THE BIOLOGY OF BLADDER CANCER

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THE HUNTERIAN MUSEUM of this Royal College is a witness to the breadth of John Hunter's vision and his realization, so far ahead of his contemporaries, that a better understanding of the diseases of man could be attained when they were considered within the general context of living creatures as a whole. It was the discipline of his mind that enabled him to record the pathological changes he observed with such remarkable diligence and in itself makes him a paradigm among pathologists of his own and several generations to come. It was his apperception that the study of animals could provide the answer to some of the mysteries of morphology and disease that was the hallmark of his genius.

I hope that this lecture will capture something of this spirit in its attempt to show, within the limited context of the biology of the urothelium and its tumours, how the present-day medical biologists are seeking for evidence in man and other mammals that can point the way to a new appreciation of certain phenomena of disease.

The normal urothelium

UROTHELIUM IS THE specially adapted epithelium that lines the renal tract from the renal pelvis to the urethra. It is formed by transitional cells, usually three to four layers thick, separated from the submucosa by a well-defined basement membrane. Although some of the general properties of this tissue, such as its permeability[1] and its sensitivity to tumour induction by chemical carcinogens in experimental animals, have been studied for many years[2], it is only more recently that attention has been focused on the cellular basis of its function and the biology of urothelial cancer.

The structure of normal bladder epithelium is best seen in thin resin-embedded sections (Fig. 1). The luminal surface of the bladder is lined with large cells, each of which covers about 12 to 20 underlying intermediate cells. There is considerable variation of the nuclear size, with the nuclei of the surface cells generally being much larger than those in the intermediate and basal layers. Transmission electron microscopy reveals that, in rats and mice, differentiated cells in contact with the urine have a very complex structure with characteristic stack-like arrays of membrane (fusiform vesicles) in the cytoplasm. It is possible that in these animals these vesicles are stores of surface membrane. In man and several other species the fusiform vesicles are far less numerous. The second feature present in most species is the telolysosomes that are the final stage of the autolytic process whereby

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damaged and effete organelles are removed from the cytoplasm. The luminal membrane is an asymmetrical, trilaminar membrane made up of a lattice of repeating sub-units[3]; it is the biophysical properties of this membrane and the tight junctions that join contiguous surface cells together that give the epithelium its particular permeability characteristics to water and electrolytes. It has been assumed that, owing to its low permeability to water and ions, bladder epithelium prevents changes in the composition of the urine. In fact, there is good evidence to show that there is considerable exchange of molecules across the epithelium in both directions, although the net movement is very small. Nevertheless, certain lipid-soluble substances can penetrate across the epithelium very rapidly.

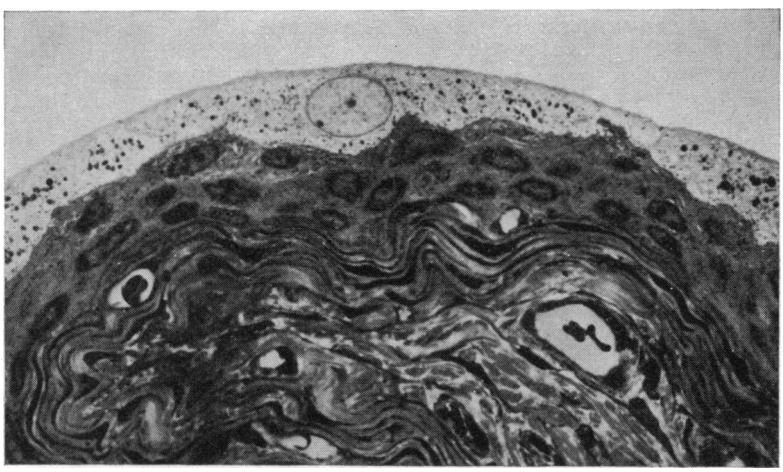

Fig. 1 Normal mouse bladder showing polyploid surface cell. Toluidine blue. × 36.

Examination of the nuclei of the urothelium shows that this tissue is polyploid—that is, it contains nuclei with twice or four times the normal DNA content and twice or four times the normal number of chromosomes. Polyploidy occurs in other tissues, such as the liver, where it increases as the organ ages. In the bladder epithelium the polyploidy is established in embryonic life. A second feature that sets urothelium apart from all other epithelia is its slow turnover rate. The mitotic index is about 0.01%, and a conservative estimate suggests that some of the cells could have a life span of more than 200 days. Maybe it is this combination of cellular longevity and the evolution of the large surface cells, perhaps for reasons of mechanical stability, that has necessitated some of the genetic information to be reduplicated.

Response of urothelium to injury

Although the bladder epithelium is normally very stable, it starts to divide in response to several forms of injury. Surgical trauma induces division in the vicinity of the wound; radiation, infection, over-distension, and chemical injury by carcinogens and non-carcinogenic compounds all cause widespread mitotic activity thoughout the epithelium. In Leeds we have made a detailed study of two of these types of injury in experimental animals and have learnt something of the sequence of change that occurs as the epithelium responds to the acute and chronic chemical injury.

