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Effect of Promoters on Incidence of Bladder Cancer in Experimental Animal Models

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Multistage models of carcinogenesis proposed to account for the observed patterns of tumor development in the skin, liver, lung, bladder and other organs involve initiation of neoplastic change in a few cells by a threshold dose of carcinogen followed by conversion of these latent tumor cells into an autonomous cancer by further doses of the same and/or other carcinogens, and/or noncarcinogenic promoting agents. In the rat urinary bladder, neoplastic change can be initiated by a few weeks treatment with low doses of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) or *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) or by a single, low dose, intravesicular instillation of *N*-methyl-*N*-nitrosourea (MNU). Very few animals treated thus will develop bladder cancer unless exposed subsequently to some further regime which will promote tumor growth from the initiated cells.

Many different factors will stimulate tumor growth in the initiated rat bladder, including further low doses of complete bladder carcinogens, dietary factors such as metabolites of tryptophan or deficiency of vitamin A, the food additives saccharin and cyclamate and some alkylating agents such as cyclophosphamide and methylmethane sulfonate. New and published evidence is reviewed which supports the belief that these and other factors are promoters or later stage carcinogens in the bladder. The difficulties of defining a promoter and of identifying markers of promotion, i.e., of distinguishing the second from the later stages of carcinogenesis in the urinary bladder, are discussed with reference to the action of promoters in the mouse skin initiation/promotion model. However, in terms of their effect on an initiated population on the risk of developing cancer, it is suggested that such a distinction is largely irrelevant. Since both second and later stage carcinogens accelerate tumor development in an initiated urothelium, they both have the potential to lower the age at which bladder cancer becomes symptomatic. They are thus as important as are initiating carcinogens in determining the patterns of age-related neoplastic disease in any population.

Introduction

Multistage models of carcinogenesis have been proposed to account for the observed patterns of tumor development in the skin, liver, lung, bladder and other organs (*1-10*). Such models involve initiation of neoplastic change in a few cells by a threshold dose of carcinogen, followed by conversion of these initiated tumor cells into an autonomous cancer by further doses of the same and/or other carcinogens, and/or noncarcinogenic promoting agents.

In the rat urinary bladder, neoplastic change can be initiated by a few weeks treatment with low doses of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) (*11, 12*) or *N*-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT) (*13, 14*) or by a single, low dose, intravesicular instillation of *N*-methyl-*N*-nitrosourea (MNU) (*15, 16*). Very few of the animals treated with an initiating dose of such a carcinogen will develop bladder cancer in their natural lifespan unless they are exposed subsequently to some further regime which will promote tumor growth from the initiated tumor cells. The initiating event is generally believed to be rapid and to involve an interaction of the initiating carcinogen with the DNA of the target cell to produce a permanent mutagenic event (*2, 17*). By contrast, promoting agents do not necessarily interact with the DNA and thus are not necessarily mutagens; promotion is thought to involve epigenetic as opposed to genetic events (*18*). Furthermore, prolonged or repeated exposure of the initiated cell to promoters is required to achieve tumor growth (*2*).

The concept of tumor promotion was developed

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from studies on the mouse skin model by using the diterpene series of promoters present in croton oil (*1, 2, 4, 19, 20*). The extension of this concept to any other tissue has become partly a problem of semantics, i.e., the study of the meaning of the word as well as its original derivation. It was realized only recently that the mechanism of carcinogenesis in other epithelial tissues has much in common with carcinogenesis in the mouse skin, even though other carcinogens and other promoters are involved, but it is not yet clear if every observation made with the mouse skin model can be rigidly applied to other tissues. For example, from studies with the polycyclic hydrocarbon class of carcinogens in the mouse skin it was concluded that initiation is a rapid, "permanent" event which will persist more or less indefinitely; initiated cells can thus remain dormant but still be susceptible to promotion many months after the initiating event (*2*). This may well prove to be substantially true not only for the skin but also for other tissues and other carcinogens, but the rigid concept of permanence may yet have to be modified to take into account the well recognized fact that much potentially initiating carcinogen-induced DNA damage is in fact not permanent, but is eliminated by enzymatic repair mechanisms (*21*). Whether or not the initiating damage is permanent may depend on the carcinogen and the exact nature of the interaction between it and the genome. Thus in the bladder, there appears to be a considerable decay in the number of persisting initiated cells in the first 6 weeks following treatment with initiating doses of FANFT (*14*) whereas, by contrast, the initiating effect of MNU does persist undiminished for at least 25 weeks (*16, 22*).

If promotion is to be strictly defined by reference to the mouse skin model, then only compounds equivalent to 12-O-tetradecanoylphorbol-13-acetate (TPA) which bring about the second stage in a multistage process can be considered, and any factors such as ethylphenylpropriolate which act at the third or later stages in carcinogenesis must be excluded from the discussion. The argument is further confused, however, for recently incomplete first- and second-stage promoters have been defined; for example the calcium ionophore A23187 is a first-stage promoter and mezerein a second-stage promoter in skin carcinogenesis (*23*). When attempting to define the biochemical and cellular mechanisms which underlie early stages of tumor growth, a narrow definition of promotion in terms of biochemical and biological events is clearly essential. In terms of human disease, however, if continuous exposure to a factor increases the risk of developing a particular cancer and conversely, if removal of that factor decreases the risk, it is largely irrelevant whether the agent happens to act predominantly at the second stage, i.e., as a promoter, or at one of the later stages in the carcinogenic process.

