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Polyploidy in Mammalian Urothelial Cells

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Summary. The mitotic indices and the extent of polyploidy in urothelial cells of baboons, dogs and swine were studied. All three species had very low mitotic activity in vivo but short-term culturing of these cells in vitro stimulated mitosis thus enabling chromsome counts. Tetraploid cells were found in the urothelium of all three species, and higher ploidies also in dog and swine. There were substantial differences in the proportions of diploidy and higher ploidies among the three species and among individuals within each species. Dog urothelial cells were predominantly tetraploid (70%) while more swine cells were diploid (68%). Baboon urothelial cells had only two ploidy classes and 92% were diploid.

Key words: Mitotic index - Polyploidy - Urothelium - Baboon - Dog - Swine.

The transitional epithelium lining the urinary bladder has been found to have a high proportion of polyploid cells in various rodents: Mouse (9, 17, 25), rat (19), and guinea pig (20). Studies of human urothelium yielded conflicting results (10, 18, 24). Diploid and tetraploid cells were most common in rodent urothelium but higher ploidies were also found, especially in the large surface cells. Three of these ploidy determinations (17, 24, 25) were made by chromosome counts; the others were based on measurements of the nuclear size and DNA content of interphase nuclei.

The more direct approach to ploidy determination by chromosome counting has been limited by the very low mitotic activity of bladder epithelium in vivo (6, 7, 9, 17, 19, 21, 24), with very few chromosome spreads found in direct preparations. Chromosome analysis of solid tumours with low mitotic activity presented a similar problem which has been circumvented by short-term culture of the tumour cells in vitro (11, 15, 16). This procedure can now be used with urothelial cells, as a result of recent improvements in methods for isolating these cells and culturing them in vitro (1, 2, 12, 23). As a part of our studies on the aetiology of bladder cancer and the interaction of urothelial cells with carcinogens, we needed additional information on the chromosome complement of potential test cells. Such information would also be of interest in extending knowledge of ploidy characteristics beyond the rodent group. We have used short-term culture and chromosome counting to examine urothelial cell ploidy, and we report the results with animals of three different orders, baboon, dog, and swine.

MATERIALS AND METHODS

Animals

Adult animals of both sexes of unknown age and breeding were used. Bladders were obtained from baboons, Papio hamadryas, and dogs, Canis familiaris, through the cooperation of investigators in thoracic surgery. Swine, Sus scrofa, bladders were obtained from a local abbatoir.

Preparation of Cell Culture

Dogs were killed by exsanguination under pentobarbital anaesthesia, baboons by pentobarbital overdose, and swine by a captive bolt. Bladders were removed as soon as possible after death of the animals. Urothelial cells were placed in short-term culture as described (2). Briefly,

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the mucosal layer was stripped off and treated with 0.25% trypsin and 0.02% EDTA overnight at 2°C. After an additional hour at 37°, the suspension was stirred 10 minutes at room temperature. The dispersed cells were washed once by centrifugation and plated in plastic dishes or flasks in RPMI medium 1640 supplemented with 8% tryptose phosphate broth and with 20% calf serum for dog and baboon cells, or with 20% swine serum for the swine cells. Cultures were incubated at 37° in a humidified atmosphere of 5% CO_2 in air and were monitored by phase-contrast microscopy. Separate cultures were prepared from the urothelium of 8 dogs, from 8 swine, and from 4 baboons. For comparison, cell cultures were prepared also from the prostate of one baboon, the kidney cortex of one dog, and the skin from a second dog.

Chromosome Study

As soon as cell divisions became frequent, colchicine was added to the culture medium at a final concentration of 0.05 μg/ml. After 2-3 hours at 37°C the cells were harvested with trypsin, given a hypotonic treatment with culture medium diluted with three volumes of water, and then fixed in methanol-acetic acid (3:1). The cell suspension was dropped onto ice-chilled slides, air dried, and stained with Giemsa.

For ploidy determination, chromosome counts were made at 1000 x magnification, from all intact countable metaphase spreads as they were encountered. An effort was made to score at least 200 spreads per animal.

Mitotic Activity

For determination of the mitotic activity in vivo without mitotic arrest, the bladder mucosa was stripped off as usual and stirred in 0.25% trypsin, 0.02% EDTA at room temperature for three successive periods of 20 minutes each. At the end of each stirring, the tissue fragments were allowed to settle, and the cell suspension was removed to a tube containing growth medium and replaced with fresh enzyme solution. The urothelial cells were collected by centrifugation and fixed in methanol-acetic acid (3:1) without hypotonic treatment. The cells were spread by dropping the suspension on slides, air-dried and stained with Giemsa. The cells were counted under 200 x magnification. The mitotic activity is the number of dividing cells expressed as a percentage of the total cells.

