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Canine Urinary Bladder Epithelial Cells: Preparation for Cell Culture by Enzyme Dispersion

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Summary. In a qualitative and quantitative study of enzymic dispersion of cells from the mucosal layer stripped from canine urinary bladder, trypsin was found to be equal or superior to the other enzymes tested for dispersal of urothelial cells specifically. Collagenase or collagenase plus trypsin served to disperse the whole tissue. A procedure for recovering the urothelial cells as a single-cell suspension and establishing them in culture is presented. The morphology, culture behaviour, and chromosome complement of these cells is described.

Key words: Urinary bladder - Mucosa - Enzymic dispersion - Urothelial cells - Tissue culture.

Methods for growing bladder cells, particularly urothelial cells, in vitro have potential value for a variety of studies, as has long been recognized (3). There have been several reports of the successful culture of normal urothelial cells using explant techniques (1, 2, 6, 7, 15, 16, 17). In another study, instillation of trypsin solution into the rat bladder was used to prepare urothelial cells for culture (9). Reliable, convenient procedures for preparation of single-cell suspensions from bladder tissue would allow quantitation and replicate plating, and would also provide an additional approach to selecting from among the several cell types which form the bladder wall. With these ends in mind, we have compared the application of several enzymes to dispersal of cells from bladder mucosa. Our particular interest was in the cells of the transitional epithelium, and the selection and characterization of these cells is emphasised in this report. We have described some aspects of the morphology of these cells in culture (18).

MATERIALS AND METHODS

Bladders were removed aseptically, 5 to 60 min after death, from adult dogs of unknown age or breeding, which had been killed by exsanguination under sodium pentobarbital anaesthesia. The bladder was cut open and laid flat. The mucosal layer was stripped from the underlying muscle with forceps and cut into pieces of less than one square centimeter. Several pieces were placed in each of several tared sterile vials which were closed and weighed quickly. In some cases, pieces of the mucosa were fixed in neutral buffered formalin for histological examination.

Some of the weighed fragments in each experiment were treated with 0.25% trypsin (Difco 1:250, Difco Laboratories, Inc., Detroit, Mich.) and 0.02% ethylenediaminetetraacetic acid (EDTA) in a diluent containing 0.137 M NaCl, 0.00268 M KCl, 0.0081 M Na_2HPO_4, and 0.00147 M KH_2PO_4.
Other enzymes were used to treat fragments as follows: 0.1% crystalline trypsin (Worthington Biochemical Corp., Freehold, N.J.); 0.1% collagenase, types I, II, III and IV (Worthington); 0.05% bacterial elastase (Calbiochem, La Jolla, Cal.); 0.05% hog-pancreas elastase (Sigma Chemical Co , St. Louis, Mo.); and 0.1% Pronase (Calbiochem). These were dissolved in phosphate-buffered saline (PBS) containing 0.137 M NaCl, 0.00268 M KCl, 0.00646 M Na_2HPO_4, 0.00147 M KH_2PO_4, 0.00090 M $CaCl_2$, and 0.00049 M $MgCl_2$.

In the early experiments, two different schedules of enzyme treatment were compared. In one, the tissue was stirred in the enzyme solution at room temperature for three successive periods of 20 minutes each. At the end of each interval, 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 cells were recovered from the combined fluids by centrifugation at 1500 rpm for five minutes. In the other schedule, which was adopted as standard, the tissue fragments were kept in the enzyme solution overnight at 2° C. The suspension was then brought to 37° C for one hour, and then either stirred gently for 10 minutes, or pumped with a pipette to suspend the cells. The residual tissue was allowed to settle, and the cell suspension was removed. The residue was washed with growth medium which was added to the cell suspension, and the cells were recovered by centrifugation as above.

The cells were then suspended in growth medium (RPMI 1640 with 20% calf serum (v/v), 8% tryptose phosphate broth (v/v), and 50 µg Gentamicin/ml). A sample was diluted with 0.04% trypan blue solution and counted in a Cytograph (Bio/Physics Systems, Inc., Mahopac, N. Y.) to determine cell number and proportion dead. Cells were plated at densities of 1 to 2 x 10^4 live cells/sq. cm in plastic Petri dishes or flasks. Cover slips were placed in some dishes before adding the cell suspension. Medium was changed when it became acid, or at least once per week. The living cultures were observed by phase contrast microscopy, both directly and with time-lapse cinematography. Cells grown on cover slips were fixed in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, and stained with May-Gruenwald-Giemsa.

