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Effect of N-Ethyl-N-Butyl-Nitrosamine on the esophageal mucosa of the rat. Histometric investigation of early tumor stages

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Summary. 80 male Wistar rats received an oral ad libitum application of N-ethyl-N-butyl-nitrosamine in a concentration of 0.18 g per litre of drinking water. The changes induced in the esophageal mucosa and determined at three intervals (up to 48 days, up to 91 days, and up to 112 days after commencement of carcinogen exposure) were compared by microscopy with the results from a control group of 10 male Wistar rats of the same age. Several histomorphometric parameters were investigated with the aid of a Leitz ocular micrometer. The earliest localized changes found were an increase in the thickness of the epithelium and the horny layer, and an elongation of the papillary bodies and a widening of the parabasal cellular layer. Later - with a substantial increase in the rate of mitosis in all layers of the epithelium, there was a significant thickening of the non-papillomatous and papillomatous epithelium, an enlargement of the nuclei, especialli in the middle and upper layers of the epithelium and a thickening of the horny layer, parts of the latter being parakeratotic. The papillomatous changes corresponded in some cases to moderate epithelia1 dysplasias. As expected, no fully-developed invasive carcinomas occurred in the early period investigated. The histometric data permit the conclusion to be drawn that the lesions described are demonstrable not only at the exophytic-papillomatous epithelium but also in multifocally localized form at the flat, non-papillomatous mucosa, and that they can definetely be regarded as the expression of an incipient field cancerification.

Key words: Carcinogenesis - Histomorphometry - Esophagus - Rat

Introduction

Since the fundamental work performed by Druckrey et al., and other groups of researchers it has been known that there are particular nitroso compounds which can be used to produce esophageal carcinomas in experimental animals (Schoental and Magee, 1962; Druckrey, 1963, 1967; Magee and Schoental, 1964; Thomas and Schmal, 1965; Horie et al., 1965; Pozharisski, 1973; Takeuchi et al., 1974; Nakamura et al., 1974; Lijinski and Taylor, 1975, Reznik et al., 1975; Levinson et al., 1979; Hodgson et al., 1982; Iizuka et al., 1982; Reuber, 1982). No conclusive, definitive answer can yet be given to the question as to why particular nitrosamines produce cancer exclusively or predominantly in specific organs («organo-tropism») and others do not do so, or why, if the latter do so, then in quite different organs (Dontenwill and Mohr, 1962; Süss, 1965; Sander, 1971; Tannenbaum, 1979; Miller and Miller, 1981). A possible explanation is given by the diazoalkane theory, according to which alpha oxidation and subsequent dealkylation into diazoalkane takes place not only in the liver, but is also possible in other organs, e.g. in the bladder, the lung, the stomach, the kidney, the esophagus and other organs (Argus and Hoch-Ligeti, 1961; Keberle et al., 1963; Druckrey et al., 1967; Poland and Kende, 1977; Hecker, 1978; Hecker, 1981). In which organ the «toxification» of the nitrosamine and the development of cancer occurs in individual cases accordingly depends, inter alia, upon the chemical structure of the substance used, but also upon the species. Thus, for example, the N-ethyl-N-butyl-nitrosamine used in the present study can be administered to proceduce esophageal cancers in the rat, but can also be used to produce cancers of the forestomach in the mouse (Schmahl et al., 1963; Druckrey et al., 1967).

No detailed comment can be made here on the complex molecular-biological aspects of tumor genesis, which are currently the subject of intensive research. As representative of this, we mention the «information hypothesis on tumor genesis» which includes, as causes of tumor genesis, changes in the information content by

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mutation or transformation of DNA or RNA at the level of transcription or translation, as well as changes in the intra and extra-cellular regulatory mechanisms, perhaps by the mutation of regulatory genes or through the loss of regulatory proteins (Magee and Schoental, 1964; Miller and Miller, 1981; Hecker, 1978, 1981, 1983, 1984).

In the present morphological study, attention was chiefly focused upon the earliest demonstrable changes in the esophageal mucosa of the rat under the influence of N-ethyl-B-butyl-nitrosamine. In most reports the preliminary stages receive little mention, or are incidental to the study. A systematic histometric investigation of these pre-neoplastic changes has not yet been performed.

Materials and methods

80 male Wistar rats with an average body weight of 420 (±36 g) received an oral ad libitum application of N-ethyl-N-butyl-nitrosamine (Atlanta-Chemic- und Handelsgesellschaft, Heideberg, FRG) in a concentration of 0.18 g per litre of drinking water. According to reports by Druckrey et al. (1967) and Thomas et al. (1969) the aplication of this carcinogenic substance in animal experiments with rats can produce carcinomas of the esophagus after an exposure period averaging 200-240 days (Druckrey, 1967; Thomas and So, 1969). N-ethyl-N-butyl-nitrosamine has hitherto been used only rarely as a chemical carcinogen (Schmäl et al, 1963; Druckrey et al, 1963, 1967; Sons et al, 1985). In a earlier experiment our working group had already used this chemical carcinogen in order to demonstrate that rats with an operatively produced esophageal stenosis developed a statistically significant greater number of esophageal papillomas than control rats (Sons et al., 1985): the tumor induction commenced earlier in the rats that had undergone surgery, and the preferred site was in the area of the stenosis.

In order to enable a histomorphometric investigation of the gradual changes in the esophageal mucosa under the influence of this carcinogenic substance to be performed, the rats were divided into three groups of approximately equal size (II to IV), and at specific intervals, namely 48 days, 91 days and 112 days after commencement of carcinogen exposure, the rats were killed by the intramuscular injection of T 61. 1 ml injection solution T 61^R ad us, vet. contains 0.2 g embutramide, 0.05 g mebezonium iodide, and 0.005 g tetracaine hydrochloride in aqueous solution. Immediately after death, the organs were removed and dissected. The esophagus, forestomach and stomach were cut open and, after cleaning, loosely spread out on a sheet of cork and secured with pins. Fixation in 4% aqueous formaldehyde solution was then carried out for 2 days. The sections, with a thickness of 6 µm, were stained with hematoxylin-eosin. Five specimens were taken from each esophagus: the first specimen from the upper end of the esophagus, the third from the midle of the esophagus, and the fifth from the lower end of the esophagus; the second and fourth specimens were taken at locations between these respective points.