RESULTS

The mitotic activities are shown in Table 1. There was some variation among animals but activity was very low in all three species. None of these animals had any apparent bladder infection or tumour. The short-term culturing of urothelial cells greatly increased their mitotic activity (Tables 2-4), thus making possible the chromosome study. For dog and baboon cells, 3-5 days of culture was sufficient. Cells from two dogs were studied after first passage. The swine urothelial cells were slower to attach to the culture dishes and active division was seen in one to two weeks.

Table 1. Mitotic activity in epithelial cells of the normal urinary bladder of three mammalian species

Species	Animal	Number of cells scored	Number of mitoses observed	Mitoses (per cent of total cells)
Dog	1	40,000	0	<0.002
	2	57,450	12	0.02
	3	80,000	0	<0.001
	4	57,646	15	0.03
	Total	235,096	27	0.011
Swine	1	66,786	6	0.009
	2	83,991	3	0.004
	3	72,015	6	0.008
	4	101,140	34	0.034
	5	72,724	9	0.01
	Total	396,656	58	0.015
Baboon	1	68,405	5	0.007

Typical chromosome spreads are shown in Figures 1-3, and the distribution of chromsome numbers of the urothelial cells from individual animals in Figures 4-6. The chromosome numbers cluster around the diploid, tetraploid and octoploid values. The deviations from the exact numbers may be true cell properties or may be counting errors or artifacts due to chromosome loss or gain during the slide preparation, or both. The cells around each ploidy class were grouped together to estimate the proportion of cells in each class (Tables 2-4) Diploid and

Table 2. Distribution of ploidy classes in dog urinary bladder epithelial cells in short term cultures

	Culture (Animal)	Culture stage	Days in culture	Number of cells	Percent of cells 2n	3n	4n	6n	8n
1.	2176	1st passage	7	153	22.9	-	72.5	-	4.6
2.	3276	Primary	3	162	22.2	5.2	71.6	-	0.6
3.	3476	Primary	3	167	13.2	3.5	83.2	-	-
4.	7777	Primary	3	207	91.3	-	8.7	-	-
5.	7877	Primary	3	187	3.0	0.5	96.0	0.5	-
6.	8377	1st passage	6	239	5.0	-	87.0	-	8.0
7.	8677	Primary	3	208	13.5	-	84.6	-	1.9
8.	9077	Primary	4	262	29.0	1.0	68.0	0.4	1.5
Mean:					25.0	1.2	71.0	0.1	2.3

Table 3. Distribution of ploidy classes in swine urinary bladder epithelial cells in short term cultures

	Culture[a] (Animals)	Days in culture	Number of cells	Percent of total cells 2n	3n	4n	6n	8n	16n
1.	2177	4	212	85.8	-	12.7	1.0	0.5	-
2.	3977	8	257	76.3	0.4	22.9	0.4	-	-
3.	4177	15	248	85.5	0.4	13.3	-	0.8	-
4.	4277	15	286	33.9	0.4	62.9	0.4	2.4	-
5.	7377	7	252	64.0	0.4	31.0	-	4.0	-
6.	7677	7	257	52.0	0.4	46.0	-	-	-
7.	9477	7	262	72.9	-	25.2	-	1.2	0.4
8.	9577	7	267	70.0	0.4	28.0	0.4	0.8	0.4
Mean:				67.6	0.3	30.2	0.3	1.5	0.1

[a] All cultures were primary

Table 4. Distribution of ploidy classes in baboon urinary bladder epithelial cells in short term cultures

	Culture[a] (Animals)	Days in culture	Number of cells	Percent of cells 2n	4n
1.	5876	4	194	90.5	9.5
2.	577	3	181	91.2	8.8
3.	877	5	180	90.0	10.0
4.	2477	4	174	97.6	2.4
Mean:				92.3	7.7