When the cell layers became confluent, they were dispersed with 0.05% trypsin in the EDTA-containing trypsin diluent, at 37° C. The cultures were monitored by phase-contrast microscopy to determine the length of time required for the trypsin to loosen all of the cells from the dish. The cells were recovered by centrifugation, suspended in growth medium, counted, and plated in new dishes at concentrations of 1 to 5 x 10^4 live cells per sq. cm.

For chromosome studies, cells in culture in growth medium were treated with colchicine at a final concentration of 0.5 µg/ml for two to three hours. After harvesting with trypsin, the cells were treated with hypotonic culture medium (growth medium diluted with three volumes of water), fixed in methanol-acetic acid (3 : 1), air-dried, and stained with Giemsa (10).

RESULTS

The mechanical stripping of the mucosal layer from the underlying muscle was quite reproducible. Separation occurred at the interface between the tunica propria and the muscle. The thin, almost transparent, membrane which was stripped off consisted chiefly of a fibrous layer with embedded cells and was lined with transitional epithelium (Fig. 1). Small blood vessels occurred in the fibrous layer and small pieces of muscle were sometimes attached.

Histological examination of the residual tissue after enzyme treatment showed that trypsin, elastase, or Pronase caused dispersion of the epithelial cells while leaving the fibrous layer largely intact, as illustrated by the section of tissue remaining after trypsin treatment in Figure 2. When muscle cells were present, they were also dispersed by these enzymes. The endothelial cells lining the blood vessels were partially separated. The collagenases, on the other hand, dispersed the whole tissue, although separation of the urothelial cells was incomplete and usually yielded clumps of cells. Fig. 3 illustrates the results of a short treatment with collagenase, type I; with the urothelial layer coming off in sheets or clumps, and the underlying fibrous tissue starting to disintegrate.

The cell types found in culture corresponded to those dispersed by the enzymes, as judged by tissue examination. Trypsin and elastase yielded cultures which were almost entirely epithelial (Fig. 4-6), while cultures prepared with collagenase contained both epithelial and fibroblastic cells (Fig. 7). Most of the cells which attached after Pronase dispersion were epithelial.

Within each of these two groups of enzymes, which differed qualitatively in the kinds of cells released, there were quantitative differences as well. The values for numbers of live cells released per mg of tissue (wet weight) are given in the left portion of Table 1. Because of the substantial differences in cell yield from one animal to another, the results for each enzyme were also compared to the yield obtained using trypsin with tissue from the same bladder. These values relative to the standard trypsin treatment are given in the right portion of Table 1, and provide some normalization of the non-enzymic factors among the different animals.

Crystalline trypsin and hog-pancreas elastase were similar to crude trypsin in cell yield, while bacterial elastase was less effective. Pronase treatment produced more cells, but fewer of them attached to the culture dish. The proportion of trypsin-dispersed viable cells