Ethylsulphonylnaphthalene-1-sulphonamide (ENS) was originally produced as a potential anticonvulsant and diuretic, but it never entered clinical trial because routine screening in animals had shown that it induced bladder cancer. Later it was observed by Bonser and Clayson[4] that giving a single dose of ENS to mice could cause the bladder epithelium to become hyperplastic within a few days. This agent has been a valuable tool in revealing the immediate response to acute injury[5] and the adaptation to chronic chemical damage[6]. Immediately after giving a single dose of ENS there are signs of activation of the lysosomes, a system of cell organelles which are concerned with the breakdown of damaged cytoplasmic components and the activation of hydrolytic enzymes. In a few cells this damage is progressive and they die, but in many the injury sets in train the sequence of RNA and DNA synthesis that is the necessary prelude to cell division. The division is unusual as it takes place not only in the simple basal layer but also in the highly differentiated polyploid surface cells. A similar process takes place after surgical wounding. The epithelium in its repair tends to produce an excessive number of cells and become hyperplastic. They tend to stabilize in this state for some time though the immediate effects of injury have long since passed. During the phase of acute proliferation the average time for the urothelial cells to pass through the cycle of events between divisions (cell cycle) is about 20 hours. This time is within the range of several other dividing tissues in mice and indicates that the long dormancy of the cells does not affect their speed of division when they are recalled to active proliferation. Furthermore, the diploid and tetraploid cells have identical cell cycle times. The recall to proliferative activity of dormant urothelium when injured is similar to that seen in other organs in which there is normally little cell division—for example, in the liver after partial hepatectomy and in the salivary glands when stimulated with isoprenaline (isoproterenol)[7].

The continuous feeding of ENS in the diet will eventually produce bladder cancer in mice, but this process takes many months. ENS inhibits renal carbonic anhydrase and induces alkalosis, which causes

calcium oxalate and phosphate concretions to develop in the bladder; they are often present after about 10 weeks on this diet. After ENS has been fed for 10 weeks the bladder epithelium in many animals has undergone a remarkable alteration of its form (Fig. 2). The differentiated surface cells are no longer present and the whole epithelium is composed of fairly simple cells containing few organelles and with uniformly diploid nuclei. Though the chemical injury is being applied continuously, this abnormal epithelium may be relatively stable, with few mitoses. Another sign of the dedifferentiation is the loss of alkaline phosphatase activity, which is normally present in intermediate and basal cells, from the epithelium, although it is strongly positive in the inflammatory cells in the submucosa of these animals. At this stage bladder cancer is not inevitable, and returning the animal to a normal

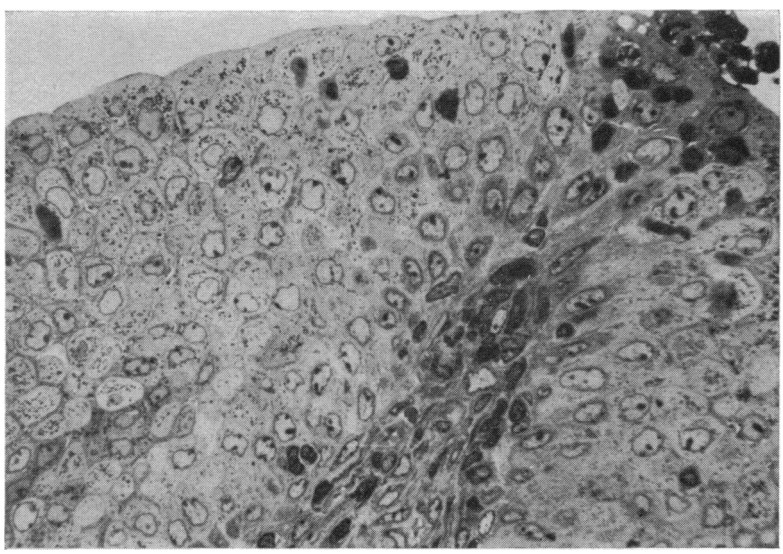

Fig. 2. Hyperplasia of bladder epithelium induced by feeding ENS to mice for 10 weeks. Note the inflammatory cells migrating through the epithelium. Toluidine blue. × 256.

diet will be followed by resolution of the chronic hypertrophic cystitis. A more detailed examination of the hypertrophic cells reveals the absence of the organelles characteristic of normal urothelium. The mitochondria undergo progressive destruction owing to the formation and swelling of dense inclusion bodies inside their cristae.

This change is not a particular reaction to ENS but is thought to be a non-specific response to chronic irritation that so far has been observed only in mice. This type of lesion found in one species and not

another is typical of the epiphenomena observed in carcinogenesis research that should put the research worker on his guard against making general statements that really are applicable only to a special circumstance in one species of animals. Despite these cautionary remarks, the recovery of the hyperplastic cystitis induced by feeding ENS is of some interest. When ENS is withdrawn from the diet the urine rapidly returns to its normal acidic pH and the cells lining the luminal surface make an attempt to differentiate and begin to elaborate new cell organelles. However, this attempt is often abortive and ends in the cells undergoing a cystic degeneration and being shed. The underlying cells repeat this process until a layer of cells that can differentiate normally is exposed to the urine. (Fig. 3).

