

## Decrease in Le<sup>x</sup> Expression in Esophageal Adenocarcinomas Arising in Barrett's Epithelium<sup>1</sup>

Ulla Engel,<sup>2</sup> Ronda McCombs, Patricia Stranahan, David Pettijohn,<sup>3</sup> and Esther Hage

State University Hospital, Copenhagen, Denmark [U.E., E.H.], and University of Colorado Cancer Center, Denver, Colorado 80262 [R. M., P. S., D. P.]

### Abstract

Fifty esophageal adenocarcinomas were investigated for their expression of Le<sup>a</sup>, Le<sup>x</sup>, and Le<sup>a</sup>-Le<sup>x</sup>. Among the 50 adenocarcinomas, 17 cases developed in Barrett's epithelium. Those 17 differed from the other 33 cases by expressing much less Le<sup>x</sup>. Fifty-nine percent of Barrett's adenocarcinomas were Le<sup>x</sup> negative compared with 24% of the non-Barrett's carcinomas. All Barrett's adenocarcinomas showed less than 50% Le<sup>x</sup> whereas 50% of non-Barrett's carcinomas showed between 50 and 100% expression. The statistical correlation coefficient for this association was  $P < 0.001$ . Normal gastric cardia epithelium showed the same Le<sup>x</sup> expression in both groups. In the Barrett group, Le<sup>x</sup> expression decreased from normal through intestinal metaplasia and dysplasia to adenocarcinoma. This progression was not seen in the non-Barrett group. Loss of Le<sup>x</sup> expression may prove useful in following patients with Barrett's epithelium in evaluating progression toward a malignant process. No difference in expression of Le<sup>a</sup> and Le<sup>a</sup>-Le<sup>x</sup> was found between Barrett's and non-Barrett's carcinomas.

### Introduction

Carcinogenesis has been described as a molecular disease of cell membrane glycoconjugates (1, 2). Studies have demonstrated that although cell surface glycosylation varies with cell and tissue type, changes in relation to transformation have some common themes. These appear to be either incomplete oligosaccharide synthesis or neosynthesis (3–9).

Studies in one of our laboratories have focused on aberrant glycosylation of mucins and other large glycoproteins in non-small cell carcinomas of the lung and cancers of the gastrointestinal tract (10–12). Initially, we described expression of a previously unidentified extended Lewis antigen (Le<sup>a</sup>-Le<sup>x</sup>) in

squamous cell lung carcinoma which is recognized by Mab<sup>4</sup> 43–9F. Le<sup>a</sup>-Le<sup>x</sup> is suspected to be exclusively associated with glycoproteins in contrast to other Lewis antigens which are glycoprotein/glycolipid carbohydrate moieties (13). These studies were extended to include the use of additional Mabs to identify expression of other Lewis antigens which have each, individually, been described as tumor-associated carbohydrate antigens by others (1, 14–17). Utilization of a panel of biomarkers on patient specimen was initiated in each case to increase the probability of concordant aberrant glycosylation which would lead to patterns which might have diagnostic and/or prognostic significance. Furthermore, emerging patterns could alter patient management decisions.

The current study focuses on adenocarcinomas developing at the gastroesophageal junction, either in Barrett's epithelium (which is columnar epithelium located in the distal esophagus generally believed to occur secondary to chronic reflux) or in non-Barrett's gastric cardia mucosa. Several investigations have shown that Barrett's epithelium is premalignant. These columnar epithelial cells transform first into dysplastic cells and then, in many instances, progress into adenocarcinomas (16–18).

We utilized Mabs against Le<sup>a</sup>-Le<sup>x</sup>, Le<sup>a</sup>, and Le<sup>x</sup> to assess expression in premalignant epithelium and within the adenocarcinomas arising in both gastric cardia mucosa and Barrett's epithelium.