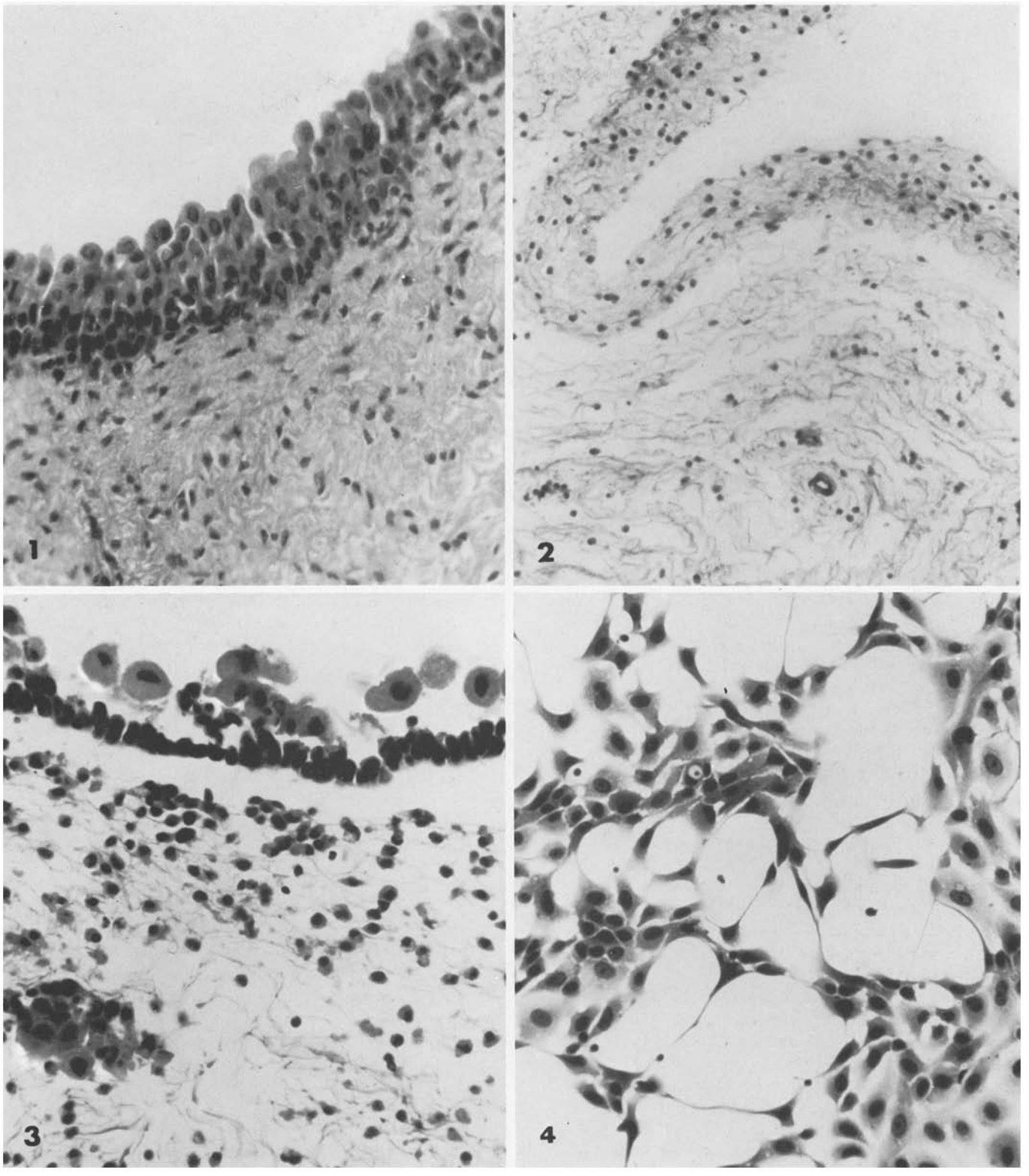

Fig. 1. Section of mucosal layer stripped from canine bladder. No enzyme treatment. The urothelial layer is distinct from the fibrous matrix with its scattered fibroblastic cells. H & E, X 420

Fig. 2. Section of tissue like that of Figure 1, but which was prepared after treatment with trypsin as described in the text. The urothelial cells are gone. The fibrous matrix is loosened, but not dispersed. H & E, X 420

Fig. 3. Section of tissue like that of Figure 1, but prepared after a short treatment with collagenase (60 minutes, 23° C). The urothelial layer has separated but not dispersed, and the fibrous matrix is partially digested. H & E, X 420

Fig. 4. Cells released from canine bladder mucosa by trypsin treatment, and maintained in culture for 1 day. Note the loose aggregation of the cells into monolayer islands, and the long processes. May-Gruenwald-Giemsa, X 220

