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# Induction of Transitional Cell Carcinoma of the Urinary Bladder in Rats by Feeding N - [4-(5-nitro-2-furyl)-2-thiazolyl] Formamide

## Histological and Ultrastructural Findings

H.-D. Adolphs, J. Thiele, H. Kiel, and L. Steffens

Department of Urology, University of Bonn; Institute of Pathology, Medical School, Hannover; Department of Laboratory Animals, Chemie Grünenthal GmbH, Stolberg; and Department of Urology, St. Antonius-Hospital, Eschweiler, FRG

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Summary. After feeding with 0.188% N-[4-(5-nitro-2 furyl)-2-thiazolyl] formamide (FANFT), transitional cell carcinoma of the urinary bladder was induced in all female Wistar rats tested. Histological changes of the urothelium consisted of various degrees of hyperplasia and dysplasia. An infiltrating transitional cell carcinoma first appeared after 8 months. These results are compared with the findings of other authors, and divergencies of the tumour induction rates are discussed with respect to strain, sex and weight of experimental animals as well as concentration and amount of ingested carcinogen. Electron microscopy shows microvillous transformation of the luminal plasma membrane and appearance of a thick fluffy cell coat (glycocalyx). These changes are explained by an altered function of the Golgi complex occurring during malignancy and leading to a loss of the specific discoid vesicles of the urothelial cells.

Key words: Chemical carcinogenesis - Bladder carcinoma - Light and electron microscopy - Membrane transformation - Antigenicity.

N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT) is known to exercise a strong, specific carcinogenic action on the transitional epithelium of the urinary bladder in the hamster (5), the dog (11), the mouse (7, 10, 31, 32, 33, 44), and the rat (6, 8, 9, 20, 23, 28, 34, 35, 39, 43). FANFT-induced tumours closely resemble neoplasms of the urinary bladder in man (8, 29) and thus provide a suitable model for studying new methods of chemotherapy (7, 31, 32, 33).

However, the reliability of this tumour model is still the subject of controversy. Although several authors, especially Ertürk and co-workers (8, 9) regard FANFT as the most potent carcinogen at present available for inducing urinary bladder neoplasms, particularly in rats, others report only a weak induction rate. These latter authors disapprove of the FANFT-tumour-model chiefly because of unreliability in respect of the varying induction rates and fluctuating as well as non exposure-dependent tumour weights (42).

This study was performed firstly to evaluate the degree of usefulness of the FANFT-tumour in the rat as an experimental model for bladder cancer and secondly to elucidate some of the morphological alterations to the transitional epithelium occurring during malignant transformation.

## MATERIAL AND METHODS

45 female Wistar rats (Han/Bö), aged about 35 days, weighing 70-90 g, were fed a standard diet (Herilan M/R 204, Eggersmann Comp.; Rinteln, Germany) mixed with 0.1888 percent FANFT (Saber Laboratories Inc.; Morton Grove, Ill. /USA). All animals were