Complete carcinogens can bring about all stages of the carcinogenic process if a sufficient dose is given over a long enough period of time, i.e., they act both as initiators, promoters and later stage carcinogens. However, not all complete carcinogens are necessarily equally effective at the different stages of carcinogenesis and, indeed, there is no *a priori* reason why they should be, for the biochemical events underlying the initiation and later stages of carcinogenesis differ quite fundamentally. Some carcinogens are more effective at the first, initiating stage but are poor promoters, for example 2-acetylaminofluorene (2-AAF) in its effect on the rat liver (*24*), whereas other carcinogens or the same carcinogen in other tissues, may be weak initiators but powerful late stage carcinogens, for example 2-AAF in the rat bladder (*25*). Thus many compounds possess a spectrum of carcinogenic activity, and it is rare to find a compound which is a "pure" initiator or one which is a "pure" promoter. Many agents generally regarded as promoters, e.g., croton oil, also have some, albeit weak, initiating activity (*26-28*).

Factors Identified as Affecting the Second Stage (Promotion) and/or Later Stages of Carcinogenesis in the Urinary Bladder

In models other than the mouse skin, very few late-stage carcinogens have been investigated in sufficient detail to say whether they meet all the criteria which have been defined for a promoter in skin. In experimental rat bladder models a number of compounds have been identified which significantly increase the tumor incidence after treatment with an initiating dose of carcinogen. Of these, sodium cyclamate after MNU (*29*), sodium saccharin after MNU (*16, 29*), after FANFT (*14*) or after BBN (*30, 31*) tryptophan after FANFT in the rat and mouse bladder (*14, 32*) and after 4-aminobiphenyl and 2-naphthylamine in the dog (*33*) and phenacetin after BBN (*12*), can all be claimed to act as late-stage carcinogens and possibly as promoters for the development of transitional cell carcinoma in a previously initiated urothelium. The evidence for regarding these chemicals as promoters in the urinary bladder is already published and is primarily based on their ability to increase the tumor yield above that produced by an initiating dose of carcinogen alone. On the published evidence they may well act

as second-stage rather than later-stage carcinogens, but the data are far from unequivocal (*34*).

Other factors which also increase the time-related tumor yield in carcinogen-treated bladders include an as yet unidentified component of normal rat urine (*35-39*), presence of the male sex hormone, testosterone (*40, 41*), deficiency of vitamin A (*42*) and nonspecific irritation of the bladder mucosa either by a calculus (*35*) or by calcified and/or live ova of the parasite *Schistosoma haematobium* (*43*).

The effect of *S.haematobium* infection is interesting as it illustrates the effect that irritation can have on the induction of bladder cancer. In experimental animals, egg deposition which occurs in the bladder during *S.haematobium* infections in general leads to extensive inflammation followed by fibrosis of the bladder wall, polyp formation, and ulceration plus hyperplasia of the urothelium, but urothelial carcinoma does not usually follow simple infection with *S. haematobium* alone. [Well differentiated, papillary carcinomas were reported in one Capuchin and one Talapoin monkey and in the ureter of one of 21 baboons infected with *S.haematobium* (*44*), but subsequent experiments showed that similar lesions in Capuchin monkeys had a restricted growth potential and did not survive transplantation; furthermore the primaries regressed in the third or fourth year after infection (*45*).] In our study, in addition to infecting animals with *S.haematobium* cercariae, weekly low doses of BBN were used to give a threshold carcinogenic stimulus to the urothelium (*43*).

None of the five baboons given BBN alone developed neoplastic urothelial lesions in the 2½-year experiment nor did those treated with *S.haematobium* alone, although the latter group developed various degrees of urothelial hyperplasia. Of the 10 animals treated both with BBN and *S.haematobium*, four developed neoplastic disease of the bladder (Fig. 1) and three had multiple endophytic papillary downgrowths of the urothelium into the walls of the ureters which passed into and through the muscle layers (Fig. 2). Although the BBN treatment alone was subcarcinogenic in the 2½ years duration of this experiment, this period represents only a quarter or less of the normal lifespan of this species and it