[a] All cultures were primary

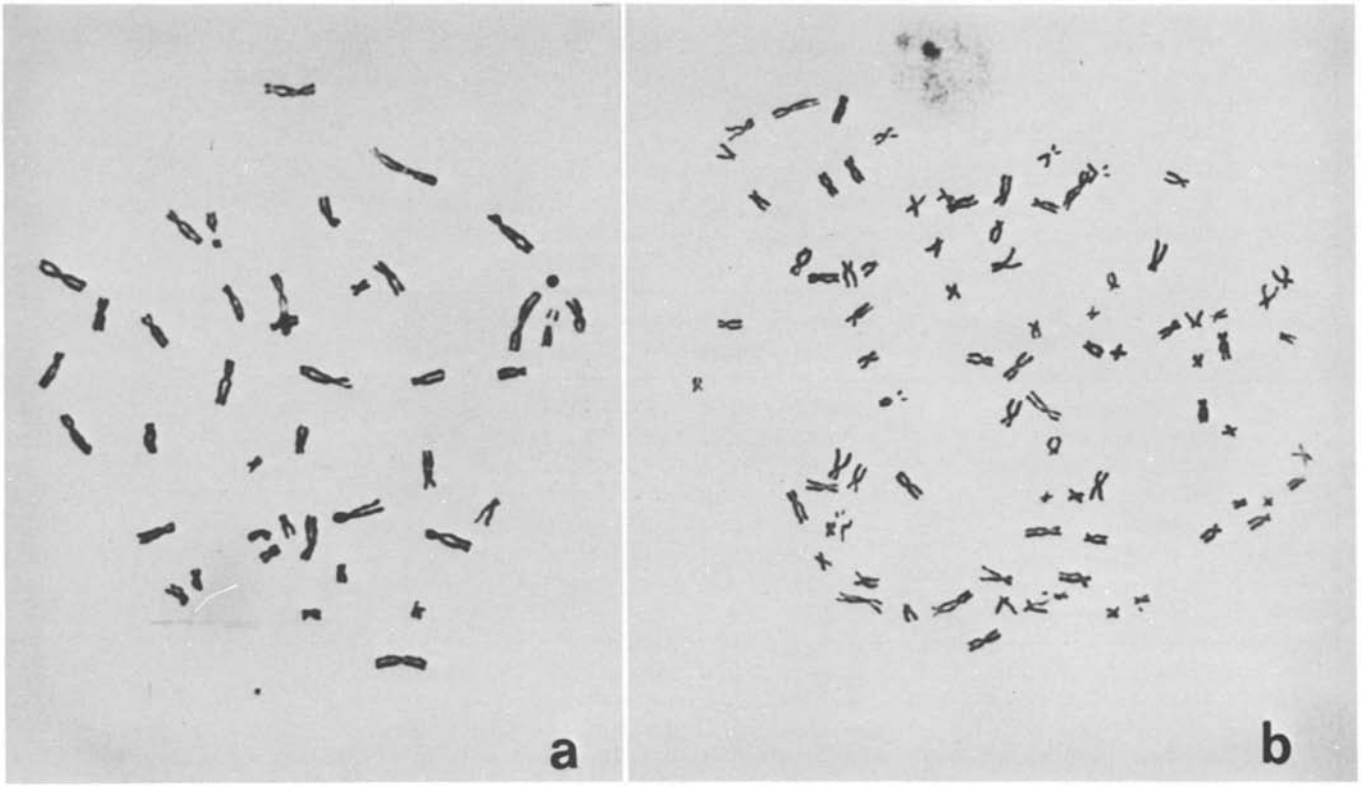

Fig. 1 a and b. Representative metaphase figures of various ploidy classes in baboon urothelial cells. a Diploid (2n = 42) X580; b Tetraploid (4n = 84) X530

tetraploid cells predominate in all three species. Despite substantial individual variation, there is a characteristic pattern for each species. In the dog, tetraploid cells were the principal class in 7 of 8 animals studied. Five of the 8 animals studied had only about 20% diploid cells, while 2 had even lower values of 3-5% and 1 a relatively high 91%. The number of tetraploid cells varied inversely with the diploid. Some animals had a small proportion of octaploid cells and there were a few cells with 3n and 6n numbers. In contrast, the cells cultured from canine kidney cortex were 97.2% diploid and 2.8% tetraploid. Skin cells cultured from a second dog were 98.4% diploid, 1.6% tetraploid.

The ploidy of swine urothelial cells also varied from animal to animal but, unlike the dog, the swine cells were predominantly diploid. Only one animal had fewer than 50% diploid urothelial cells. Most of the remaining cells were tetraploid, although a small proportion (0.5 - 4.9%) was octoploid. There were a few triploid and hexaploid counts and two animals had a few cells with 16n chromosome number.

Unlike the other two species, baboon urothelial cells were of only two ploidy classes, i.e., diploid and tetraploid. Diploid cells were the predominant type, with a mean value of 92%. In the 4 animals studied, the number of tetraploid cells ranged from only 2.4% to a maximum of 10%. Epithelial cells cultured from a baboon prostate were 97.6% diploid, 2.4% tetraploid.