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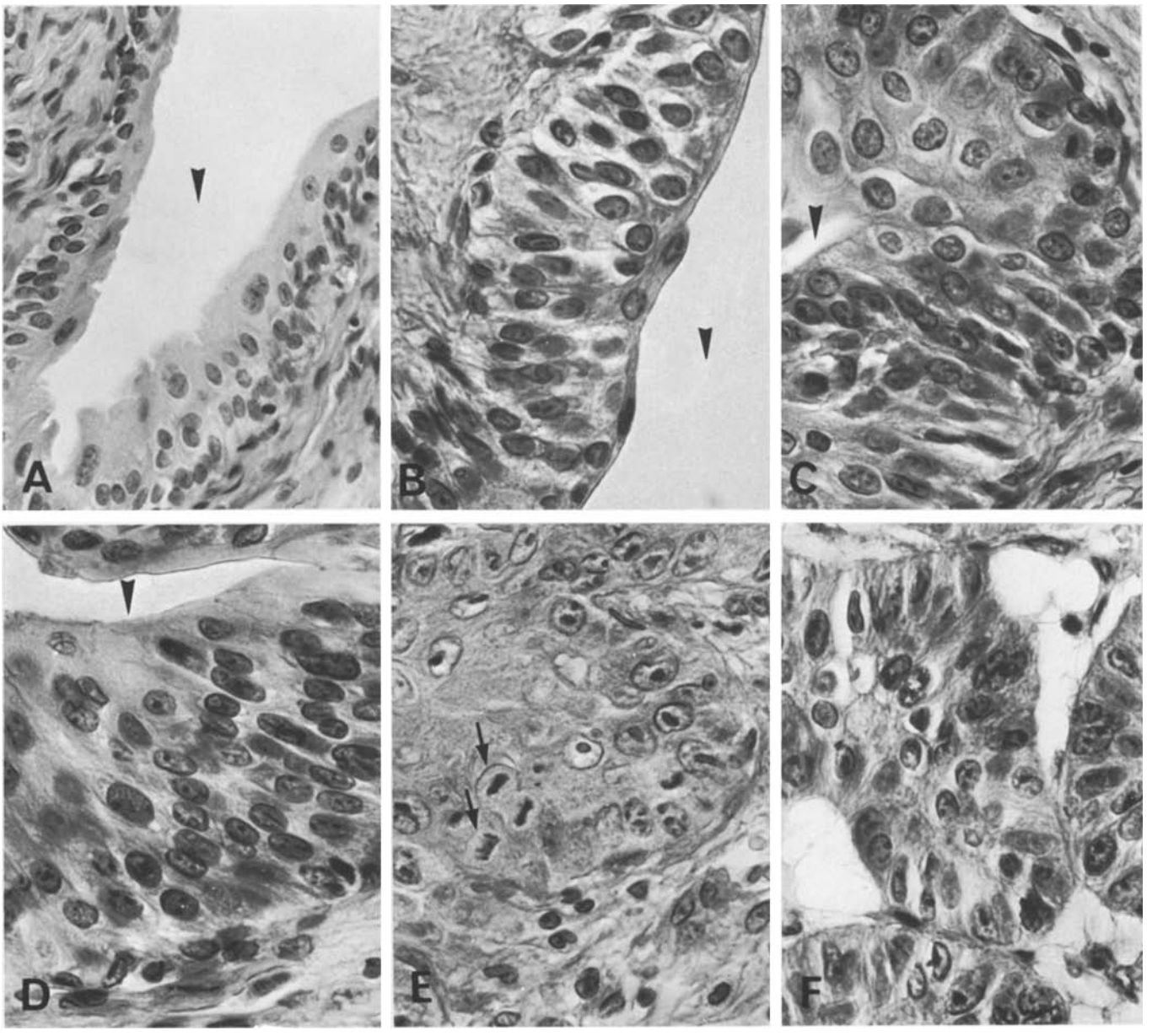


Fig. 1 A-F. Alterations in the transitional epithelium of the urinary bladder after feeding with FANFT (lumen of urinary bladder is indicated by arrowhead). A) Normal transitional epithelium of a control rat showing three layers with larger superficicial cells (cf. Fig. 2 A). B) 1 month, hyperplastic epithelium with hyperchromatic, slightly enlarged nuclei. C) 2 months, nodular areas of dysplastic cells with extension into the lamina propria. D) 4 months, severe dysplasia with large hyperchromatic nuclei containing distinct nucleoli. E) 6 months, intraepithelial carcinoma with atypical cells displaying mitotic figures (arrows). F) 8 months, papillary transitional cell carcinoma with strands of atypical cells infiltrating the submucosa

