Quantification of oral epithelial hyperplasia in rats after topical application of the carcinogen 4-nitroquinoline 1-oxide

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Hyperplasia of the palatal epithelium was quantified in two groups of rats exposed thrice weekly to the carcinogen 4NQO for 2 weeks and 2 months, respectively. The lengths and areas of the epithelial layers were measured with a computerized line-following device. In the group treated for 2 weeks the maximum area of the nuclear layer was nearly three times and the maximum length of the epithelial/connective tissue interface almost twice the normal at the end of the carcinogen application period. The maximum area of the cornified layer was three times and the maximum lengths of the epithelial surface and the keratin/nuclear layer interface almost one and a half times the normal 1 week after painting with 4NQO. Thereafter the lengths and areas decreased gradually in both experimental groups. The area of the cornified layer and the length of the epithelial/connective tissue interface in the group treated for 2 months were significantly larger than those in animals treated for 2 weeks. These variables may be two of several indicators of prognostic significance in the assessment of dose-related premalignant epithelial hyperplasia. □ *Animal study; dysplasia; malignancy; oral pathology; premalignant hyperplasia*

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In recent years several attempts have been made to provide quantitative or semiquantitative data of morphologic changes in the epithelium of the oral cavity during disease or during experimental exposure to exogenous stimuli, including carcinogens (1). These attempts include multifactorial studies involving the weighting of various histologic features based on photographic standards or the use of discriminant analysis (2–6). Recently, stereologic techniques have been applied to histologic sections of epithelial hyperplasia of hamster cheek pouch and tongue after application of carcinogenic and non-carcinogenic substances (7–12). In these studies cytologic and histologic determinants of defined epithelial components were used, such as number and size of cells and nuclei, area of epithelial compartments, length of interfaces, and length-to-length and area-to-length ratios.

In a previous study (13) it was demonstrated that a 2-month application (thrice weekly) of the ultimate carcinogen 4-nitroquinoline 1-oxide (4NQO) resulted in a morphologically more pronounced epithelial hyperplasia of the palatal mucosa than did a 2-week application. It is, however, unsatisfactory that conventional grading and comparison of degrees of epithelial hyperplasia depend on subjective morphologic evaluation. On the basis of the recognition of the dose-related difference in hyperplastic response to 4NQO it was considered worthwhile to explore the use of quantitative methods for the evaluation of degrees of epithelial hyperplasia after a short-term (2 weeks) and a long-term (2 months) application of 4NQO for the purpose of evaluating at a later stage the suitability of the method to distinguish between non-neoplastic and neoplastic epithelial hyperplasia.

The histologic factors selected for the quantitative study of the sequential changes in the rat palatal epithelium were the lengths of 1) the epithelial surface, 2) the keratin/nuclear layer interface (keratin interface), and 3) the epithelial/connective tissue interface (epithelial interface) and the areas of 4) the cornified layer and 5) the nuclear layer. A computerized line-following device mini-computer was used for the quantification.

Materials and methods

Three- to 5-month-old inbred, white Wistar rats weighing 210–380 g were kept in Macrolon cages with stainless steel covers. Standard rat pellets and tap water were available ad libitum. Under reversible neuroleptanalgesia (14) the palatal intermolar mucosa was painted in one stroke with an 0.5% solution of 4NQO (Fluka AG, Buchs SG, Switzerland) in propylene glycol three times a week. In each treatment approximately 6 mg of the carcinogenic solution (0.03 mg (153 nmol) 4NQO) was applied. After the painting, the anesthesia was reversed, and in 5 min the animals were aroused.

The animal material (equal numbers of females and males in each group and subgroup) comprised three groups: 1) 80 animals treated with 4NQO for 2 weeks. Subgroups of eight animals were killed at the end of week 1 and 2 and 1, 2, 5, 8, 12, 16, 20, and 26 weeks after the cessation of carcinogen application. 2) 48 animals treated with 4NQO for 2 months. Subgroups of eight animals were killed 2, 6, 10, 14, 16, and 24 weeks after the end of carcinogen treatment. 3) 32 animals painted with saline for 2 months were used as controls. Subgroups of eight animals were killed at regular intervals during the experiment. Animal groups treated with the vehicle only were omitted, since a previous study has shown that no differences could be traced, clinically or microscopically, between solvent-treated and untreated controls (13).