DISCUSSION

The mitotic indexes of baboon, dog, and swine urothelium were all very low, the mean values for each species being about 0.01% (Table 1). These findings are consistent with observations in other mammals. The urinary bladder epithelium was found to be a tissue of slow turnover, as indicated by its low rate of ^{3}H-thymidine labelling and its paucity of mitoses, in all the species in which it was studied: Rat (19), mouse (6, 9, 17), guinea pig (21), and human (24). Cooper (17) has given the figure 0.01% as being generally characteristic of the mitotic index of mammalian urothelial cells, and has estimated that some of them have a life span of more than 200 days. However, the urothelium has a remarkable capacity to respond to injury with greatly increased mitotic activity. The response may be local, such as in the immediate vicinity of a wound (13), or widespread, such as that following cell death caused by a toxic substance in the

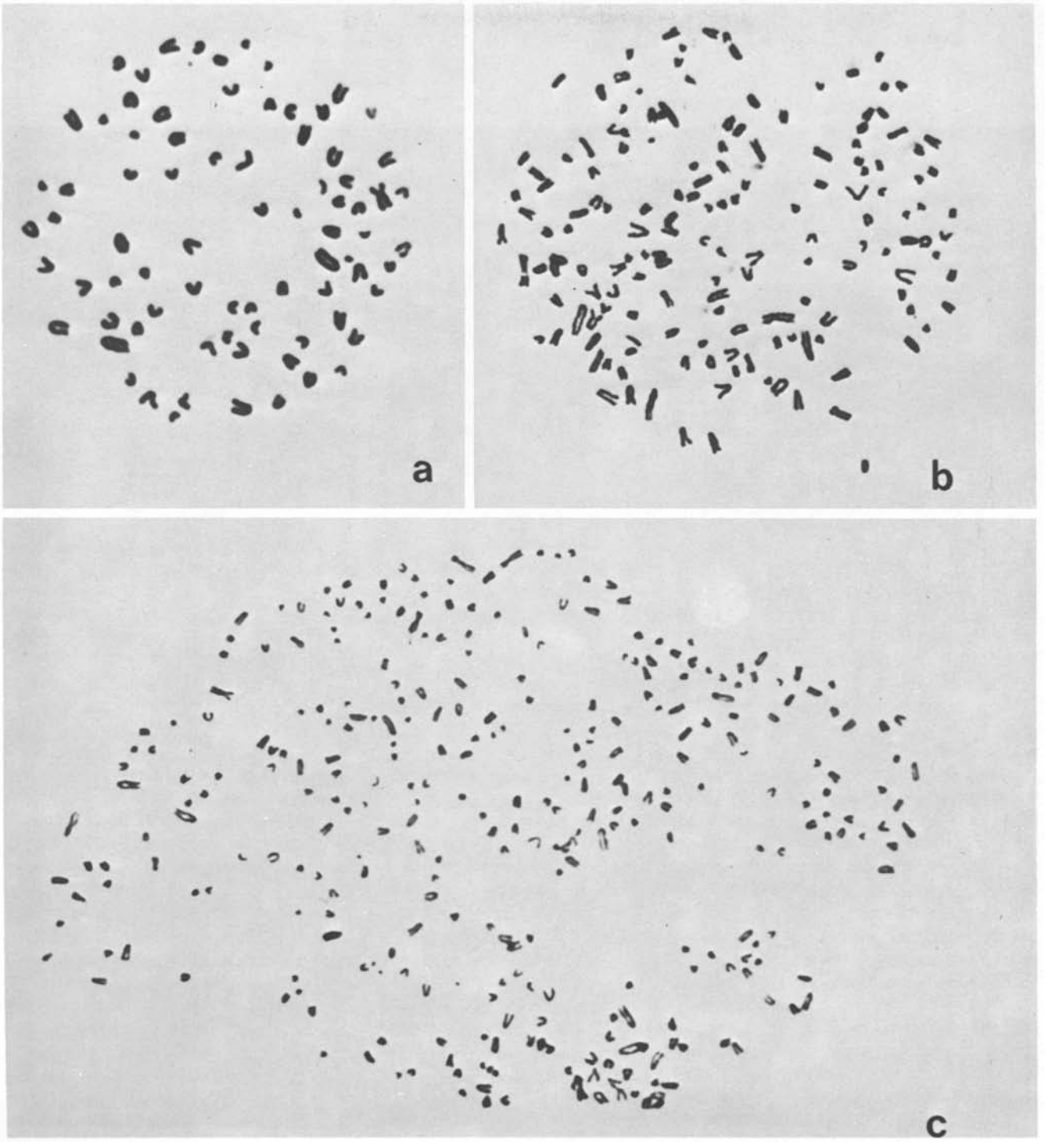

Fig. 2 a-c. Representative metaphase figures of various ploidy classes in dog urothelial cells. a Diploid (2n = 78) X700; b Tetraploid (4n = 156) X670; c Octoploid (8n = 312) X320

urine (17, 19). The vigorous mitotic activity which we observed in cultured urothelial cells is probably a manifestation of this same cellular ability. The mitotic rate of dog bladder cells increased from about 0.01% in vivo to about 1% after 2 to 3 days in culture (2), a 100-fold increase. Similar increases were found with swine and baboon cells in the present study.

By taking advantage of this burst of mitotic activity in short-term culture, we were able to obtain an ample number of dividing cells for the determination of ploidy by chromosome counts.