All the animals exposed to carcinogen (Groups II to IV) were kept in the same room in individual cages at an ambient temperature of 22° Celsius and 55% air humidity, and were fed with standard rat feed (Altromin, Messrs. Altromin, Lage/Lippe, FRG).

The following histometric data were obteined with the aid of a Leitz eyepiece micrometer: width of the epithelium, length of the papillary bodies, thickness of the horny layer and the parabasal cellular layer, the number, length and width of the cell nuclei, and the number of mitoses. As papillomas were found in the animals of groups III and IV, in these two groups the above-mentioned histometric parameters were determined separately for papillomas and for esophageal mucosa that was non-papillomatous.

As a control group, 10 male Wistar rats of the same body weight (Group I) were used; they were kept under identical experimental conditions in another room, but were not exposed to carcinogen, and were killed after 112 days. With these animals the same histomorphometric parameters were investigated.

For the statistical evaluation the Mann-Whitney-Wilcoxon U test was used. The lower confidence level was specified as $p=0.01$.

Results

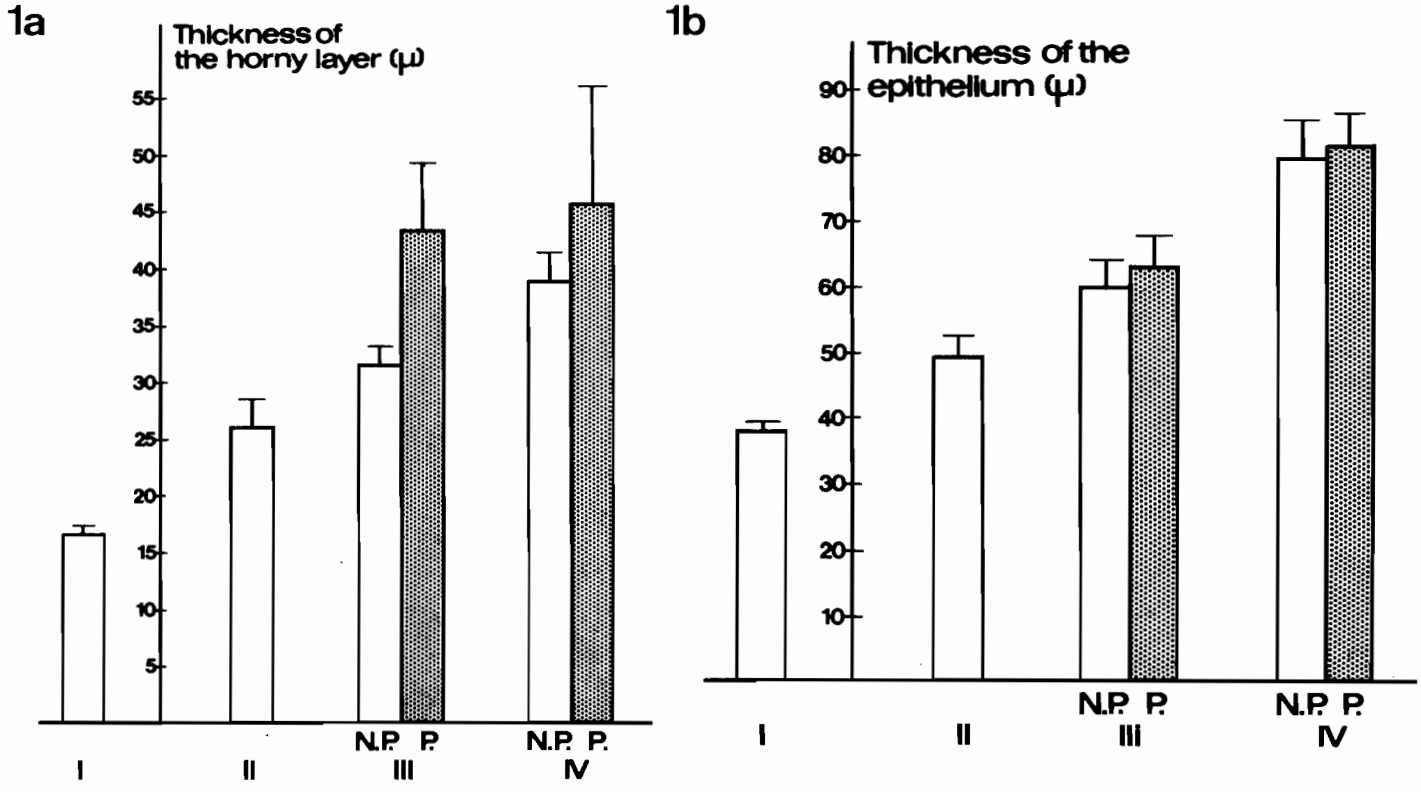
The earliest histometrically demonstrable changes (Group II) in comparison to the control group (Group I), found after 48 days exposure to carcinogen, were the following localized lesions: an increase in the thickness of the epithelium and the horny layer, an elongation of the papillary bodies, and a widening of the parabasal cellular layer (Table 1 and 2, Fig. 1). Thus, the average thickness of the horny layer in Group II increased to 25.6 µm in comparison with 16.5 µm in Group I (control group not exposed to carcinogen), the average width of the epithelium increased to just under 50 µm in comparison with 37.7 µm, the average length of the papillary bodies increased to 26.6 µm in comparison with 13.2 µm, and the average thickness of the parabasal cellular layer increased to 18.4 µm in comparison with 13.4 µm.

