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Enhancement of formation of the esophageal carcinogen benzylmethylnitrosamine from its precursors by *Candida albicans*

(chemical carcinogens/esophageal cancer/fungus infection/nitrosamine/nitrosation)

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ABSTRACT **Previous studies in Linxian, an area of China with a high incidence of esophageal carcinoma, showed that fungal infections are common in the esophageal epithelium of patients with either premalignant changes or early esophageal carcinoma. Fungi of the genus *Candida* were the most frequent invaders. In these areas nitrate and nitrite are often present in high concentrations in drinking water and staple grains. The present studies have established the ability of *Candida albicans* to augment the nitrosative formation of the esophagus-specific carcinogen, benzylmethylnitrosamine (NBMA; *N*-nitroso-*N*-methylbenzylamine). Stationary *C. albicans* cultures, with pH held at 6.8, were incubated with the precursors of NBMA, benzylmethylamine (BMA; *N*-methylbenzylamine) and $NaNO_2$. There was a significant increase in the amount of NBMA formed in these cultures, compared to precursors-only controls. The amount of NBMA synthesized depended on fungal cell number. Exponentially growing cultures were also able to cause NBMA formation. The identity of the NBMA was confirmed by high-performance liquid chromatographic coelution with authentic NBMA in three solvent systems and by mass spectroscopy. Boiled cells and conditioned medium in which cells had been incubated were not effective in enhancing nitrosation. Cultured *Candida* released acidic metabolites that reduced the pH of the medium when only a low concentration of buffer was present. Spontaneous nitrosation of BMA was enhanced under these acidic conditions. Thus, *C. albicans* infecting the esophageal epithelium could cause local formation of NBMA by both cell-mediated catalysis and extracellular decrease in pH.**

Esophageal carcinoma is the second most common cancer in China, causing 157,000 deaths every year. In Linxian, a county in Northern Honan province, the incidence of esophageal cancer is 178 per 100,000 people per annum, with a death rate of 132 per 100,000 (1). Pathological investigations in Linxian showed that fungal infections were often associated with hyperplasia or dysplasia of the esophageal epithelium in patients with premalignant changes or early carcinoma (2). More than half of the fungi found in the epithelium were *Candida* species; *Candida albicans* was isolated from the hyperplastic epithelium of a patient with esophageal carcinoma *in situ* (2). Anti-*candida* antibody titers were also high in the infected patients (3; unpublished data). An association between fungal infections and esophageal carcinoma was suggested.

Are *Candida* infections of the esophagus causally linked to development of cancer in this tissue? Stimulation of hyperplasia, release of tumor-promoting agents, and formation of carcinogens are possible mechanisms by which fungi could contribute to the appearance of tumors. Fungi and other microorganisms may enhance formation of nitrosamines from naturally occurring precursors (4–6), and certain nitrosamines are specific inducers of esophageal tumors in rodents (7, 8). It is possible that *Candida* participates in the causation of esophageal cancer in China by enhancing localized formation of carcinogenic nitrosamines in the epithelium. Therefore, we have investigated whether *C. albicans* in broth culture can enhance the formation of the carcinogen benzylmethylnitrosamine (NBMA; *N*-nitroso-*N*-methylbenzylamine)—which is esophagus-specific in rodents—from its precursors, benzylmethylamine (BMA; *N*-methylbenzylamine) and sodium nitrite.

MATERIAL AND METHODS

Media and Chemicals. Ingredients for Sabouraud's medium were obtained from Difco. Media were prepared from Sabouraud's broth powder (30 g/liter) or by adding 30 g of bactodextrose and 10 g of neopeptone per liter of distilled water. Chemicals used were BMA (Aldrich), $NaNO_2$ (Fisher), NBMA (Chemical Repository of the National Cancer Institute), ammonium sulfamate (Fisher), and glass-distilled methanol, ethyl acetate, isopropanol, and acetonitrile (Fisher or Waters Associates).