### Materials and Methods

**Tumor Specimens.** Slides from 50 esophageal adenocarcinomas (41 men and 9 women, ages 20–82 years) were obtained from the Department of Pathology, State University Hospital, Copenhagen Hospital Cooperation. The material consists of 17 adenocarcinomas developed in Barrett's epithelium and 33 other gastric cardia adenocarcinomas (1979–1990). All adenocarcinomas were surgical specimens. The diagnosis of esophageal adenocarcinomas was made if more than half of the tumor's length was above the gastroesophageal junction. The diagnoses of Barrett's esophagus in the 17 cases were based on the presence of Barrett's epithelium proximal to the gastroesophageal junction. From each surgical specimen, 15–30 blocks were taken representing resection lines, nontumorous mucosa, and pathological mucosa, whereas 1–10 blocks were taken of tumor and surrounding tissue. From each surgical specimen, one to six sections of nontumorous gastric cardia and one to three slides were chosen as representative. From the 17 Barrett cases, areas of Barrett's epithelium with intestinal metaplasia and, if present, dysplasia were chosen as well. Tumor differentiation in both groups included adenocarcinomas which were poorly, moderately, or well differentiated.

Received 6/21/96; revised 12/23/96; accepted 1/6/97.

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<sup>1</sup> This research was supported in part by funding from Onkologisk Forskningsenhed, State University Hospital, Copenhagen Hospital Cooperation, Denmark and by funding from Architect Holger Hjortenbergs and wife Dagmar Hjortenbergs and by DOD AIBS 2549, P50CA58187 from the U.S. National Cancer Institute and Howard Hughes Medical Institute Grant 71109-500802 to the University of Colorado-Hughes Undergraduate Biological Sciences Educational Initiative.

<sup>2</sup> Present address: Nakskovvej 108, 2500 Valby, Denmark.

<sup>3</sup> To whom requests for reprints should be addressed, at University of Colorado Cancer Center, Box B188, 4200 East 9th Avenue, Denver, CO 80262.

<sup>4</sup> The abbreviation used is: Mab, monoclonal antibody.

Table 1 Le<sup>x</sup> expression in 17 patients with Barrett's esophageal adenocarcinoma, dysplasia, intestinal metaplasia, and normal cardia and in 33 patients with non-Barrett's adenocarcinoma

Adenocarcinoma	% Positive cells	Barrett's adenocarcinoma (%)	Dysplastic (%)	Intestinal epithelium (%)	Normal metaplasia (%)	Non-Barrett's cardia (%)
	0	59	0	0	0	18
	1-10	18	71	59	18	18
	11-50	23	23	35	18	12
	51-80	0	6	6	64	30
	81-100	0	0	0	0	22

**Antibodies.** The three monoclonal antibodies recognizing Le<sup>a</sup> (Co-514), Le<sup>x</sup> [(P12)], and Le<sup>a</sup>-Le<sup>x</sup> (43-9F) were individually applied to each section, and both immunofluorescence and immunoperoxidase staining were performed on serial sections from each case. Each Mab was purified from serum-free culture media (RPMI 1640) of the respective hybridomas as described previously (6, 19). In some experiments, the antibody-containing media were used without purification. Purified Mabs were applied at a concentration of about 2 µg/ml (diluted in PBS), whereas Mabs in serum-free culture media were applied after dilution of 1:40 (in PBS).

For immunofluorescence, each tissue section was incubated with primary antibody at room temperature for 1 h in a humidity chamber. After 1 h the tissues were vigorously washed three times with PBS and secondary antibody was applied. For immunofluorescence, the secondary antibody was FITC- or tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse polyclonal IgG/IgM applied at a concentration of 1 µg/ml (Sigma, St. Louis, MO). Again, tissues were incubated for 1 h in humidity chambers at room temperature. After incubation, the tissues were thoroughly rinsed in PBS and several drops of antifade coverslip mounting media were applied to the sections, and the slides were coverslipped and stored in the dark at 4°C until they were evaluated.