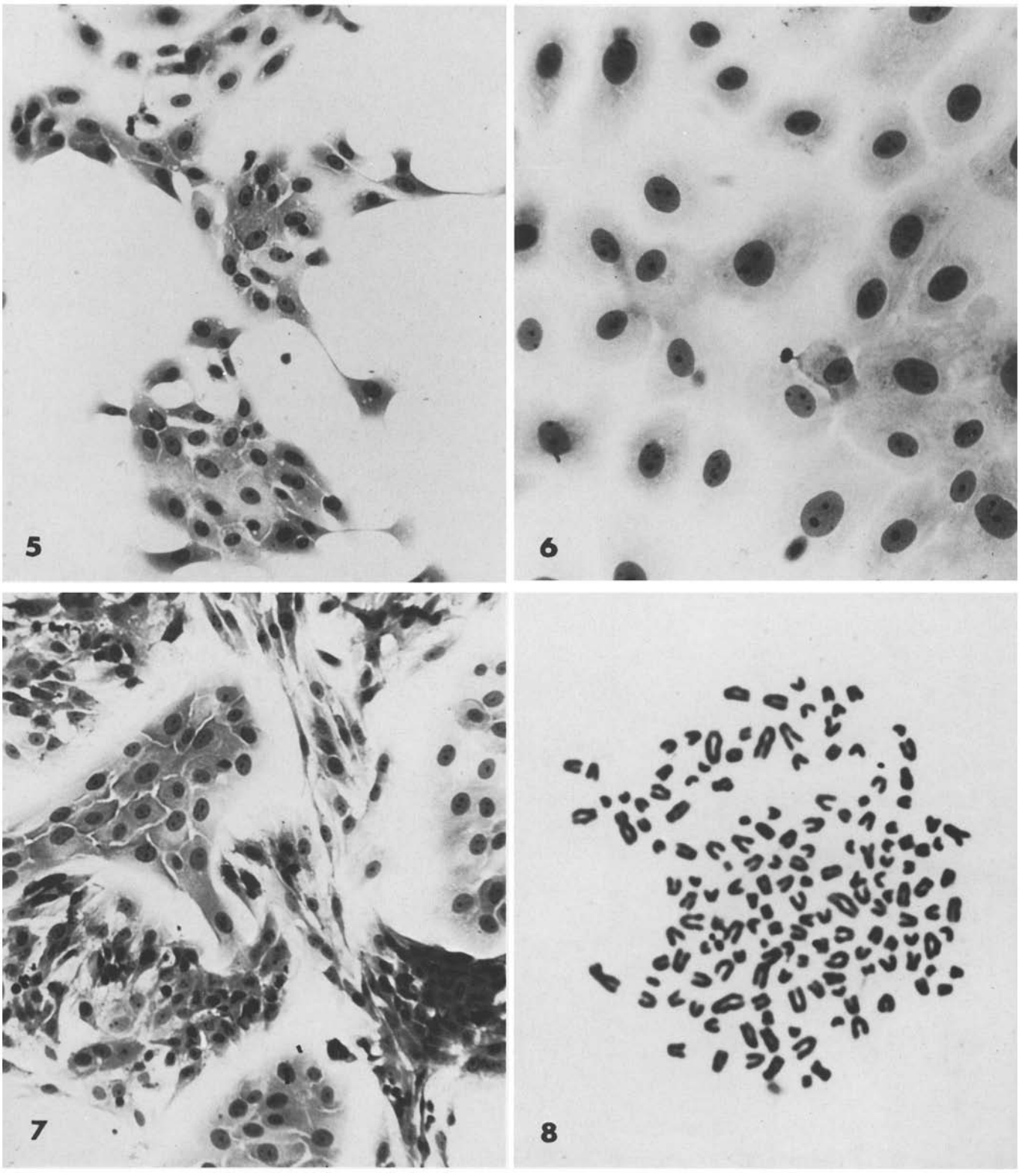

Fig. 5. Cells from the same culture as in Figure 4, after two days in vitro. Note the closer association of the cells. May-Gruenwald-Giemsa, X 220

Fig. 6. Cells from another culture like that of Figure 5, also after two days in vitro. These cells have spread more rapidly and have already formed a confluent monolayer of thinly spread cells. May-Gruenwald-Giemsa, X 430

Fig. 7. Cells released from canine bladder mucosa with collagenase, type I; and maintained in culture for two days. Note the presence of fibroblastic as well as epithelial cells. May-Gruenwald-Giemsa, X 220

Fig. 8. Chromosomes in a metaphase spread of a near-tetraploid urothelial cell from a culture six days old (including one passage). X 1200