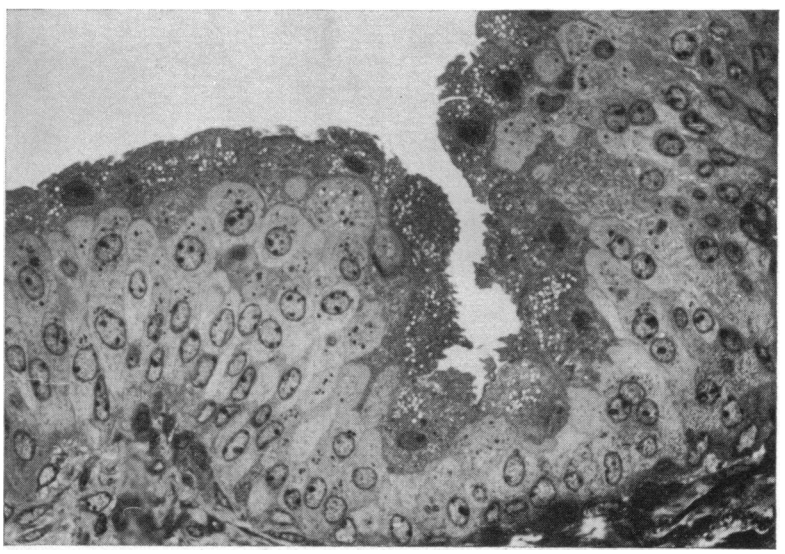

Fig. 3. Mouse bladder showing cystic degeneration of luminal cells of hyperplastic epithelium 4 weeks after withdrawing ENS from the diet. Toluidine blue. × 256.

Cyclophosphamide. The second experimental model we have investigated is the acute cystitis in rats that follows exposure to the alkylating agent cyclophosphamide[8]. This reaction is analogous to the acute haemorrhagic sterile cystitis that may complicate treatment of malignant diseases in man with cyclophosphamide. Unlike the acute response to ENS, a single injection of cyclophosphamide causes a rapid and widespread necrosis of the urothelial cells. By 24 hours there are many areas where the surviving epithelium is only one cell thick and others where it is denuded to the basement membrane. At this time the permeability of the bladder is increased, although within a further

24-48 hours it returns once more to its normal level. Examination of the ultrastructure during healing shows that restoration of the cell contacts one with another is established early. The cells forming the surface, even though they are not differentiated and are quite small relative to the normal luminal cells, unite together with tight junctions. Islands of new luminal cells can be recognized when the injury has not been widespread (Fig. 4). The cells responsible for the repair after injury with cyclophosphamide seem to be the diploid cells in the

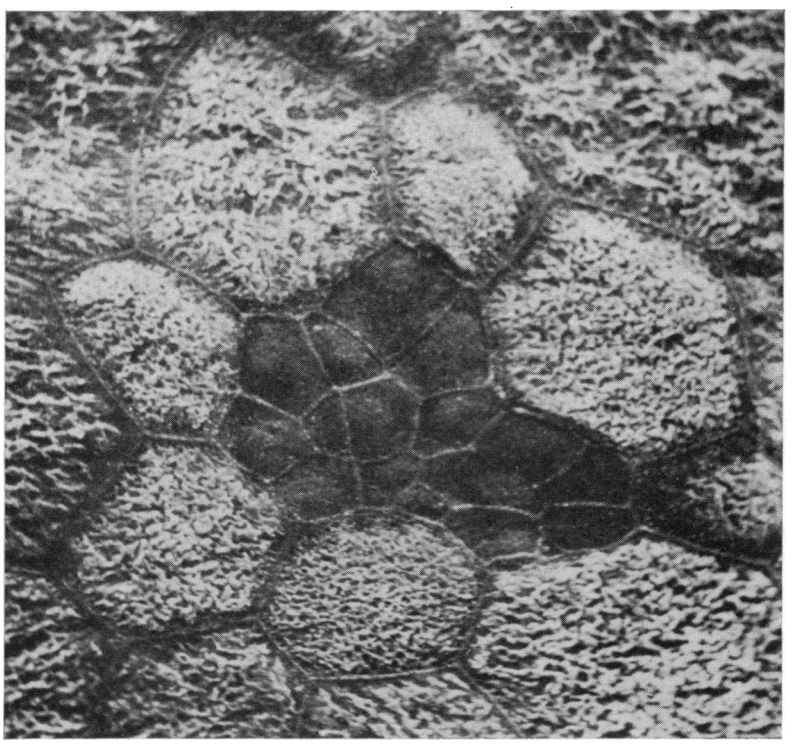

Fig. 4. Scanning electronmicrograph of the bladder of a rat treated with cyclophosphamide 10 hours previously. A number of surface cells have been shed and the underlying cells adapt to contact with the urine. × 3,700. (Fulker.)

deeper layers of the urothelium and, as with the repair of damage induced by ENS, the formation of binucleate cells appears to be an intrinsic step in the production of nuclei of higher ploidy. Some cells with very large nuclei are to be seen in the hyperplastic epithelium; they arise as a consequence of the residual action of the alkylating agent on DNA replication[9]. Similar abnormal cells can be seen in the urine of patients treated with cyclophosphamide.