For the immunoperoxidase technique, slides were preincubated at room temperature with PBS and BSA (15 min). Primary antibody was then applied and the sections were incubated overnight at 4°C. The peroxidase-conjugated secondary antibody, P260 (DAKO), was then applied in a dilution of 1:20, and the sections were incubated at room temperature for 60 min, developed in 0.04% 3-amino-9-ethylcarbazol for 10 min (Sigma), and counterstained with hematoxylin for 2 min. Aquamount coverslip media were applied and the sections were coverslipped.

On a routine basis, H&E sections were obtained adjacent to those sections which were evaluated using both immunohistochemistry methods.

**Evaluation.** Evaluation of the patient specimen was on the basis of the fraction of positively stained tumor cells as well as the fraction of positively stained dysplastic or nondysplastic Barrett's epithelial cells and nontumorous gastric cardia mucosa. Semiquantitatively, each specimen was placed into one of the following groups: 0% positive cells, 1-10% positive cells, 11-50% positive cells, 51-80% positivity, and 89-100% positivity. The recording of positive tumor cells was based on all of the investigated tumor tissue, estimating the fraction and counting of 5-10 high-power fields. The intensity of the fluorescent reaction product for each was also evaluated each time (0-4+). Only reaction products of 4+ were recorded as positive. With each experiment, a known positive carcinoma control and a negative control were processed. Also, nonspecific reactivity was evaluated by paralleling a section with PBS,

replacing primary antibody to each of the oligosaccharide epitopes.

Reevaluation of the tissues prepared for fluorescent microscopy was possible for up to 2 months, at which time the reaction product faded dramatically. Immunoperoxidase preparations are stable at room temperature for many years. These have been filed along with the patients original slides. Photomicrographs were taken on a Leitz Dialux 20 microscope with a UFX-II photographic attachment. Filter cube N for FITC incident light microscopy was used for immunofluorescent studies.

**Statistical Analysis.** Statistical evaluation of the data was performed using the Student *t* test. A two-tailed analysis was carried out. Only results with *P* < 0.05 were regarded as significant. These analyses were performed in the Biostatistics Core Laboratory of the University of Colorado Cancer Center.

## Results

Le<sup>x</sup> expression by tumors arising in Barrett's epithelium is markedly decreased when compared with adenocarcinomas arising in gastric cardia mucosa (Table 1). Fifty-nine percent of the Barrett-associated adenocarcinomas do not express this epitope, whereas only 18% of non-Barrett adenocarcinomas are negative. Additionally, when Le<sup>x</sup> expression was present in the Barrett adenocarcinomas, the percentage of cells expressing the epitope was significantly reduced compared with the non-Barrett cases. All Barrett adenocarcinomas had less than 50% Le<sup>x</sup>-positive tumor cells as shown in Fig. 1. Most of the non-Barrett cases contained between 50 and 100% of the tumor cells expressing Le<sup>x</sup>, as demonstrated in Fig. 2. This difference between Barrett's and non-Barrett's cases was statistically significant (*P* < 0.001).

Gastric cardia epithelium in both patient groups expressed similar percentages of Le<sup>x</sup>, and the areas of junctional type epithelium did not show any clear differences from nontumorous gastric cardia. Table 1 shows the progressive loss of the Le<sup>x</sup> epitope by abnormal cells as intestinal metaplasia occurs and progresses to dysplasia in patients with Barrett's esophageal adenocarcinoma. The decrease of Le<sup>x</sup> did not correlate with patient age or with tumor differentiation (*P* > 0.5). Moreover, we observed no morphological changes between Le<sup>x</sup>-expressing cells and cells which were nonexpressors within the same tumor.

The present study also examined expression of Le<sup>a</sup> and Le<sup>a</sup>-Le<sup>x</sup> by normal, metaplastic, dysplastic, and cancer cells of each of the 50 cases. Expression of Le<sup>a</sup> and Le<sup>a</sup>-Le<sup>x</sup> was similar among both subsets of patients (results not shown).

