THE CELLS OF NORMAL HUMAN URINE

A QUANTITATIVE AND QUALITATIVE STUDY USING A NEW METHOD OF PREPARATION

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Despite the many reports of the finding of malignant cells in various body fluids, dating back to Beale, of London, in 1860, the use of exfoliative cytology as a diagnostic procedure did not find much application until the publication in 1943 of the monograph by Papanicolaou and Traut on the diagnosis of uterine cancer by examination of the vaginal smear. Following this classical work the principle has been applied to many regions of the body and now finds widespread acceptance, particularly in the United States, as an aid to diagnosis and for screening in cancer-prevention clinics.

In a survey of the status and future trends of exfoliative cytology Papanicolaou (1952) emphasized the need for more basic studies of the normal cell population in the various body fluids. Of the diverse types and several origins of the cells in urine relatively little is known, although pathological samples have been examined on a large scale. Papanicolaou's paper in 1947 on the examination of samples from 240 clinical cases was followed by several others (Schmidlapp and Marshall, 1948, 1950; Chute and Williams, 1948; Harrison, Botsford, and Tucker, 1951), and in 1952 Crabbe, reporting from this laboratory, gave the results of using the Papanicolaou technique as a screening procedure for 1,000 workers in the dyestuffs industry who may at one time or another have been exposed to bladder carcinogens.

The Papanicolaou technique consists essentially of smearing the sediment from centrifuged urine over an albuminized slide, and, after fixation and staining, the whole smear, which covers most of the slide, is examined under the microscope. The latter process is both laborious and time-consuming owing to the very wide dispersion of the cells. A 50-ml. sample of normal urine produces a quantity of deposit on centrifugation requiring about five to 10 slides to accommodate it when smeared.

It is apparent that if the cells could be concentrated on a slide within an area of a few square millimetres the examination could be carried out in a very short time. In addition, the diagnosis would be strengthened by the fact that it would be based upon virtually the whole cell content of the sample rather than a fortuitous small fraction of the whole represented by one or two smears.

In the present paper a method for the concentration of the cells of urine is described and the results of its application to quantitative and qualitative study of the cell population of normal male urine are given.

Technique

The sediment which is deposited on centrifuging normal urine consists largely of mucus.* Removal of this mucus permits free suspension of cells and enables a high degree of concentration to be achieved by centrifugation. A simple procedure is described below by which this may be effected: it depends upon the fact that adding absolute alcohol to the sediment results in fixation of cells without irreversibly precipitating the mucus. It then becomes possible to dissolve the mucus by diluting with water without fear of disrupting the cells.

Determination of the Rate of Cell Loss into the Urine.—(1) About one and a half hours' output of urine in a 70-ml. centrifuge tube marked as shown (Fig. 1) is adjusted to pH 6 by adding N/2 HCl or N/2 NaOH and centrifuged for 10 minutes at 1,750 g.† (2) The liquid is gently drawn off and 15 ml. absolute alcohol added and mixed thoroughly. After five minutes distilled water is added slowly with shaking to fill the tube. (3) After centrifugation for 10 minutes at 1,750 g. the liquid is drawn off and about 1 ml. benzidine/hydrogen peroxide solution is added to

* The term "mucus" is used throughout this paper to mean the whole of the mucilaginous material of the normal urinary sediment, and is broadly justified by the protein reactions of the cell-free sediment and by the action of 33% acetic acid on an aqueous solution of this sediment (see Harrison, 1947).

† Centrifugal force is expressed throughout as a multiple of normal gravity (g.).

"stain" erythrocytes, and well mixed. The centrifuge tube is stoppered and left for half an hour. (4) Dilution with about 30 ml. 20% alcohol is followed by centrifugation for 10 minutes at 1,750 g. and the liquid drawn off to leave about 1 ml. (5) Cell clumps are broken up by drawing the suspension vigorously in and out of a pipette connected to a hypodermic syringe. (6) The suspension is now centrifuged again for five minutes at 1,750 g., the liquid is drawn off to either 0.05 or 0.1 ml., and cell counts are carried out using a Neubauer chamber.