2-Acetylaminofluorene. A third pattern of injury noted in the study of the action of carcinogens on the urothelium is a far more insidious process than the previous examples. 2-Acetylaminofluorene causes bladder cancer in mice and rabbits, but continuous feeding for many weeks may induce only slight hyperplasia which might be dismissed as relatively trivial on the basis of light microscopy. Electron microscopy demonstrates that the cells have sustained an increased rate of organelle turnover so that the production of new organelles keeps pace with the damage and autolysis of the effete organelles (Fig. 5). Under these circumstances the cells can survive for long periods and the requirement for new cell production seems to be slight.

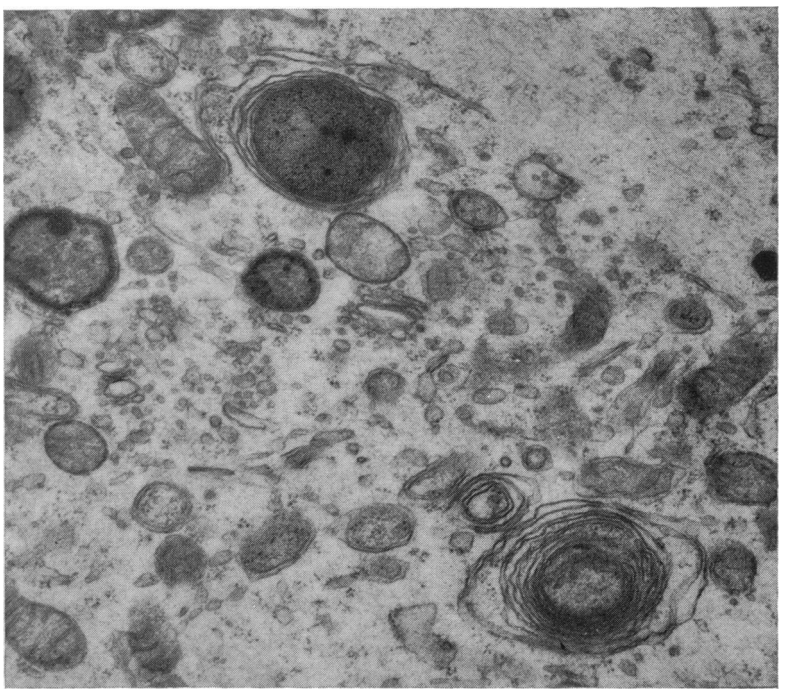

Fig. 5. Mouse bladder after feeding 2-acetylaminofluorene in the diet for 20 weeks showing lysosomal activity with membrane whorls surrounding degenerating organelles. × 22,100.

These briefly described examples serve to indicate some of the information about urothelium that is being acquired from studies in experimental animals. The principal characteristics of the urothelium that need to be borne in mind when considering its pathology and its tumours are as follows: the epithelium is innately stable but has the ability to respond to injury rapidly by reversion to active division,

a reaction that often leads to hyperplasia; the basal cells have a greater potential for proliferation than the well-differentiated cells, which appear able to sustain only one or two 'emergency' divisions; some forms of injury produce considerable subcellular damage, with little change visible with the light microscope; and finally, it is reasonable to comment that contact of the undifferentiated cell with the urine appears to be the key stimulus to induce its differentiation.

UROTHELIAL CANCER IN MAN

It is very difficult to obtain fresh normal urothelium in man under the rigid conditions required to be absolutely sure about its structure in great detail; so we have had to compromise and accept samples taken at the time of surgery for non-malignant conditions such as prostatectomy as being representative of the normal state. For this reason we are less certain of our baseline observations in man than in animals. Our research is being carried out on three fronts: a detailed analysis of the relationship between a tumour's state of differentiation as defined by light microscopy and the underlying changes in its subcellular organization; study of the cytogenetics and proliferative activity of the tumour cell population; and more recently we have begun to consider the immunological reactions of the host against the tumour.

Classification

Anderson classifies bladder tumours as being well, moderately well, or poorly differentiated, their general growth being described as fronded, fronded and solid, or solid and the extent of invasion noted[10]. Fulker *et al.*[11], from their electron microscopical investigation, consider that changes in the submicroscopic organization of the surface of the cells in contact with the urine and the way in which the cells become less intimately joined together are the most consistent features of dedifferentiation. The main changes are summarized in Table I. Varia-

TABLE I

ULTRASTRUCTURAL CHANGES ASSOCIATED WITH DEDIFFERENTIATION*

Ultrastructure	*Normal*	*Well-differentiated tumours*	*Undifferentiated tumours*
Luminal membrane	Ridges	Microvilli	Smooth
Surface junctions	Tight with occasional desmosomes	Tight with frequent desmosomes (Fig. 7)	Often open (Fig. 8)
Telolysosomes	Frequent	Frequent	Rare
Glycogen	Frequent	Frequent	Rare
Nuclei	Oval, often invaginated	Oval, often invaginated	Round
Cell arrangement	Orderly	Orderly	Disorderly
Fibrils	—	Often in large quantities	Small quantities only
Cellular interdigitation	Highly complex	Highly complex (Fig. 6)	Simple (Fig. 9)

*From Fulker and Anderson (reported to the International Bladder Cancer Conference, Leeds, 1971).

tions in the organelle content of the cells during dedifferentiation are difficult to measure quantitatively; these investigators are attempting to devise a system of morphometric analysis that is applicable to this problem. In relation to these changes, it is of some interest to consider the findings of Fellows and Marshall[12], who have measured the efflux of tritiated water and radioactive sodium across the bladder in various conditions. They observed that patients with "normal" bladders and bladders containing well-differentiated tumours had the same permeability. Poorly differentiated tumours and urinary infection were both associated with an increased permeability of the bladder, which correlated with the decreased adhesion of the cells in these conditions.