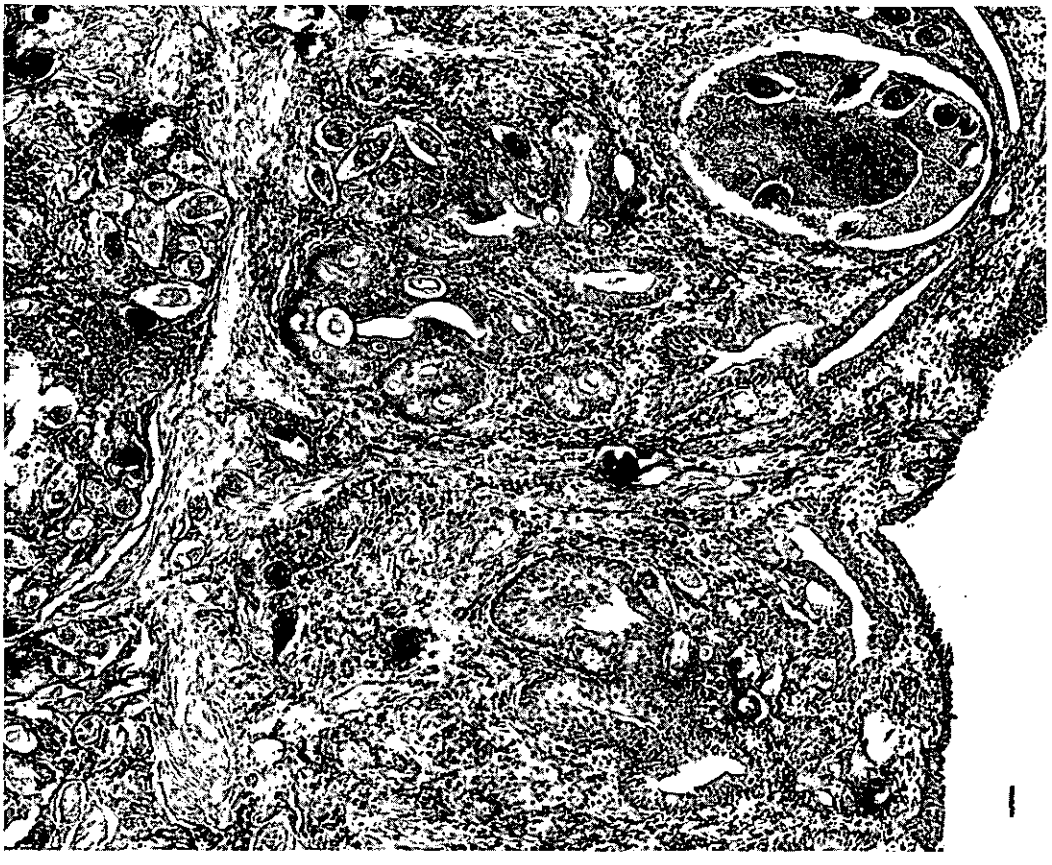

FIGURE 1. Adenocarcinoma of the urothelium in a baboon treated with 5 mg BBN/kg/week and infected with *S.haematobium*, killed 2½ years after infection. Neoplastic downgrowths of urothelium have penetrated deeply into the lamina propria and into the superficial muscle layer of the bladder wall. Wax-embedded; H & E; × 100.

FIGURE 2. Downgrowth of dysplastic urothelium which has penetrated the circular muscle layers in one ureter of a baboon treated as the animal illustrated in Fig. 1 with BBN and infected with *S.haematobium*. Epon-embedded; Toluidine Blue; × 200.

would be rash to conclude that the monkey is immune to BBN. This bladder carcinogen is effective in several other species (*40, 46-48*) and would doubtless have proved so in the baboons if the animals had been kept alive for a further 10 years or so. The *S.haematobium* infection probably acted by reducing the latent period before tumor growth commenced, thus bringing forward the time at which urothelial carcinoma developed to within the time scale of the experiment. In all probability, the "promoting" effect of *S.haematobium* in this system is in fact a propagating stimulus attributable to increased urothelial cell turnover in response to mechanical irritation. We suggest that the primary effect of *S.haematobium* in the induction of bilharzial bladder cancer in countries such as Egypt, is to lower the age-related cancer incidence following exposure to some as yet unidentified initiating carcinogen, by acting at a late state in the carcinogenic process. In the West, the peak age of bladder cancer incidence is in the seventh decade, whereas in Egypt it is in the fifth decade. The effect on the bladder of calculi, an indwelling catheter, or any other local irritant could well act in the same way and lower the age at which bladder cancer becomes symptomatic in any particular individual.

Another compound which may be added to this list of late stage carcinogens or promoters of carcinogenesis in the bladder is methylmethane sulfonate (MMS). MMS has been reported to produce tumors of the nervous system in rats after IP injection (*49*) and local tumors after subcutaneous injection (*50*). We have investigated its ability to act both as a complete carcinogen and as a promoting agent when applied directly to the urothelium in our MNU/rat model. For the present work an inbred strain, the F344 rat was used instead of the outbred Wistars used for previous experiments with the MNU/bladder model. Animals were given six intravesicular doses of 2.5 mg MMS either alone, or following a single threshold/initiating intravesicular dose of 0.3 mg MNU. Appropriate untreated controls and a control group given the 0.3 mg MNU only were set up in parallel, and the number of macroscopically visible tumors produced in 2 years is show in Table 1. The final number of neoplasms

Table 1. Promotion of methylnitrosourea (MNU)-induced bladder cancer with methylmethane Sulfonate (MMS) in female F344 rats.

Treatment (intravesicular instillation)	Number of animals	Number of tumors[a]	Incidence of tumors,%
1 × 0.3 mg MNU	30	7	23
6 × 2.5 mg. MMS	32	2	6
1 × 0.3 mg MNU plus 6 × 2.5 mg MMS	37	18	49
Untreated controls	27	0	0

[a]Macroscopic observations; preliminary data from unpublished observations by R. J. Tudor, R. M. Hicks, N. Severs, and S. Barnes.

may be modified after completion of histological examination of serial sections of all bladders, but these preliminary results are included here, since they demonstrate for the first time that this compound also behaves as a very weak bladder carcinogen when used on its own, and as an effective promoting agent when applied to a previously initiated urothelium. This suggests that MMS, like 2-AAF, has weak first stage but powerful late stage carcinogenic potential for the bladder urothelium.

Another compound which we believe falls into this category is cyclophosphamide. As reported previously, 18 single IP doses given at monthly intervals (total dose 1800 mg/kg body weight over 1½ years) failed to produce bladder cancer (*29*). Similarly, 10 mg/kg body weight twice weekly for 40 weeks (total dose 800 mg/kg body weight) was not carcinogenic on its own but did produce a 35% incidence of bladder cancer following a 6-week course of a threshold dose of FANFT (*51*). When, however, rats were given lifetime treatment with cyclophosphamide by including it in the drinking water five times a week to give total doses of 1,270, 698, 475 and 230 mg/kg body weight, a low dose-related incidence of bladder cancer was obtained in male rats (*52*). At the highest dose level, seven of 37 (19%) males but only one of 35 females developed bladder cancer. No bladder cancers were found in the three groups of females given the lower doses of cyclophosphamide, but 13%, 5% and 5%, respectively, of the males developed transitional cell carcinoma. These results are consistent with the suggestion that cyclophosphamide, like 2-AAF and MMS, is a weak first-stage carcinogen but a more effective promoter or late-stage carcinogen. These observations have obvious clinical implications when it is remembered that cyclophosphamide is frequently used as only one of several agents, some of which are mutagenic, in a combined cytotoxic therapy regime for treating neoplastic disease elsewhere in the body.