With carcinogen exposure of greater duration (Group III: 91 days carcinogen exposure, and Group IV: 112 days carcinogen exposure) there was -with a considerable increase in the rate of mitosis in all layers of the epithelium- a significant thickening of the non-papillomatous and papillomatous epithelium, an enlargement of the nuclei, especially in the middle and upper epithelial layers, and a thickening of the horny layer, some of the latter being parakeratotic (Tables 1 and 2, Fig. 1). Morphometry of the nuclei revealed no substantial change in the nuclear length but significant increases in the nuclear width, especially in the middle und upper epithelial layers. During histometric examination of the papillomas an especially marked elongation of the papillary bodies was observed. In the papillomatous mucosa in Groups III and IV the rate of mitosis was higher than that of the non-papillomatous

Table 1. Thickness of the epithelial layer and the horny layer, length of the papillary bodies, and thickness of the parabasal cellular layer in μ (mean values and standard deviations from the mean value). I = control group not exposed to carcinogen, II - IV = rats exposed to carcinogen. NP = non-papillomatous esophageal mucosa, P = papillomatous esophageal mucosa.

Group	Duration of carcinogen exposure (days)	Thickness of epithelium (μ)	Thickness of horny layer (μ)	Length of papillary bodies (μ)	Thickness of parabasal cellular layer (μ)
I	–	37.8 ± 1.5	16.5 ± 0.7	13.2 ± 1.2	13.5 ± 0.6
II	48	49.4 ± 2.5	25.6 ± 1.9	20.6 ± 2.1	18.4 ± 0.9
III_{NP}	91	59.3 ± 3.6	31.4 ± 1.6	32.3 ± 2.2	17.1 ± 1.7
III_P	91	62.4 ± 4.5	42.9 ± 7.9	640.9 ± 107.7	19.5 ± 0.9
IV_{NP}	112	79.4 ± 5.8	38.8 ± 2.1	44.0 ± 3.0	19.1 ± 0.7
IV_P	112	81.1 ± 5.3	47.7 ± 7.9	778.1 ± 130.3	22.7 ± 1.4
Significance					
$p < 0.0001$		I/III_{NP}, III_P, IV_{NP}/IV_P	I/II, III_{NP}, III_P, IV_{NP}, IV_P	I/II, III_{NP}, III_P IV_{NP}, IV_P	I/II, III_{NP}, III_P, IV_P
		II/III_{NP}	II/III_{NP}	II/III_{NP} III_{NP}/III_P	–
		III_{NP}/IV_{NP}	–	IV_{NP}/V_P	–
$p <$		I/II	IV_{NP}/IV_P	–	IV
$p <$		–	–	III_{NP}/IV_{NP}	–

Fig. 1. a-h. Various histometric parameters of the rat esophagus, dependent upon the duration of carcinogen exposure. Group I = control group of normal animals not exposed to carcinogen; Groups II, III and IV = rats exposed to carcinogen (48 days, 91 days and 112 days respectively). N.P.= non-papillomatous esophageal mucosa. P = papillomatous esophageal mucosa. O = upper third of the epithelium. M = middle third of the epithelium. B = basal third of the epithelium.