A) x 500, B-F) x 800

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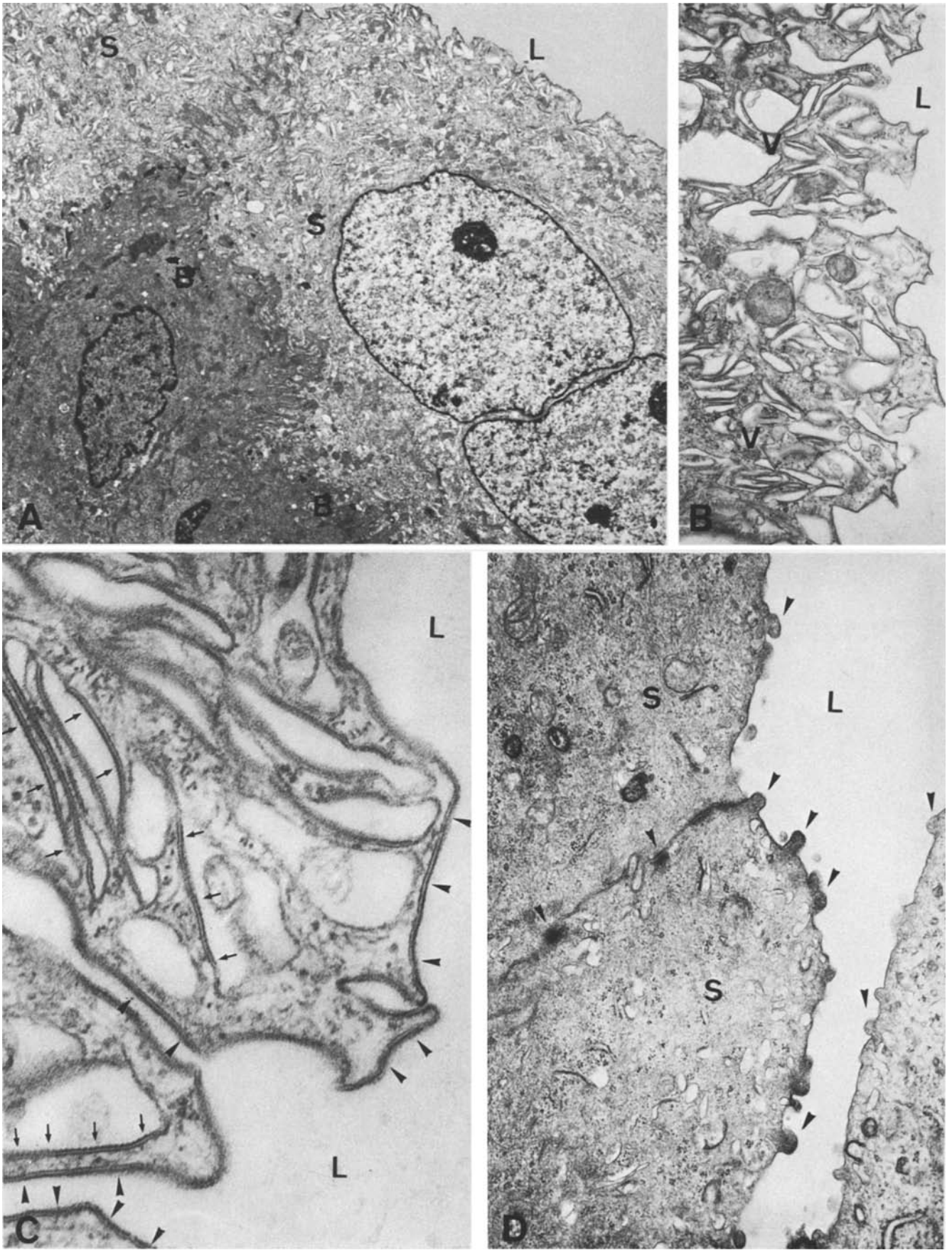
Fig. 2 A-D. Electron microscopy of normal and dysplastic transitional epithelium. A) Survey with lumen ▶ of urinary bladder (L) and superficial cells (S) with large nuclei and cytoplasm filled by numerous vesicles. Basal cells (B) display a more dense cytoplasm. x 5,000. B) Luminal portion of superficial cell with lumen (L) and many discoid vesicles (V). Luminal plasma membrane with sharp angulations and excavations. x 18,000. C) Higher magnification of the luminal plasma membrane with asymmetry showing a thick dense leaflet on its luminal face (arrowheads, L) and a thinner leaflet on the cytoplasmic side. The discoid vesicles below are bounded by a similar asymmetric membrane which has a thick luminal leaflet (small arrows) and a thinner leaflet on the cytoplasmic border, thus providing asymmetric membrane material for