In general, such factors as vitamin A deficiency, *S.haematobium* infection, etc. have not been regarded by their investigators as promoters, but rather as late stage carcinogens comparable to turpentine or ethylphenylpropriolate in the mouse skin model, although urine has been referred to as a tumor promoter (*38, 39*). There may or may not prove to be a sound basis for making a distinction between the first group of compounds discussed above such as saccharin, tryptophan, etc., as promoting factors, and the second group, calculi, vitamin A deficiency etc., as later-stage carcinogens, but as yet the experimental evidence is insufficient for this distinction to be regarded as unequivocal.

Biological Markers of Promotion

In the Mouse Skin

Numerous biochemical and biological phenomena have been investigated as markers of promotion in the mouse skin following application of TPA. Those which appear to correlate well with promotion include the induction of the enzyme ornithine decarboxylase (ODC) and a consequent increase in polyamine synthesis, the induction of hyperplasia and of dark basal keratinocytes, the inhibition of normal tissue differentiation and alteration in the normal pattern of gene expession (*17, 18, 53-57*).

None of these phenomena, however, are unique markers for promoters. Thus although all skin promoters are hyperplastic agents, the converse is not true. Similarly, growth promoting stimuli in general will induce ODC and polyamines. For example, partial hepatectomy (*58, 59*), isoproterenol in the salivary gland (*60*), growth-promoting hormones (*61-63*) and epidermal growth factor (*64*) will all stimulate ODC and polyamine synthesis in their target tissues. The induction of ODC is probably a marker for cell proliferation rather than for second stage carcinogenesis. Since factors which stimulate cell proliferation also accelerate tumor growth, it is likely to be associated with the later stages of carcinogenesis rather than with the second stage exclusively.

Currently much emphasis is being placed on the ability of TPA but not of the later stage "promoter" mezerein, to induce the appearance of large numbers of dark, basal keratinocytes in skin (*18*). These dark keratinocytes are also induced by the incomplete promoter, the calcium ionophore A23187 (*23*). However, these results were obtained in a mouse strain, the Sencar, which is peculiarly sensitive to TPA promotion and they may or may not hold for other strains or other species. Slaga and his colleagues have suggested that dark keratinocytes may be primitive stem cells since they are also found in large numbers in embryonic and new born mouse skin (*53, 54*). Dark cells are also found in papillomas and carcinomas (*53, 54*) and must therefore be induced by complete carcinogens as well as by TPA.

Many published studies demonstrate that the diterpene series of promoters delay or prevent terminal differentiation of cell lines in culture. They increase the time, i.e., the number of cell divisions required before a cell can leave the cell cycle and enter the pathway for terminal differentiation and subsequent death. *In vivo*, if the basal stem cells of epithelial tissues are affected by a promoter in this way, then after each cell division the daughter cells

will be recruited, even if only temporarily, to the dividing stem cell population, the tissue will become hyperplastic and fail to differentiate normally, and the predominant cell population will appear relatively undifferentiated. If in addition, the original stem cells had been previously subjected to an initiating event, the new increased cell population will carry this defect in their genome and the chances of it being expressed as a new phenotype automatically will be greater than in a smaller cell population.

Although supression of terminal differentiation has been regarded as a characteristic property of promoters, recently evidence has been published which demonstrates that many mouse skin cells become resistant to terminal differentiation after exposure *in vivo* to an initiating dose of carcinogen (*65*). Thus the regulation of normal differentiation, in particular the development of the proliferative block which permits terminal differentiation, may be disturbed primarily by the initiating event and not by promotion, although *in vivo* the cell division which accompanies promotion may be a necessary factor before the failure to differentiate is made evident.

One of the significant properties of promoters is thought to be their ability to alter the normal pattern of gene expression, thereby permitting expression of normally repressed areas of the genome with the consequent appearance of new phenotypes (*17*). At the same time this increases the chance of expression of any latent initiating damage to the DNA which may have occurred in an area of the genome not normally expressed in that particular cell type. This disturbance of gene expression is reflected in the bizarre metaplasias seen in many tumors and has given rise to the concept of cancer as a disease of maladjusted differentiation. Many of the "new" phenotypes seen in cancers have accordingly been claimed as markers of neoplastic transformation, whereas in fact, they are more often epiphenomena and not causally implicated in the development of neoplastic growth.

At present, even in the mouse skin there is thus no single specific marker for a second-stage carcinogen (promoter) which will distinguish it unequivocally from a complete or later-stage carcinogen, other than its ability or inability to function at a particular point in a strictly defined temporal sequence in a multistage process of carcinogenesis.

In the Rat Urinary Bladder

As a model for the study of multistage carcinogenesis, the rat bladder has been developed far more recently (*14, 29, 30, 66*) than was the mouse skin model (*1*), and the direct effects of promoters and other late-stage carcinogens on the urothelium have been less systematically studied than they have in skin. Such studies as have been made show considerable similarity between the rat bladder and the mouse skin models.