Since in this rat model there is a predilection for carcinomas to develop in the lateral parts of the intermolar palate (13, 15, 16), these areas were selected for the quantitative study. Immediately after decapitation, the heads were fixed in Gendre's fluid for 2 h, followed by 10% buffered formalin for 4–5 days. The upper jaw was demineralized in ethylenediaminetetraacetic acid (EDTA) at 4°C. Seven-micrometer-thick paraffin sections were cut frontally through the palate at the level of the first molars. The sections were stained with hematoxylin–eosin and periodic acid-Schiff counterstained with aniline blue and orange G solution (to increase the definition of the cornified layer). From one histologic slide per animal, 1-mm-wide zones from each side of the palate were magnified 300 times by the use of a projector. The lateral border of each of these zones was 0.5 mm medial to the free gingival margin. The epithelial surface, the boundary between the cornified and the nuclear layer (keratin interface), and the basement membrane (epithelial interface) were traced (Fig. 1).

The first step in the quantification was the digitization of the lines representing the epithelial surface, the keratin interface and the epithelial interface. The digitization process involved assigning values in a two-dimensional coordinate system to equally spaced points on each of these lines. This was accomplished with a PDP-11 mini-computer (Digital Equipment Corp.) programed to convert lines presented to a Vidissector camera (International Telephone and Telegraph Corp.) into coordinate information. The computer program was based on a line-following algorithm of Coleman et al. (17). There were approximately six coordinates per micrometer in the tissue. The closed lines—that is, those representing the boundaries of the cornified and nuclear layer areas—had the same start- and end-coordinates. The sequential coordinate representation of each of the lines was labeled and stored on magnetic discs for later use.

The mini-computer was programed to determine the lengths of non-closed lines (that is, those representing the epithelial surface and the keratin and epithelial interface) by an extended application of the Pythagorean theorem to the coordinates representing the closed lines. Similarly, the coordinates representing the closed lines were

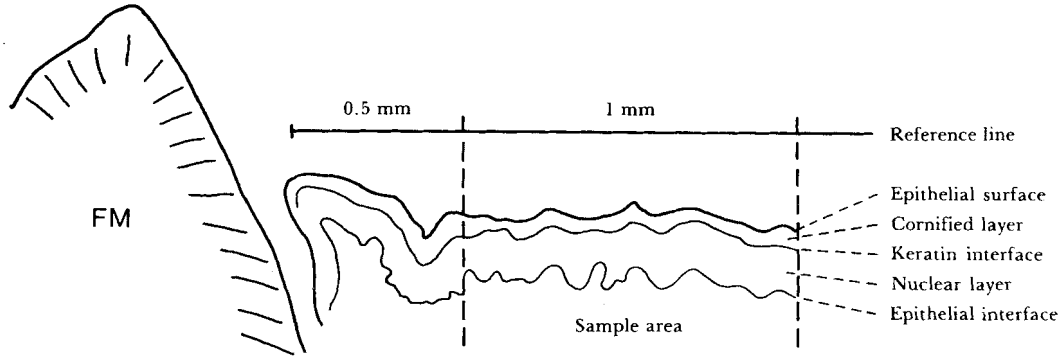


Fig. 1. Tracing from a histologic section showing the lateral part of the palatal epithelium and the sample area. The reference line is a straight line drawn parallel to the main direction of the sample area. The factors measured are indicated. FM = first molar.

used to determine the enclosed areas in accordance with the formula of the area of a polygon. The relative areas and lengths (in camera coordinate units) were then converted to the absolute or real values they had in the tissue. The lengths of the epithelial surface, the keratin interface, and the epithelial interface and the areas of the cornified layer and the nuclear layer of the bilateral zones in each animal were assessed and related to 1 mm of the reference line.

As the control group factors did not vary significantly during the experiment, these data were pooled. The significance of the differences between the respective factors in the control and experimental animals and those between the two experimental groups was assessed by a modified Student's t test (the Welsch solution to the Fisher-Behrens problem (18)); a p value less than 0.05 was considered significant.

Before running the quantification program the reliability of the drawing procedure and the computer calculation procedure (the total technical variation) was examined by computerizing five drawings of the same slide of normal, slightly changed (slight hyperplasia and minor degree of basal membrane folding), and heavily changed (marked hyperplasia and heavy folding) epithelium, respectively (Table 1). The total technical variation did not exceed 3.7%. The reliability of the computer procedure alone was correspondingly assessed, computerizing five copies of an area of normal, slightly changed, and heavily changed epithelium. The variation was at most 0.8%.