Table 1. Enzymatic dispersion of cells from dog bladder mucosa

Enzyme	n[a]	Live cells per mg[b] ($\times 10^{-6}$)		Cell yields relative to those obtained with trypsin in the same experiment (percent)			
		Mean	Std. dev.	Mean	Std. dev.	Std. dev. of mean	Significance[c]
Trypsin, crude	22	4.4	2.8	(100.)			
Trypsin, cryst.	4	5.6	5.6	109.	43.	21.	0.5
Elastase, hog pancreas	9	4.1	1.8	101.	26.	9.	0.5
Elastase, bacterial	5	2.6	2.5	58.	26.	12.	0.001
Collagenase type I	14	3.3	3.3	75.	72.	19.	0.2
Collagenase type II	6	8.7	4.9	136.	62.	25.	0.05
Collagenase type III	8	5.0	4.0	69.	36.	13.	0.01
Collagenase type IV	6	6.2	3.4	82.	22.	9.	0.05
Pronase	8	8.2	5.2	150.	54.	19.	0.001
PBS with EDTA	2	0.45		50.			
PBS	4	2.0	2.1	38.	21.	11.	0.001

[a] Number of trials [b] Live (trypan blue exclusion) single cells recovered per mg wet weight of tissue

[c] Probability that the difference in yield from that with trypsin is due to chance, as determined with the t-test

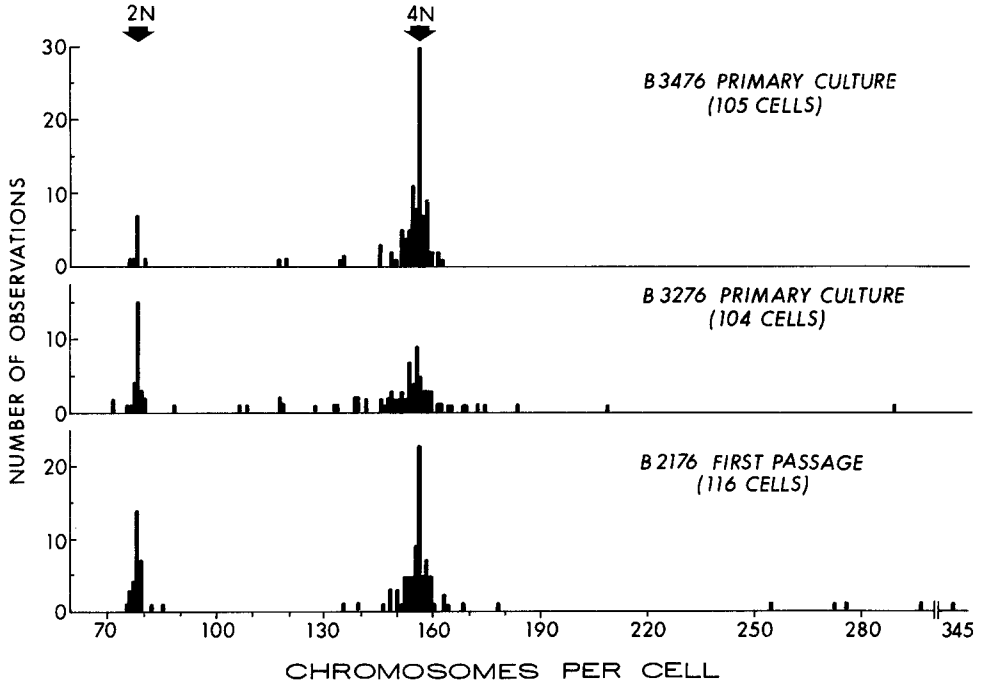

Fig. 9. Distribution of chromosome numbers counted in metaphase spreads of canine urothelial cells in culture

(viability indicated by trypan blue exclusion) which attached to the dish was estimated by measuring total floating cells after two or three days of culture. The unattached cells were from 40-80% of those placed in the dish, indicating an attachment of 20-60% in various experiments. Treatment of the tissue with trypsin at room temperature for three successive 20-minute intervals also yielded useful numbers of live cells, but the procedure was less satisfactory than the overnight treatment at 2° C. The yield of cells was usually smaller and the proportion which attached to the dish and survived was less.

