ABO (H) ISOANTIGENS IN BLADDER TUMOURS - A NEW TECHNIQUE OF QUANTITATIVE ANALYSIS

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ABO(H) ISOANTIGENS IN BLADDER TUMOURS - A NEW TECHNIQUE OF QUANTITATIVE ANALYSIS

by

© V. Srinivas, M.B.,B.S.

A Thesis submitted in partial fulfillment of the requirements

for the degree of Master of Science

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ABSTRACT

Bladder tumours are the second most common urological malignancy seen and twenty-five percent of all superficial tumours ultimately become invasive.

The Specific Red Cell Adherence Test (SRCA) appears most promising with a potential to predict which superficial bladder tumour(s) will ultimately become invasive.

The major drawbacks of the test in its present format are:

1. inability to quantitate the test.
2. variability of antigen expression in tissue sections.
3. weak reaction in blood group 'O' patients.

The objectives of this research project were:

1. to develop a new technique for quantitative analysis of ABO(H) isoantigens.
2. to improve the sensitivity of the test in blood group 'O' patients.

The project was carried out in three phases:

Phase 1: A new technique of obtaining viable single transitional cell suspensions from bladder biopsies with preservation of ABO(H) isoantigens was developed. Toluidine blue was used to confirm the presence of transitional cells and trypan blue stain to determine the viability and cell count. Cell separation was carried out successfully in 20 cases.

Phase 2: A modified SRCA test with final staining procedure was developed to quantitatively measure the ABO(H) isoantigens in the single cell suspension. Bombay blood which contains a high titre of

anti H antibodies was used successfully in blood group 'O' patients.

Phase 3: A double blind clinical study was carried out in a total of 15 patients; 8 with normal bladder and 7 with bladder tumour. The biopsies were subjected to cell separation and quantitative analysis as worked out in phases 1 and 2.

In the 8 patients with normal bladder, the mean antigen positive cell count was 88% and in the 7 patients with bladder tumour, the mean antigen positive cell count was 33%. The mean antigen positive counts between these two groups were significantly different at the $P < .001$ level.

A new technique for quantitative analysis of ABO(H) isoantigens in bladder tumours has been developed and this should help in standardizing the test and making it applicable in different prospective clinical trials.

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LIST OF ABBREVIATIONS

1.	SRCA	Specific red cell adherence
2.	HBSS	Hanks' balanced salt solution
3.	DNAse	Deoxyribonuclease
4.	FANFT	N - {4 - (5 - nitro-2-furyl)-2-thiazolyl} formamide
5.	AUM	Asymmetric unit membrane
6.	DNA	Deoxyribonucleic acid
7.	ELISA	Enzyme linked immunosorbent assay

BACKGROUND

Epidemiology of bladder tumours

Bladder cancer is a world wide problem and its incidence varies as much as tenfold in different countries and wide differences in incidence may occur even within a single country. In terms of incidence, bladder cancer annually affects 1676 Canadians (1) more than the number who develop leukemia or cancer of the stomach. Over fifty new cases of bladder tumour are seen in Newfoundland annually (1).

Bladder cancer annually accounts for 9000 deaths in the United States (2) and 900 deaths in Canada (3). This is similar to the number attributed to cancer of the cervix, rectum or ovary.

Bladder cancer is a disease of advanced age; currently in Canada the mean age at diagnosis is 68 years. However an appreciable number of persons under age 65 are also affected (1).

Epidemiological studies have contributed to the recognition of many possible or probable aetiological factors such as (4):

1. Occupation - chemicals, dye industry, leather processing
2. Habits - smoking, ? coffee drinking
3. Diet - bracken fern, nitrosamines, tryptophan metabolites, non-nutritive sweeteners
4. Medications - phenacetin
5. Chronic bladder irritation - bilharzial cystitis

Although bladder cancer may represent a spectrum of diseases with different causes, the complexity of the problem of causation

has been increased by evidence that bladder carcinogenesis may be a multi-stage process of initiating and promoting factors. Initiation may follow a single exposure to a carcinogen, at a dose level that is too low to induce manifest cancer; promotion may then follow a varying period of exposure to a non-carcinogenic agent. Initiation is regarded as irreversible, but promotion may be prevented or inhibited before the development of autonomous tumour. The implications of these concepts are considerable. Investigators have demonstrated apparent synergistic action of saccharin in the induction of bladder tumours in N-methyl - N-nitrosurea treated rats (5).

Diagnosis of bladder tumours

Bladder tumours are rarely identified as incidental findings in asymptomatic patients either on routine health examinations or autopsies. Bladder tumours commonly produce symptoms.

Gross painless haematuria as the presenting symptom is seen in a majority of patients. A smaller number have only symptoms of vesical irritability and occasionally a few patients present with progressive renal failure due to ureteral obstruction caused by infiltrative tumour (6). Intravenous pyelogram and a cystoscopic examination accompanied by a bladder biopsy will enable the diagnosis to be made in nearly all cases.

Classification of bladder tumours

Transitional cell carcinoma accounts for over 90% of all bladder tumours. In 1922 Broders first formalized the concept of grading bladder tumours by recognizing four grades of transitional cell growth that ranged from grade 1 carcinoma to grade 4. Grade 1 and 2 are often referred to as low grade and grade 3 and 4 as high grade lesions (7). The grade of a bladder tumour may be regarded as providing a visual estimate of the growth potential of the lesion, in that the probability of bladder wall invasion is proportionately greater with a higher tumour grade (8). Analysis of survival in terms of tumour grade generally demonstrates that the rates diminish as tumour grade increases regardless of the method of treatment. At present there is no uniformly accepted principle of grading and furthermore, different pathologists may interpret pathological material differently (4).

The formal clinical staging of bladder tumours began with the work of Jewett and Strong in 1946 (9) and at present, although there is no universal agreement, three main staging classifications are in use:

1. Jewett-Strong Marshall classification (9).
2. The International Union Against Cancer developed the TNM staging system (10).
3. Whitmore classification (11).

Table No. 1 illustrates these classifications.

TABLE NO. 1

STAGING CLASSIFICATION OF BLADDER TUMOURS

DEPTH OF INVASION	JEWETT-STRONG-MARSHALL CLASSIFICATION	UICC	WHITMORE
In situ (mucosa)	0	TIS	Superficial
Submucosal	A	T1	Superficial
Superficial muscle	B1	T2	Superficial
Deep muscle	B2	T3	Deep
Perivesical	C	T3	Deep
Pelvic fixation or invading adjacent organs	D1	T4 N1-N4	Metastatic
Distant metastasis	D2	M1	Metastatic

Key: T - tumour

N - lymph node

M - metastasis

The stage of a bladder tumour may be regarded as an indication of the extent to which the growth potential of a particular lesion has been expressed at the time of diagnosis. The tendency for urothelial tumours to be multicentric in time and space has long been recognized. No practical system for classifying bladder tumours in relation to multicentricity has yet been proposed. Such a system should ideally incorporate some indication of the rate at which new bladder tumours develop and the possibility of extravesical urothelial tumours. Clinical recognition of the problem of multicentricity in time and space is reflected in the increasing use of random biopsy sampling of the apparently uninvolved bladder wall, which is done at the time of biopsy of existing tumour(s) in an effort to define pre-neoplastic or early neoplastic changes.

Treatment and prognosis

The local and regional treatment of bladder tumours has included an array of surgical and radiotherapeutic procedures. Conservative treatment is defined as treatment limited to a part or parts of the bladder and radical or aggressive treatment as that which is directed to the entire bladder (4).

The management of bladder tumours entails two components -- the existing tumour(s) and new tumour formation. Seventy percent (70%) of all superficial bladder tumours recur and in roughly a third of these cases the tumour ultimately becomes more aggressive and invasive (12).

Superficial tumours are treated by local transurethral resection with 5 year survival rates of roughly 75% (13). However once muscle invasion occurs, the prognosis becomes poor and combined irradiation with radical surgery results in 5 year survival rates of 30 - 40% (14).