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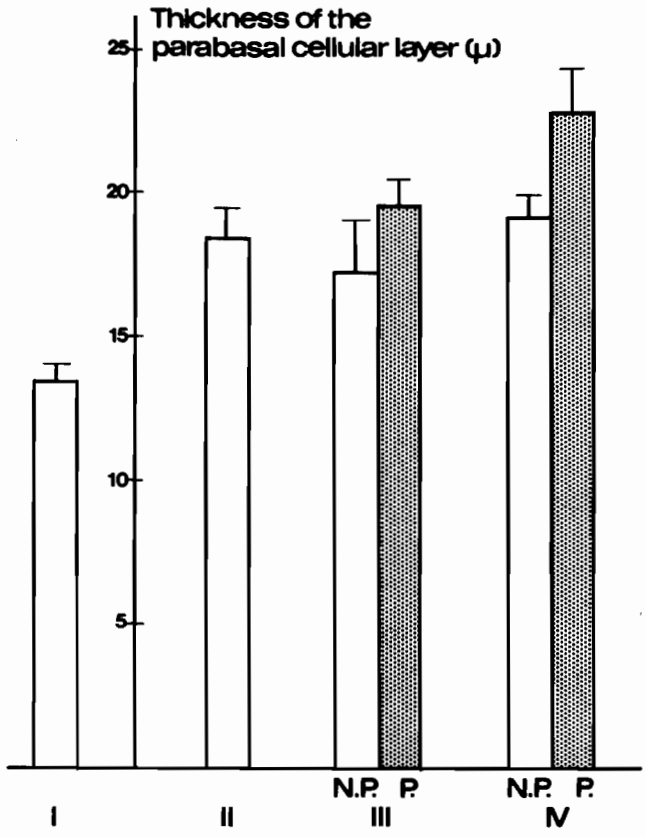
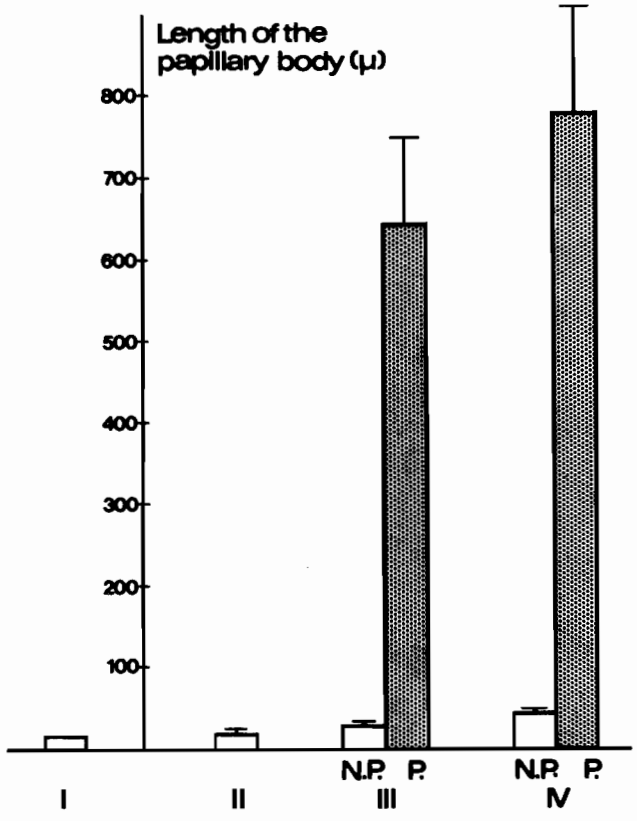
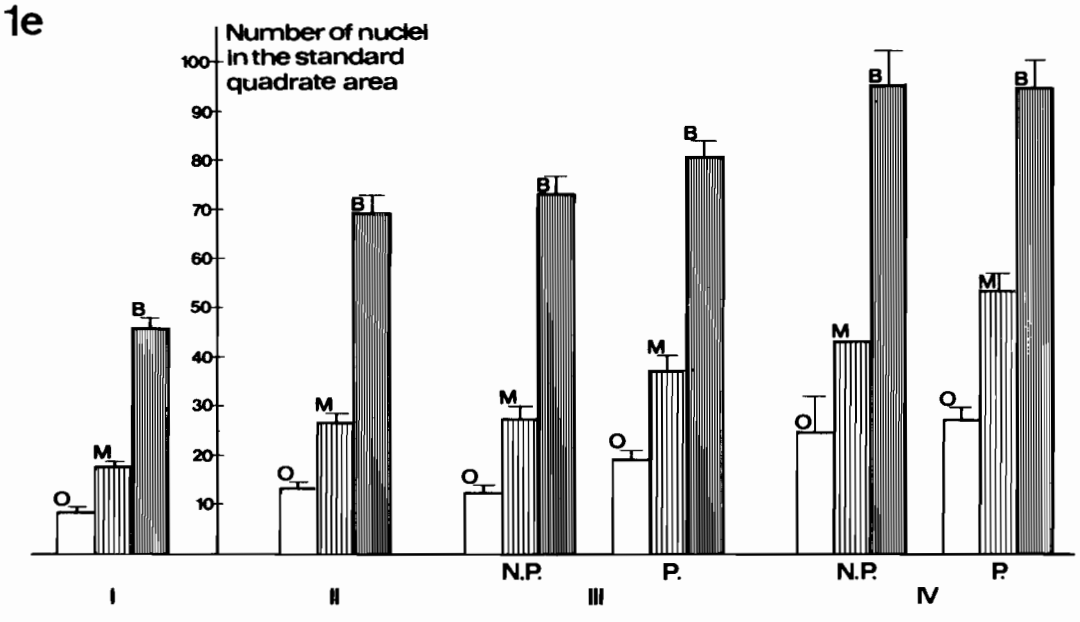

Thickness of the
parabasal cellular layer (μ)
25
20
15
10
5
N.P.
P.
N.P.
P.
I
II
III
IV

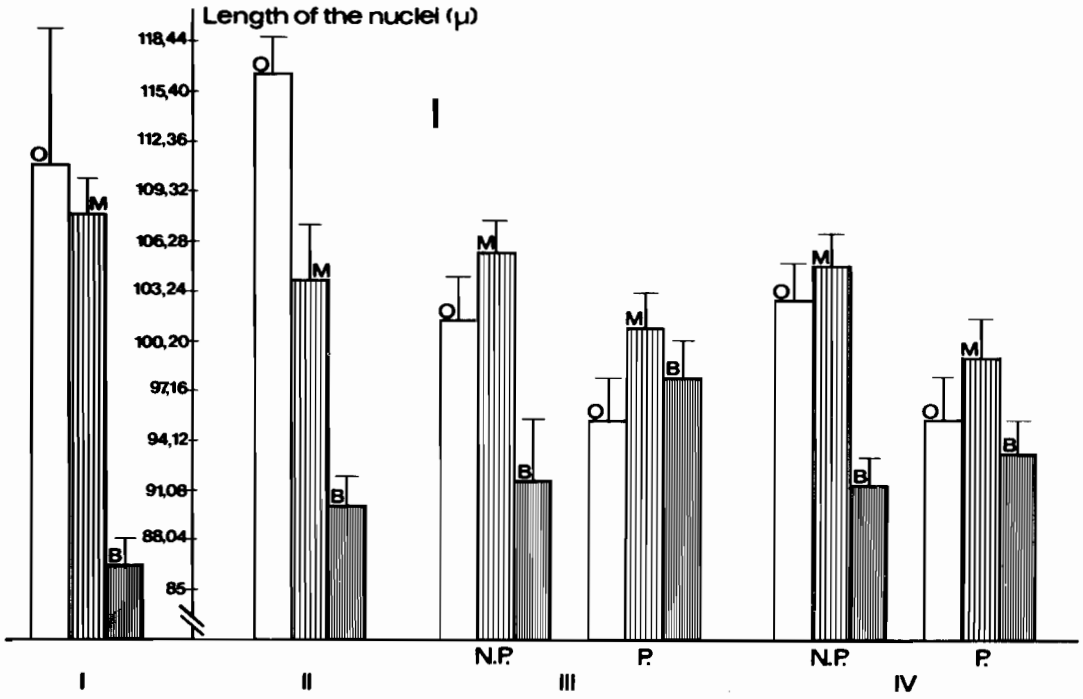
1d


Length of the
papillary body (μ)
800
700
600
500
400
300
200
100
N.P.
P.
N.P.
P.
I
II
III
IV

