary fats could directly promote free radical propagation via lipid peroxidation (55). Conversely, low-risk diets—such as those rich in fruits, vegetables and fiber—contain diverse antioxidants that could scavenge reactive oxygen species and limit free radical chain reactions (16,17). For example, phytic acid is a common component of fiber that can coordinate iron in a manner that inhibits hydroxyl radical formation (56). Finally, intraluminal reactive oxygen species may promote CIN by transforming dietary procarcinogens into tumor promoters (13,14).

We previously investigated the relationship between intestinal colonization with O_2^--producing enterococci and the risk for colorectal adenomas and cancer (42). In a prospective cohort study we found 40% of human stool samples from adults presenting for colonoscopy contained enterococci that produced extracellular O_2^-. No association, however, could be established between colonization with these bacteria and the risk for colorectal adenomas or cancer using multivariate modeling. Follow-up stool cultures from the same subjects one year later revealed significant changes in enterococcal flora. Eleven percent of subjects who had superoxide-producing enterococcal strains in their initial stool samples were no longer colonized with these microorganisms, whereas 14% had acquired a superoxide-producing strain when the initial culture had been negative. Heretofore unrecognized variability in enterococcal intestinal flora highlights difficulties inherent to assessing risk factors for slowly developing malignant lesions. Changes in colonic flora over time would obviously render single point-in-time measurements of intestinal contents inadequate for the determination of long-term risk (50). Despite this, our *in vitro* and *in vivo* findings support a role for extracellular O_2^- production by *E. faecalis* in colon carcinogenesis.

Oxidative stress on the colon epithelium from normal luminal bacteria such as *E. faecalis* is most likely low level, chronic and subject to modulation by diet and changing flora. It affords a plausible mechanism for CIN and may explain, at least in part, the genomic instability observed at the earliest stages of colorectal tumorigenesis (1,6). Further work, however, is needed to establish a direct link between luminal oxidative stress and the formation of adenomatous polyps and colorectal cancer.

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