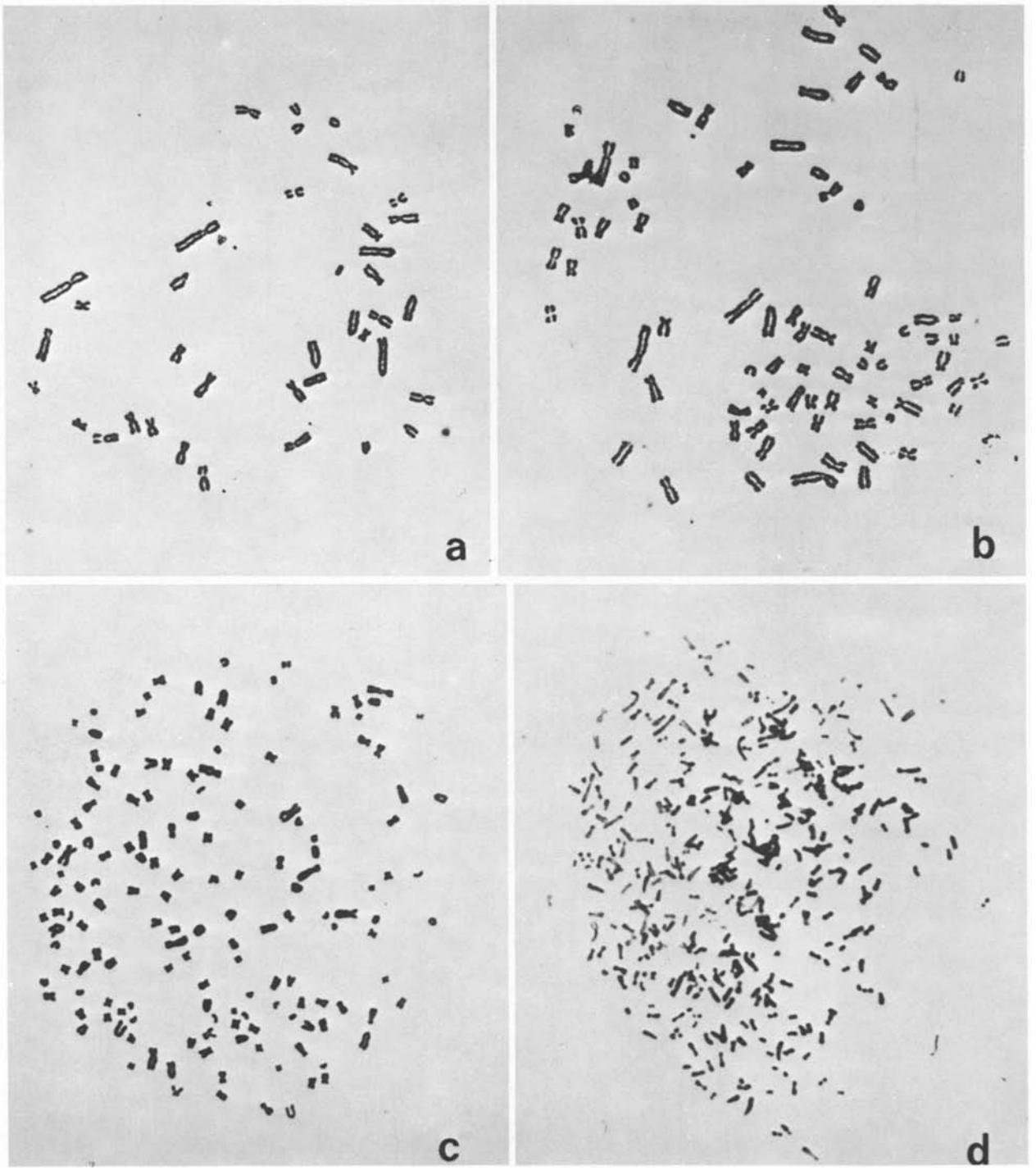

Fig. 3 a-d. Representative metaphase figures of various ploidy classes in swine urothelial cells. a Diploid (2n = 38) X520; b Tetraploid (4n = 76) X490; c Octoploid (8n = 152) X370; d 16-ploid (16n = 304) X168

Short-term culture has been accepted as an appropriate procedure in the cytogenetic study of cells of tissues in which mitoses are scarce. Fetal cells obtained by amniocentesis (22) and fibroblasts from skin (11) are each grown in culture for as long as several weeks before chromosome analysis. In the case of tumour cells, or other karyotypically heterogeneous populations, grown in vitro, the possibility of selection for particular types of cells must be considered. Kotler and Lubs (15) and Levan (16) compared the direct-preparation and short-term-culture techniques for karyotyping solid tumours. The results were in agreement, and they con-