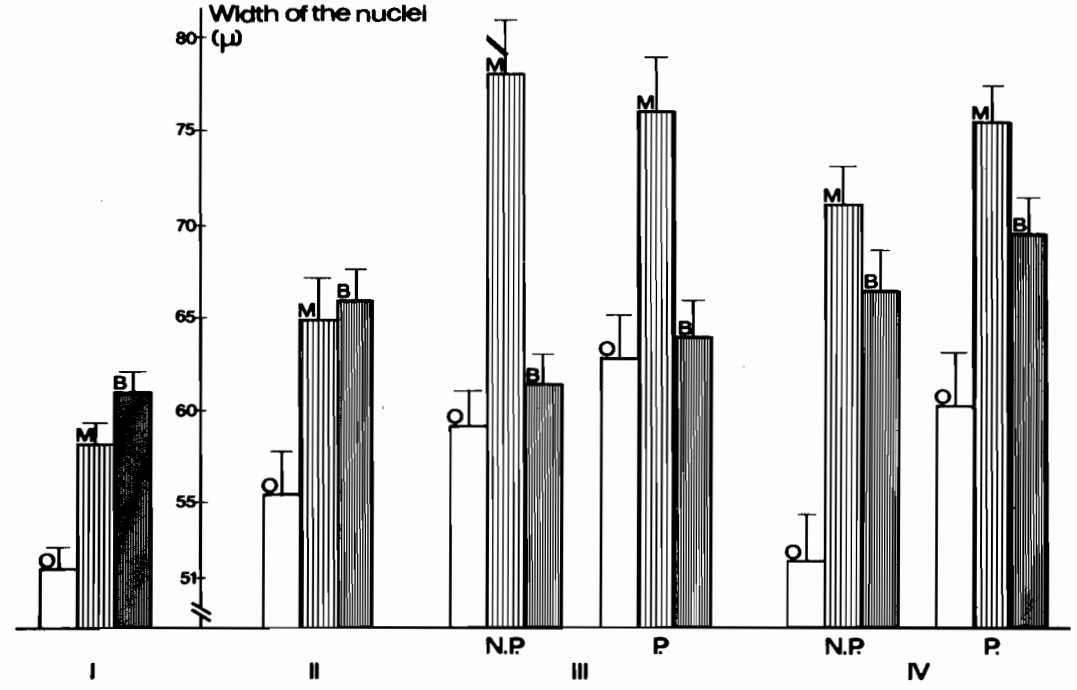
1e


Number of nuclei
in the standard
quadrate area
100
90
80
70
60
50
40
30
20
10
O
M
B
N.P.
P.
N.P.
P.
I
II
III
IV

1f


Length of the nuclei (μ)
118,44
115,40
112,36
109,32
106,28
103,24
100,20
97,16
94,12
91,08
88,04
85
O
M
B
I
N.P.
P.
I
II
III
IV

1g


Width of the nuclei (μ)
80
75
70
65
60
55
51
O
M
B
N.P.
P.
I
II
III
IV

1h

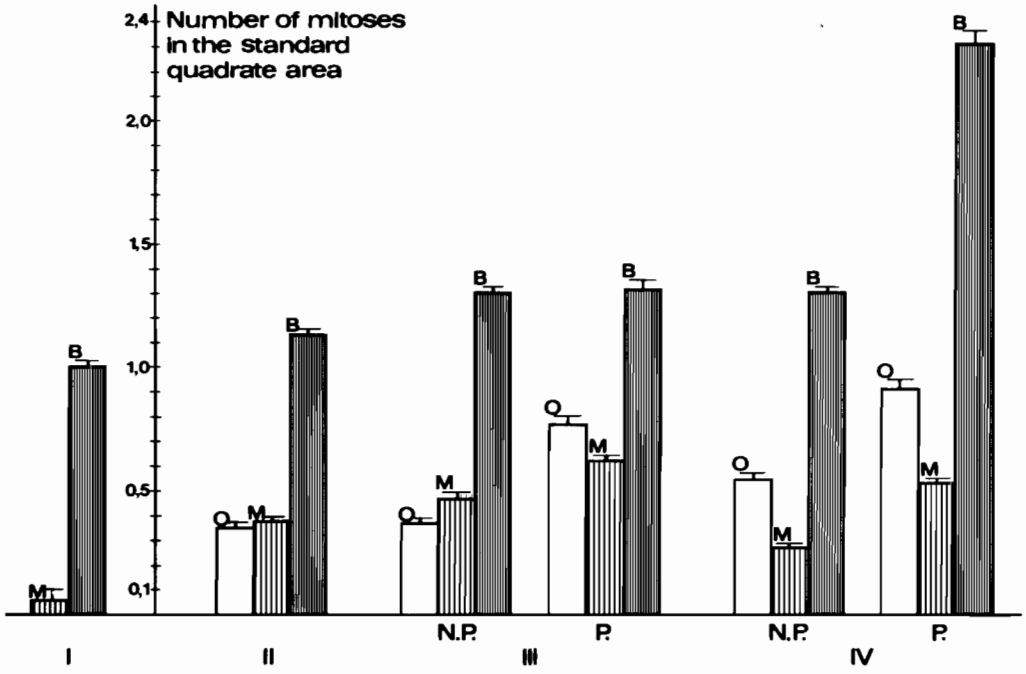


Fig. 2. Normal esophageal mucosa of the rat (no exposure to carcinogen). H.E. ×200.

Fig. 3. Early localized hyperplastic changes of the esophageal mucosa, with acanthosis, hyperkeratosis, incipient loss of polarity in the basal cells, and isolated mitoses (48 days exposure to carcinogen). H.E. ×200.