the luminal plasma membrane by a fusion process (16, 22). x 90,000. D) Luminal border of dysplastic cells (4 months of FANFT feeding) with lumen (L) and microvillous transformation (arrowheads). Two superficial cells (S) bridged by desmosomes (arrowheads) at their junction. x 18,000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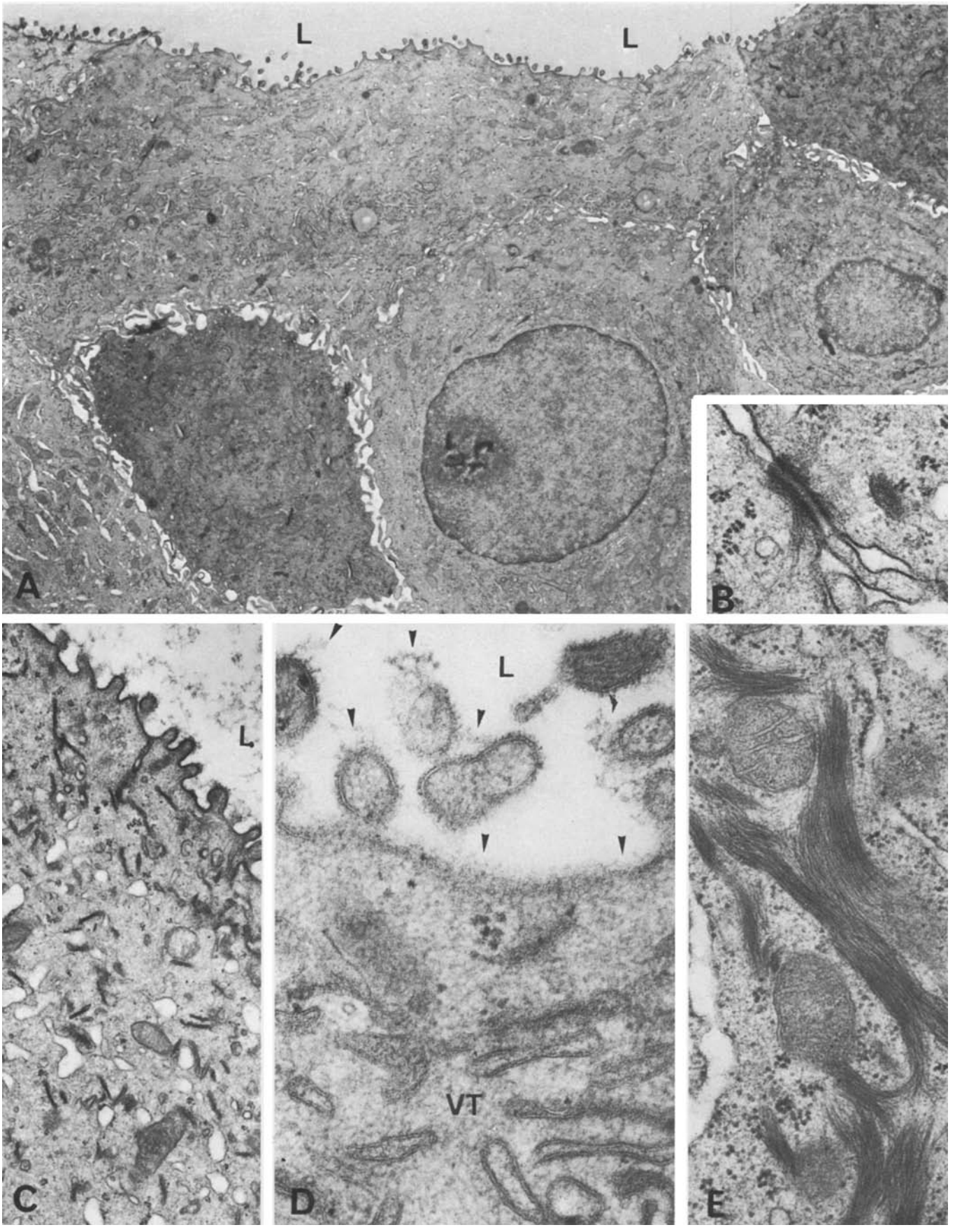
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allowed free access to water and pellet diet and caged in groups of five rats. The carcinogen exposure was of 8 months duration; afterwards the animals received a carcinogen free standard diet. The average FANFT-consumption during this period amounted to 0.195 g/week/animal (0.134-0.260 g). Thirty control rats were kept under identical living conditions, but received a FANFT-free standard diet. From these two groups of rats, one animal was sacrificed each month for investigation by light and electron microscopy.