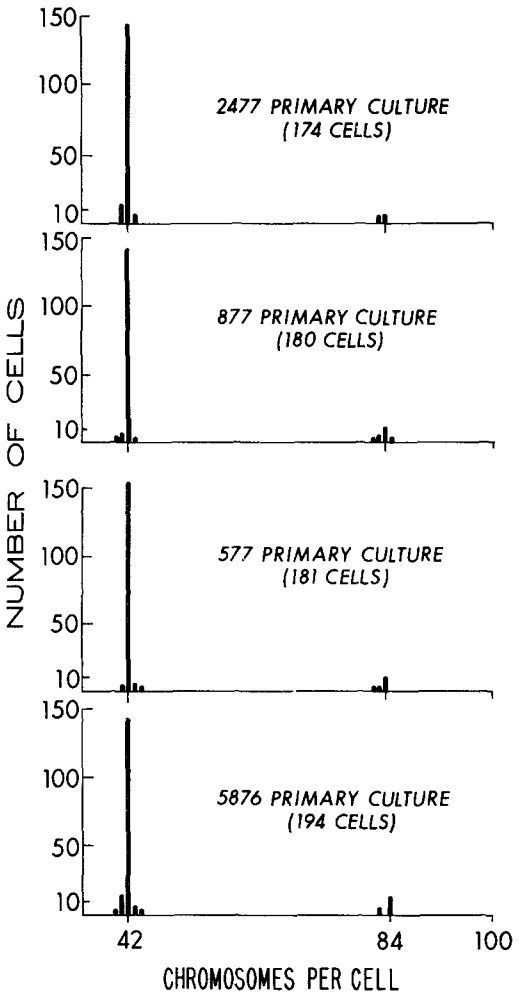

Fig. 4. Distribution of chromosome numbers per cell in primary baboon bladder epithelial cell cultures from each of 4 animals (2n = 42)

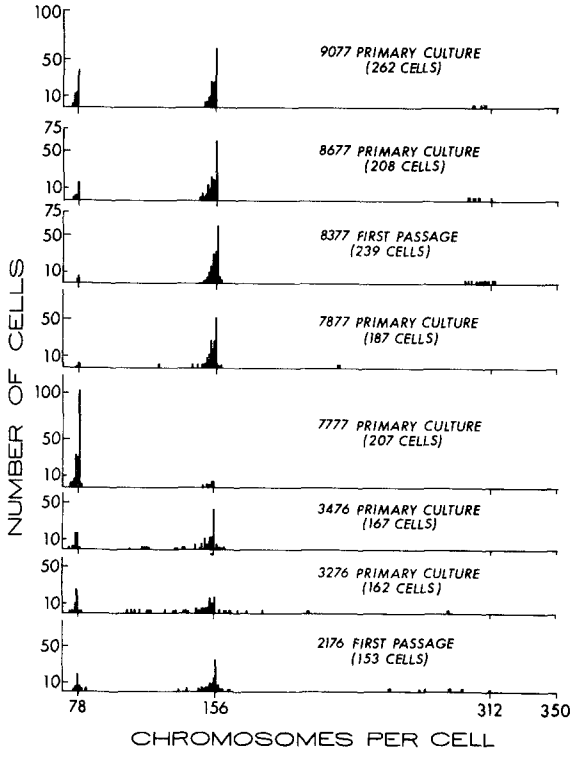

Fig. 5. Distribution of chromosome numbers per cell in dog bladder epithelial cell cultures from each of 8 animals (2n = 78)

