Objective: While clinical observation has suggested an association between gastroesophageal reflux and laryngeal carcinoma, the nature of this relationship has yet to be defined. The purpose of this study is to determine the carcinogenic potential of acid and pepsin mixtures in the hamster cheek pouch animal model.

Design: A blinded intervention study.

Subjects: One hundred male Syrian hamsters aged approximately 5 weeks.

Interventions: A control group of 20 hamsters received application of the carcinogen 9,10-dimethyl-1,2-benzanthracene (DMBA) to their cheek pouch mucosa. One experimental group (n = 20) received applications of DMBA plus hydrochloric acid, and another (n = 20) received DMBA plus an acid and pepsin solution. Latency to squamous cell tumor production, size of tumors, and numbers of tumors were compared among groups.

Results: Latency to tumor production and size of tumor were similar among groups, with both experimental and control groups developing tumors of comparable size after 12 weeks of chemical application. However, the number of tumors produced was significantly higher in the DMBA/acid and DMBA/acid/pepsin groups than in the DMBA only group at 18 weeks, with 23, 27, and 10 tumors in these groups, respectively (P < .02). Likewise, a cumulative dysplasia score was different among groups at 18 weeks with the DMBA/acid and DMBA/acid/pepsin groups scoring higher degrees of dysplasia than the DMBA only group.

Conclusion: These results suggest that application of acid and acid/pepsin mixtures may promote experimental carcinogenesis in the hamster cheek pouch.
thermore, both tobacco and alcohol, known risk factors for laryngeal cancer, are promoters of gastroesophageal reflux. The presumed pathophysiological development of reflux carcinogenesis begins with a chronic inflammation brought about by the long-term exposure of laryngeal tissues to gastric secretions. A cycle of repeated tissue damage and regeneration is set in motion. The inflammation itself is thought to be a possible mutagenic factor, and the hyperregenerative state a neoplastic promoter. The malignant transformation is thought to be the same as at other inflammatory-carcinogenic sites, i.e., burn scars and skin cancer (Marjolin ulcers) or poor oral hygiene and oral cancer.

Carcinogenesis in tissues of the upper aerodigestive tract has best been studied using the hamster cheek pouch (HCP) model. First introduced by Salley in 1954 the HCP has been the model of choice since then; it is histologically very similar to keratinizing human oral mucosa. The HCP mucosa has 4 distinct layers (from superficial to deep): keratinized epithelium, lamina propria, a muscle layer, and loose areolar tissue. Application of 9,10-dimethyl-1,2-benzanthracene (DMBA) to the HCP at varying concentrations produces an invasive squamous cell tumor in 8 to 12 weeks. Latency to tumor production is uniform within 1 to 4 weeks. Promoter studies are performed via the concurrent or serial application of the substance in question. Animals are killed and cheek pouches examined histologically to determine differences in latency to tumor production, tumor number, and tumor size. A promoter will shorten the latency and increase size and number of tumors.

### MATERIALS AND METHODS

Institutional guidelines for animal experimentation were followed for the duration of the project. One hundred male golden Syrian hamsters, aged approximately 5 weeks, were divided into 5 groups as follows: group 1, DMBA only (controls, n = 20); group 2, hydrochloric acid (HCl) solution only (positive controls, n = 20); group 3, HCl/pepsin solution only (positive controls, n = 20); group 4, DMBA/HCl solution (experimental, n = 20); and group 5: DMBA/HCl/pepsin solution (experimental, n = 20).

Solutions were prepared as follows: 0.5% DMBA, 1 g of DMBA (powder) in 200 mL of heavy mineral oil, mixed over heat; 0.1N sodium hydroxide solution, 1.016 g of sodium hydroxide in 250 mL of distilled water, 1.5 pH HCl solution, 0.1N HCl buffered with 0.1N sodium hydroxide solution to reach a pH of 1.5; and HCl/pepsin solution, 1 mg of porcine pepsin in 1 mL of HCl solution, prepared daily.

Using a small camel hair brush and a cheek retracting instrument, DMBA was applied to the right cheek pouch (Figure 1) of hamsters in groups 1, 4, and 5 twice weekly. The HCl and HCl/pepsin solutions were applied to the right cheek pouch twice daily Monday through Friday in the corresponding groups (HCl in groups 2 and 4, HCl/pepsin in groups 3 and 5). When certain groups were scheduled for applications and others were not, isotonic sodium chloride was applied to those not scheduled to assure all animals underwent the same number of mechanical applications to the cheek pouch.

All cheek pouches were inspected weekly using a headlight with eversion of the pouch. When 5 animals in any 1 group developed gross lesions at least 3 mm in diameter, these 3 animals and the 5 worst animals in the other groups were killed (3-mm lesions are a historical parameter, with cancerous lesions following these by 2-4 weeks.) From that point on the 5 worst animals in each group were killed every 2 weeks for a total of 4 killing periods.

After the animals were killed, the cheek pouches were harvested and placed in formalin. A pathologist, blinded to group designation, examined each specimen for number and size of lesions. The lesions were then examined histologically. Each was graded and scored for degree of dysplasia as follows:

<table>
<thead>
<tr>
<th>Lesion Size, mm</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;3</td>
<td>1</td>
</tr>
<tr>
<td>3-5</td>
<td>2</td>
</tr>
<tr>
<td>&gt;5</td>
<td>3</td>
</tr>
</tbody>
</table>

Grade of Dysplasia: None/mild/moderate 1 Severe dysplasia/invasive SCC* 2

*SCC indicates squamous cell carcinoma.