From the 5th month onwards the urinary bladders of all animals were examined monthly by transillumination for the presence of tumour growth (36). Twelve months after commencement of carcinogen feeding, all rats in the experimental and control groups were killed. Light microscopy and ultrastructural examination of the urinary bladders were performed and the tumour-containing bladders were weighed.

For light microscopy the bladders were fixed in Bouin's solution, paraffin embedded and processed by several standard staining methods. For electron microscopy, small tissue blocks were fixed in an aldehyde solution followed by osmium tetroxide, embedded in plastic (Epon) and ultra thin sections were cut and stained by lead citrate and uranyl acetate.

## RESULTS

Gross examination of the exstirpated urinary bladders revealed the first definite alterations of the mucosa after 5-6 months of FANFT feeding. These changes consisted of irregular foldings of the inner surface sometimes with small papillary or polypoid growths, gradually increasing during the ensuing months. After 8 months of carcinogen exposure, all experimental animals exhibited tumour-like lesions in their bladders, easily detected by transillumination. After 12 months, at the termination of the experiment, the median weight of the 30 tumour-bearing bladders was found to be 2.985 g (± 4.56); 15 control animals showed no pathological changes in their urinary bladders and the median organ weight was 0.085 g (± 0.07).

Light microscopy revealed that the three cell layers of the normal bladder mucosa (Fig. 1 A) displayed a gradually increasing hyperplasia, beginning at about the 4th week of FANFT exposure (Fig. 1 B). This hyperplasia exhibited nodular areas of focal cellular extensions into the lamina propria (Fig. 1 C). During the 2nd-4th month, formation of micropapilli with a total loss of the differentiation between basal and superficial cell layers was evident (Fig. 1 D). Hyperplastic growth was accompanied by an increasing dysplasia, with polymorphous and hyperchromatic nuclei and many mitotic figures. Some of these alterations were so marked that from the 5th-6th month small foci of intra-epithelial carcinomas were recognisable, but there was no infiltration beyond the basal membrane (Fig. 1 E). At about the 6th month, dysplastic changes and papillary growth of the mucosa predominated in one area and gave rise to gross papillary tumours consistent with inverted papillomas, with atypia of the ingrown epithelium or proliferating transitional cell papillomas. At the 8th month of FANFT exposure, all papillary tumours displayed an infiltration of the subepithelial layers of the bladder wall, characterising them as transitional cell carcinomas (Fig. 1 F). These carcinomas showed a high to moderate differentiation with various degrees of squamous metaplasia and invasive growth ranging from the lamina submucosa to the pelvic peritoneum. Only very few carcinomas had regional lymph node metastases, and there was no further metastatic spread into other organs.

Electron microscopy of the normal transitional epithelium showed sharp angulations, infoldings and protrusions of the luminal surface of the superficial cells (Figs. 2 A and B) as reviewed by Hicks (16) and Koss (22). Most conspicuous were groupings of specific discoid vesicles with an asymmetric unit membrane; these vesicles were incorporated into the luminal plasma membrane (Fig. 2 C). Following prolonged FANFT-feeding there was not only an increase in nuclear size and indentations with coarse dispersion of chromatin, but a remarkable decrease of the characteristic discoid vesicles accompanied by a gradual microvillous transformation of the luminal