After attaching to the substrate, the epithelial cells obtained with trypsin (or elastase) treatment showed a strong tendency to aggregate into monolayer groups. The single cells moved actively about the dish while changing markedly in shape, assuming rounded, polygonal, and elongated forms. Cellular processes, sometimes several cell diameters in length, were common (Fig. 4). As the cells associated into monolayer islands, they spread more thinly, the cell boundaries became less distinct, and the cell shapes became more regular (Figs. 5 and 6). Transmission electron microscopy of the cells at this stage showed the formation of punctate desmosomes and interdigitation of the lateral cell membranes (18). After the association with neighbouring cells was established, the epithelial cells were flat and thinly spread, and varied widely in diameter, from about 20 to 300 µm for mononucleated cells. Multinucleated cells were rather common and were of larger average size. The largest were over 400 µm in diameter. Only the smaller cells were seen to divide. Both multiplication and cell spreading appeared to contribute to an increase in total cell area. Even after the cells covered the dish, division continued and the layer became more crowded, but never multilayered. In some crowded cultures, patches of cells peeled off and the resulting holes were filled by the expanding monolayer. Blisters or domes were seen occasionally in which the cell monolayer was raised or separated from the substrate over several cell diameters. The sheet of cells in such domes remained one cell thick.

During the first week of culture, the urothelial cells could be removed from the substrate and dispersed with 0.05% trypsin, 0.02% EDTA at 37° C in about 15 min. With time in culture, however, the cells became more resistant to trypsinization. After several weeks, an hour or more was sometimes required to free all the cells from the dish. Perhaps because of the prolonged trypsinization time, subsequent attachment and growth were often poor. In 13 attempts at repeated passage, 5 cultures were twice transferred successfully but cell division slowed and stopped after the third transfer. One culture grew well for 16 passages before failing, and two cultures are growing well after 12 and 31 transfers, respectively. It should be emphasised, however, that confluent monolayers of primary urothelial cells were obtained routinely within a week, and that without passage, or with 1 or 2 early passages, the cells could be maintained in culture in apparent good health for 2 months or more.

In making chromosome counts, it was noted that the number of mitoses varied among different cultures, and, particularly, at different times in a given culture. Because the hypotonic treatment used to give chromosome spreads suitable for counting chromosome numbers may lead to loss of some mitotic nuclei, mitotic indices determined with this material may underestimate the true values. These indices, however, should be internally consistent and allow a comparison of the mitotic activity in different cultures, as well as providing an approximation of the true mitotic index value. In two experiments, cells which had been in culture one hour before colchicine treatment (not yet attached to the dish) showed no mitoses in 80,000 or 100,000 cells (dividing cells <0.002%). Both of these cultures grew well after cell attachment, and later became confluent. In another experiment, attached cells which had been in culture for 24 h had 13 mitoses in 57,450 cells, a ratio of 0.02%. After 72 hours in culture, the ratio had reached approximately 1%. In a fourth experiment, no divisions were seen in 80,000 attached cells after 24 h of culture (dividing cells <0.002%). In this culture, also, the index reached approximately 1% after 72 h in vitro. Fig. 8 shows a metaphase spread of a near-tetraploid cell with chromosome morphology typical for canine cells. Chromosome counts were made on three cultures. Two were primary (without passage by trypsinization) and the cells were studied three days after plating. The third culture was used two days after its first passage. The results (Fig. 9) were similar for all three. Tetraploid cells predominated, although diploid cells were also common. There were a few triploid and hypertetraploid cells.

DISCUSSION

The mechanical stripping of the mucosal layer separated the urothelial cells, together with the underlying fibrous layer, from the bulk of the muscle coat of the bladder. The separated layer contained the transitional epithelial cells, fibroblasts, capillary endothelial cells, and some muscle. By examination of the

residue after enzyme treatment, it appeared that all these cell types were dispersed to some extent by collagenase, although the urothelial cells tended to remain clumped. Most of the cells could thus be obtained as a mixed single cell suspension by collagenase action alone or supplemented with tryptic digestion to disperse the epithelial clumps. Trypsin alone, however, dispersed the urothelial layer completely but had little effect on the fibrous layer; and the single cell population which attached to the dish was epithelial in appearance. A few cultures contained small numbers of cells resembling those derived from smooth muscle. Fibroblasts were rare. The partial dispersion of blood vessels by trypsin suggested the possibility of the presence of small numbers of endothelial cells in the cultures, but these were not identified.