Evaluation of patient specimens was undertaken by both fluorescence and immunoperoxidase staining to evaluate any differences each technique might reveal in terms of epitope expression. Immunofluorescence is usually considered more

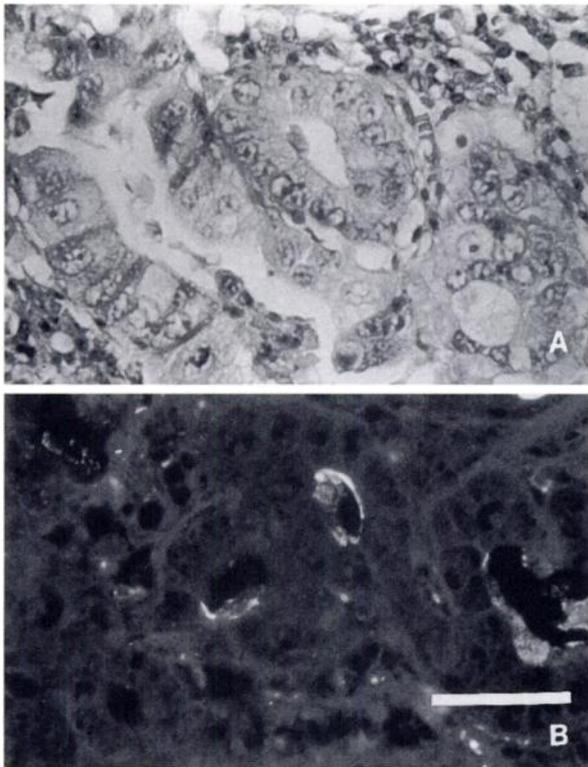


Fig. 1. Less than 50% of the adenocarcinoma cells arising in Barrett's esophagus are Le<sup>x</sup> positive. A, H&E-stained section of a patient tumor and B, FITC immunofluorescent microscopy of adjacent serial section.  $\times 200$ . Bar, 150  $\mu\text{m}$ .

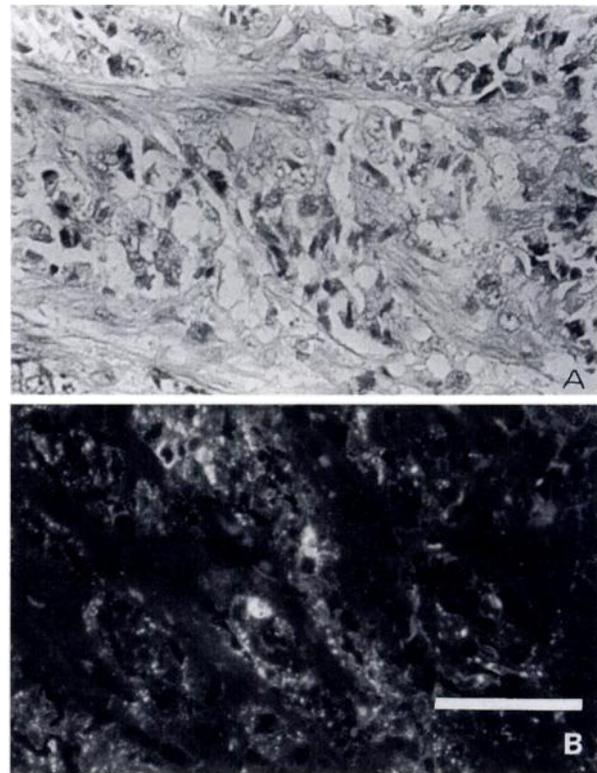


Fig. 2. Non-Barrett's adenocarcinomas (those not previously associated with reflux) contain tumor cells which are high in the expression of Le<sup>x</sup>, 80% of these cells are Le<sup>x</sup> positive. A, H&E-stained section of a patient tumor and B, FITC immunofluorescent microscopy of adjacent serial section.  $\times 200$ . Bar, 150  $\mu\text{m}$ .

sensitive compared with the immunoperoxidase technique which is more stable and may be more accurate when considering morphology. The fraction of positive cells was recorded identically with both techniques. Throughout the course of this study, parallel sections were independently evaluated by three different pathologists utilizing both techniques. Notably, no cases negative with the immunoperoxidase technique were scored positive with immunofluorescence and *vice versa*. Since the pathologists evaluating the different techniques evaluated only one technique or the other, there was no inter/intraobserver variation at this level.

