**Bacillus** Species Are Present in Chewing Tobacco Sold in the United States and Evoke Plasma Exudation from the Oral Mucosa

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Five *Bacillus* species, predominantly *Bacillus megaterium* and *Bacillus pumilus*, were isolated from two popular brands of commercially available chewing tobacco [(5.0 ± 1) × 10^6 CFU/ml of supernatant; results for four experiments]. Moreover, the supernatant of the *Bacillus* culture evoked plasma exudation from postcapillary venules in the intact hamster cheek pouch, exudation that was mediated by the kallikrein/kinin metabolic pathway. Taken together, these data indicate that *Bacillus* species contaminate chewing tobacco commercially available in the United States and elaborate a potent exogenous virulence factor(s) that injures the oral mucosa.

Habitual use of chewing tobacco is on the rise in the United States (4). This practice may be associated with oral mucosa inflammation and injury in susceptible individuals and may predispose chewing tobacco users to oral epithelial cell dysplasia and cancer (7, 15, 23). Previous studies from our laboratory showed that chewing tobacco elicits plasma exudation from the oral mucosa by activating oral keratinocytes and the kallikrein/kinin metabolic pathway (2, 5, 6, 18, 25). However, the toxic constituent(s) of chewing tobacco that accounts for these responses has not been characterized (8, 17).

A physiological disorder of the tobacco plant, termed “frenching,” has been known to the tobacco industry since colonial times (22). Although the etiology of this condition is uncertain, so-called organic toxins produced by *Bacillus* species and found in the soil where tobacco plants are grown have been implicated (22). It is conceivable that *Bacillus* spores could contaminate chewing tobacco processed for human use and germinate when placed onto the oral mucosa. These bacteria could then elaborate potent virulence factors, such as proteases, that activate oral keratinocytes and the kallikrein/kinin metabolic pathway in the oral mucosa, leading to plasma exudation and tissue injury (2, 9, 14, 16, 19, 20).

The purpose of this study was to begin to address this issue by determining whether *Bacillus* species contaminate commercially available chewing tobacco and, if so, whether they evoke plasma exudation from the intact oral mucosa.

**MATERIALS AND METHODS**

**Culture of chewing tobacco.** One box each of two popular chewing tobacco brands (moist snuff; Skoal Cherry and Skoal Spearmint [U.S. Tobacco Co., Richmond, Va.]) were purchased at a local grocery store in Chicago, Ill. One-half brands (moist snuff; Skoal Cherry and Skoal Spearmint [U.S. Tobacco Co., Richmond, Va.]) were purchased at a local grocery store in Chicago, Ill. One-half of one box of each was homogenized in a blender (9,600 rpm for 1 min). Aliquots of the homogenate of each box were placed on blood, mannitol salt, and MacConkey agar plates by sterile cotton swabs and incubated aerobically at 37°C. After 48 h, the cultures were examined and a Gram stain of the isolated colonies was performed (11, 12, 16). After an additional 48 h of incubation 13 biochemical tests were conducted to identify the isolated gram-positive, spore-forming *Bacillus* species (12). A viable count was done to determine the number of organisms present in each sample. The most frequently isolated *Bacillus* species was inoculated into 7 ml of Trypticase soy broth and incubated for 72 h (11, 16). Thereafter, the culture was centrifuged (15,000 × g), and the supernatant was filtered through a 0.22-µm-pore-size filter, snap-frozen in liquid nitrogen, and stored at −70°C until used (see below).

**Determination of macromolecular efflux from the oral mucosa.** To determine the effects of the *Bacillus* supernatant on plasma exudation from the intact oral mucosa, we used the in situ microcirculation of the hamster cheek pouch as previously described in our laboratory and by other investigators (5, 6, 13, 18–20, 25). Briefly, we used adult, male golden Syrian hamsters (mean body weight, 130 ± 1 g) that had been anesthetized with pentobarbital sodium and on which tracheostomies had been performed. The cheek pouch microcirculation was visualized with a fluorescence microscope (magnification, ×40). Macromolecular leakage was determined by extravasation of fluorescein isothiocyanate-labeled dextran (FITC-dextran; molecular mass, 70 kDa), the intravascular tracer, which appeared as fluorescent spots or leaky sites around postcapillary venules. The number of leaky sites in three random microscopic fields was counted, averaged, and expressed as the number of leaky sites per 0.11 cm^2 of cheek pouch, which corresponds to the area of one microscopic field (5, 6, 13, 18–20, 25). With a spectrophotofluorometer, the concentration of FITC-dextran in the plasma and submucosa was determined based on a standard curve of FITC-dextran concentration versus percent emission. The clearance of FITC-dextran was determined by calculating the ratio of the concentration of FITC-dextran in submucosa (nanograms per milliliter) to that in plasma (milligrams per milliliter) and multiplying this ratio by the sulfusate flow rate (2 ml/min).