Table 1. Methodologic variation derived from the drawing and the computer calculation procedures

	Normal epithelium	Slight hyperplasia	Marked hyperplasia
Epithelial surface	0.5 (0.2)	0.4 (0.3)	1.4 (0.4)
Cornified layer	3.0 (0.8)	1.3 (0.4)	3.7 (0.4)
Keratin interface	0.6 (0.3)	0.5 (0.4)	1.2 (0.6)
Nuclear layer	1.1 (0.4)	0.8 (0.1)	0.9 (0.1)
Epithelial interface	2.3 (0.5)	1.8 (0.5)	1.2 (0.4)

The figures indicate the total technical variation (that is, the variation derived from the drawing procedure plus the computer calculation procedure) and (in parentheses) the variation derived from the computer calculation procedure only. The variation is calculated as standard deviation/mean and given in percentage.

Results

The normal rat palatal epithelium is of the orthokeratinized stratified squamous cell type, with inconspicuous blunt rete pegs (Fig. 2a). After 4NQO application the epithelium developed hyperplasia, which was most marked in the group treated for 2 months (Figs. 2b and d). During the observation period the degree of epithelial hyperplasia decreased but was still obvious in both

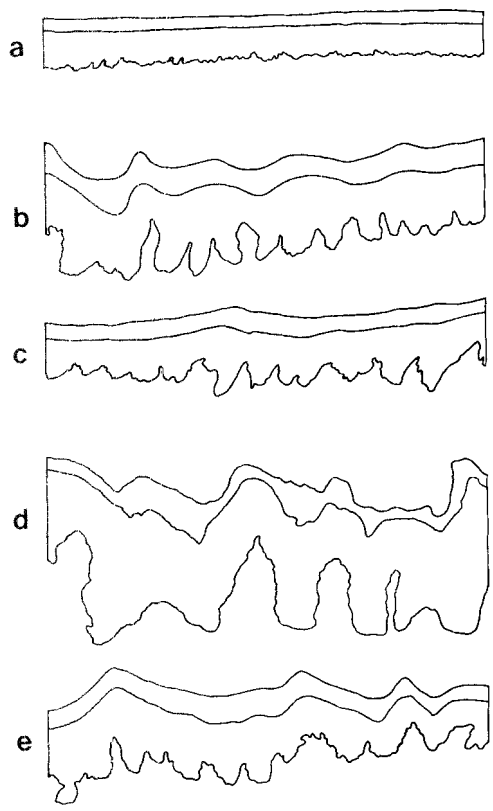


Fig. 2. Tracings of the epithelium of the normal rat palate (a), 2 weeks (b) and 26 weeks (c) after cessation of a short-term (2-week) 4NQO application, and 2 weeks (d) and 24 weeks (e) after cessation of a long-term (2-month) application. For interpretation, see Results.