◀ Fig. 3 A-E. Electron microscopy of transitional cell carcinoma after 8 months of FANFT-feeding. A) Survey with lumen of the urinary bladder (L) and superficial cells of the papillary carcinoma with villous transformation. x 5,200. B) Large desmosome with broad bundle of tonofilaments at junction of basal cells. x 46,000. C) Luminal surface (L) with many microvilli surrounded by fluffy material. x 18,000. D) High magnification with lumen (L) and microvilli in cross-section covered by electron dense flocculent material (glycocalyx, arrowheads). Luminal portion of superficial cell filled with tubular and vesicular structures without membrane asymmetry (VT, cf. Fig. 2 C). x 90,000. E) Large bundles of microfilaments surrounding mitochondria in basal cell. x 46,000

plasma membranes. These changes became evident after the 4th month of carcinogen exposure with a luminal surface containing microvilli instead of angulations and a cytoplasm with only few of those specific discoid vesicles (Fig. 2 D). Formation of microvilli was accompanied by a considerable proliferation of the basal cell layers in the ensuing 4 months. The appearance of large desmosomes in the layers below the superficial cells was also noticeable (Fig. 3 B), together with scattered bundles of dense microfilaments in their cytoplasm (Fig. 3 E) during this period of malignant transformation. At the 8th month there was complete microvillous transformation of the luminal plasma membrane (Figs. 3 A and C), which was covered by a broad fluffy coat of electron dense material consistent with a glycocalyx (Fig. 3 D). The cytoplasm of the superficial cell layers of these papillary transitional cell carcinomas was devoid of the specific discoid vesicles but exhibited many tubular and vesicular structures lacking the characteristic asymmetric unit membrane (Fig. 3 D). Consequently, the luminal plasma membrane did not show asymmetry (compare Fig. 2 C with Fig. 3 D).

## DISCUSSION

Our results demonstrate that there is a 100 percent incidence of bladder tumours in female Wistar rats after 8 months' feeding of 0.188 percent FANFT. At termination of the experiment, in all 30 experimental rats transitional cell carcinomas were demonstrable, whereas 15 control animals showed normal bladders. In contrast to these findings, in Sprague-Dawly rats (weight 70-100 g) early papillary carcinomas can be observed after only 10 weeks application of 0.188 percent of this carcinogen (9, 28). In Fischer rats (weight 50-100 g) very different rates of tumour induction have been reported; Wang et al. described transitional cell carcinomas after 20 weeks of exposure to FANFT in only 16 percent of animals (39), while other investigators observed carcinomas in all the rats after 20, 24 and 25 weeks (6, 35, 43). In addition to these strain-dependent differences in bladder tumour induction rates, the problems of varying tumour gains related to sex and/or exogenous hormone application remain unsolved. With varying carcinogenic compounds and different species of rodents, tumour incidence is higher in male than in female animals (3, 14, 21, 24, 26, 38). Conversely, other investigators found higher tumour induction rates in female animals or no differences between both sexes (19, 25, 37, 44). So far, no experimental data is available in respect of sex differences concerning tumour induction by FANFT in rats. In endocrinologically intact mice, however, no such differences were noticeable with FANFT-induced tumours (44).

The molecular events leading to the carcinogenic action of FANFT are still unknown, but experimental findings indicate that the 5-nitro group of the furan ring plays an important role in the carcinogenicity of this compound, probably by formation of hydroxyl-amino furan as reactive intermediate (12). Nitroreduction has been suggested as the initial step for the carcinogenic activation of FANFT (4).

Of further interest is the problem of whether chemical tumour induction depends on the concentration of the orally applied carcinogen or the absolute amount of the ingested compound. Ito and coworkers investigated these relations in N-butyl-N-(4-hydroxybutyl) nitrosamine-induced carcinoma of the urinary bladder in rats (19). They found an identical tumour induction rate with carcinogen feeding ranging between 0.1 and 0.01 percent, provided that lower concentrations of this compound were ingested over a longer period. Below the critical concentration of 0.01 percent, tumour gains are found to be remarkedly decreased. A similar correlation between carcinogen concentration and tumour growth is seen in mice after feeding with N-2-Fluorenylacetamide (14). So far, no systematic study has been undertaken to ascertain whether these results may be transferable to our FANFT tumour model. Observations of Williams and Murphy, however, suggest that following very low concentrations of FANFT feeding (0.1 percent), tumour development is only 40 percent after 70 weeks of exposure (42), although the total amount of carcinogen ingested by the rats ought normally to lead to a 100 percent induction rate (8, 9). These results indicate that the varying rates of bladder tumours obtained may depend, at least within certain limits, more on concentration than on total amount of FANFT ingested.