At present radical surgery is deferred until muscle invasion occurs. This accounts for the low survival rates since widespread micrometastasis invariably occurs during the interval from initial diagnosis to muscle invasion.

Identification of bladder cancer patients with initially superficial tumours who later will suffer invasive or metastatic disease is a major problem confronting urologists. New tumour formation is an ever-present risk in the bladder tumour patient as long as the bladder remains. If the nature (grade, stage and multicentricity) of the next tumour and the interval to its occurrence could be predicted, criteria for characterization of the "malignant bladder" and for "prophylactic cystectomy" could be established. Unfortunately such predictive capabilities currently do not exist. However measurement of ABO(H) cell surface isoantigens in bladder tumours appears most promising with a potential to fulfill this need.

Cell membrane alterations in bladder tumours

The surface cell membrane relates directly to physiologic functions such as cell-growth, division, movement, immunologic responsiveness and other properties. Comparisons of surface properties between normal and transformed cells have been compiled in many systems and the observed changes in surface membrane components seem to correlate well with onset and progression of tumours (15).

The carcinogen N (4 -(5-Nitro-2-furyl) - 2 -thiazolyl) formamide (FANFT) selectively produces urothelial tumours in the urinary bladder of rats (16). Thus the FANFT model of urinary bladder tumourigenesis is felt to be particularly well suited for studies of the relationship of cell surface alterations to tumour invasiveness.

The normal mammalian urinary bladder is lined by a highly differentiated epithelium. The plasma membrane at the luminal surface of the superficial cells seems morphologically unique (17). The luminal surface membrane takes on a scalloped appearance because of the alternating pattern of rigid concave plaque regions and flexible, hinge or interplaque regions. The plaque regions which constitute approximately 73% of the surface area show a thickened asymmetric unit membrane (AUM) structure in thin section and exhibit a highly ordered particulate substructure in stained preparations. In contrast the particle-free inter plaque regions show a regular symmetric unit membrane structure in thin section (18). The plaque membrane structure is asymmetric because the luminal leaflet measures approximately twice that of the cytoplasmic leaflet.

Whenever the urothelium is subjected to either mechanical or cytotoxic (FANFT) damage, the epithelium becomes hyperplastic and is composed of many layers of undifferentiated cells. The luminal surface reveals an undistinguished plasma membrane which is no longer differentiated into plaque and inter plaque regions and which no longer exhibits regions of asymmetric unit membrane structure. In benign conditions regression of hyperplasia with reversibility of the morphologic specialization within the luminal plasma membrane occurs whereas in malignant tumours this process is irreversible (19). Thus a positive correlation was observed between loss of asymmetric unit membrane of superficial cells and the onset of abnormality in the bladder.

Recent analysis suggest that the plaque particle is a glycoprotein complex. Although the function of these plaque particle glycoproteins along the plasma membrane surface is unknown at present, it is known that one of the first signs of bladder abnormality to be expressed is lack of asymmetric unit membrane specialization. This could be attributed to a lack of synthesis of the plaque particle-glycoprotein complex or a cellular deficiency in organizing the particle subunits into the correct configuration (20).

The importance of the cell surface in certain neoplastic phenomena is relatively indisputable and a study of cell surface molecules might help in understanding the behaviour of malignant cells. Thus one of the most important aspects of research into bladder cancer, is the persistent search for markers -- morphological, biochemical or

immunological-accompanying very early transformations of normal cells into neoplasia (21). The measurement of blood group ABO(H) isoantigens, which are also glycoproteins, could prove to be a very useful marker during these changes.

Characteristics of the A-B-O blood groups

Karl Landsteiner in 1900 identified the blood groups A-B-O and initiated methods of examination that are still in use:

Blood groups 'A' and 'B' are characterized by the presence of agglutinogen A and B on the red cell surface and this is determined by a corresponding gene. Blood group 'O' is characterized not merely by the absence of agglutinogen A and B but also by the regular presence of Specificity H. Specificity H is due to substance H which is distinct from A and B substances but similar in chemical structure. Specificity H occurs in unequal amounts in the red cells of the various A-B-O blood groups; the cells of group O have the highest degree of H reactivity.

H and A-B develop in parallel but the genes that determine H substance and those that determine A and B agglutinogens apparently compete for the same substrate from which the substances are formed (22).

Most epithelial and endothelial cell surfaces contain A-B-O(H) substances which in these locations are referred to as isoantigens. The isoantigens are not present in nerves, bone and cartilage.

The A-B-H blood group substances are glycoproteins with a molecular weight ranging from 300,000 to 1,000,000. The substances are composed of about 85% carbohydrates and 15% aminoacids. The carbohydrate moiety is composed of five sugars:

1. L - fucose
2. D - galactose
3. N - acetyl - D - glucosamine
4. N - acetyl - D - galactosamine
5. N - acetyl neuraminic acid (sialic acid)

These five sugars are arranged in a large number of fairly short chains attached by a co-valent linkage to a peptide backbone composed of 15 aminoacids of which threonine, serine, proline and alanine compose two-thirds (23).

The determinant chemical groups on the antigen molecule responsible for their blood specificities are:

1. N - acetyl - D - galactosamine for specificity A
2. D - galactose for specificity B
3. L - fucose for specificity H

Table No. 2 illustrates this structure. (page 14)

Reagents having anti-H specificity are most easily derived from extracts of seeds, especially the seeds of Ulex europeus, and instead of being called anti-serum, these anti-H reacting extracts are termed lectins. Lectins are a class of proteins which bind to carbohydrate residues on cell surface membranes and individual lectins having narrow ranges of action are used to determine glycoproteins on cell surfaces. In most centres, anti-H (Ulex europeus) lectin is used to perform immunological tests on 'O' group patients; however the anti-H from this source is weak.

In 1952, Bhende reported on a rare type of blood group found in Bombay. This rare gene, if homozygous, can prevent the expression of the A-B-O blood group antigens on the red blood cells. Whereas individuals with Bombay blood type have normal A-B-O genes, they are also homozygous for the inhibitor gene that prevents the ABO genes from placing their respective antigens onto the red cells. Thus the

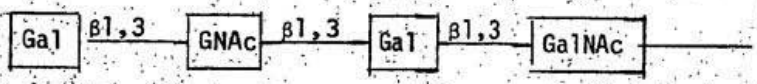
serum of these people contains a high titre of anti-H antibodies as well as anti-A and anti-B (24).

Bombay blood is extremely rare and although more than a million donors were screened in England, not a single sample was found (23). A family in St. John's, Newfoundland has been found to have this rare blood group and we have therefore used Bombay blood from this family in our study.