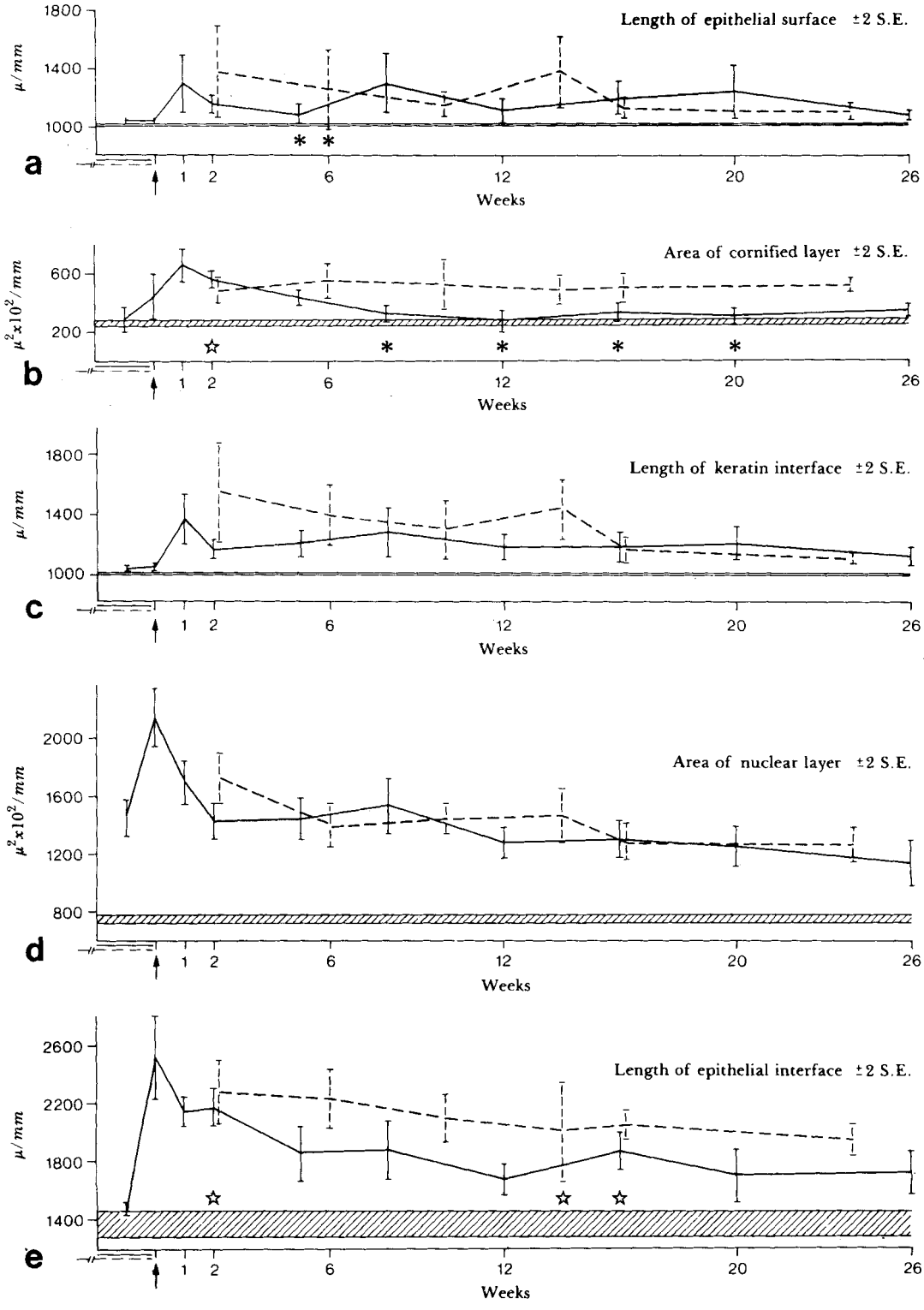
groups at the end of the 6-month observation period (Figs. 2c and e). In animals treated for 2 months a few specimens disclosed, in addition to epithelial hyperplasia, dysplasia of slight to moderate degree from week 8 on. Epithelial dysplasia was not found in animals treated for 2 weeks.

In the group treated with 4NQO for 2 weeks the initial change in the epithelium was an increase in area of the nuclear layer (Fig. 3d). It was nearly twice that of the control value after 1 week of treatment. The maxima for the area of the nuclear layer (nearly three times that of the control) and for the length of the epithelial interface (nearly twice that of the control (Fig. 3e)) were reached after 2 weeks (that is, at the cessation of 4NQO application). The area of the cornified layer had increased after 2 weeks (Fig. 3b), reaching a maximum (three times that of the control) after an additional week. This coincided with the maximum values for the length of the epithelial surface and the length of the keratin interface (both nearly one and a half times the control value (Figs. 3a and c)). All values remained significantly higher than control values throughout the study, with the exception that the area of the cornified layer approached that of the controls from the 8th week onwards.

Two weeks after termination of the 2-month carcinogen treatment, the areas of the cornified and the nuclear layers showed a twofold increase (Figs. 3b and d). The lengths of the epithelial surface, the keratin interface, and the epithelial interface were one and a half times that of the controls (Figs. 3a, c, and e). The area of the cornified layer remained constant, whereas the other values decreased gradually during the experimental period, although remaining significantly above the control values.