FIG. 1.—The main items of apparatus used, shown at about a quarter actual size. The 70 ml. centrifuge tube comes internally to a fairly sharp point, and is marked at 0.05 and 0.1 ml. The modified Pasteur pipette, used for handling the concentrated cell suspension, should have an internal diameter of 0.5 mm. in the terminal drawn-out portion. The Buchner funnel, containing a sintered glass filter of pore size 2, is used when it is desired to separate large cells from small cells. The mouthpiece is added before blowing through the filter.

Areas of 1 to 4 sq. mm. are then counted for large cells, small cells, and erythrocytes. An arbitrary value of 15 μ diameter is taken to define large and small cells: in practice the sizes of most of the cells are found to fall conveniently far from this. The benzidine treatment shows erythrocytes as black discs.

The calculation is thus: Cells counted $\times \frac{V}{\sqrt{T}}$ = loss in thousands/hour, where V = volume of suspension in ml. (0.05 or 0.1), v = volume of suspension actually counted in c.mm., and T = period of secretion of urine in hours. The time taken for two determinations in parallel is about three hours.

Preparation and Staining of Cellular Contents of Urine.—Stages 1 and 2 given above are carried out. Then (3) crystals and the grosser "dirt" particles (and unavoidably a few cells) are deposited by centrifuging for one minute at about 280 g., and are then removed with a pipette. (4) After centrifugation for 10 minutes at 1,750 g. most of the liquor is drawn off, and one half of the remaining volume of a pectin adhesive solution is added and mixed. After a further centrifugation for two minutes at 1,750 g. liquid is

removed until a volume roughly equal to that of the sediment remains. (5) After mixing, the whole is transferred by pipette to a grease-free microscope slide. The diameter of the droplet thus deposited is usually about 2 to 10 mm. As evaporation proceeds, a mat, slightly glistening spot remains on the slide which is placed horizontally in 1:1 alcohol/ether fixative.

It is sometimes convenient to make two preparations, one of the small cells (less than 15 μ in diameter) and the other of the remaining cells. The separation is carried out by means of a sintered glass filter.

If this separation is desired, the following should be carried out after stage 3:

The cleaned cell suspension is poured into a sintered glass filter funnel, pore size 2, the filtrate from which contains only the small cells; this is collected in a 70 ml. centrifuge tube. Meanwhile large cells have been accumulating on the filter; when only a small depth of liquid remains, and before it falls to zero, the filter funnel is inverted over another centrifuge tube, as shown in Fig. 1. A little 20% alcohol is then blown through the filter transferring the cells on it to the centrifuge tube.

Pectin is effective as an adhesive in minute concentration, and being a carbohydrate is relatively refractory to stains.

Staining.—From alcohol/ether the preparation is taken through the following sequence (10 seconds or longer at each stage unless otherwise stated):—

Seventy per cent. alcohol; benzidine- H_2O_2 solution, 10 minutes; 70% alcohol; 50% alcohol; Harris haematoxylin, 10 minutes; 50% alcohol; 70% alcohol containing 1% NH_4OH , one minute; 70% alcohol; orange G, one minute; absolute alcohol, irrigate to remove all surplus orange G; light green, one minute; absolute alcohol, one second; xylol. The preparations are mounted in balsam.

Small-cell Preparations.—Haematoxylin 5 minutes counterstains at half strength, or use 0.5% eosin.

For Screening for Malignancy.—Counterstains at half strength.

The time taken to make the general preparation of six urine samples in parallel is two and a half hours. To make separate small-cell and large-cell preparations of each of six samples, about four hours are required.

Composition of Solutions Employed.—The following solutions are employed:—

Pectin Adhesive.

Pectin	...	0.02%	} in water
Glycerin	...	4.0%	

Benzidine-Hydrogen Peroxide.

Benzidine	...	1.0%	} in 96% alcohol
Acetic acid	...	2.0%	

To 50 ml. of this stock solution 1 ml. 20-volume hydrogen peroxide is added. After 24 hours the mixture is centrifuged.

Haematoxylin.

Harris haematoxylin	} equal volumes
Alcohol 70%	

Orange G.

Orange G ... 5% in 50% alcohol

Light Green.