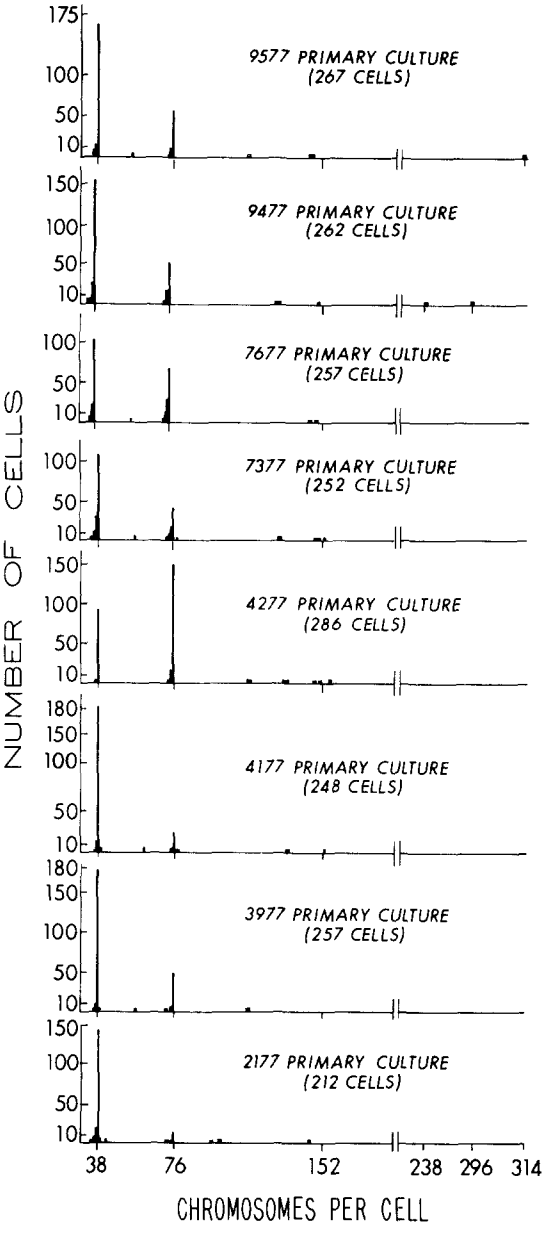

Fig. 6. Distribution of chromosome numbers per cell in primary swine bladder epithelial cell cultures from each of 8 animals (2n = 38)

cluded that short-term culture was a satisfactory and useful procedure. Our results with cells from other tissues of dogs and baboon, grown under the same culture conditions as those used for urothelial cells, showed the expected high proportion of diploidy, 97.2 to 98.4%. These data indicate that the polyploid cells are characteristic of urothelium in vivo and are not induced by the short term culture conditions. The proportions of cells in the various ploidy classes in the dog urothelial cell culture # 2176 (Table 2) were determined in passages 1, 8, 9, and 12, and they remained stable (unpublished results). The occurrence of mitoses in all ploidy classes (even 16n) argues against selection pressure for particular ploidies. We conclude that the ploidy distributions in cells in short-term culture are representative of the cells in vivo.

There were striking variations in urothelial cell ploidy, both among species and among individuals. Despite the individual variation, the differences among the three species were significant as judged by the t-test (baboon vs. dog, $p < 0.001$; baboon vs. swine, $p < 0.02$; and dog vs. swine, $p < 0.005$). The high proportion of tetraploid cells in dog bladder epithelium (75%) resembles that reported in rodents (9, 17, 19, 21, 25). In contrast, the baboon urothelium had only 8% tetraploid cells. Swine urothelium was intermediate between baboon and dog, having about 30% tetraploidy. The liver, another polyploid tissue, also shows different proportions of tetraploid cells in different species (4). Two studies of ploidy levels in cells from normal human urothelium, utilizing Feulgen microdensitometry of interphase nuclei, gave conflicting results. In one (18) a mean of 30% polyploid cells was found in an unspecified number of individuals, while another study (10) of 14 persons reported a mean of 3% tetraploidy. In a study (24) employing chromosome counting, more than 90% of the cells in normal human bladder epithelium were found to be diploid, and no polyploidy was seen. The absence of polyploidy was indicated also by the failure to detect two or more Y-bodies in any interphase nuclei after quinacrine staining. Microdensitometry may overestimate polyploidy somewhat, depending on the proportion of cells in the G2 and S phases of the cell cycle; while the very brief incubation in vitro in the last study (24) might not allow polyploid cells to divide, and thereby underestimate their number. However, these reports and the lack of double Y-bodies (24) suggest that the proportion of polyploid cells in human urothelium is low, a conclusion consistent with our findings in the baboon, another primate.

The individual variation in proportion of diploid cells was great, particularly in dog (3-91%) and in swine (34-86%). Considerable variation in ploidy proportions was found also in mouse urothelial cells (9). The presence of a few aneuploid, triploid, and hexaploid cells in the dog and swine urothelium was rather surprising, but aneuploidy and triploidy were seen also in cells of rapidly growing livers of rats after partial hepatectomy (14). There were indications of some hexaploidy in mouse urothelial cells (9).

Work with rodent urothelium indicated that ploidy distributions are relatively stable in adult animals. The ploidy of urothelial cells in mouse bladder mucosa is largely established during fetal life (25) although Farsund (9) observed some postnatal changes, with a decrease in the relative number of diploid cells and an increase in tetraploid cells. He observed no major changes in the proportions of different ploidy classes after eight weeks of age.

The role of polyploidy in urothelial cells, and the reasons for individual variation, are unknown. Levi et al. (17) suggested that polyploidy may have evolved with the requirements for large size and mechanical stability of the urothelial cells, especially those at the luminal surface, for a long life span, and for the prolonged maintenance of an effective transport barrier. Brodsky and Uryvaeva (3) have speculated that polyploid cells in liver are functionally more efficient than diploid cells, while Evans (8) suggested that the liver polyploid cells may have a selective advantage due to a greater resistance to mutagens and carcinogens. A greater resistance to chemical induction of recessive mutants has been reported for tetraploid as compared to diploid Chinese hamster cells (5). If such enhanced resistance of polyploid cells is a general phenomenon, it might be especially important not only in liver, where some procarcinogens are metabolically activated, but also in the bladder epithelial cells exposed to excreted mutagens and carcinogens.