Fungal Cultures. *C. albicans* (group A) from the Infectious Disease Service, Department of Medicine, Memorial Sloan–Kettering Cancer Center, was cultured for 48 hr on Sabouraud agar before inoculation into Sabouraud's broth. After incubation at 37°C for 48 hr, 0.1–0.5 ml of this culture was transferred to 10 ml of fresh medium. For experiments with stationary-phase cells, these cultures were incubated for 48 hr before addition of 1 ml of $NaNO_2$ (10 mg/ml) and 1 ml of BMA (neutralized with HCl; 30 mg/ml in 0.5 M sodium phosphate buffer) and 1 ml of buffer of appropriate concentration. Unless otherwise noted, the concentration of buffer in the medium was 0.12 M. For the experiments with exponentially growing cells, the precursors of NBMA were added 8 hr after inoculation. Precursors-only controls consisted of BMA and $NaNO_2$ added to medium without *Candida*. Triplicate cultures were prepared in all cases and were incubated 24–48 hr.

The pH of the cultures was determined at the beginning and at the end of incubation with the precursors and in most experiments was found to vary by not more than 0.1 pH unit during this time. Cell numbers were counted before and after incubation with the precursors; the values listed in the table are the final cell counts. Cell viability was determined with methylene blue (1:10,000) and was found to be 99% in 48-hr cultures

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Abbreviations: NBMA, benzylmethylnitrosamine (*N*-nitroso-*N*-methylbenzylamine); BMA, benzylmethylamine (*N*-methylbenzylamine); HPLC, high-performance liquid chromatography.

before addition of the precursors and 98% after further culture with the precursors for 48 hr.

Boiled *Candida* were prepared by washing cells from 10-ml stationary cultures three times with 0.15 M NaCl, followed by resuspension in NaCl and boiling for 15 min. The boiled cells were washed again with NaCl and suspended in 10 ml of broth. Conditioned medium was obtained by filtration of 48–68 hr stationary cultures of *Candida*.

At the end of the incubations, the cells were removed by Millipore filtration (0.2 or 0.45 μm), and 0.2 ml of 0.1 M ammonium sulfamate was added to each tube to prevent further nitrosation.

Extraction and High-Performance Liquid Chromatography (HPLC) Determinations. Aliquots (5 ml) of filtered medium were extracted three times with 20 ml of ethyl acetate. After reduction in volume by rotary vacuum evaporation, the ethyl acetate extract was Millipore-filtered and evaporated just to dryness under N_2. The residue was dissolved in 50–200 μl of methanol and stored at −80°C prior to analysis. Extraction of 5-ml samples of Sabouraud's medium, including 25, 100, or 200 μg of NBMA indicated 95.4 ± 6.9% recovery of the nitrosamine by this method.

The extracted material was analyzed for NBMA content by HPLC with a Waters Associates chromatograph, a solvent flow of 1 ml/min and UV detection at 254 nm. Three solvent systems were employed for identification of NBMA by coelution: methanol/0.006 M potassium phosphate buffer, pH 6.5, 50:50 (vol/vol); isopropanol/0.006 M potassium phosphate buffer pH 7.8, 30:70 (vol/vol); and acetonitrile/water, 40:60 (vol/vol). The methanol system was used for routine separation of NBMA.

Identification of NBMA isolated from cultures was confirmed by gas chromatography/mass spectrometry with a LKB_{2091} GC-MS and ionization potential of 70 eV.

For quantification of the amounts of NBMA formed, the 2 ml containing the NBMA was collected during elution of the sample extracts; determinations made with extracts to which a known amount of NBMA had been added showed that 97–100% of the NBMA was recovered in this volume. A 40-μl aliquot of this fraction was rechromatographed, and the area of the NBMA chromatographic peak was estimated as the product of the peak height times the width at half peak height. Standard curves of peak area as a function of NBMA concentration were prepared with authentic NBMA.

RESULTS

Incubation of BMA and $NaNO_2$ in Sabouraud's medium for 24–48 hr resulted in the spontaneous formation of the nitroso derivative NBMA to an extent dependent on pH (Fig. 1). At pH values above 7.4, the amounts of NBMA detected were very low and near the limit of sensitivity of the methods employed. Increasing amounts were found with greater acidity over the pH range 7.4–3.5

With pH controlled at 6.8, the presence of *C. albicans* in the stationary phase of growth enhanced the formation of NBMA 2- to 4-fold, compared with precursors-only control cultures (Table 1, experiments 1–3). Similarly, with pH controlled at 5.8 or 7.0, *C. albicans* in stationary phase enhanced the formation of NBMA 2.5-fold (data not shown). The amount of NBMA formed depended on the number of cells (Table 1, experiments 1 and 2).