Light green ... 0.2% in 96% alcohol

The Rate of Cell Loss into the Urine

Using the method described above, the rate of cell loss has been determined on 12 men of ages varying from 18 to 47, none of whom was aware of any previous history of kidney, bladder, or prostatic complaints or of macroscopic haematuria. Part or all of a two-hour sample was used, secreted during the period 7.30 a.m. to 9.30 a.m. in each case. Subjects were asked to ensure that the urine stream did not come into contact with the prepuce. Table I shows the rates of cell loss obtained.

TABLE I
RATE OF CELL LOSS IN THOUSANDS/HOUR

Subject No.	Squamous, Transitional, and Degenerate Cells	Small Epithelial Cells and Leucocytes	R.B.C.s (1,000/hour)
I	6.2	34.0	2.8
II	31.1	76.6	13.0
III	10.1	54.6	3.7
IV	33.0	177.1	1.5
V	14.8	46.7	0.1
VI	74.8	88.3	0.8
VII	148.9	165.0	3.7
VIII	31.6	62.3	1.1
IX	45.2	28.2	1.2
X	21.6	136.6	1.0
XI	17.3	56.6	0.1
XII	12.2	206.6	2.8

It appears from this limited series that the output of blood cells and exfoliation proceed at very different rates in different individuals. In the same individual, however, the output appears to be constant from day to day. The variation in total cell output as indicated by a repeat determination on another day on each of the 12 subjects was from 0.5 to 20.5%.

The results of a preliminary assessment of diurnal changes in the rate of cell loss in one individual is shown in Figs. 2a and 2b. The red cell output appears to be increasing with the day's activities. The peaks occurring in Fig. 2b for the period 6-8 a.m. might well be due to sloughing off of cells which have accumulated in the upper layers of the epithelium during the night.

The Normal Urine Cell Picture

Samples were obtained from the same subjects. All urines were produced between 7.30 a.m. and 9.30 a.m. By means of the technique described, the cells were separated from extraneous matter and segregated into two groups, those greater than about 15 μ diameter (mainly squamous and transitional) and those less than about 15 μ diameter.

Figs. 2a and 2b.—Diurnal changes in the rate of cell loss from the urogenital system. In this preliminary experiment the changes occurring in one individual on two days (broken line and continuous line) are indicated. Fig. 2a refers to red blood cells. Fig. 2b refers to large epithelial cells and to small epithelial cells and leucocytes.

FIG. 2a

FIG. 2b

They were then mounted and stained. Figs. 3 and 4 show typical fields of these large-cell and small-cell preparations.

The Large Cells.—Examples of the main types of cells to be found in the large-cell preparation are shown in Fig. 5. Table II shows that the proportions of the various kinds of large cells exfoliated vary greatly between individuals, although, so far as this has been studied, there appears to be remarkable consistency in the cell picture of urine from the same subject at different times.

The figures in Table II were obtained indirectly by carrying out differential counts on stained preparations of the large cells of normal male urine and applying these proportions to the directly obtained rate of large-cell loss for each individual given in Table I, column 2. About 250 cells were

FIG. 3.—A typical preparation of large epithelial cells from normal male urine. $\times 350$. Cells smaller than about $15\ \mu$ diameter have been removed by filtration.

counted in each case. The last two columns show the reaction to staining with 5% orange G followed by 0.2% light green.

The individuality of the cell picture is evident in one or more of the following: (i) the total quantity of cells present (see Table I); (ii) the occurrence of multinucleate cells (see Fig. 5); (iii) the relative proportions of squamous, transitional, "early degenerate," and "late degenerate" cells (see Fig. 5); and (iv) the reaction to staining with 5% orange G followed by 0.2% light green (see Table II).

All of these differences invite speculation, not least the reaction to orange G and light green. For example, Subject II shows 91.9% of the cells stained green, Subject XI shows 76.0% stained yellow. The cells which stain yellow are mainly the degenerated type: a count of 973 yellow-staining cells from 20 normal male subjects shows that 2.1% are squamous, 3.7% are transitional, 16.8% are "early degenerate" cells, and the remainder, namely 77.4%, are "late degenerate" cells. These figures are in accord with the preferential staining of cornified tissues by orange G. The

FIG. 4.—A typical preparation of small cells from normal male urine. $\times 1,750$. Cells larger than about $15\ \mu$ have been excluded by filtration. A clearly defined red blood cell is seen bottom left.