It is also likely that the weights of the experimental animals are a limiting factor. In Wistar rats weighing 180-200 g, 0.188 percent FANFT feeding over a period of 59 weeks results in a bladder carcinoma in only 33 percent of the animals (23). Applying 2-Acetyl-aminofluorene to rats, tumour gains are considerably higher if feeding starts in weanling animals, whereas there is a significant decrease in older rats (27).

Our histological findings demonstrate that infiltrating transitional cell carcinoma first appears after 8 months. This long induction period may be related to differences in the

strains of rats and commencement of FANFT feeding as discussed above. Alterations of the bladder mucosa preceding cancer development consist of various degrees of hyperplasia associated with increasing dysplasia of the transitional epithelium, comparable with observations made by Tiltman et al. (34) and Ertürk et al. (9). Several authors (6, 8, 9, 43) describe epidermoid- or adenocarcinomas in addition to transitional cell tumours, which are exclusively found in our series. These differences may probably be associated with different strains of rats, e.g., Fischer rats (6, 43), Sprague-Dawly (9) or Wistar rats as in our model.

Electron microscopy shows a progressive alteration of the luminal surface membrane towards microvilli formation covered by a thick fluffy coat (glycocalyx). Biological characteristics of malignant cells are closely associated with significant changes of structure and function of plasma membranes. Plasma membrane alterations play a major role in the abnormal social behaviour of the cancer cell expressed by loss of contact inhibition, differences in surface charge, decrease in enzyme activity and, most important, changes of antigenicity (30, 41). These changes are unspecific since they are observed in virus and carcinogen induced tumours as well as in spontaneously transformed cells, and largely account for the infiltrative growth and metastatic spread of tumour cells. The fluffy mucopolysaccharide-containg surface coat (glycocalyx) is thought to represent the morphological substrate for the increased negative surface charge of cancer cells (30). Further membrane alterations during malignant transformation are indicated not only by the appearance of microvilli but also by the loss of the asymmetric unit membranes of discoid vesicles and luminal plasma membranes. Thus our results extend the findings of Hicks and Wakefield (18) and Jacobs et al. (20) on FANFT-induced bladder tumours so far as alterations of the apical cell membrane are concerned. These obvious changes of plasma membranes and vesicular structures are attributable to an altered function of the Golgi complex, which is assumed to be responsible for the synthesis of the glycocalyx and for the asymmetric membranes of the discoid vesicles fused into the luminal plasma membrane (17, 22). Findings regarding other ultrastructural features in addition to membrane alterations in normal, dysplastic and malignant transitional epithelium confirm the sparse reports pertaining to FANFT-induced (28, 43) or nitrosamine-induced (1, 19) bladder tumours. Most remarkable, however, is the fact that electron microscopy shows the transitional cell carcinoma of our FANFT tumour model to be identical to the papillary carcinoma of man (13). This is a further indication for the usefulness of this model for studying new methods of therapy in papillary carcinoma of the urinary bladder.

In experimental tumour induction, the existence of tumour specific antigen (TSA) is of great interest. Whereas in tumours induced by the same virus common antigenicity generally exists, most chemically induced tumours show no common TSA (40). Conversely, common antigenicity is observed in certain spontaneously arising as well as in chemically induced tumours of the same histological type (2, 15). Employing immunological absorption and precipitation experiments Daly et al. found common TSA also in FANFT-induced bladder tumours of the rat (6). Hence, in addition, this experimental tumour model seems to be suitable for experimental elucidation of questions regarding immunoprophylaxis and immunotherapy.

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Hans-Dieter Adolphs, M. D.
Department of Urology
University of Bonn
D-5300 Bonn - Venusberg
Federal Republic of Germany