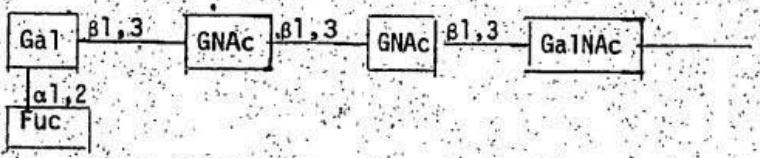
TABLE 2

CHEMICAL STRUCTURE OF THE DETERMINANT GROUPS OF THE A-B-H BLOOD GROUP SUBSTANCES

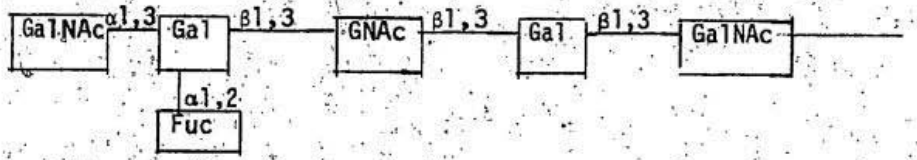
Precursor



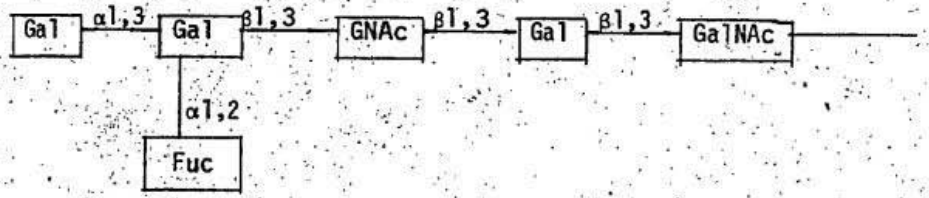
H substance



A substance



B substance



Key: Gal = D-galactose

GalNAc = N-acetyl-D-galactosamine

Fuc = L-fucose

GNAc = N-acetyl-D-glucosamine

α1,2 = Alpha 1,2 linkage

β1,3 = Beta 1,3 linkage

Specific Red Cell Adherence Test

Coombs in 1956 described the mixed cell agglutination reaction for examining the presence of A and B isoantigens on epidermal cells (25). The test was subsequently modified slightly and referred to as the specific red cell adherence test (SRCA) (26). The SRCA test was used to test the isoantigens in tissue sections from carcinoma of the cervix (26,27) where it was found that the cells lose their isoantigens as they undergo changes from cellular atypia to anaplasia.

Encouraging results utilizing the specific red cell adherence test in bladder tumours began appearing in the urological literature in 1975 (28). Since then numerous retrospective studies have been carried out on paraffin embedded tissue blocks of patients with bladder tumours who have had a 5 year follow up (29, 30, 31). It was found that in over 80% of superficial tumours that ultimately became invasive the isoantigens were absent at the time of initial diagnosis while the tumour was still superficial. On the other hand only 10% of superficial tumours that remained superficial lost their isoantigens (32).

The principle of this test is that bladder epithelial cells possessing the respective isoantigen should adsorb the appropriate anti sera (antibody) and consequently, after being washed, possess free extending "receptors" with affinity for the respective antigen. Red blood cells of the same blood group added at this stage should combine with the extending antibody receptors on the treated epithelial cells and give rise to red cell adherence (25) (Figure 1). Conversely if the epithelial cells have lost their isoantigen or if antiserum of a different blood group is used, red cell adherence will not occur.

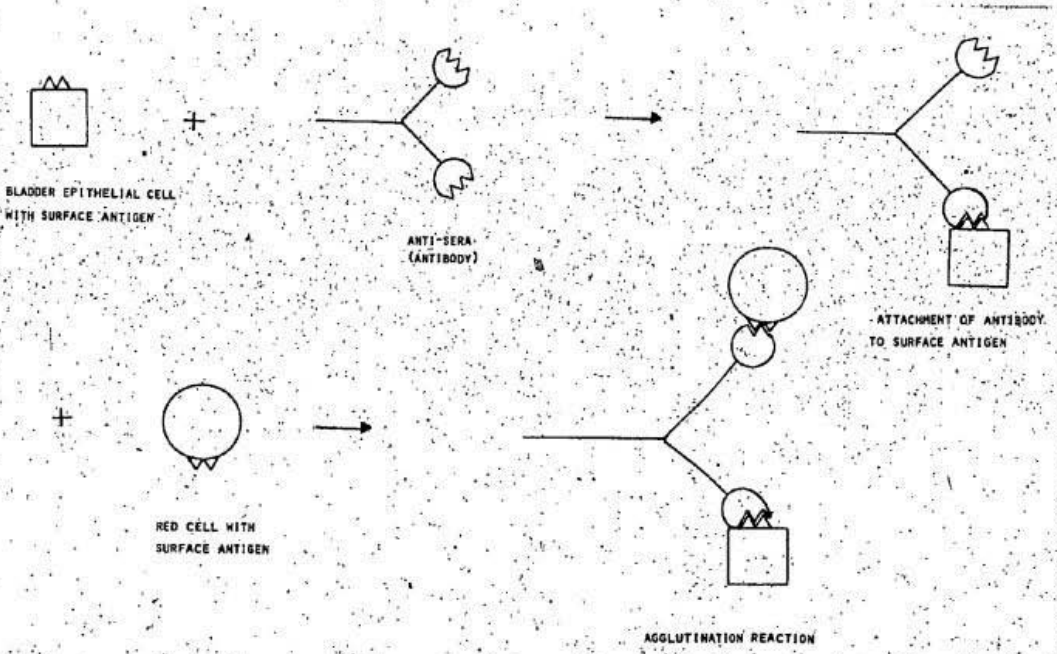


Figure 1

Principle of the SRCA test.

The specific red cell adherence (SRCA) has been used to detect blood group A, B and O(H) isoantigens on the cell surface of various tumours. Investigators have reported the reliability of SRCA in detecting the anaplastic potential of cancer of the lung, cervix, pancreas and stomach (26, 27, 33, 34). Other investigators have applied this principle to bladder carcinomas also and reported a correlation between the presence or absence of cell surface isoantigens and prognosis in superficial bladder tumours (28, 29, 30, 31). The basis for this test is that cell surface isoantigens, normally expressed on epithelial cells, are not expressed on cells of most bladder tumours that have a high malignant potential, even those that are histologically well differentiated and superficial initially.

Clinical studies (35, 36, 37) reveal that most high grade, invasive tumours are antigen negative, while superficial, low grade tumours are either antigen positive or antigen negative. Approximately 80 percent of patients with antigen negative, low grade, superficial tumours subsequently have invasive lesions while only about 10 percent with antigen positive superficial tumours do so. False positive tests have been encountered in patients having prior radiotherapy (31) and false negative tests have occurred in patients with blood group 'O'(30), presumably due to the weakness of H antigen expressed on 'O' group cells and the use of Ulex europeus anti-H extract to perform the test in 'O' group patients.

SRCA test

At present the SRCA test is performed (30) by incubating deparaffinised 5 micron thick bladder biopsy blocks with appropriate antisera (anti A, anti B, Ulex europeus anti H) in moist petri dishes for one hour. Excess reagent is then removed by washing the slides twice, for 10 minutes each time, in tris-buffer saline, pH 7.4. The slides are then incubated with red blood cells of the same blood group for 15 minutes. The slides are then inverted carefully on a wooden applicator in petri dishes filled with enough buffered saline to cover the inverted surface of the slide. After 10 minutes the slides are moved gently to a clear area of the petri dish and are examined and photographed while inverted in saline (Figure 1)(page 16).

The intensity of red cell adherence to the epithelial cells is graded from 0 to 4 plus and this indirectly reflects the amount of ABO(H) isoantigen present in the biopsy specimens.

Antiserum of a different blood group is used in the control slides. For example in blood group A patients, anti-B antisera is used.

Drawbacks of SRCA

The major drawbacks of the SRCA test in its present format are:

1. Inability to quantitate the test
2. Variability of isoantigen expression in tissue sections
3. Weak reaction in blood group O(H)
4. Difficulty in maintaining permanent records

1. Inability to quantitate the test

At present the SRCA test is interpreted by grading the red cell adherence from 0 to 4 plus. This is based on the intensity of red cell adherence to the epithelial surface. Thus subjective variation in interpreting and reporting the adherence is likely and a standardized result cannot be obtained from different laboratories. Moreover in some patients the red cell adherence is graded from 1+ to 3+ and it is not possible to categorize this group as positive or negative.

2. Isoantigen expression in tissue sections

Since fixed tissue sections are being used, variability in the method of fixation will reflect in altered isoantigen preservation. It is only the epithelial cell layer, which is being tested for isoantigens but a full thickness biopsy is being used. Since the epithelial cells are attached to each other, the third surface which may also contain isoantigen is not available to take part in this reaction.

3. Weak reaction in blood group 'O'

In blood group 'O' patients a large number of false negative reactions occur (38, 39) because of the weak anti-H expression of Ulex

europeus extract. Since 45% of the population is blood group 'O' this will create a problem.