TABLE II
RATE OF LARGE CELL LOSS IN THOUSANDS/HOUR

Subject No.	Transitional	Squamous	Early Degenerate	Late Degenerate	Staining Reaction	
					% Green	% Yellow
I	1.9	1.1	1.7	1.5	67.7	32.2
II	11.2	12.1	6.3	1.5	91.9	8.1
III	2.2	1.1	1.7	5.1	41.2	58.8
IV	12.4	3.6	15.8	1.1	70.5	29.5
V	3.9	5.5	3.6	1.9	77.1	22.9
VI	10.5	5.0	30.0	29.2	61.0	39.0
VII	20.6	31.5	77.0	19.8	83.3	16.7
VIII	16.4	7.6	1.0	7.3	74.6	25.4
IX	26.2	3.5	4.0	11.3	79.6	20.4
X	9.3	1.6	2.2	8.6	52.9	47.1
XI	2.8	1.0	1.7	11.8	24.0	76.0
XII	4.1	1.3	0.7	6.0	43.5	56.5

common occurrence in some subjects of apparently undegenerate squamous and transitional cells which nevertheless give a strong yellow reaction could perhaps be taken to show that this reaction is a more sensitive indication of degenerative changes in these types of epithelium than structural criteria. A further point of interest is the occurrence of groups consisting of about six or more structurally identical cells which have developed together and peeled off en masse, in which one or two cells show a strong yellow reaction,

FIG. 5.—The cell types common to all preparations of the large cells of normal male urine. $\times 495$. (i) Squamous epithelial cells. (ii)a and (ii)b Transitional epithelial cells. (iii) "Early degenerate" cell: a squame in the early stages of degeneration: the nucleus is pyknotic. (iv) "Late degenerate" cell. The nucleus has disappeared and the cytoplasm has a crinkled appearance. Figures for the rates at which these cells are lost are given in Table II.

although all their fellows stain strongly green. It is tentatively suggested that there is a relation between the state of degeneration of the cells and their affinity for the yellow stain, but signs of degeneration may be difficult to recognize morphologically in some cases.

The Small Cells.—Examples of the main types of small cells are shown in Fig. 6. Table III gives some indication of the rates at which they are lost into the urine: it is evident that there is great variability between individuals.

FIG. 6.—The principal cell types common to all preparations of the small cells of normal male urine. $\times 1,550$. Evidence is submitted suggesting that type (i) originates in the kidney; (ii) is probably a degenerate form of one or more of the other types; (iii), (iv), and (v) represent comparatively well-defined examples of a group which includes numerous types intermediate to these; (vi) is a polymorphonuclear leucocyte. Figures for the rates at which these cells are lost are given in Table III.

The figures in Table III were obtained indirectly by carrying out differential counts on stained preparations of the small cells of normal male urine and applying these proportions to the directly obtained rate of small-cell loss for each individual given in Table I, column 3. The average number of cells counted in each case was 280. Rates for loss of red cells are repeated from Table I.

TABLE III
RATE OF SMALL CELL LOSS IN THOUSANDS/HOUR

Subject No.	Cell Types (Fig. 6)			Polymorphs	R.B.C.s
		ii	iii, iv, v		
I	3.6	17.1	13.3	—	2.8
II	5.1	55.8	15.8	—	13.0
III	5.5	17.2	21.0	10.8	3.7
IV	2.4	20.9	22.5	131.5	1.5
V	1.9	29.0	9.8	6.0	0.1
VI	6.1	32.0	32.0	18.9	0.8
VII	1.4	29.3	10.7	123.9	3.7
VIII	1.8	10.4	7.1	43.1	1.1
IX	0.6	15.5	8.9	3.1	1.2
X	5.4	43.9	57.5	29.8	1.0
XI	1.5	10.2	7.0	37.0	0.1
XII	8.8	53.0	38.8	106.0	2.8

FIG. 7a.—Application to cytological diagnosis. Urine from a case of carcinoma of the bladder. $\times 660$. A smear of the urinary sediment was prepared according to the standard method of Papanicolaou. Many fields showed no cells; this field showed the most cells out of 15 fields taken at random. For comparison with Fig. 7b.