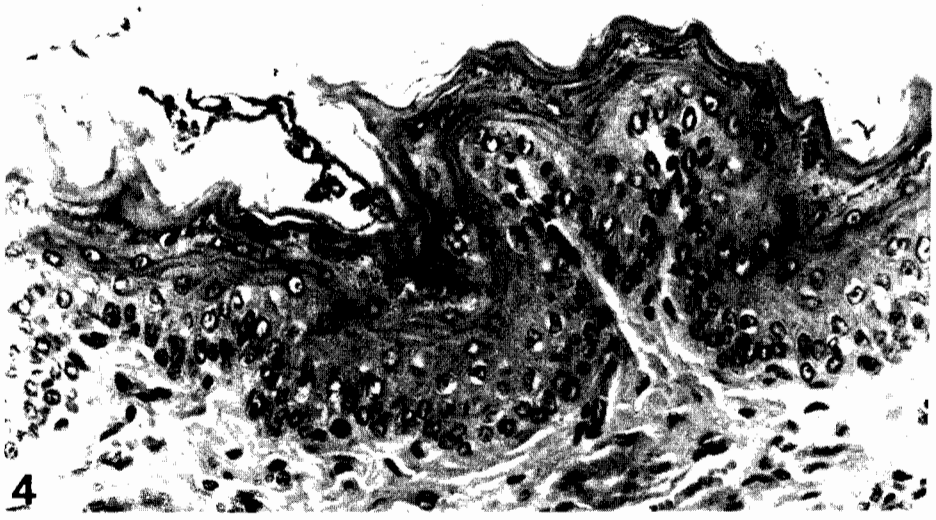


Fig. 4. Incipient exophytic growth with elongation of the papillary bodies, slight hyperplasia of the basal cells, acanthosis and hyperkeratosis (91 days carcinogen exposure). H.E. ×250

Fig. 5. Localized hyperplasia of the layer of basal cells, with loss of polarity in the basal cells and incipient endophytic growth, isolated mitoses, polymorphism of cells and nuclei, hyperkeratosis, slight acanthosis (48 days carcinogen exposure). H.E. ×300

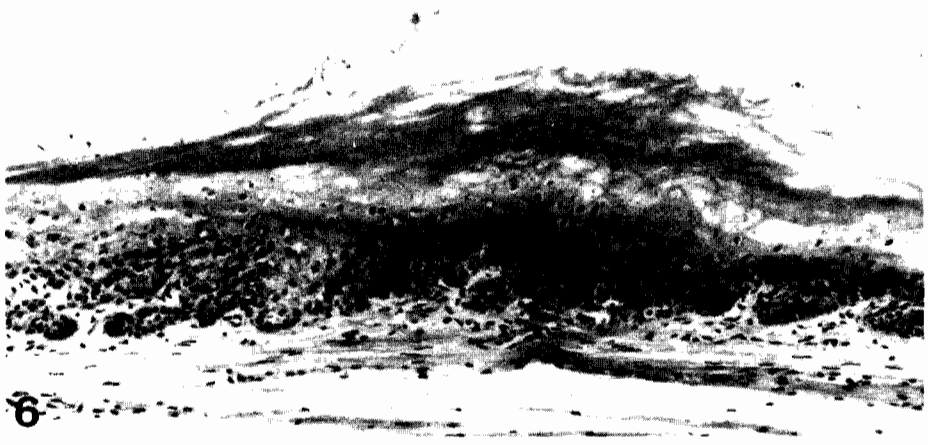


Fig. 6. Hyperplasia of the basal cells, loss of polarity in the basal cells, moderate polymorphism of the cells, mitoses, and incipient, partly digitiform endophytic growth and distinct hiperkeratosis (91 days carcinogen exposure). H.E. ×80

Table 2. Number of cell nuclei and mitoses per standard quadrate area, and average width of nuclei and maximum length of nuclei (μ) (mean values and standard deviation from the mean value). I = control group not exposed to carcinogen, II - IV = rats exposed to carcinogen. NP = non-papillomatous esophageal mucosa, P = papillomatous esophageal mucosa. B = basal third of the epithelium, M = middle third of the epithelium, O = upper third of the epithelium.