4. Difficulty in maintaining permanent records

After completion of the test, the slides are interpreted while still inverted over saline in petri dishes. Photographs are occasionally taken to maintain permanent records. Thus a routine technique of maintaining permanent records which will enable the slides to be cross-checked by independent investigators is not available.

OBJECTIVES

The main objectives of the research project were to:

1. Develop a new technique for quantitative analysis of ABO(H) isoantigens in bladder tumours. This would help in standardizing the test and making it applicable in different prospective clinical trials.

2. Improve the sensitivity of the test in 'O' group patients so that the final results could be interpreted uniformly in all blood groups.

3. Improve the technique of final interpretation so that the slides could be maintained as permanent records and cross-checked by independent investigators.

RESEARCH PLAN

The research plan was divided into three phases:

Phase 1: to develop a technique of obtaining a high yield of viable solitary transitional epithelial cells from bladder mucosal biopsy specimens.

Phase 2: to develop an immunological technique for quantitative analysis of ABO(H) isoantigens in these single cell suspensions. To utilize a final staining technique, after completion of the test, to enable permanent records to be maintained.

Phase 3: to conduct a double blind clinical study, using this new technique for quantitative analysis, in a total of 15 patients with normal bladder and bladder tumour.

PHASE 1

PREPARATION OF SINGLE-CELL SUSPENSION

Materials and method

Six bladder specimens were obtained from human autopsies performed four to eight hours after death. Three fresh dog bladders were then used and finally twenty fresh normal human bladder biopsy specimens were obtained using a Storz biopsy forceps during trans-urethral surgery. The specimens were transported in cold Hanks' balanced salt solution (HBSS) (Flow laboratoreis # 18-100-54) and cell separation was done immediately in the following manner:

(1) The bladder mucosal specimens were weighed and incubated in a metabolic shaker for 10 minutes at 37°C. with 10 ml. HBSS containing 0.15% collagenase (collagenase type IA - Sigma # C0891).

(2) The supernatant, containing blood cells, was discarded and the bladder fragments re-suspended in 10 ml. HBSS containing 0.15% collagenase and 133 U DNAse/ml (DNAse I Type 1 - Sigma # D4763). Incubation was continued with gentle stirring at 37°C. for 30 minutes.

(3) This procedure was repeated twice except that shearing forces were applied every 10 minutes. This was accomplished by drawing the suspension of bladder fragments into a 10 ml. plastic syringe fitted with a 3 mm. polyethylene tube.

(4) Supernatant fluid of the third harvest containing the isolated epithelial cells was centrifuged at 1000 G for 10 minutes.

(5) The pellet was then resuspended in 2 ml. HBSS and processed for microscopy and immunological analysis.

Staining technique

The presence of transitional cells was confirmed by a standard-toluidine blue staining technique (40):

(1) A drop of cell suspension was allowed to air dry on a slide.

(2) 1 - 2 drops of toluidine blue was added onto the slide (Fischer Scientific # T-161).

(3) After 30 seconds the slide was washed with water.

(4) 1 drop of permount mounting medium was put on the slide.

(5) Cover slip was placed on the slide.

(6) Slide was examined to confirm the presence of solitary transitional epithelial cells.

Calculation of yield and viability

The percentage of viable cells was determined by trypan blue staining (41) and the yield calculated in a haemocytometer (40):

(1) Final cell harvest was diluted with 2 ml. HBSS.

(2) Cell suspension solution was mixed with 0.4% Trypan blue stain in the ratio of 4:1 (Grand Island Biological Company # 525).

(3) The mixed solution was allowed to stand at room temperature for 7 minutes.

(4) A haemocytometer was used to count the number of viable and non viable cells. Trypan blue penetrates only those cells whose membranes are damaged, thus all the cells stained blue were non-viable and the unstained cells viable.

(5) The total yield of cells with percentage viability was then calculated from this count.

Results

Cell separation could not be achieved in the initial six experiments using human autopsy specimens. The doses of various reagents were altered and the period of incubation was also changed but this did not alter the results. It was then concluded that in these autopsy specimens, the bladder epithelial cells were probably being autolysed in the interval from death.

Cell separation worked well in the 3 fresh dog bladder specimens.

In the twenty experiments carried out on fresh human bladder biopsies, separation of epithelial cells was accomplished as confirmed by toluidine blue staining (Figure 2).

The yield of epithelial cells from approximately 100 mg. of tissue was of the order of nine million cells; the cell suspensions used for testing had a concentration of 4.53×10^6/ml (standard deviation $\pm 0.86 \times 10^6$). Eighty-four percent of these cells were viable in the trypan blue test.

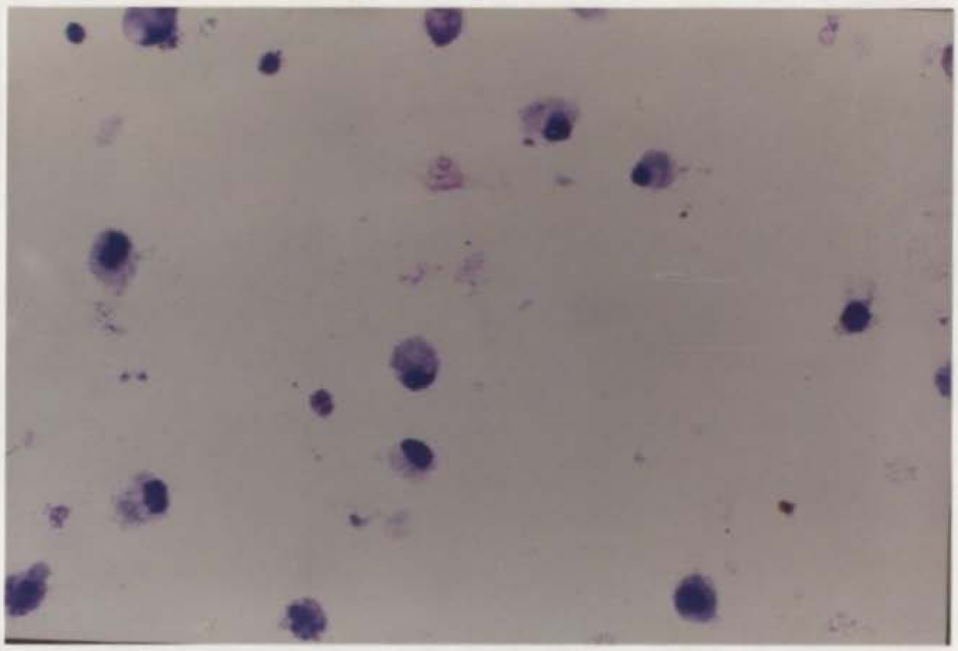


Figure 2

Solitary transitional epithelial cells stained with toluidine blue (x 250).

PHASE 2

IMMUNOLOGICAL TECHNIQUE FOR QUANTITATIVE ANALYSIS

Materials and methods

A modified specific red cell adherence test was performed on the single cell suspension. The technique was similar to that described previously (30) except that:

(1) solitary transitional epithelial cells were used instead of tissue paraffin sections.

(2) Bombay blood anti-H antisera was used in blood group 'O' patients instead of Ulex europeus extract.

(3) A final staining technique was used to facilitate counting and maintenance of permanent records instead of examining the wet preparations.

Biopsies were obtained from 10 patients of different blood groups and single cell suspensions were prepared utilizing the technique developed in phase I. The modified SRCA test was then performed.

(1) Slides with a drop of cell suspension were allowed to air dry overnight.

(2) Slides were covered with appropriate antisera - Anti A, Anti B (Ortho Diagnostics), anti-H (Bombay blood); and incubated in moist petri dishes at room temperature for 60 minutes.

(3) Excess antibody was removed by washing the slides twice, for 10 minutes each time, with tris-buffer saline pH 7.4.