### Discussion

A number of anti-Lewis antibodies have been evaluated as probes for cancer markers (10, 20–22). Previously, changes in expression of Lewis antigens have been observed in human intestinal metaplasia, gastric adenomas and gastric carcinomas (9, 23, 24). Human gastric cancers show an enhanced expression of Le<sup>a</sup> and loss of ABH (17). Distal colon cancers have been shown to express Le<sup>b</sup> and Le<sup>y</sup> aberrantly (7). Sialyl-dimeric Le<sup>x</sup>, an oncodevelopmental carbohydrate antigen, has been shown to be expressed in human colorectal carcinomas, on both glycolipids and mucin proteins, and long and short chain Le<sup>x</sup> antigens are significantly enhanced in colonic carcinoma (13). It has been postulated that several discrete cell populations at different stages of progression of tumors show variable patterns of glycosylation, and a single tumor can show mosaicism in the expression of carbohydrate antigens (14).

The present study corroborates the findings of others,

expands the hypothesis of mosaicism, and confirms changes in epitope expression with epithelial transformation (23, 24). Furthermore, this study suggests possible differences in carbohydrate expression by malignant cells when tumors vary etiologically. Those adenocarcinomas of the distal esophagus which were preceded by intestinal metaplasia and dysplasia and clinically evolved secondary to chronic gastric reflux (Barrett's) contained subsets of transformed cells which progressively lost their Le<sup>x</sup> cell surface epitope. Non-Barrett adenocarcinomas arising without documented Barrett's epithelium and symptoms which suggested the presence of gastric epithelium within the distal esophagus retained the expression of the Le<sup>x</sup> molecule (Table 1). Documentation of the gradual decrease in Le<sup>x</sup> expression from normal gastric cardia via intestinal metaplasia via dysplasia to invasive adenocarcinoma was possible in the Barrett cases. These changes are not present in the non-Barrett adenocarcinomas studied. In this analysis, it is possible that a Barrett-derived adenocarcinoma was misassigned if the carcinoma obliterated the preexisting metaplasia. Also, it is possible that a carcinoma arising in gastric cardia could invade nearby Barrett's epithelium and be incorrectly assigned. However, any misassignments should tend to blur any real differences in Barrett- and non-Barrett-derived adenocarcinomas. Such error in assignments, if they occur, would not be expected to artifactually create differences in marker expression that do not exist. Thus, the uncertainty should not compromise our general results.

Presently, it is unknown whether the decrease in Le<sup>x</sup> expression occurs early in the evolution of Barrett's epithelium.

Longitudinal studies of patients with Barrett's esophagus are under way to explore further the evolution of these changes comparing junctional-type Barrett's epithelium with normal gastric cardia and specialized type to determine whether a decrease occurs in junctional epithelium and progresses through specialized epithelium. This study suggests that Le<sup>x</sup> expression may be a useful tool in following patients with Barrett's epithelium, since a decrease of Le<sup>x</sup> expression by these cells appears to herald the onset of progressive disease.

### Acknowledgments

A special thanks to laboratory technician Kirsten Jorgensen for continual dedication to this project, Dr. Iffat Rahim for assistance in manuscript preparation, and Dr. Phil Archer for statistical analysis of the data.

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U Engel, R McCombs, P Stranahan, et al.

*Cancer Epidemiol Biomarkers Prev* 1997;6:245-248.

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