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REFERENCES

1. Berky, J. J., Zolotor, L.: Development and characterization of cell lines of normal and mouse bladder epithelial cells and 2-acetyl-

aminofluorene-induced urothelial carcinoma cells grown in monolayer tissue culture. In Vitro 13, 63 (1977)
2. Bonar, R.A., Reich, C.F., Sharief, Y.: Canine urinary bladder epithelial cells: Preparation for cell culture by enzyme dispersion. Urological Research 5, 87 (1977)
3. Brodsky, W.Y., Uryvaeva, I.V.: Cell ploidy: Its relation to tissue growth and function. International Review of Cytology 50, 275 (1977)
4. Carriere, R.: The growth of liver parenchymal nuclei and its endocrine regulation. Internation Review of Cytology 25, 201 (1969)
5. Chasin, L.A.: The effect of ploidy on chemical mutagenesis in cultured chinese hamster cells. Journal of Cell Physiology 82, 299 (1973)
6. Clayson, D.B., Cooper, E.H.: The immediate response of bladder epithelium to injury by chemical carcinogens. British Journal of Urology 41, 710 (1969)
7. Cooper, E.H.: The biology of bladder cancer. Annals of the Royal College of Surgeons of England 51, 1 (1972)
8. Evans, I.H.: Polyploidization in rat liver: The role of binucleate cells. Cytobios 16, 115 (1976)
9. Farsund, T.: Cell kinetics of mouse urinary epithelium: I. Circadian and age variations in cell proliferation and nuclear DNA content. Virchows Archiv B Cell Pathology 18, 35 (1975)
10. Fossa, S.D.: Feulgen-DNA-values in transitional cell carcinoma of the human urinary bladder. Beiträge zur Pathologie 155, 44 (1975)
11. Harnden, D.G.: Skin culture and solid tumor technique. In: Human Chromosome Methodology, J.J. Yunis (ed.), p. 167. Academic Press, New York, London 1974
12. Hashimoto, Y., Kitagawa, H.S.: In vitro neoplastic transformation of epithelial cells of rat urinary bladder by nitrosamines. Nature 252, 497 (1974)
13. Hepperlen, T.W., Stinson, W., Hutson, J., Bartone, F.F.: Epithelialization after cystotomy. Investigative Urology 12, 269 (1975)
14. Hsu, T.C., Pomerat, C.M.: Mammalian chromosomes in vitro: III. On somatic aneuploidy. Journal of Morphology 93, 301 (1953)
15. Kotler, S., Lubs, H.A.: Comparison of direct and short-term tissue culture technics in determining solid tumour karyotypes. Cancer Research 27, 1861 (1967)
16. Levan, G.: The detailed chromosome constitution of Benzpyrene-induced rat sarcoma. A tentative model for G-band analysis in solid tumours. Herditas 78, 273 (1969)
17. Levi, P.E., Cowan, D.M., Cooper, E.H.: Induction of cell proliferation in the mouse bladder by 4-Ethylsulphonylnaphthalene-1-sulphonamide. Cell Tissue Kinetics 2, 249 (1969)
18. Levi, P.E., Cooper, E.H., Anderson, C.K., Williams, R.E.: Analysis of DNA content, nuclear size, and cell proliferation of transitional cell carcinoma in man. Cancer 23, 1074 (1969)
19. Locher, G.W., Cooper, E.H.: Repair of rat urinary bladder epithelium following injury by cylophosphamide. Investigative Urology 8, 116 (1970)
20. Martin, B.F.: The effect of distension of the urinary bladder on the lining epithelium and on its histochemical reaction for alkaline phosphatase. Annales d'Histochimie 7, 51 (1962)
21. Martin, B.F.: Cell replacement and differentiation in transitional epithelium: a histological and autoradiographic study of the guinea pig bladder and ureter. Journal of Anatomy 112, 433 (1972)
22. Nadler, H.L., Ryan, C.A.: Amniotic cell culture. In: Human Chromosome Methodology, J.J. Yunis (ed.) p. 185. Academic Press, New York, London 1974
23. Roszell, J.A., Douglas, C.J., Irving, C.C.: Polyamine-stimulated growth of cultured rat urinary bladder epithelial cells. Cancer Research 37, 239 (1977)
24. Sandberg, A.A.: Chromosome markers and progression in bladder cancer. Cancer Research 37, 2950 (1977)
25. Walker, B.E.: Polyploidy and differentiation in the transitional epithelium of mouse urinary bladder. Chromosoma 9, 105 (1958)

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