(4) Excess saline was wiped off and the slides were incubated at room temperature for 15 minutes with red blood cells of the same group.

(5) Slides were then inverted carefully on a wooden applicator in a petri dish filled with enough buffered saline to cover the inverted surface of the slides.

(6) The slides were left like this for 10 minutes to enable the unattached red blood cells to precipitate to the bottom of the dish.

(7) The slides were examined in this position, while still wet, to confirm the presence of red cell adherence.

(8) Slides with antisera of a different blood group were used as controls in each case.

Numerous fixation and staining techniques were then tried in an attempt to develop the best technique to fix the red cells onto the slide and at the same time maintain the red cell adherence to the antigen-antibody complexes on the solitary epithelial cells. This would enable a correct count to be obtained and maintenance of these slides as permanent records.

<u>1st. staining technique</u>

(1) The slides were air dried.

(2) The slides were dipped in stock Giemsa, diluted 1:20, for 20 minutes (Fishcer Scientific # SO-G-28).

(3) The slides were rinsed briefly in buffer pH 6.86 (Fischer Scientific # B-78).

(4) The slides were air dried.

(5) 1 drop permount was placed on the slides.

(6) Cover slip was placed on the slides and the slides examined.

2nd. staining technique

(1) The slides were air dried.

(2) The slides were fixed in methanol for 5 minutes and dried.

(3) Giemsa staining was carried out as above.

3rd. staining technique

(1) The slides were air dried.

(2) The slides were covered with 40 drops Wright's stain solution (Fischer Scientific # SO-W-16).

(3) After 3 minutes, 20 drops Giordano buffer (Fischer Scientific # SO-B-122) pH 6.4 was added onto the slides.

(4) The solutions were gently mixed by blowing on the slides.

(5) After 6 minutes, the stain was washed off the slides with distilled water.

(6) The slides were air dried.

(7) 1 drop permount (Fischer Scientific # SO-P-15) was placed on the slides.

(8) Cover slip was placed on the slides and the slides examined.

4th staining technique

(1) The slides were air dried.

(2) The slides were fixed by dipping in methanol for 5 seconds.

(3) The slides were air dried.

(4) Wright's staining was carried out as above.

5th staining technique

(1) The slides were dipped in methanol while still wet.

(2) The slides were then air dried.

(3) Wright's staining was carried out as above.

6th staining technique

(1) The slides while still wet were sprayed with cytoprep fixative (Fischer Scientific # 12-570-10) by holding the nozzle 5 - 6 inches from the slide surface. With a continuous flow, the spray was moved from one end of the slide to the other.

(2) The cytoprep fixative was allowed 5 minutes to dry and Wright's staining was carried out as above.

7th staining technique

(1) The slides were air dried very quickly by directing the air current from a hair dryer onto the surface of the slide.

(2) The slides were fixed by dipping in methanol.

(3) The slides were air dried.

(4) Wright's staining was carried out as above.

8th staining technique

(1) The slides were dried very quickly by heating the undersurface of the slide.

(2) The slides were fixed by dipping in methanol.

(3) The slides were air dried.

(4) Wright's staining was carried out as above.

Results

The modified SRCA test carried out on single-cell suspensions showed red cell adherence when the wet preparations were viewed.

With blood group O(H) samples, Bombay blood anti-H antisera gave satisfactory positive results. However, the first seven fixation and staining techniques did not preserve the red blood cells or maintain the red cell adherence to the antigen antibody complexes on the epithelial cells.

1st. staining technique: red blood cells were washed off the slide.

2nd., 3rd. and 4th. staining techniques: haemolysis of the red blood cells occurred.

5th. staining technique: the red blood cells were washed off the slides.

6th. staining technique: very few red blood cells were preserved; the majority were haemolysed.

7th. staining technique: a few red blood cells were preserved but many were haemolysed. The red cell adherence to the antigen-antibody complexes on the epithelial cells was greatly distorted.

8th. staining technique: this was suitable for fixing the red cells onto the slides and for maintaining the red cell adherence to the antigen-antibody complexes on the epithelial cells. The only drawback in this technique was that the histological morphology of some of the epithelial

cells became distorted; but even in the cells that were morphologically distorted, the surface isoantigen was well preserved as evidenced by red cell adherence.

The final Wright's stain in this technique differentially stained the epithelial cells and the red cells. This allowed those epithelial cells with red cells adherent to their surface to be classified as antigen positive (Figure 3) and those epithelial cells without any red cells adherent to their surface as antigen negative (Figure 4). The exact criteria for classifying a cell as antigen positive is discussed later.

Thus it was possible to differentially count the antigen positive and negative cells and at the same time maintain permanent records.

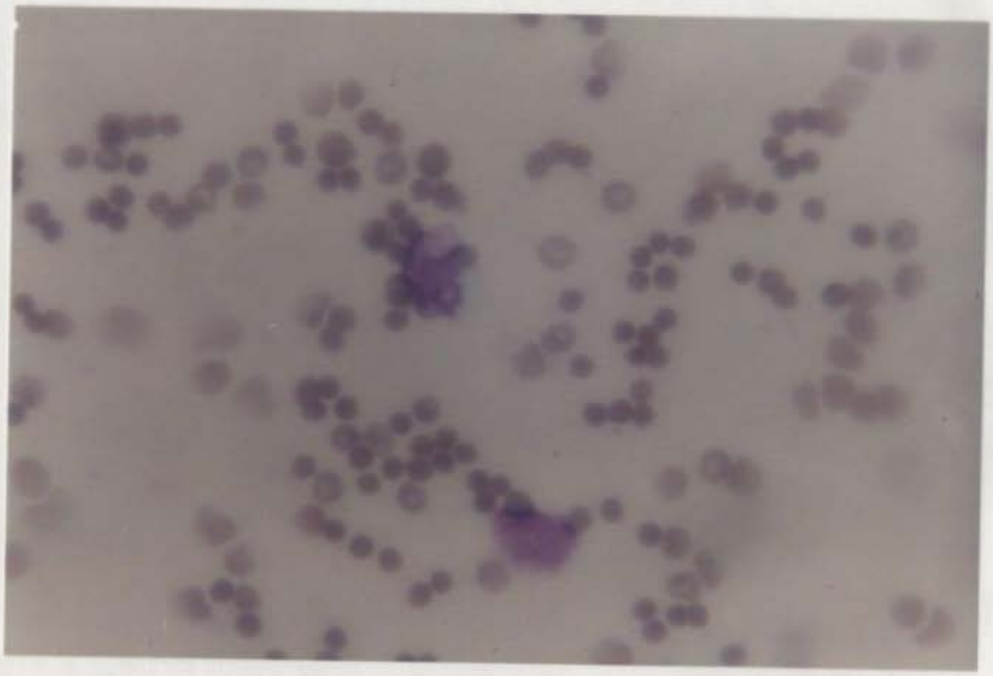


Figure 3

Antigen positive epithelial cells demonstrating red cell adherence (Wright's stain x 400).