The experimental design has been used previously in our laboratory (5, 6, 18–20, 25). After the buffer was suffused for 30 min (the equilibration period), FITC-dextran was injected intravenously and the number of leaky sites and the clearance of FITC-dextran were determined for 30 min. Next, the *Bacillus* supernatant (1:1,000 dilution in bicarbonate buffer, pH 7.4) was suffused over the cheek pouch for 20 min. The number of leaky sites was determined every minute for 20 min and at 5-min intervals for 60 min thereafter. The clearance of FITC-dextran was determined before the application of the supernatant and every 5 min after for 60 min. In another group of animals, 1 µM Hoe140 or 1 µM NPC 17647, both structurally distinct selective Bradykinin B2 receptor antagonists (2, 5, 19–21), was suffused over the cheek pouch for 30 min before the check pouch was suffused with the *Bacillus* supernatant (1:1,000 dilution in bicarbonate buffer, pH 7.4) for 20 min. The observer of the cheek pouch microcirculation was unaware which hamster belonged in which treatment group. In preliminary studies, we determined that suffusion of bicarbonate buffer (vehicle) or of a 1:1,000 dilution of the bacteriological medium in bicarbonate buffer (pH 7.4) for the entire duration of the experiment or for 20 min, respectively, was associated with no visible leaky-site formation and no significant increase in the clearance.
of FITC-dextran (four animals; P > 0.5). The concentration of the Bacillus supernatant used in these experiments was based on preliminary experiments. The concentrations of Hoe140 and NPC 17647 used in these experiments were based on previous studies in our laboratory (5, 19, 20).

**Chemicals and drugs.** FITC-dextran was obtained from Sigma Chemical Co. Hoe140 and NPC 17647 were gifts from Aventis Pharmaceuticals and Nova Pharmaceutical Corporation, respectively. All drugs were dissolved in saline. Drugs were prepared fresh before each experiment and were diluted in saline to the desired concentrations.

**Data and statistical analyses.** When a test compound was suffused over the cheek pouch, we determined the maximal change in the number of leaky sites and the clearance of FITC-dextran and noted it as the response to that compound. Data are expressed as means ± standard errors of the means, except for body weight, which is expressed as mean ± standard deviation. Statistical analysis was performed using two-way analysis of variance and the Newman-Keuls test for multiple comparisons (StatView; SAS Institute Inc., Cary, N.C.) on a personal computer. A P value of <0.05 was considered statistically significant.

**RESULTS**

**Culture of chewing tobacco.** Gram stain of cultures of both brands of chewing tobacco revealed large gram-positive, spore-forming bacilli in all samples (four separate experiments were conducted in duplicate). Biochemical analysis of these isolates revealed the presence of five distinct species of Bacillus, i.e., eight isolates of Bacillus megaterium, six of Bacillus pumilus, five of Bacillus brevis, two of Bacillus licheniformis, and one of Bacillus subtilis in the four experiments. The mean colony count was (5.0 ± 1.0) × 10^6 CFU/ml of supernatant (7.0 × 10^6 CFU/ml for experiments 1 and 2, 3.0 × 10^6 CFU/ml for experiment 3, and 2.1 × 10^6 CFU/ml for experiment 4). No other bacteria were isolated from either brand of chewing tobacco.