Group	Duration of carcinogen exposure (days)	Third of epithelium	Number of cell nuclei per standard quadrate area	Number of mitoses per standard quadrate area	Length of cell nuclei (μ)	Width of cell nuclei (μ)
		B	45.8 ± 1.8	1.0 ± 0.1	86.5 ± 2.3	60.9 ± 1.3
I	–	M	17.6 ± 0.7	0.06 ± 0.05	108.2 ± 1.8	58.2 ± 1.0
		O	8.7 ± 0.5	0	111.0 ± 8.1	51.4 ± 1.3
		B	68.8 ± 3.3	1.1 ± 0.2	89.9 ± 2.0	66.2 ± 1.4
II	48	M	26.7 ± 1.3	0.4 ± 0.1	105.3 ± 1.9	64.8 ± 2.1
		O	13.3 ± 0.9	0.3 ± 0.1	115.7 ± 1.9	55.4 ± 2.3
		B	73.0 ± 3.0	1.3 ± 0.2	91.7 ± 1.9	61.4 ± 1.6
III_{NP}	91	M	27.3 ± 2.3	0.5 ± 0.2	105.6 ± 1.8	78.2 ± 2.7
		O	12.5 ± 1.2	0.3 ± 0.1	101.5 ± 2.5	59.3 ± 1.9
		B	80.7 ± 3.2	1.3 ± 0.2	97.8 ± 2.3	63.8 ± 2.1
III_P	91	M	37.3 ± 2.8	0.6 ± 0.2	100.9 ± 2.2	76.0 ± 2.8
		0	19.4 ± 1.6	0.8 ± 0.2	95.3 ± 2.5	62.8 ± 2.4
		B	95.2 ± 6.3	1.3 ± 0.2	91.3 ± 1.6	66.3 ± 2.1
IV_{NP}	112	M	43.1 ± 6.8	0.3 ± 0.1	104.8 ± 2.0	71.0 ± 2.0
		O	24.4 ± 7.0	0.5 ± 0.1	102.7 ± 2.4	51.9 ± 2.4
		B	93.8 ± 5.3	2.3 ± 0.3	93.3 ± 2.2	69.4 ± 2.3
IV_P	112	M	52.8 ± 3.4	0.5 ± 0.1	99.1 ± 2.3	75.3 ± 2.0
		O	27.6 ± 2.5	0.9 ± 0.3	95.3 ± 2.9	60.3 ± 2.7
Significance						
$p < 0.0001$		I/II, III_{NP}, III_P,	I/II II/III_{NP}	–	–	
		IV_{NP}, IV_P, II/III_{NP}	–	–	–	
	B	III_{NP}/IV_{NP}	–	–	–	
$p < 0.001$		–	I/IV_P	I/III_P	I/II, IV_{NP} II/III_{NP}	
$p < 0.01$		–	–	II/III_{NP}	III_{NP}/IV_{NP}	
$p < 0.0001$		I/II, III_{NP}, III_P,	–	–	I/III_{NP}, III_P, IV^{NP},	
		IV_{NP}, IV_P, II/III_{NP}	–	–	IV_P, II/III_{NP}	
	M	III_{NP}/IV_{NP}, III_{NP}/III_P	–	–	–	
$p < 0.001$		III_P/IV_P, IV_{NP}/IV_P	–	–	–	
$p < 0.01$		–	I/IV_P	I/III_p, IV_P	I/II	
$p < 0.0001$		I/II, III_{NP} III_P, IV_P	–	I/III_P, IV_P	I/III_P	
		III_{NP}/IV_{NP}, IV_{NP}/IV_P	–	I/III_P, IV_P	I/III_P	
$p < 0.001$	O	III_{NP}/III_P	–	–	I/III_P	
$p < 0.01$		I/IV_{NP}, III_P/IV_P	I/III_P, IV_{NP}	I/III_{NP}	I/III_{NP}/IV_P	

Fig. 7. Nodular proliferation with localized widening of the parabasal cellular layer, hyperkeratosis, enlargement of nuclei, a greater number of mitoses, and irregular ramification of the papillary bodies (112 days carcinogen exposure). H.E. ×100

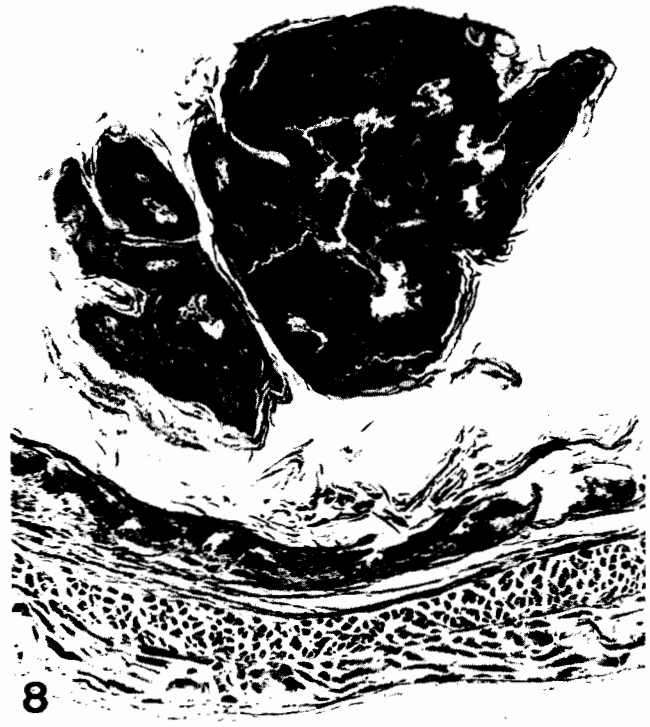


Fig. 8. Papilloma of the esophageal mucosa as an example of exophytic growth with marked acanthosis, hyperkeratosis and demonstrable mitoses (112 days carcinogen exposure). H.E. ×50

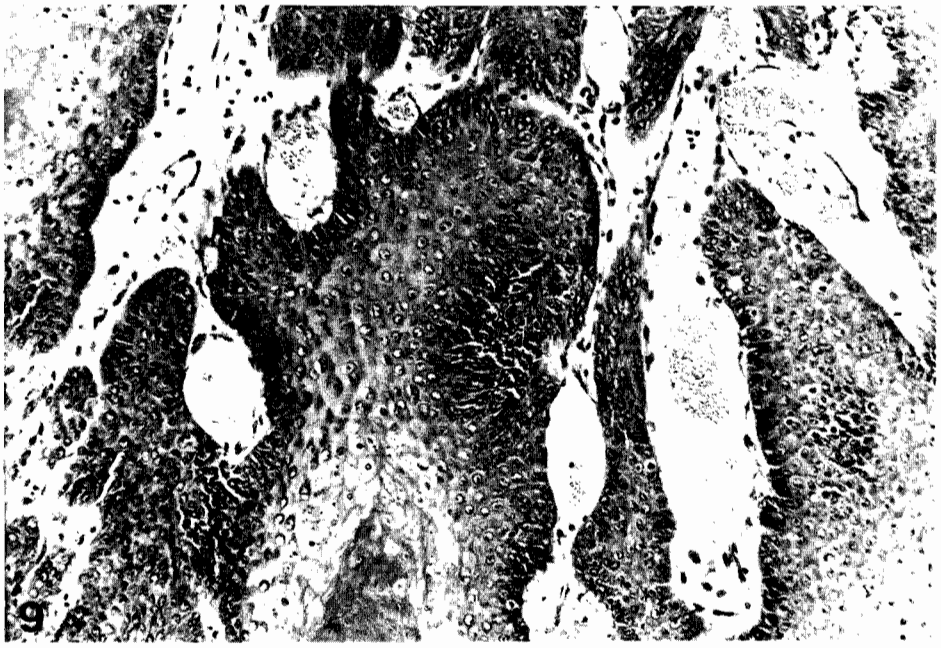


Fig. 9. Cross-section through a papilloma with ramose, fibrous, well-vascularized stroma and hyperplastic squamous epithelium with acanthosis, hyperkeratosis, polymorphism of cells and nuclei, and demonstrable mitoses (112 days carcinogen exposure). H.E. ×110

mucosa. The histologic changes corresponded in some cases to moderate epithelial dysplasias. As expected, fully-developed invasive carcinomas were not observed during the period of the investigation. Examples of the histologic changes demonstrated are shown in Figures 2-9.