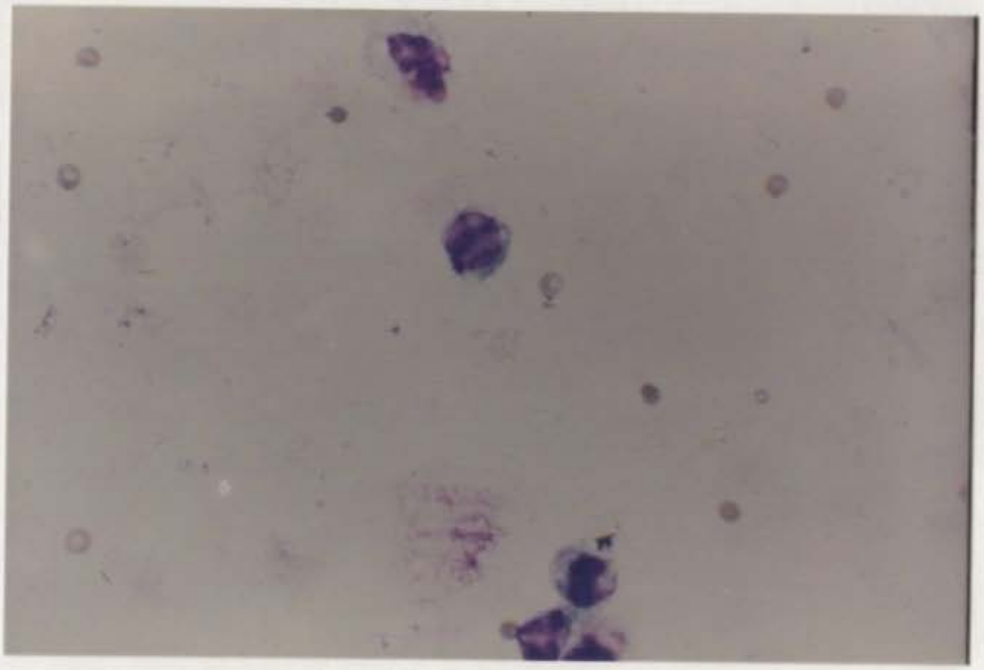


Figure 4

Antigen negative epithelial cells demonstrating no red cell adherence (Wright's stain x 400)

PHASE 3

DOUBLE-BLIND CLINICAL STUDY

Materials and methods

A double-blind clinical study was carried out on a total of 15 patients with normal bladder and bladder tumour. The study was double-blind in that the histology of the specimen was not known while counting the antigen positive cell counts; and the pathologist who finally examined the histology did not know the results of the antigen positive cell counts.

The biopsies obtained from these patients were subjected to single cell separation as developed in phase 1.

The following protocol was then followed:

Protocol

A drop of single cell suspension was smeared on four slides.

Slide # 1: This slide was stained with toluidine blue to confirm the presence of transitional epithelial cells. This showed that similar transitional cells were present on the other 3 slides.

Slide # 2: This slide was used as a control for the SRCA test by using antiserum of a different blood group.

Slides # 3 and 4:

The SRCA test was performed in duplicate on these 2 slides.

After completion of the SRCA, the final fixation and staining technique as developed in phase 2 was used. This consisted of heating, methanol fixation and Wright's staining. The cell counts were then carried out.

Cell count

(1) In each slide 200 epithelial cells were counted.

(2) The number of red cells adherent to each of these epithelial cells was recorded.

(3) All those epithelial cells with one or more red cells adherent to their surface were counted as antigen positive cells.

(4) The mean antigen positive cell count from the two test slides was calculated.

(5) The mean antigen positive cell count was expressed as a percent.

(6) In each case, graphs were plotted demonstrating the individual number of red cells adherent to each of these 200 epithelial cells. The epithelial cell count is on the ordinate and the red cell adherence on the abscissa.

After completion of the trial, the histology in these 15 cases was interpreted independently by a pathologist and the results correlated.

Results

A representative sample of patients belonging to different blood groups and different histological categories illustrates the final outcome of this new technique of quantitative analysis.

Patient no. 1

Slide no. 1

Solitary transitional epithelial cells stained with toluidine blue (Figure 5).

Slide no. 2

SRCA control slide showing solitary epithelial cells with no adherent red cells(Figure 6).

Slide no. 3

SRCA test slide showing solitary epithelial cells with adherent red cells (Figure 7).

Graph of the two antigen positive counts from slide no. 3 and slide no. 4. This graph illustrates the individual number of red cells adherent to each of the 200 epithelial cells (Figure 8).

Final antigen positive count -93%

Blood group -A positive

A

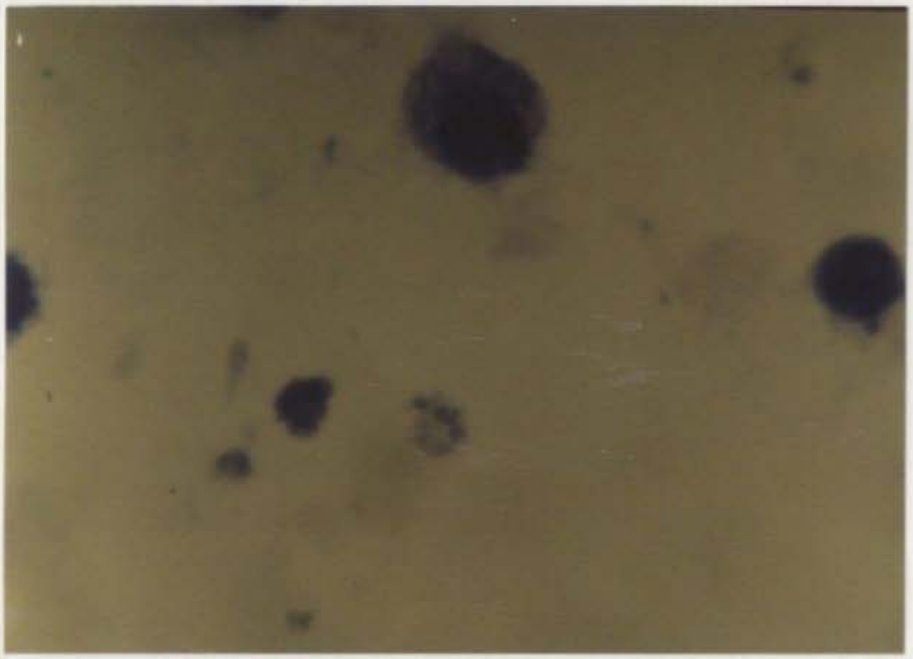


Figure 5

Solitary epithelial cells from patient no. 1 stained with toluidine blue (x 400).

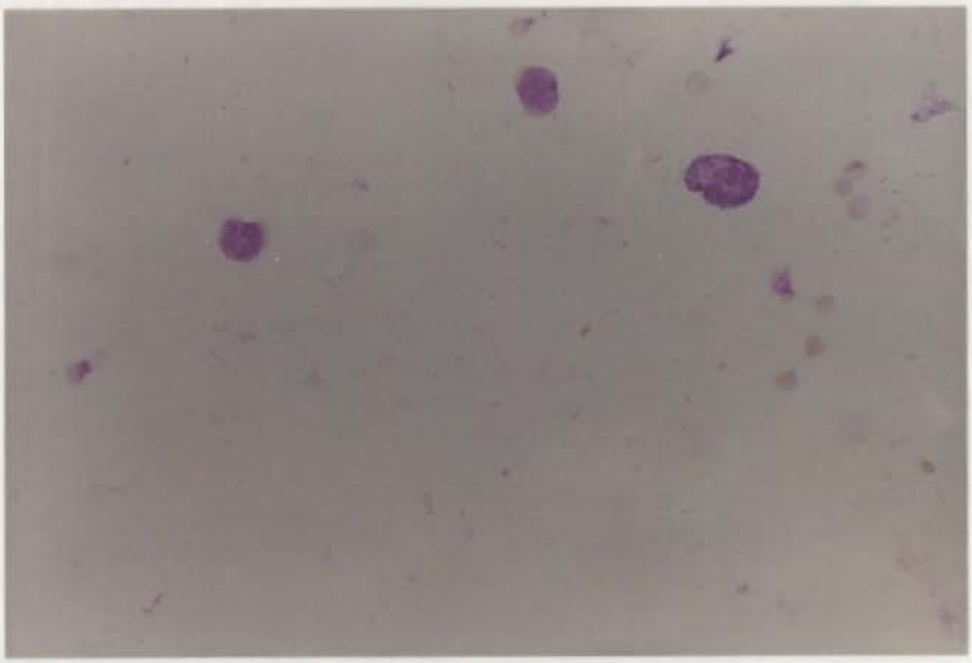


Figure 6

SRCA control slide from patient no. 1 stained with Wright's stain (x 250).