Induction of ODC. Direct application to the urothelium by intravesicular instillation of the complete carcinogen FANFT or its major urinary metabolite, 2-amino-4-(5-nitro-2-furyl)-2-thiazolyl (ANFT) causes a rapid induction of ODC; similarly ODC activity in the urothelium was induced by FANFT administration PO (*67*). These results indicate that the urothelium, like skin, may be expected to react to treatment with other complete carcinogens by an induction of ODC which appears at the time of cell proliferation in the neoplastic tissue. To some extent this has been confirmed using three urothelial cells lines derived from rat bladder carcinomas. When these cancer cells were treated *in vitro* with the bladder carcinogens MNU, ANFT, or *N*-butyl-(3-carboxypropyl)nitrosamine (BCPN), ODC was induced (*68*). ODC was induced in this system to a similar extent by the second or later stage carcinogen saccharin and by 3-hydroxyanthranilic acid, a metabolite of 3-tryptophan. Epidermal growth factor (EGF) and insulin also induced ODC; but no aforementioned inducer was anywhere near as effective as was TPA (*68*). On the basis of these results, ODC induction appears to be concomitant with tumor promotion in the bladder as well as in the skin and furthermore TPA may well prove to be an effective promoter of carcinogenesis in the urothelium. With the *in vitro* cancer cell culture system, ODC was also shown to be inducible by an unidentified, heat-stable, filterable factor in rat urine which the authors related to previous reports of promoting activity in rat urine (*36-38*).

Induction of Urothelial Hyperplasia. Many of the agents identified as second- or later-stage carcinogens for the bladder also have the ability to induce urothelial hyperplasia when used on their own. Thus mechanical irritation from bladder calculi and from *S.haematobium* ova has been widely reported to cause local regenerative hyperplasia. Similarly, vitamin A deficiency causes an increased mitotic activity in the basal cells of the urothelium leading to hyperplasia and ultimately to epidermalization (*67, 70, 71*). High concentrations of dietary saccharin cause focal hyperplasia of the urothelium with increased numbers of cells entering into mitosis (*72*). Contrary to a previous report (*14*), this was later confirmed by Fukushima and Cohen (*73*), who ob-

served an increased uptake of tritiated thymidine into focal areas of the bladder urothelium in saccharin-treated rats. Urine, as well as promoting tumor growth, also induces hyperplasia in the urothelium of heterotopically transplanted rat bladders (*74*). However, since urine does not cause hyperplasia in the normal bladder *in situ*, the explanation for its hyperplastic effect on heterotopic bladders is not necessarily related to its tumor-promoting potential.

Cyclophosphamide has long been known to produce severe atypical hyperplasia following cytotoxic damage to the urothelium (*75-77*). MMS (*78*) and 2-AAF (*25*) likewise produce urothelial hyperplasia. As discussed above, these last three compounds are also weak complete carcinogens and after prolonged application will produce cancer of the urothelium, but their hyperplastic action is probably associated with their late-stage carcinogenic activity rather than with their first stage initiating activity.

Induction of Dark Basal Cells. Dark basal cells, comparable to those reported by Slaga et al. (*18*), in embryonic and promoted skin and in skin cancers are a feature of neoplastic rat urothelium treated with complete carcinogens such as BBN, FANFT, MNU (Fig. 3), and 2-naphthylamine (Fig. 4). They are seen in both papillary (Fig. 5) and nodular tumors and in areas of atypical but not necessarily hyperplastic urothelium within 2 weeks of completing a carcinogenic treatment with MNU (Fig. 3). They are also present in small numbers in the human fetal bladder (Fig. 6). A few dark basal cells are occasionally found in normal, adult, untreated rat urothelium particularly in females, but they do not appear to be significantly increased in number by prolonged treatment of the animal with saccharin. They are present in some saccharin-treated animals (*72*), but not with sufficient regularity to be treatment-related. Dark cells are also seen in association with squamous metaplasia in vitamin A-deficient rats, and in that situation they reflect increased numbers of tonofilaments and ribosomes in the keratinising tissue. We have not yet observed dark basal cells in areas of simple urothelial hyperplasia induced by treatment with MMS alone or cyclophosphamide alone although there were dark basal cells in transitional cell tumors induced by MMS only. The effect of 2-AAF on the staining characteristics of the basal urothelial cell layer does not appear to have been reported. At the moment, therefore, there is no positive evidence to suggest that the induction of dark basal cells can be regarded as a

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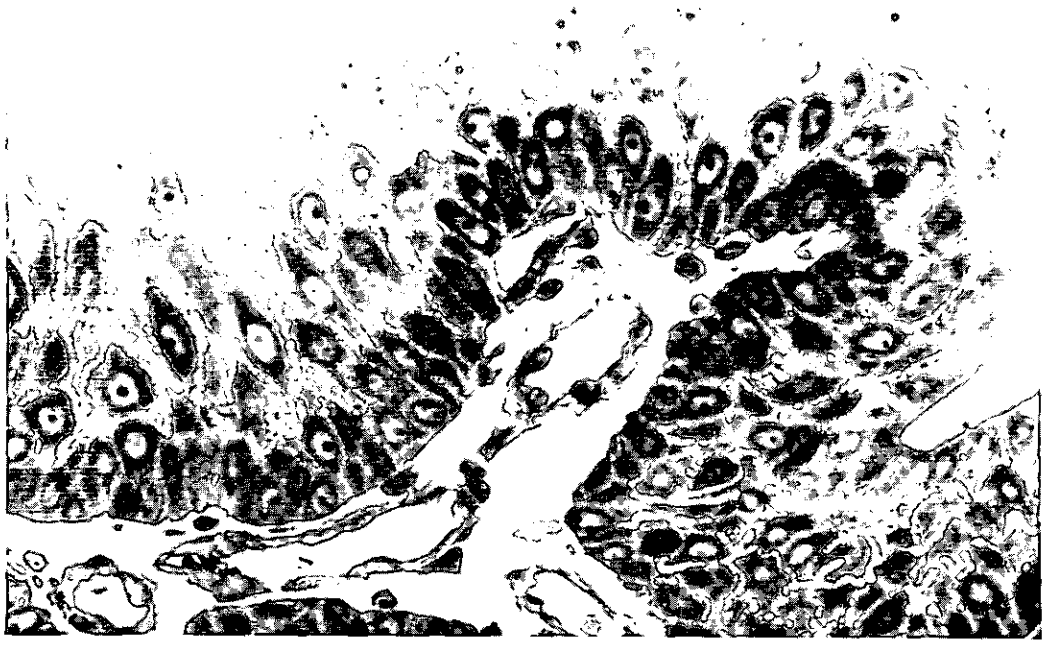