A different approach to the separation of urothelial (and other epithelial) cells was reported by Owens et al. (15). They utilised the tendency of the epithelial cells to remain in clumps after collagenase digestion, separated the clumps by settling, and plated them as small explants. After attachment, remaining fibroblasts were removed by selective trypsinization.

Elastase from porcine pancreas gave results similar to those obtained with trypsin (Table 1), but was more expensive. Crystalline trypsin, also, did not appear to be superior to the crude (and cheaper) preparation. The reasons for the difference between pancreatic and bacterial elastase were not explored. The larger number of cells obtained with Pronase might warrant further investigation of digestion conditions or subsequent cell washing, to seek ways to improve the plating efficiency of the bladder cells obtained by its use.

It is not suprising that some cells were obtained by treatment of the bladder mucosa with buffered saline alone, since such cells are continuously shed into the urine. The failure of most of the cells to attach and grow in culture is in keeping with our observation, in preliminary experiments, that human urothelial cells from urine or bladder washings seldom attach to the culture dish. Addition of EDTA to the saline did not improve the cell yield.

Among the different types of collagenase, cell yields were similar with types I, III and IV. Average yields were higher with type II, but the wide variation among animals made the difference between types I and II of uncertain significance. (The probability of a chance variation is 0.09, t-test.) Type I is described by the supplier as being the normal balance of clostridial "collagenase" enzymes, while type II is high in clostridiopeptidase.

With a given enzyme, there was considerable variation in cell yields from one animal to another. Factors contributing to the variation probably included differences in age and health of the source dogs, as well as some variation in time from death to processing of the tissue. There was some difference among animals in the ease with which the mucosal layer was stripped off.

Examination of the histological sections of tissue remaining after trypsin (or elastase) treatment suggested that the principal cell type dispersed was that of the transitional epithelium. Since the predominant cell type in these cultures was epithelial, we tentatively identify it as the urothelial cell. The size variation and the occurrence of multinucleated cells in the cultures are consistent with the characteristics of urothelial cells in vivo. Blisters or domes which were common in some cultures resembled those described in a dog kidney cell line (11). They differed from those described in mammary epithelial cell cultures (14) in lacking any cells attached to the dish below the dome. We have not yet examined the genesis or possible physiological activity of these structures in the bladder cell cultures.

The frequency of polyploidy in the cultured cells (Fig. 9) is consistent with the frequent occurrence of polyploid cells in the transitional epithelium of the urinary bladder in several species (4, 8, 12, 13, 19). The proportion of cells of different ploidy in these cultures may not reflect exactly the distribution in the original tissue, since there may be some selection with respect to those cells which survive tissue dispersal, attach to the dish, and undergo division under culture conditions. However, the large number of tetraploid cells in primary cultures argues strongly for their presence in vivo. Later passages have not yet been examined, but it is of interest that the same pattern of ploidy persists at least through the first culture transfer.

As might be expected, the freshly dispersed cells showed little mitotic activity, although they were alive as judged by trypan blue exclusion; and 20-60% had the capacity to attach to the culture dish. The mitotic rate remained low as the cells first attached to the dish. After about two days in culture, however, mitotic activity increased greatly, as it does in urothelial cells in vivo after stimulation by trauma (5), or exposure to certain chemicals (4).

Our best results in the preparation of urothelial cell cultures were obtained with the overnight treatment of the mucosal layer with 0.25% crude trypsin, 0.02% EDTA, in the cold, followed by warming and stirring; a procedure which allowed the routine preparation of primary cultures. While we have had only

moderate success with repeated transfer of these cells, they grow well to primary monolayers, have a predictable burst of mitotic activity starting at about 2 days, and survive for long periods as primary or low-passage cultures with epithelial morphology and in apparent good health. The availability of single-cell suspensions facilitates plating of replicate cultures. These cells may, therefore, be of value in the study of proximate carcinogens and other agents affecting the bladder lining.

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