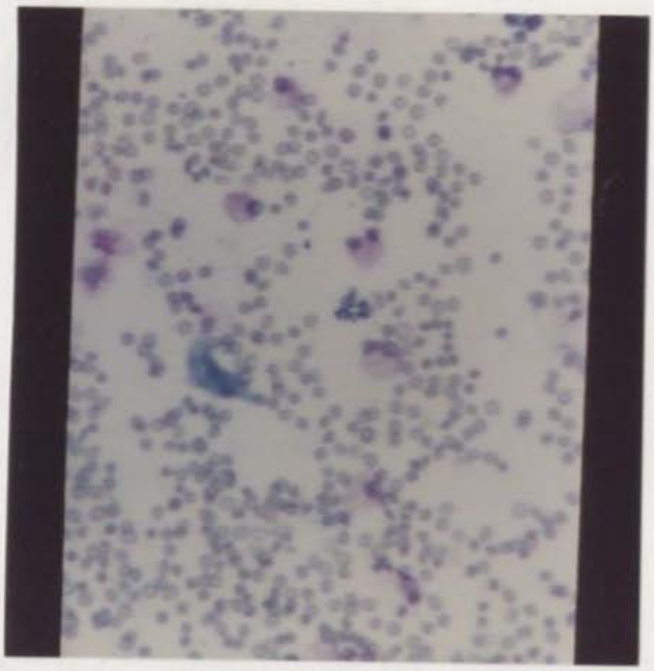


Figure 7

SRCA test slide from patient no. 1 stained with Wright's stain (x 250).

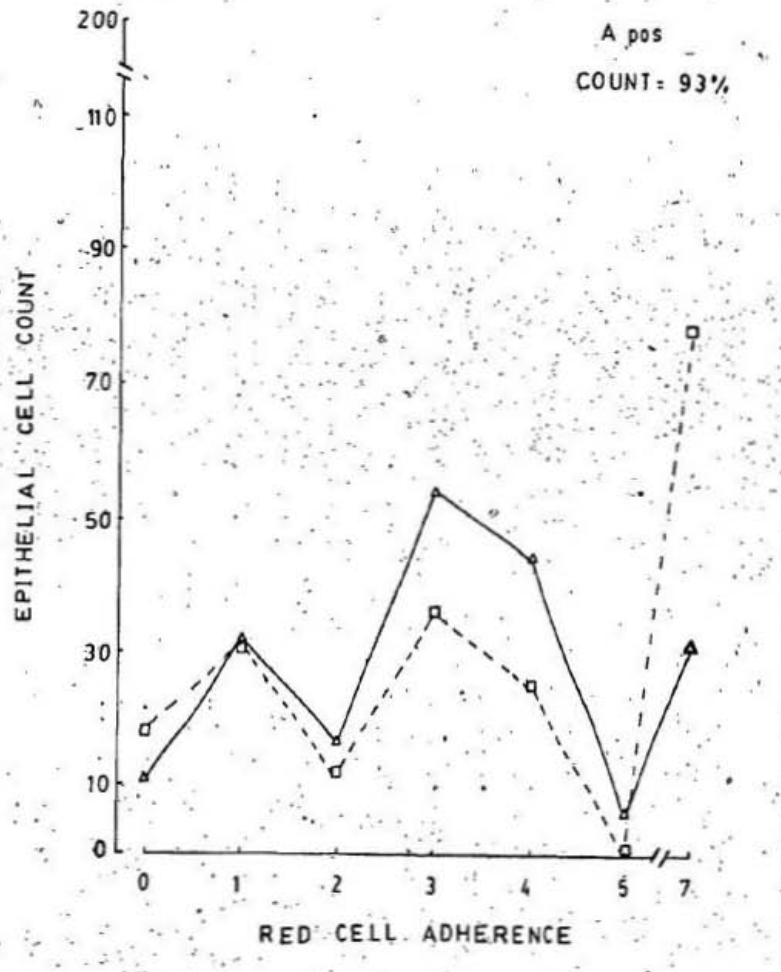


Figure 8

Graph of antigen positive cell count.

Key: The two duplicate counts from slide 3 and slide 4 are shown by Δ——Δ and □- - - -□

Patient no. 2

Slide no. 1

Solitary transitional cells stained with toluidine blue (Figure 9).

Slide no. 2

SRCA control slide - solitary epithelial cells with no adherent red cells (Figure 10).

Slide no. 3

SRCA test slide - solitary epithelial cells with adherent red cells (Figure 11).

Graph of antigen positive cell count shows a 95% positive count in a blood group 'O' patient (Figure 12).

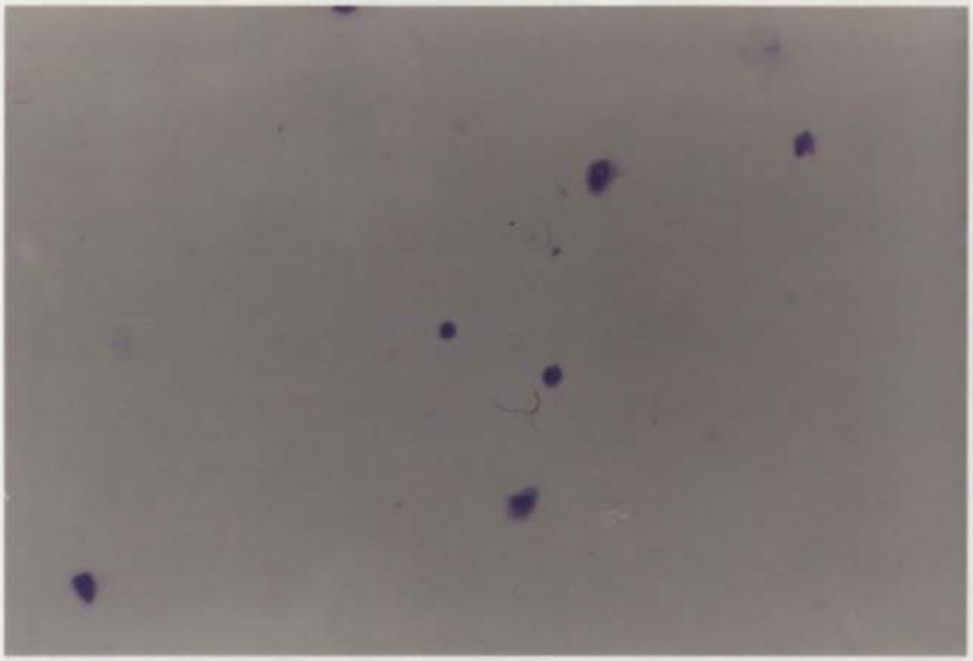


Figure 9

Solitary epithelial cells from patient no. 2 stained with toluidine blue (x 250).

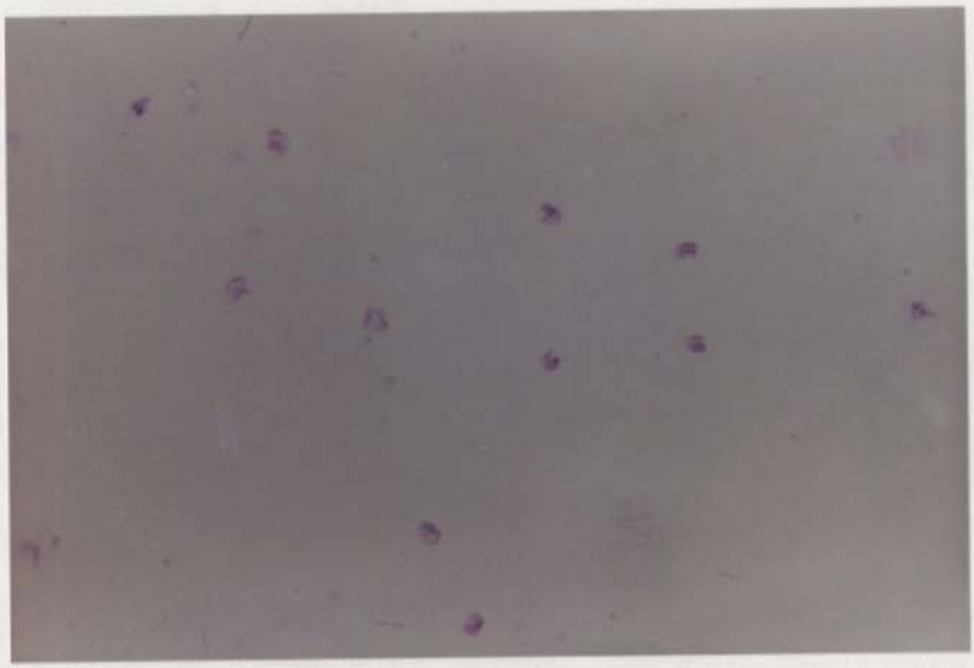


Figure 10

SRCA control slide from patient no. 2 stained with Wright's stain (x 250).