FIGURE 3. Dark basal cells in an area of flat, dysplastic urothelium lining the bladder of a rat 2 weeks after completing a fully carcinogenic course of MNU (four doses, 1.5 mg each). Epon-embedded; Toluidine Blue; × 800.

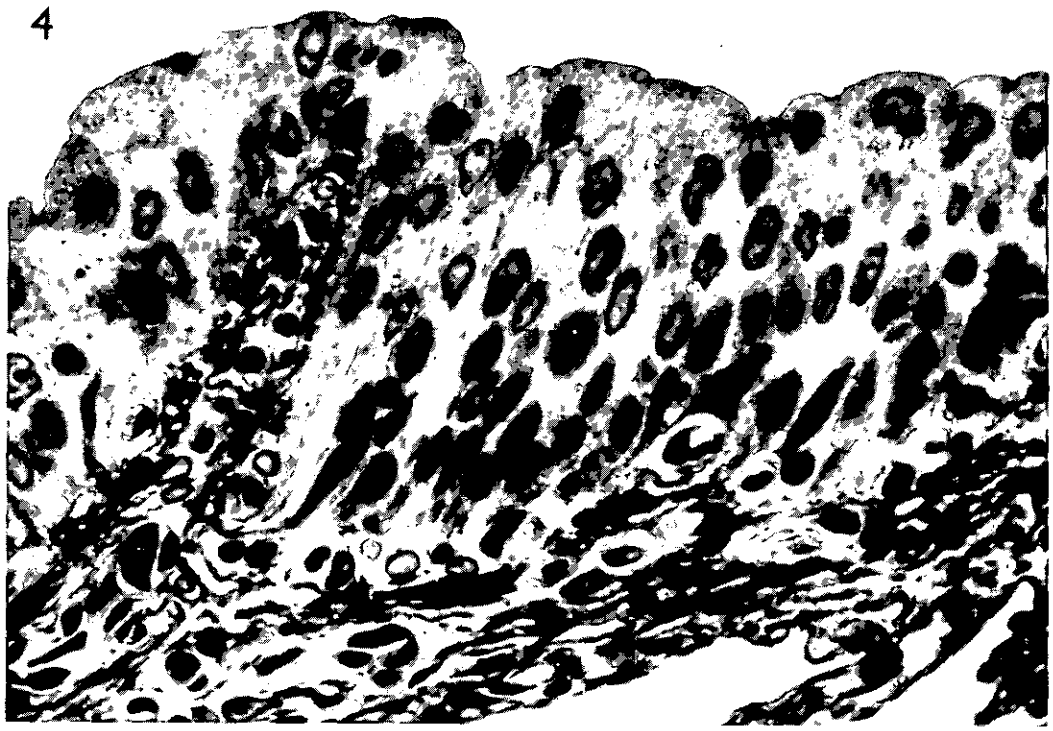

FIGURE 4. Darkly staining basal cells in the mildly hyperplastic and dysplastic urothelium lining the bladder of a rat treated with 2-naphthylamine (300 mg/kg/week for 57 weeks). Epon-embedded; Toluidine Blue; × 800.