Inclusion of BMA and $NaNO_2$ in the culture medium inhibited the growth rate of *Candida* cells during the exponential phase: 4×10^4 cells per ml cultured with precursors multiplied at about 1/10th the rate of controls over 36 hr. Nevertheless,

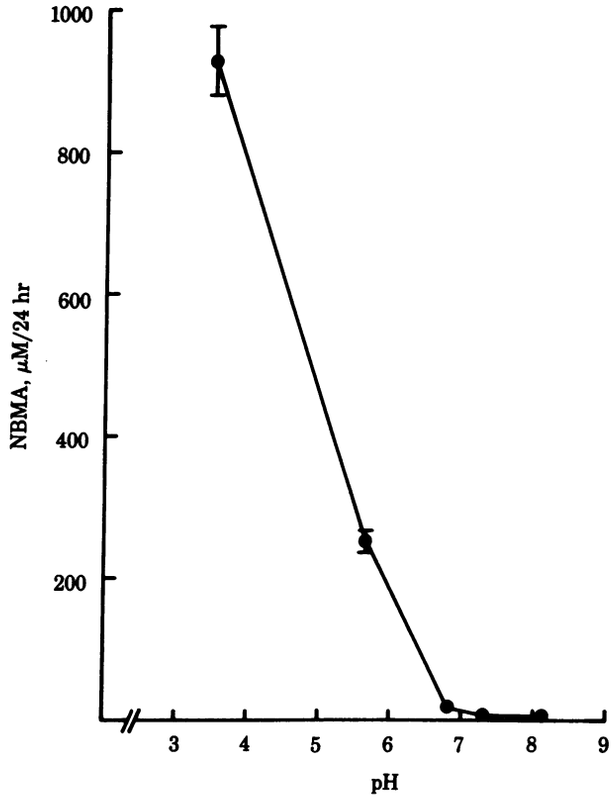

FIG. 1. pH dependence of NBMA formation from precursors.

exponentially-growing cultures were as able as stationary ones to catalyze nitrosamine formation (Table 1, experiment 3).

The nitrosating activity of the cells was destroyed by boiling (Table 1, experiments 1 and 3). Conditioned medium in which cells had been cultured for 48–68 hr and then removed by filtration did not enhance nitrosation at controlled pH (Table 1, experiments 1 and 2). In order to determine whether metabolites in the conditioned medium had catalytic activity masked by buffer, nitrosation in such medium without buffer was compared with that in control medium buffered at the same pH(Table 1, experiment 4). No difference was observed.

The identity of the NBMA formed in a culture of *Candida* with BMA and nitrite was confirmed by coelution in three HPLC solvent systems with standard NBMA and by mass spectroscopy of the isolated material (Fig. 2 *A* and *B*).

Incubation of stationary *Candida* cells in Sabouraud's medium in the absence of buffer resulted in reduction of the pH to about 5.0 after 48 hr. That this effect was due to production of acidic metabolites was shown by incubating boiled cells in the absence of buffer: the pH did not drop below 7.4, and only trace amounts of NBMA (about 2 μg/5 ml) were formed (Table 1, experiment 4). In a simulation of physiological conditions, a stationary culture of 1.2×10^8 cells per ml was adjusted to pH 7.4 with 0.025 M sodium phosphate buffer—approximately equivalent to the buffering capacity of human plasma. MBA and $NaNO_2$ were added to these cultures and to control media. After 48 hr, the precursors-only cultures had a pH of 7.2 and contained 8.4 ± 2.7 μg of NBMA per 5 ml. The *Candida*-plus-precursor cultures had decreased in pH to 6.1 and had produced 121.3 ± 19.6 μg of NBMA per 5 ml.