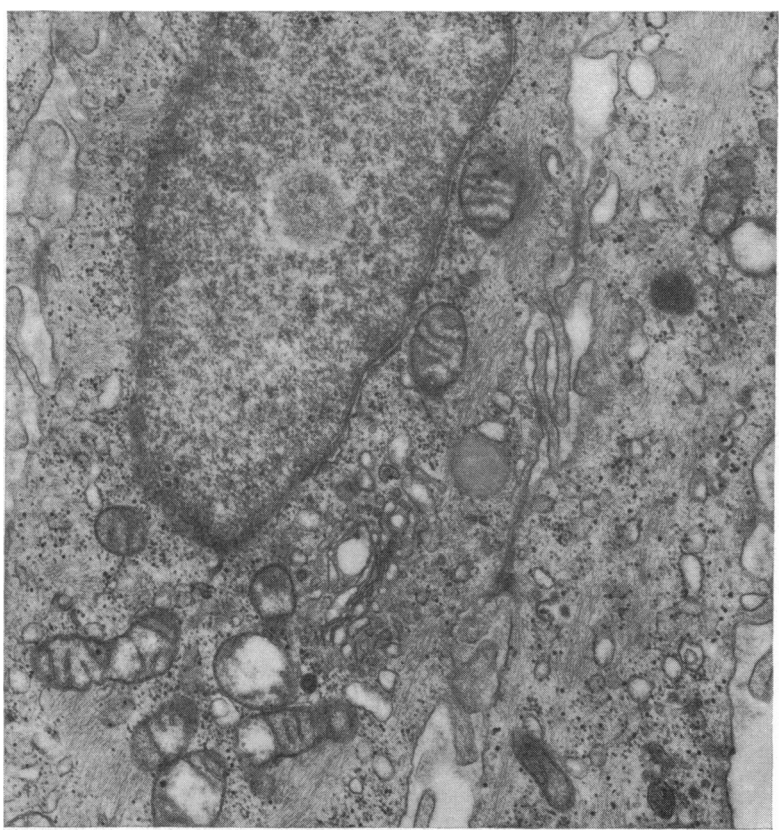

Fig. 6. Well-differentiated bladder tumour showing cellular interdigitation. × 22,400. (Fulker.)

Histological grading and DNA content

Examination of the cell populations that make up a tumour has been undertaken to try to establish the extent of the variation within a

particular histological grade. A difficulty that besets the urological surgeon is to know, among patients who present initially with tumours with low-grade malignancy, which ones will behave in a typical fashion and in time evolve towards a more malignant tumour. Clearly, from the point of view of selection of the best line of treatment, this predictive information would be invaluable, and at present it cannot be gained from histological examination alone. Levi *et al.*[10] commenced this line of inquiry by measuring the DNA content of the tumour cell

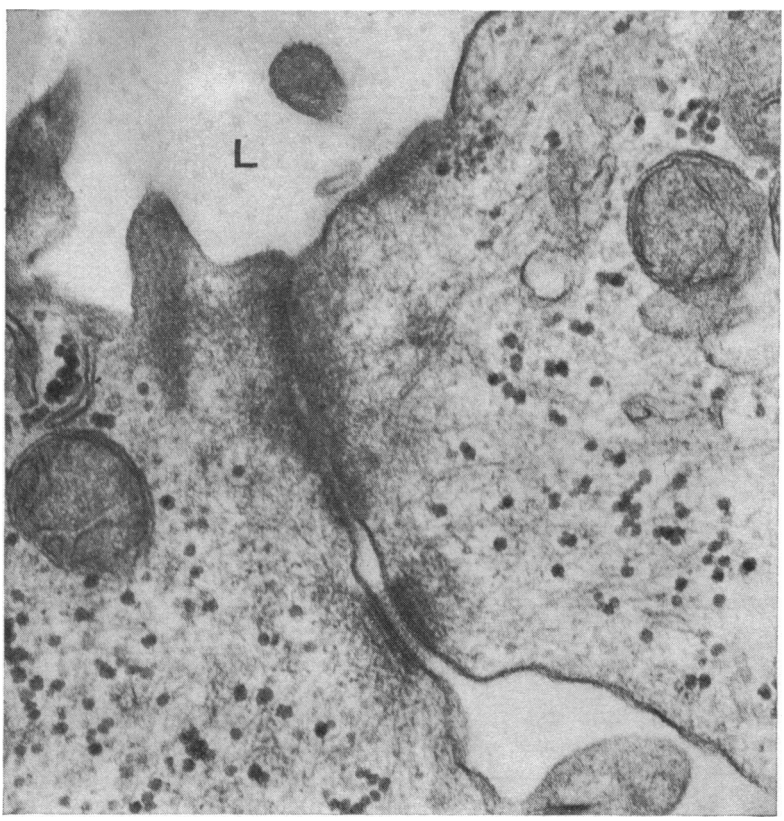

Fig. 7. Well-differentiated bladder tumour showing a tight junction at the luminal surface with a desmosome below it. L = bladder lumen. × 70,000.