**Determination of macromolecular efflux from the oral mucosa.** Suffusion of saline (vehicle) alone for the entire duration of the experiment evoked no visible leaky-site formation and no significant increase from baseline in the clearance of FITC-dextran (Fig. 1) (four animals; P > 0.5). Repeated suffusions of B. megaterium supernatant (1:1,000 dilution in bicarbonate buffer, pH 7.4) for 20 min, each with 30-min suffusions of saline B. megaterium supernatant (1:1,000 dilution in bicarbonate buffer, pH 7.4) for 20 min, each with 30-min suffusions of saline alone for 30 min, like suffusion of saline alone, evoked no visible leaky-site formation and no significant increase from baseline in the clearance of FITC-dextran (each group contained four animals; P > 0.5). Suffusion of Hoe140 and NPC 17647 (1 μM each) for 30 min significantly attenuated B. megaterium supernatant-induced leaky-site formation and the increase in clearance of FITC-dextran from the cheek pouch (Fig. 1) (each group contained four animals; P < 0.05 in comparison to results for B. megaterium supernatant alone).

**DISCUSSION**

This study produced two new findings. First, we found that five distinct Bacillus species, predominantly B. megaterium and B. pumilus, contaminate, in relatively large numbers, two popular brands of chewing tobacco commercially available in the United States. Second, suffusion of a diluted B. megaterium supernatant onto an intact hamster cheek pouch, an established animal model for studying the mechanisms underlying the deleterious effects of smokeless tobacco in the oral mucosa (5, 6, 18), significantly increases macromolecular efflux from the cheek pouch.

This response was mediated by local elaboration of bradykinin, because Hoe140 and NPC 17647, two structurally distinct selective bradykinin B2 receptor antagonists (2, 5, 19–21), significantly attenuated B. megaterium supernatant-induced responses. Taken together, these data indicate that Bacillus species contaminate commercially available chewing tobacco and elaborate a potent exogenous virulence factor(s) that activates the kallikrein/kinin metabolic pathway in the intact oral mucosa. This, in turn, evokes plasma exudation from postcapillary venules, which leads to interstitial edema and tissue dysfunc-
tion. Whether Bacillus species contaminate other brands of commercially available chewing tobacco remains to be determined.

The mechanisms underlying bradykinin production by B. megaterium supernatant in the intact hamster cheek pouch were not elucidated. Nonetheless, the results of this study indicate that a potent bacterium-derived exogenous virulence factor(s) activates the kallikrein/kinin metabolic pathway in the oral mucosa to elaborate bradykinin, a potent edemagenic mediator (2, 25). Previous studies have implicated proteinases released by oral bacteria, such as subtilisin and gingipain RgpA, in bradykinin-induced oral mucosa injury and inflammation in vivo (9, 14, 18–20). It remains to be determined whether Bacillus species contaminating commercially available chewing tobacco elaborate proteinases that directly activate the kallikrein/kinin metabolic pathway in the oral mucosa or whether a virulence factor(s) released by these bacteria stimulates oral keratinocytes, the first cells in the oral mucosa exposed to chewing tobacco and Bacillus species, to elaborate proteinases that activate this metabolic pathway (9, 14, 18–20). Likewise, the role of various chemical constituents of chewing tobacco in modulating the germination and expression of virulence factors by Bacillus species in the oral mucosa should be addressed (8, 17).

Notwithstanding the findings described above, the relevance of the present acute experimental study of the intact hamster cheek pouch to oral mucosa inflammation and injury observed in susceptible habitual chewing tobacco users is uncertain; the results of this study do not include the number of bacteria required to elaborate sufficient quantities of soluble virulence factors to injure the oral mucosa or whether Bacillus species spores and bacteria adhere to oral keratinocytes (2, 7, 9, 14–16, 19, 20). However, habitual users consume relatively large quantities of chewing tobacco continually and spit it out rather than rinsing their mouths thoroughly after each application (4, 7, 23). This practice creates a local environment in the oral mucosa that is conducive to the germination of Bacillus species spores and to the elaboration of soluble virulence factors (9,
12, 16). Certain Bacillus species, including B. licheniformis and B. pumilus, isolated from chewing tobacco in the present study, have been shown to cause opportunistic infections and pulmonary inflammation in humans (1, 3, 10, 24). The results of the present study extend these observations by showing that Bacillus species contaminating commercially available chewing tobacco elicit oral mucosa inflammation in experimental animals. Clearly, additional experimental and clinical studies are warranted to address these issues.

In summary, we found that five distinct Bacillus species, predominantly B. megaterium and B. pumilus, contaminate in relatively large numbers chewing tobacco commercially available in the United States. Once germinating, these bacteria elaborate a potent exogenous virulence factor(s) that activates the kallikrein/kinin metabolic pathway in the intact oral mucosa, leading to plasma exudation and tissue dysfunction.

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