In an additional experiment, when precursors alone were cultured at pH 8.0 without buffer, the amount of NBMA produced was 1.3 μg/5 ml. In the *Candida*-plus-precursor culture

Table 1. Formation of benzylmethylnitrosamine from its precursor in the presence and absence of *C. albicans*

Exp. no.	pH[a]	Components	Cells,[b] no. × 10^{-7}	Incubation,[c] hr	NBMA,[d] μg/5 ml
1	6.8	MBA + $NaNO_2$	—	48	30.7 ± 5.1
		MBA + $NaNO_2$ +	7.4		62.1 ± 3.3
		live stationary cells[e]	26		110.2 ± 22.6
		MBA + $NaNO_2$ +	—		34.1 ± 18.9
		conditioned medium[f]			
		MBA + $NaNO_2$ + boiled cells[g]	7.4		32.0 ± 4.0
2	6.8	MBA + $NaNO_2$	—	48	31.7 ± 1.5
		MBA + $NaNO_2$ +	7.1		58.0 ± 15.1
		live stationary cells	13		93.0 ± 7.0
		MBA + $NaNO_2$ +	—		31.9 ± 5.6
		conditioned medium			
3	6.8[h]	MBA + $NaNO_2$	—	24	14.0 ± 2.0
		MBA + $NaNO_2$ + live	2.1		59.8 ± 19.1
		stationary cells			
		MBA + $NaNO_2$ +	0.069 to 1.0		37.5 ± 8.0
		exponential cells[i]			
		MBA + $NaNO_2$ + boiled cells[j]	5.6		14.2 ± 4.0
4	6.5	MBA + $NaNO_2$ +	—	36	25.2 ± 6.8
		conditioned medium (no buffer)			
	6.6	MBA + $NaNO_2$ (0.15 M buffer)	—		26.4 ± 1.0
	7.4	MBA + $NaNO_2$	—		1.9 ± 0.1
	7.4	MBA + $NaNO_2$ + boiled	4.3		2.0 ± 0.1
		cells (no buffer)			

[a] pH at beginning of incubation with precursors, maintained with 0.12 M sodium phosphate buffer unless otherwise noted.
[b] Number of cells per ml at end of incubation.
[c] Incubation time after addition of precursors.
[d] Average of 3 determinations ±SD
[e] Cultured 48 hr before addition of precursors.
[f] Medium of stationary culture from which cells had been removed by filtration.
[g] Stationary phase cells (48-hr culture) boiled for 15 min before addition of precursors.
[h] The final pH in the experiment was 6.5 in the live-cell incubations.
[i] Culture 8-hr old before addition of precursors; cell numbers are those at beginning and at end of incubation with precursors.
[j] Cells cultured 36 hr before boiling for 15 min.

in which pH fell from 8.0 to 6.8, the NBMA produced was 73.0 μg/5 ml, an increase of more than 50-fold over the control.

DISCUSSION

The precursors of BMA are naturally occurring. MBA has been identified in a variety of foods, especially vegetables, at levels as high as 16 ppm (9). Nitrite and its precursor nitrate were commonly found in the drinking water and food in areas of China with a high incidence of esophageal cancer, and salivary nitrite was significantly higher than normal in patients with esophageal epithelial dysplasia or carcinoma (10). As a typical nitrosation reaction, the formation of NBMA from BMA and $NaNO_2$ occurs most readily under acidic conditions but also proceeds to some extent at pH levels close to neutrality. NBMA was formed from its precursors in human gastric juice at pH values of 2.5–7 (10). Because of its chemical structure, BMA is more readily nitrosated than strongly basic amines such as dimethylamine (11).

Catalysis of nitrosamine formation has been reported for several fungi and secondary amines, including dimethylnitrosamine by *Saccharomyces cerevisiae* (5); dihexylnitrosamine by *S. cerevisiae* and dibutyl-, diphenyl-, and dihexylnitrosamine by *Saccharomycopsis lipolytica* (6); dimethylnitrosamine by *Aspergillus oryzae;* and *N*-nitrosodiphenylamine by a fungus and a yeast isolated from garden soil and cow manure, respectively (4). Chinese studies demonstrating the presence of nitrosamines in fungus-contaminated food have been reviewed by Yang (10). Nitrosamine was found in cornmeal inoculated with *Aspergillus niger*, in the stomach contents of rats fed moldy corn bread, and in nitrite-treated corn bread infected with species of *Fusarium* and *Aspergillus* (12, 13). In the latter study, NBMA was one of the nitrosamines identified.