populations; with a suitable instrument this can be done rapidly and accurately. The patterns of distribution give information about the organization of the population. This work showed that well-differentiated tumours have a modal DNA content (the most frequent value) close to that of normal diploid cells. In some of these tumours there is evidence of bimodality, with the second mode at twice the value

of the main mode, suggesting the coexistence of two cell populations within the same tumour. As the tumours dedifferentiated it was generally found that the modal DNA content increased, and in anaplastic tumours frequently no distinct mode could be observed, indicating the great variety of cell lines within the tumour. Levi and her colleagues attempted to get an impression of the proliferative activity by studying the uptake of tritiated thymidine, a specific precursor for DNA synthesis, into dispersed tumour cells in vitro. These experiments showed that as a rule the well-differentiated tumours had a low rate of proliferation, while it was higher in the poorly differentiated tumours. (The average percentages of DNA-synthesizing cells in well-differentiated, moderately well-differentiated, and poorly differentiated were 2.6 ± 0.6, 35 ± 0.9, and 5.5 ± 1.5 respectively.) There were exceptions to this rule, but the significance of unexpectedly high or low rates of proliferation is still obscure.

Tumour proliferation and chromosome analysis

Looking for an alternative way to investigate tumour proliferation we decided to use a stathmokinetic test, in which cells entering mitosis for a 2–4-hour period were arrested in metaphase by the action of a colchicine derivative (Colcemid, Ciba) administered as a 10-mg dose intravenously before the tumour was resected. This approach confirmed our general impression about tumour proliferation and also drew attention to the increase of local variation in proliferation within poorly differentiated tumours that could not always be explained by signs of anoxia and cell death. The proliferative activity in 61 tumours examined by this test was as follows: well-differentiated, 0.59 ± 0.45; moderately differentiated, 0.99 ± 0.96; poorly differentiated, 1.89 ± 1.85 mitoses/100 cells/hour. The extent of the variation can be appreciated from the standard error given with each of the means. This test not only proved a valuable way to learn about tumour cell proliferation but opened up the possibility of making detailed chromosome analyses of bladder tumours which had previously been unsatisfactory owing to the low yield from the well-differentiated tumours. Spooner[13] has made a full analysis of the chromosome constitution of 61 tumours. They fell into two main groups: those with chromosome numbers closely related to the normal diploid compliment of 46 chromosomes and those with higher chromosome numbers. In the first group 10 well-differentiated tumours were observed to have no detectable chromosome abnormality; the remainder in this near-diploid range had abnormalities with the loss or gain of 1 or 2 chromosomes and various rearrangements of the chromosomes. Within this group were all the well- and moderately differentiated tumours, as well as 25% of the poorly differentiated tumours. On the other hand, tumours with high chromosome numbers were all poorly differentiated; no consistent abnormality was seen in

all tumours, and the types of rearrangements of chromosomes were comparable to those described in the cervix, ovary, and several other solid tumours in man.

As with the DNA measurements, chromosome analysis indicated evidence of bimodality in the cell populations. It is possible that the instability that leads to the formation of a second population with twice the modal chromosome number is a step in the evolution towards a cell line in the hypotetraploid range that will eventually outgrow all others.