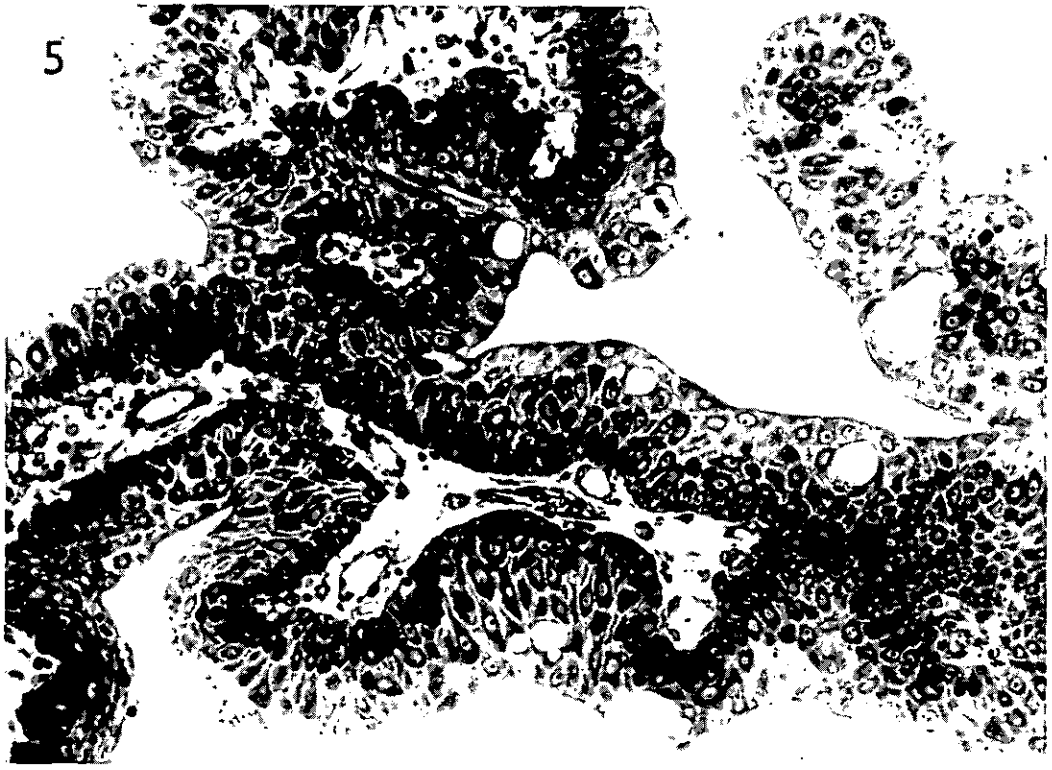

FIGURE 5. Dark basal cells in the urothelium of a papillary transitional cell carcinoma of the rat bladder, induced by MNU. Epon-embedded; Toluidine Blue; × 350.

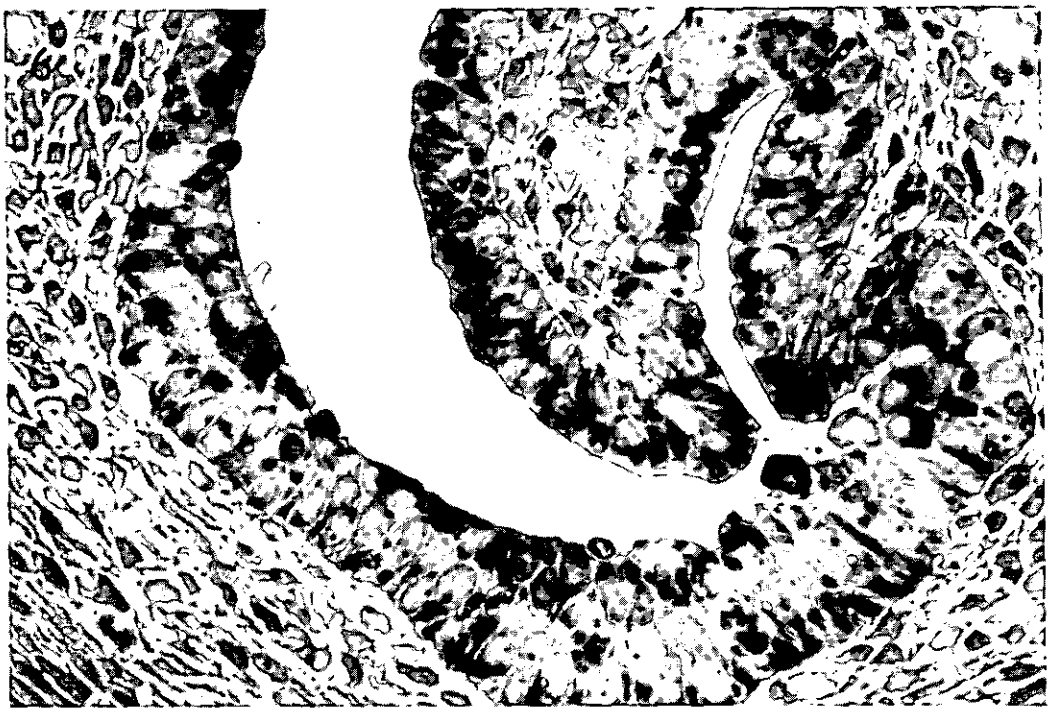

FIGURE 6. Part of a section through the bladder of a 17 week human fetus. The urothelium is composed of two to three layers of roughly cuboidal cells some of which, both in the basal and superficial layers, have darkly staining cytoplasm. Epon-embedded; Toluidine Blue; × 600.

marker for promotion rather than for any other stage of carcinogenesis in the urinary bladder, but since there has been no systematic attempt to correlate dark cells with promotion in this tissue, this conclusion may have to be revised in the light of further work.

Inhibition of Normal Differentiation and the Production of Atypical Phenotypes. In saccharin-induced hyperplasias there is a failure of terminal differentiation but we did not observe any metaplasias in our saccharin-treated rats. By contrast, rat bladder tumors produced by initiation with MNU followed by promotion with saccharin exhibited a wide variety of differentiation patterns both within the tumor and in adjacent, non-neoplastic areas of the urothelium (*6, 79*). This may reflect the ability of saccharin to increase the change of random transcription of the genome.

Adequate supplies of vitamin A have long been known to be essential for the normal differentiation of epithelial tissues including the urothelium. Vitamin A deficiency alone leads first to hyperplasia of the urothelium in which the normal differentiation of the tissue is suppressed, and then to epidermalisation with formation of a stratum granulosum containing keratohyalin granules and a keratinized horny layer (*70, 71*). These same changes were also reported in bladder cancer induced with methylcholanthrene as the initiating carcinogen in vitamin A-deficient animals (*42*). In this situation, squamous metaplasia is not indicative of neoplastic transformation or promotion *per se*, but is the consequence of the direct effect of vitamin A deficiency on gene expression.

In female rats, the addition of testosterone alone has no effect on the differentiation of the urothelium, but if the animals have been pretreated with BBN, testosterone increases the incidence of hyperplasias, cancers and of squamous metaplasias (*41*). Squamous metaplasias were found in a significant number of bladder tumors in 2-AAF-treated mice (*80*). In our MMS-treated animals, the two tumors induced by multiple doses of MMS alone both had areas of squamous metaplasia, but no metaplasias were seen in other animals with hyperplastic urothelia but no tumors. Cyclophosphamide causes first cytotoxic damage and strips the urothelium from the basal lamina, but the subsequent hyperplasias are both undifferentiated and dysplastic. Furthermore, the normal progression from diploid basal, through tetraploid intermediate to octaploid or hyperploid superficial cells which are characteristic of the normal urothelium (*15*), is disrupted by cy-

clophosphamide, and temporarily there is aneuploidy with many hyperpolyploid nuclei (*81*).