These results led to the suggestion that Chinese esophageal cancer may be caused by nitrosamines arising in fungi-contaminated food. Our findings indicate an additional possibility: that carcinogenic nitrosamines, such as NBMA, are generated *in situ* in the esophagus by the action of *Candida* and other fungal species infecting this site. Fungal infection may enhance nitrosation by two separate additive mechanisms: decrease in pH through production of acidic metabolites and cell-mediated catalysis. The cell-mediated nitrosating activity requires live *Candida*; neither boiled cells nor conditioned medium had any effect. An enzymatic catalysis is suggested. Nitrosation of dimethylamine by rat intestinal flora (14, 15) and of dimethylamine and piperidine by *Escherichia coli* (16) was also found to be partially heat-labile. In some other microbial nitrosation systems, however, boiled cells were as effective as live ones (6, 17, 18).

The effect of fungal infections on local epithelial pH is not known. Experimental irritative exudates may have a pH of 6.95 (19). *C. albicans* produces acetic, lactic, succinic, and citric acids during its metabolic cycle (20). In our experiments, stationary

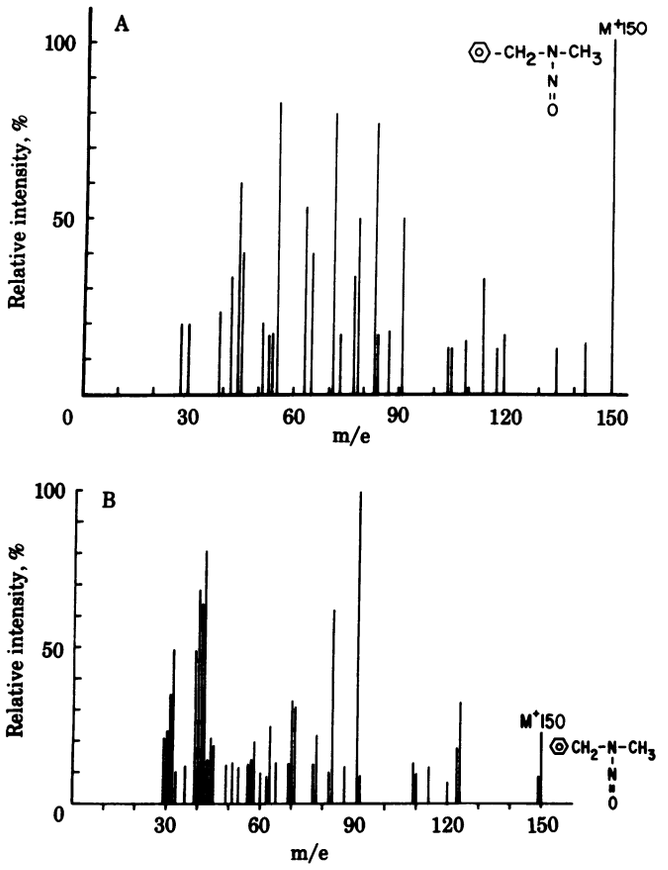

FIG. 2. (*A*) Mass spectrum of NBMA formed in culture of *C. Albicans* precursors with BMA and $NaNO_2$. *m/e* fragment ions with 30, 42, 77, 91, 120, and M^+ 150. (*B*) Mass spectrum of authentic NBMA.

cultures of *Candida* attained a pH of 5 in the absence of buffer and 6.1 in the presence of plasma-equivalent buffer concentration. Because esophageal epithelium heavily infected with *Candida* is highly thickened with no blood vessels, acidic products might accumulate; a pH as low as 6.8 seems possible. On this basis pH 6.8 was chosen for our study of cell-mediated nitrosation. Our results indicate that the combined effect of cell-mediated nitrosation and reduction in pH of the epithelial fluid from 7.4 to 6.8 could cause a 30- to 50-fold enhancement of rate of formation of NBMA from its precursors. Generation of nitrosamine in close proximity to the target tissue, especially under conditions of poor fluid circulation, could result in a concentration sufficient to initiate tumorigenesis. Similar considerations pertain also to other hygiene-related cancers, such as those of the penis and uterine cervix (21).

This hypothesized etiological mechanism, while speculative at present, is useful because it leads to experimentally testable predictions—for example, the occurrence of NBMA in fungi-infected esophagi of humans and domestic animals in the areas of China with a high incidence of esophageal cancer. It also offers hope of prevention of these cancers by elimination of the conditions leading to fungal infection.

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