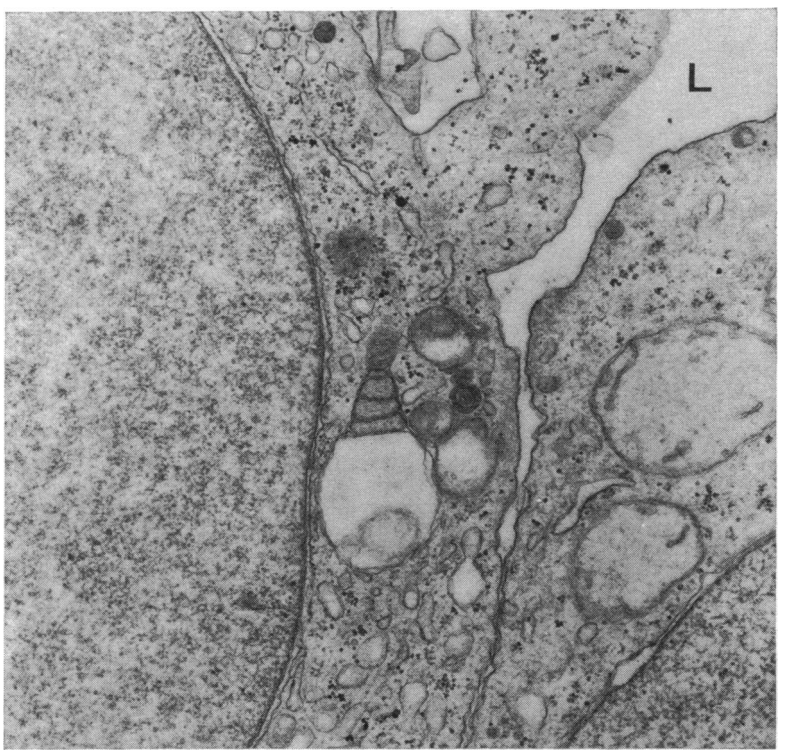

Fig. 8. Poorly differentiated bladder tumour showing an intercellular space open to the bladder lumen. L = bladder lumen. × 22,400. (Fulker.)

Analysis of repeated samples from a few patients with recurrent tumours has given some interesting information. From one subject six biopsy specimens were taken over a period of 21 months; the patient had multifocal well-differentiated tumours. The presence of 'marker' chromosomes—that is, chromosomes with readily identifiable abnormality—showed that his tumours were of the same clonal origin and

underwent very little deviation from one biopsy to the next. In contrast, in another patient the evolution of change could be observed; an abnormal population at first appeared as a minor component of the tumour, but as time went by this minor element gradually became the predominant cell population. Tumours arising in patients after radiotherapy did not have any specific chromosome abnormalities, although about 5% of the blood lymphocytes of these patients contained distinct chromosome aberrations of radiation injury, as was demonstrated when they were stimulated to divide by incubation with phytohaemagglutinin.

These studies have given grounds for some generalizations about human urothelial tumours. It is probably true that bladder tumours form a continuous spectrum from the apparently benign papilloma to the most malignant anaplastic tumour. Once a high chromosome number has been attained invasion and progression seem to be inevitable, but there is a far wider pattern of behaviour of the near-diploid tumours. It is within this near-diploid group that the surgeons and the pathologists alike meet their greatest problems. So far it seems that chromosome analysis cannot help to predict the tumour's behaviour. The pathologist is well aware of the behaviour of the anaplastic tumour and does not require DNA or chromosome analysis to confirm his findings. These investigations have made us aware of the atypical tumour —that is, poorly differentiated with a low chromosome number or anaplastic with a low proliferation—but only time will tell if these observations have a biological significance in terms of the tumour's evolution. Perhaps the most important practical point that has emerged is the attention that must be paid to areas of focal dedifferentiation, however small, within a well-differentiated tumour, for these may signal the onset of the development of a new clone of cells whose properties may be more aggressive than those of the tumour in which they have arisen.

Control of progression

As far as the proliferation studies are concerned the data indicate that the tumours are endowed with a potential for growth that far exceeds what is known to be the case from clinical observation. If it were assumed that each mitotic figure was able to give rise to two viable cells, then some of the anaplastic tumours ought to be able to double their mass within 2–3 days, and well-differentiated tumours in about 20 days. The discrepancy between the potential rate of growth and the true rate of growth is due to the loss of cells in the tumour. Cell loss occurs as a result of desquamation, intrinsic metabolic faults in the tumour cells, death in mitosis (particularly in cells with high chromosome numbers), anoxia (a common feature of anaplastic tumours), and immune attack by the host.

Immunological mechanisms. Evidence supporting the idea that there may be immunological mechanisms controlling the progression of bladder cancer in man are still at a very tentative state. Nevertheless, taken in conjunction with firmer evidence coming from the study of other tumours such as melanoma, neuroblastoma, and certain skin tumours in man, this line of research looks to be the most promising, particularly as it may give the basis for an immunological approach to the therapy of cancer. In our group Tanaka *et al.*[14] undertook a painstaking reassessment of over 1,000 biopsy specimens from the bladder in patients with a variety of urothelial diseases and estimated the incidence of lymphocytic and plasma cell infiltration (Table II). In recurrent biopsies some patients maintained a consistent infiltration of the tumour sample, in others it was an ephemeral reaction. Infection

TABLE II

INFILTRATION OF DIFFERENT CELL TYPES SEEN IN LESIONS OF THE URINARY BLADDER[14]

		Carcinoma grade		
	*Benign lesions**	*I (Well-differentiated)*	*II (Poorly differentiated)*	*III (Anaplastic)*
No. of specimens examined	265	355	244	200
Lymphocytes (diffuse)	6 (2.3%)	23 (6.5%) P†=0.0098	17 (7.0%) P=0.0091	28 (14.0%) P=0.0005
Lymphocytes (aggregate formation)	29 (10.9%)	61 (17.2%) P=0.0185	34 (13.9%) P=0.0131	35 (17.5%) P=0.0295
Plasma cells	2 (0.75%)	20 (5.6%) P=0.0006	6 (2.5%) P=0.1172	7 (3.5%) P=0.0369
Eosinophils	6 (2.3%)	4 (1.1%) P=0.9236	1 (0.40%) P=0.9900	6 (3.0%) P=0.4162

*Mainly cystitis cystica and glandularis, leukoplakia, fibrosis, or epithelial hyperplasia.