The following criteria were used in counting. Cell type i: a very round granular nucleus and high nuclear/cytoplasmic ratio; cell type ii: no nucleus visible, granular cytoplasm, sometimes vacuolated. The cell types designated iii, iv, and v in Fig. 6 have not been found to be sufficiently characteristic to permit individual classification: they have therefore been counted together, along with the various intermediate types which occur.

Counts of cells of 8 to 30 μ diameter to be found in fresh urinary sediment have been carried out for many years by Addis (1948), who gives 650,000/24 hours as the normal rate of loss of epithelial and white blood cells, and assumes that most of the epithelial cells are derived from the kidney. In the present study a preliminary attempt has been made to indicate the origin of the cell types referred to above by making routine pre-

parations (Section II) of the following material (1) macerated normal human kidney tissue suspended in cell-free urine and set aside for an hour; (2) urine removed from a human ureter at operation; (3) normal female urines. In addition (4) smears were made of prostatic fluid of three subjects. In (1) and (2) most of the cells were similar to the cells referred to above as type i. A few of these were found in the female urine and none in the prostatic smears. It thus seemed reasonable to consider that the type i cells of urine have originated in the tubules of the kidney.

The type ii cells are very probably degenerative forms and are often found to be breaking up. The numbers in which they occur are not connected with deterioration of the urine sample, as when fresh samples are used the output is consistent from day to day at the level characteristic

FIG. 7b.—Application to cytological diagnosis. The same urine sample from a case of carcinoma of the bladder as shown in Fig. 7a $\times 660$. The urine was treated according to the short method advocated. A field taken at random.

for the individual. The fact that they hardly ever stain preferentially with orange G indicates that the cells of which they are degenerate forms do not originate from a region in which cornification occurs.

The invariable presence of red cells in all individuals is of interest (see Table III), particularly as the figures given represent the minimum number present. Ghosts and broken red cells were not included. All those counted were unmistakably identified by the benzidine reaction. The mode of entry of blood cells into urine presents an interesting problem. The greater ability of polymorphs to pass from the blood into urine is demonstrated by the change of the R.B.C./polymorph ratio from about 1,000/1 in blood to about 1/30 in urine.

Application to Cytological Diagnosis

Mention has been made of the ease with which the whole cellular contents of a sample of urine may be examined, and of the possibility of improved accuracy in diagnosis thus presented. Figs. 7a and 7b give some idea of the appearance of preparations made from the same sample by the smear technique and by the method described above.

It seems possible that the principle of fixation of cells followed by solution of extraneous material may be applied with advantage to other body fluids or washings used for cytological diagnosis. The use of mechanical segregation by means of a filter may also find application where it is desired to examine cells of a particular size occurring in small proportions, and would be of value where pus, blood, or bacteria are crowding out the cells under examination.

With regard to renal diagnosis, Foot and Papanicolaou (1949) have shown that single cells of the tubule epithelium become malignant, though their fellows appear normal and the lumen remains patent. If, as is now submitted, the cells of the small-cell preparation contain identifiable renal cells, there seems a reasonable chance that its use might enable early identification of malignancy in the kidney.

Summary

A method is described whereby the number and type of cells in a sample of urine may be accurately determined. The method depends upon the

solution and removal of mucus after the urine sediment has been treated with absolute alcohol. The cells may then be concentrated by centrifugation and counted by means of a standard blood counting chamber, or they may be segregated mechanically according to size and mounted and stained for microscopic examination.

The use of pectin as an adhesive is described, and staining procedures are given.

The method was used to investigate the rates of exfoliation of epithelial cells and of the loss of leucocytes and erythrocytes into the urine in 12 normal males. The results of a preliminary experiment on the diurnal changes in the rate of cell loss are also given.

An indication of the normal urinary cell picture has been established by differential counts on stained preparations of the cells from the same 12 subjects.

Evidence is submitted suggesting that the origin of certain of the small cells of the urinary sediment is the epithelium of the kidney.

The possible application of the method to cytological diagnosis is briefly discussed.

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