Irritation of the bladder mucosa frequently leads to squamous metaplasia of the urothelium. This may be seen following irritation by a calculus or in man from other sources of local irritation such as an indwelling catheter or chronic bacterial cystitis, all of which provide a proliferative stimulus to the urothelium. If the urothelium in addition has previously undergone neoplastic transformation, such local irritation will accelerate the development of urothelial tumors and at the same time predispose to focal areas of squamous metaplasia. An example of this in man is the development of bilharzial bladder cancer, in which squamous cell carcinoma is the predominant type. Interestingly, irritation of the urothelium does not always result in squamous metaplasia but alternatively may produce mucous metaplasia. For example, cystitis cystica with mucous metaplasia developed in our *S.haematobium*-infected baboons, and in those animals receiving additionally an initiating does of BBN, both mucous metaplasias and adenocarcinomas were found. Unlike the response of the human urothelium to *S.haematobium* infection, none of these infected baboons developed squamous cell carcinoma of the urothelium. It seems probable that both mucous and squamous metaplasia are alternative phenotypes to the transitional cell phenotype normally found in the urothelium, which are relatively easily induced and expressed in the rapidly proliferating tissue, and that neither should be regarded automatically as a pre-malignant condition or even as a marker of promotion.

The development of long and/or pleiomorphic microvilli on the urinary face of cells in carcinogen-treated bladders, for some time has been thought to represent the fortuitous expression of a new phenotype which appeared to be a convenient marker for neoplastic transformation in the bladder (*6, 13, 79, 82-84*). More recently, such microvilli have been observed on urothelial cells after treatment with either saccharin alone (*73*) or with cyclophosphamide alone (*85*), which suggested they might be associated with the later stages, rather than the initiation of carcinogenesis in this tissue. However, they have now been observed after treatment of the bladder with formalin, and after wounding the urothelium by surgery or freezing (*86*). All these treatments produced reversible regenerative hyperplasia of the urothelium and the appearance of the microvilli coincided with the time of maximum cell proliferation. Thus, the production of microvilli in a carcinogen-treated bladder, like the induction of ODC in skin, is an epiphenomenon and a marker for cell proliferation rather than a specific marker for neoplastic transformation or promotion of tumor growth.

Conclusions

The evidence is overwhelming that in experimental animal models carcinogenesis in the bladder is a multistage process involving both early initiating and later promoting and/or subsequent events (*10, 34*). As Doll and Peto (*87*) point out, if cells have to experience both early and late events before cancer can develop, then elimination of exposure to either early or later carcinogens will reduce the eventual risk of cancer, and the only determinant of which is the more "important" is which is more easily avoided. The distinction between early and late stage carcinogens, however, is far more than just academic. There is a marked difference between the effect of terminating exposure to a predominantly first-stage carcinogen and the effect of terminating exposure to a promoting or later-stage carcinogen, as is shown both with experimental rat models and human epidemiological data (*9*). Because, in general, first-stage initiating carcinogens are also complete carcinogens, even after exposure is terminated the incidence of tumors continues to increase with time, as evidenced in rats by the increasing risk of liver cancer after terminating exposure to 2-AAF (*24*) and in the human population by the continued rise of 2-naphthylamine-induced bladder cancer long after exposure to this chemical was dicontinued (*88*). By contrast, when exposure to a predominantly later-stage carcinogen is discontinued, the relative risk of developing cancer does not rise further but either decreases or remains the same as when exposure was terminated. Thus in the rat, bladder cancer risk does not rise after stopping exposure to 2-AAF (*25*); similarly, in man, the risk of developing lung cancer decreases within a few years of stopping smoking because cigarette smoke, though it does have some initiating activity, is predominantly a later-stage carcinogen for the lung (*9, 89*). Cigarette smoking also approximately doubles the risk of bladder cancer in man (*87*), but no experimental evidence is available from work with animal models to indicate whether cigarette smoke affects predominantly the first stage or the later stages of the biogenesis of bladder cancer.

With the mouse skin model it has proved possible to separate late stage carcinogens into promoters, which work at the second stage, and still later-stage carcinogens which are capable of advancing the progress of cancer development in cells which have already been subjected to two or more events in the right time sequence. In the bladder, no such rigid

distinction can be made as yet, although available evidence suggests that saccharin and some other compounds may be promoters, whereas local irritation from calculi, or *S.haematobium* ova and some dietary factors such as vitamin A deficiency, may be later stage carcinogens. So far, no unequivocal cellular, subcellular or biochemical marker is available which will unequivocally distinguish the action of a promoter from that of a later stage carcinogen in the urinary bladder. In terms of human cancer prevalence, the distinction between early and late-stage carcinogens is clearly useful because of the immediate effect that removal of late stage carcinogens has on the subsequent relative risk of developing cancer. It is debatable, however, whether emphasis on narrowly defined second-stage promoters to the exclusion of factors affecting the later stages of carcinogenesis, will prove to be equally rewarding. All late-stage carcinogens have the potential to accelerate tumor development and lower the age at which cancer becomes symptomatic. It is thus important to attempt to reduce exposure not just to second-stage, skin-type promoters but to all factors acting at later stages in the carcinogenic process.

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