A comparison of the two experimental groups demonstrated that, with the exception of the values obtained 2 weeks after the termination of painting, the area of the cornified layer and the length of the epithelial interface in the group treated with 4NQO for 2 months were significantly larger than those in the 2-week group (Figs. 3b and e). No significant differences, however, could be demonstrated between the lengths of the epithelial surface, the lengths of the keratin interface, or the areas of the nuclear layer of the two experimental groups (Figs. 3a, c, and d). The ratios of the area of

Fig. 3. The lengths of the epithelial surface (a), the keratin interface (c), and the epithelial interface (e) and the areas of the cornified layer (b) and the nuclear layer (d) of the palatal epithelium in two groups of rats during and after treatment with 4NQO for 2 weeks (unbroken line) and 2 months (broken line). The hatched areas indicate measures of controls ± 2 SE. The arrow indicates cessation of carcinogen treatment. After the end of carcinogen application no significant difference was found between each of the experimental groups and controls (asterisk) and between the two experimental groups in 3b and 3e (star).


Length of epithelial surface ±2 S.E.
μ/mm
1800
1400
1000
a
1 2 6 12 20 26
Weeks
Area of cornified layer ±2 S.E.
μ²x10²/mm
600
200
b
1 2 6 12 20 26
Weeks
Length of keratin interface ±2 S.E.
μ/mm
1800
1400
1000
c
1 2 6 12 20 26
Weeks
Area of nuclear layer ±2 S.E.
μ²x10²/mm
2000
1600
1200
800
d
1 2 6 12 20 26
Weeks
Length of epithelial interface ±2 S.E.
μ/mm
2600
2200
1800
1400
e
1 2 6 12 20 26
Weeks

epithelium to the length of the epithelial interface, the area of epithelium to the length of the keratin interface, and the length of keratin interface to the length of the epithelial interface were likewise not significantly different in the two experimental groups.

Discussion

The quantitative histologic data presented here 1 to 3 weeks after the start of the 2-week 4NQO application illustrate the sequential stages in the development of chemically induced epithelial hyperplasia. The events coincided with an increase in the rate of cell proliferation, as shown previously (19). The very early stages of epithelial hyperplasia are consistent with the results reported by Craig & Franklin (20) using a hyperplasiogenic, non-carcinogenic agent (turpentine) and those of Eveson & MacDonald (7) using a carcinogenic dose of DMBA, in both studies with the hamster cheek pouch as target tissue. However, in the later stages there is a clearcut difference between the effect of the hyperplasiogenic, carcinogenic substances (present study; 7) and the hyperplasiogenic, non-carcinogenic agent (20) in that the latter results in a fully reversible hyperplasia in half a year.

Application of 4NQO to the rat palatal mucosa for 2 months resulted in a significantly thicker cornified layer (hyperkeratosis) and a significantly longer (more heavily folded) basement membrane than did painting for 2 weeks. The area of the nuclear layer and the lengths of the epithelial surface and the keratin interface were, on the other hand, unrelated to the duration of the application period. These findings may suggest that the area of the cornified layer (degree of hyperkeratosis) and the length of the basal membrane (degree of basal membrane folding) may be indicators of prognostic significance for the assessment of dose-related premalignant epithelial hyperplasia. However, further studies using carcinogenic and non-carcinogenic hyperplasiogens are needed.

It has recently been suggested that area to interface and interface to interface ratios can be used to distinguish between turpentine-treated and carcinogen-treated hamster cheek pouch epithelia (8–11). It is of interest that the ratios of the interfaces in non-dysplastic hyperplasia induced by turpentine or DMBA in these studies did not differ, whereas the ratios for hyperplasia *with* dysplasia induced by DMBA were significantly different. In the present study neither the area to interface nor the interface to interface ratios differed significantly in the two experimental groups. However, since the ratios are based on mean values, the few possible instances of epithelial dysplasia are not likely to penetrate and reflect a significant difference between groups of animals subjected to a short- and a long-term application of 4NQO.

The methods worked out here have shown a very high degree of reliability. The variation derived from the drawing procedure and the computer calculation were well within acceptable limits. With the equipment at hand the amount of preparatory work is within reason even when large amounts of material are dealt with. It is believed that the method used here, or the use of more elaborate software programs permitting similar data to be generated semiautomatically, provides a reliable mean for quantifying the effect of hyperplasiogenic agents and enables truly objective comparisons to be made between groups of experimental animals. In studies of carcinogenesis no single morphologic feature has yet been found completely to characterize the preneoplastic process. However, we do agree with Franklin & Smith (10) in that the ability to ascribe numerical values to histologic features that are usually only subjectively evaluated may indeed be useful in both diagnostic and research work.

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