Discussion

There are, in all, only a few studies which relate to the induction of tumors of the esophageal mucosa in experimental animals. Napalkov and Pozharisski (1969) used an oily solution of N-methyl-N-nitrosoaniline (MNA) which was administered to rats by means of a feeding tube five or six times weekly up to the 598th day of the experiment (Napalkow and Pozharisski, 1969). They too found, as in our investigation, a thickening of the epithelial layer as well as hyperkeratosis, often combined with parakeratosis, as early histological changes. Hipertrophy and proliferation of the papillae of the mucosal connective tissue beneath the foci of leucoplakia caused the development of verrucous lesions which they described as leucokeratosis. In the subsequent stage they observed polypoid outgrowths of the mucosa and papillomas. In their series of experiments, these authors first discovered malignant tumors of the esophageal mucosa after the 459th day of the experiment, and in general after the 508th day. These tumors were mainly growing exophytically.

Ito et al. (1971) administered N-nitrosopiperidine to male Wistar rats for 20 weeks (Ito et al., 1971). In their study of carcinogenesis they found that hyperplastic and papillomatous changes of the epithelium were precursors of esophageal cancer in the rat.

The histogenesis of esophageal cancer induced through chemical carcinogens ist also the subject of the study by Thomas and So (1969) (Thomas and So, 1969). The early non-neoplastic changes described by them were an edematous change of the mucosa with necroses, hemorrhages and inflammatory infiltrates which, at a later stage, developed into pre-neoplastic changes. The latter consisted of a thickening of the layer of basal cells, and of a cone-shaped encroachment upon the stroma, in which the basal membrane nevertheless remained intact. In addition they observed exophytic thickening of the squamous epithelium with severe hyperkeratosis. The carcinoma of the squamous epithelium in their experimental material developed mainly from papillomatous changes, and more rarely from hyperplasia of the basal cell, in which the basal membrane was perforated and a «reticulate infiltration» of the stroma and the muscular layers subsequently occurred. These reported results of the study agree with our findings that pre-neoplastic lesions can develop both from exophytic-papillomatous changes and on account of an endophytic growth.

Reuber (1977, 1982) likewise described hyperplastic changes as a preliminary stage of esophageal carcinoma induced in the rat by diethylnitrosamine (Reuber, 1977, 1982). The earliest changes found by him were areas of hyperplasia in the layer of basal cells, which developed into hyperplastic nodules. In addition, he observed that hyperplastic cells that arose in the squamous layer of the mucosa met with little or no resistance from the adjacent tissue. According to his observations, after their transmutation to malignancy, small carcinomas grew in hyperplastic nodules with the hyperplastic cells being replaced by cancerous cells. After the administration of DEN the formation of carcinomas was only extremely rarely observed in papillomas.

Takeuchi et al. (1974) also induced hyperplasia, hyperplastic nodules, papillomas with malignant changes, and carcinoma of the esophagus in Donryu rats to which

N-butyl-N-nitrosourethane was administered in drinking water (Takeuchi, 1974). The rats also developed carcinomas in the forestomach, pharynx and oral cavity. In their series of experiments Stinson et al. (1978) induced mainly papillary carcinomas of the esophagus in the rat by administering N-Methyl-N-benzylnitrosamine (Stinson, 1978). Cardesa et al. (1982), reporting on their series of experiments, described exophytic and endophytic lesions of the esophageal mucosa as pre-neoplastic changes after the injection of 2.6-dimethyl-nitrosomorpholine (Cardesa, 1982).

Finally, Gibel (1968) indicated two courses of carcinogenesis in that carcinomas could develop both on the basis of papillomas and through the in-situ-carcinoma mode (Gibel, 1968). In this respect our experiment does not permit any conclusions to be drawn, because the development of invasive tumors was not awaited. Here there is a clear comparison with the epithelium of the urinary tract in humans where, according to Koss (1985), the in-situ carcinomas, rather than the papillomas, are the precursors.

Conclusions

In the majority of reports relating to the histogenesis of nitrosamine-induced esophageal tumors, the emphasis is placed upon the microscopy of the stages of development of the exophytic lesions which lead to the squamous epithelial papilloma and the papillary carcinoma. The results of our investigation suggest that in future animal experiment studies more attention should be paid to progressive pre-neoplastic and neoplastic changes, induced by nitrosamines, in the flat and endophytically proliferating esophageal mucosa. Even in comparison with the known stages of development of human esophageal cancer, those flat and endophytic lesions which can be produced in animal experiments appear to be of particular interest, as verrucously exophytic early carcinomas of the esophageal mucosa are observed more rarely than endophytic or intra-epithelial pre-neoplasias (Mandard et al., 1964; Borchard and Sons, 1984; Sons and Borchard, 1984; Lohe and Borchard, 1985; Sons and Borchard, 1986).

Our histometric data permit the conclusion to be drawn that the lesions described are demonstrable not only at the exophytically papillomatous epithelium, but also in multifocally localized form at the flat non-papillomatous mucosa, and that they can be regarded as the expression of an incipient field cancerification.

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