†The P value was calculated by comparing the frequency of infiltration of a particular cell type seen in the benign lesions with that seen in carcinomas.

of the urine was not significantly associated with lymphocytic infiltration; specimens showing mixed polymorphonuclear and lymphocytic infiltrations are not included as positive in Table II. On the basis of the three-year survival lymphocytic infiltration did not appear to have any prognostic significance and contrasts with what has been claimed in breast cancer and seminoma, where lymphocytic infiltration appears to indicate an improved prognosis. However, this does not mean to say that the lymphocytic infiltration is without biological significance. The cellular and humoral factors that control cancer growth by their cytocidal effects are relatively weak and may be only of minor importance among the various factors that determine the three-year survival of a patient with bladder cancer.

Certainly these findings should not be dismissed too readily, as the observations of Bubenik and his colleagues[15, 16] have provided evidence that lymphocytes and serum from patients with bladder cancer can produce cytotoxic effects in vitro both on autochthonous bladder cancer cells (cancer cells and lymphocytes or serum from the same patient) or on allogeneic bladder cancer cells (cancer cells from one patient and lymphocytes or serum from another). There is a cross-reaction in that the lymphocytes from a patient with a bladder cancer may be cytotoxic

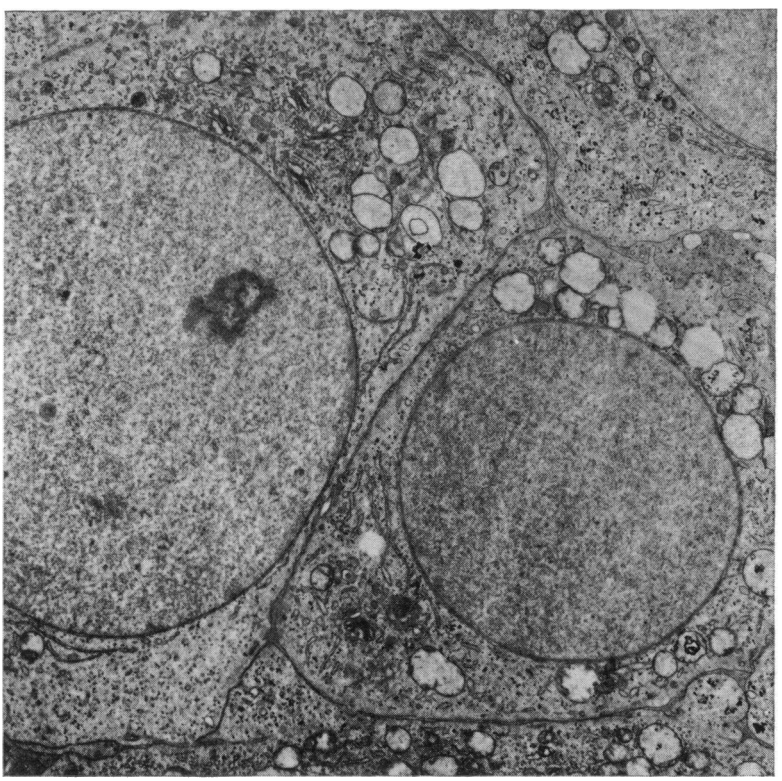

Fig. 9. Poorly differentiated bladder tumour showing lack of interdigitation between the cells. The large 'clear' spaces are swollen mitochondria. × 22,400. (Fulker.)

for several different bladder cancers. However, this cytotoxicity is specific for bladder cancer, and the lymphocytes have no reactivity against other forms of cancer or normal cells in tissue culture.

In some patients there are blocking antibodies in the serum that are capable of inhibiting the cytotoxic action of the lymphocytes. Recently O'Toole (personal communication) has demonstrated that

lymphocyte cytotoxicity that was present before radiotherapy disappeared rapidly during treatment; it then became strongly positive shortly after the completion of treatment and remained positive for 3–4 months. However, the lymphocytes of patients treated by irradiation 1½–10 years previously had no cytotoxicity except when there was a recurrence. There seems to be a variation in this reaction according to the state of the tumour. It was found that advanced tumours were often associated with non-reactive lymphocytes. On the other hand, in patients treated by a novel technique in which the intravesical pressure was raised to cause an ischaemic necrosis of the tumour[17] there was an increase in the proportion of patients with cytotoxic lymphocytes and a marked lymphocytic infiltration of the necrotic tumour tissue. These results suggest that lymphocyte toxicity may be greatly influenced by the rate of release of the tumour-associated antigens from the tissues.

Conclusion

This lecture has dealt with only a few aspects of the biology of human bladder cancer. Its occurrence as a recurrent growth of relatively low grade malignancy offers special opportunities for study of its evolution in a fashion that is rarely encountered with other solid tumours. A major challenge to the biologists is to try to understand the nature of widespread field change that may be present throughout the urothelium in some patients with urothelial cancer. The reason for distribution of the tumours in the urothelial tract and their sequence of appearance is still an open question. Unfortunately, as problems of this character present great difficulties for experimental design and execution it is likely that they will remain a mystery for some time to come.

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*Reference No. 2 is a general review article and contains all the references to the background work to the present study.