End points were therefore latency to cancer production, size of squamous cell carcinoma (SCC) lesions, number of SCC lesions, and a dysplasia score for each animal. An SCC lesion was defined as one with neoplastic change at least completely through the epithelium, abutting the lamina propria. Clinically, this would have been equivalent to a lesion graded “carcinoma in situ.” A t test was used for statistical analysis.

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**Figure 1.** Photograph of a hamster with the right cheek pouch exposed using a cheek retractor instrument and forceps.
While the HCP model has been used extensively to study the carcinogenic potential of other substances, it has never been used to address the role of gastric secretions in the development of cancer. There have been no prospective clinical studies nor any animal models that confirm or deny the association of reflux laryngitis and larynx cancer. The objective of the current study is to clarify this association in an experimental setting using the HCP model.

**RESULTS**

Gross ulcerations were first noted in groups 1, 4, and 5 at week 2. These healed, only to be replaced by papillomatous-appearing lesions in weeks 6 through 10 (Figure 2). Five 3-mm lesions were present first in group 5 at 12 weeks. These 5 animals and the 5 worst animals from each of the other groups were killed at that point. Killing periods were therefore at 12, 14, 16, and 18 weeks.

With the exception of 1 tumor in group 2 at 12 weeks, all cheek pouches in groups 2 and 3 appeared essentially unchanged on gross and microscopic inspection throughout the study. Squamous cell tumors were present in groups 1, 4, and 5 at 12 weeks (Figure 3). Therefore latency to tumor production was essentially equal between control and experimental animals. The average size of tumors among groups is depicted graphically in Figure 4, which shows an equivocal relationship between groups 1, 4, and 5. Figure 5 compares the number of squamous cell tumors in each killed group at each killing period. For the first 3 killing periods, the number of tumors was approximately the same among groups. However, in the last period, at 18 weeks, there were many more tumors present in groups 4 (23 tumors) and 5 (27 tumors) than in group 1 (10 tumors) (P<.02). Figure 6

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**Figure 2.** Histologic section of hamster cheek pouch mucosa showing a typical papillomatous lesion (hematoxylin-eosin stain; original magnification ×10). Note the adjacent normal mucosa with layers including (superficial to deep) keratinized epithelium, lamina propria, muscle, and loose areolar tissue.

**Figure 3.** Histologic section of hamster cheek pouch mucosa showing a cancerous lesion with dysplastic cells extending through the lamina propria.

**Figure 4.** Comparison of average tumor size among hamster groups. DMBA indicates 9,10-dimethyl-1,2-benzanthracene; HCl, hydrochloric acid; and pep, pepsin.

**Figure 5.** Comparison of tumor number among hamster groups. SCC indicates squamous cell carcinoma; DMBA, 9,10-dimethyl-1,2-benzanthracene; HCl, hydrochloric acid; and pep, pepsin.
depicts a gross cheek pouch specimen with multiple papillomatous and cancerous lesions spread over its surface. The cumulative dysplasia score is also different among groups. At week 18, group 1 had a score of 88; group 4, 155; and group 5, 136 (Figure 7).

Both mechanical and chemical irritation of the hamster cheek pouch (HCP) have been shown to be carcinoma promoters. Chen and Squier demonstrated enhancement of tumor production in the HCP with the concurrent application of nicotine, and Elzay did the same with application of alcohol. Renstrup et al showed that DMBA-induced tumors arise more quickly at the borders of a cheek pouch ulcer created by chronic mechanical irritation.

Several studies have examined the different properties of gastric juice components. Animal studies with short-term exposure of the esophagus to mixtures of hydrochloric acid, pepsin, bile, and pancreatic enzymes have consistently shown pepsin, at a pH of 1.0 to 3.0, to be the most injurious component. Hydrochloric acid solution, at this same pH, is also very inflammatory. Short-term exposures of the injured animal larynx have similarly shown pepsin to be the component that most actively prevents healing.

In the current study, the HCl and HCl/pepsin solutions used alone seemed to have very little inflammatory effect visible under gross and microscopic inspection in groups 2 and 3. The HCP mucosa is keratinized, which may provide some degree of protection from these solutions. The true vocal cords, on the other hand, are not keratinized and may be more prone to inflammation and injury from chronic exposure to gastric secretions. Interestingly, in groups 4 and 5, where both DMBA and an HCl or HCl/pepsin solution were applied, there was more inflammation grossly than in group 1 where DMBA was used alone. This suggests some sort of synergistic relationship between the DMBA and synthetic reflux solutions.

Latency to tumor production and size of tumor were essentially equal among groups 1, 4, and 5. Likewise, for the first 3 killing periods, number of tumors and dysplasia scores were roughly equal among these groups. However, at week 18 there was a significant difference between group 1 and groups 4 and 5. The experimental groups seem to have developed a significantly higher number of tumors and a higher dysplasia score than did the control group at week 18. These results suggest that there may well be a promotional effect beginning to occur near week 18. Perhaps it took 18 weeks for the effect of the reflux mixtures to take hold, with “field cancerization” setting in at this point. However, it is difficult to draw any firm conclusions given there were only 15 animals for comparison at week 18.

Future experiments should be modified to help clarify what effect, if any, these synthetic reflux mixtures have on the carcinogenic process. The acid load and pepsin concentration can likely be increased in order to produce a more profound inflammatory effect on the HCP mucosa, which seems somewhat resistant to even a twice-daily application schedule. Also, the experiment should be extended out beyond the 18-week point in this study. It would be very interesting to know if the differences between groups seen at week 18 continue through weeks 20, 22, and 24.

Though preliminary, the above data indicate that HCl and HCl/pepsin mixtures may promote carcinogenesis in the HCP. While tumors did not seem to grow faster or larger under the influence of acid/pepsin mixtures, those animals under chronic acid/pepsin exposure did develop significantly more tumors than those not exposed.

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