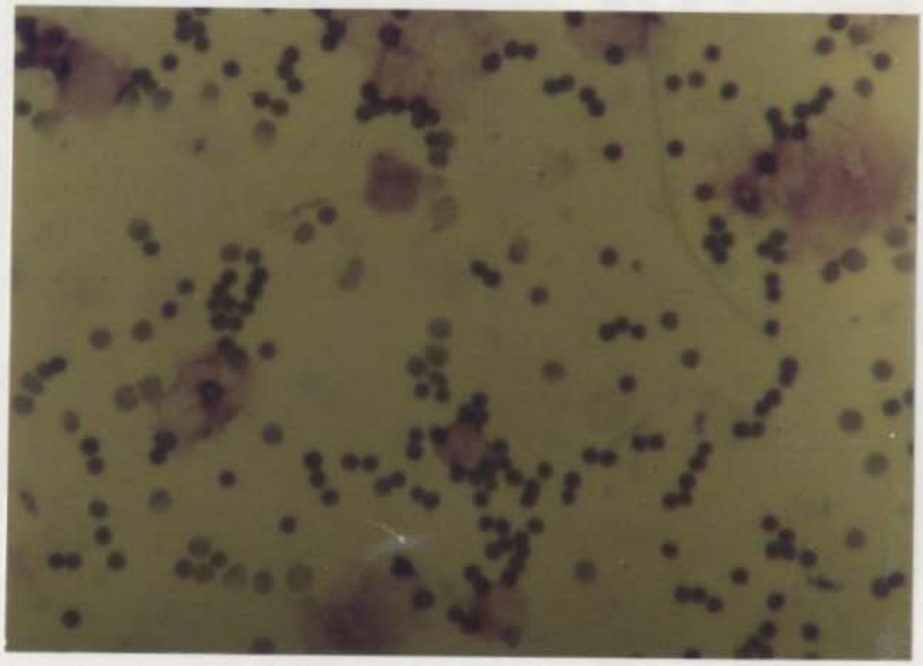


Figure 11

SRCA test slide from patient no. 2 stained with Wright's stain (x 400).

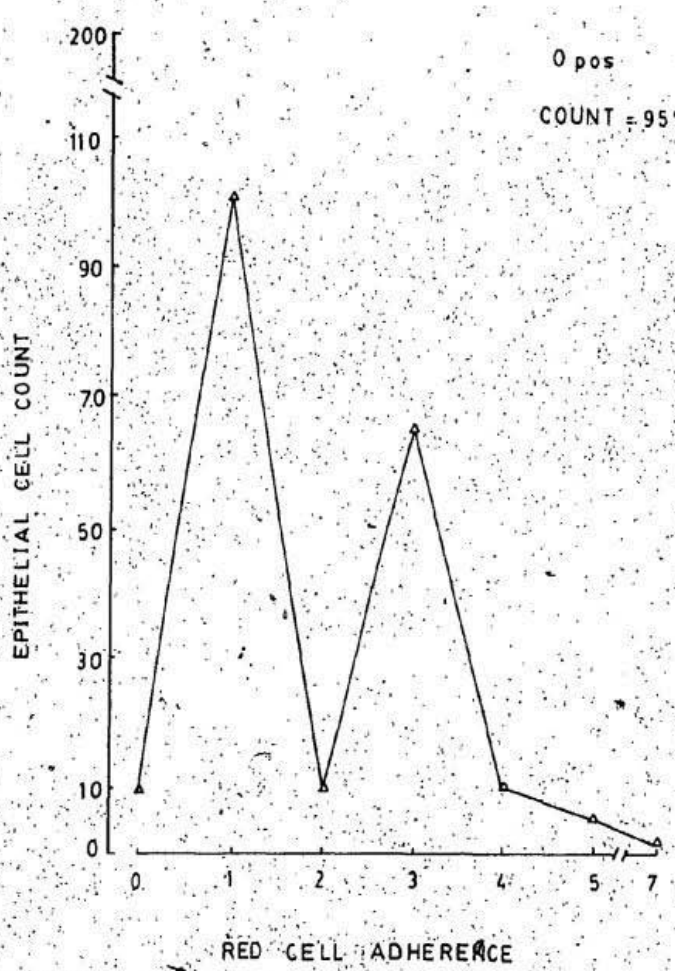


Figure 12

Graph of antigen positive cell count.

Patient no. 3

Slide no. 2

SRCA control slide showing solitary epithelial cells with no adherent red cells (Figure 13).

Slide no. 3

SRCA test slide showing red cells adhering to solitary epithelial cells (Figure 14).

Graph of antigen positive cell counts from test slide no. 3 and slide no. 4.

Antigen positive count - 99%

Blood group - B positive (Figure 15).

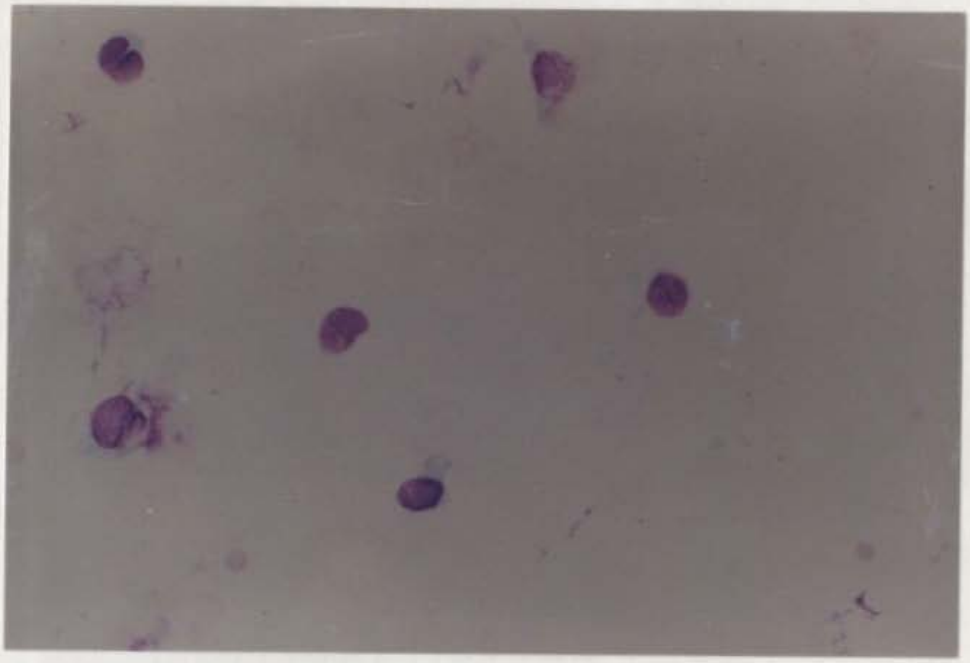


Figure 13

SRCA control slide from patient no. 3 stained with Wright's stain (x 400).

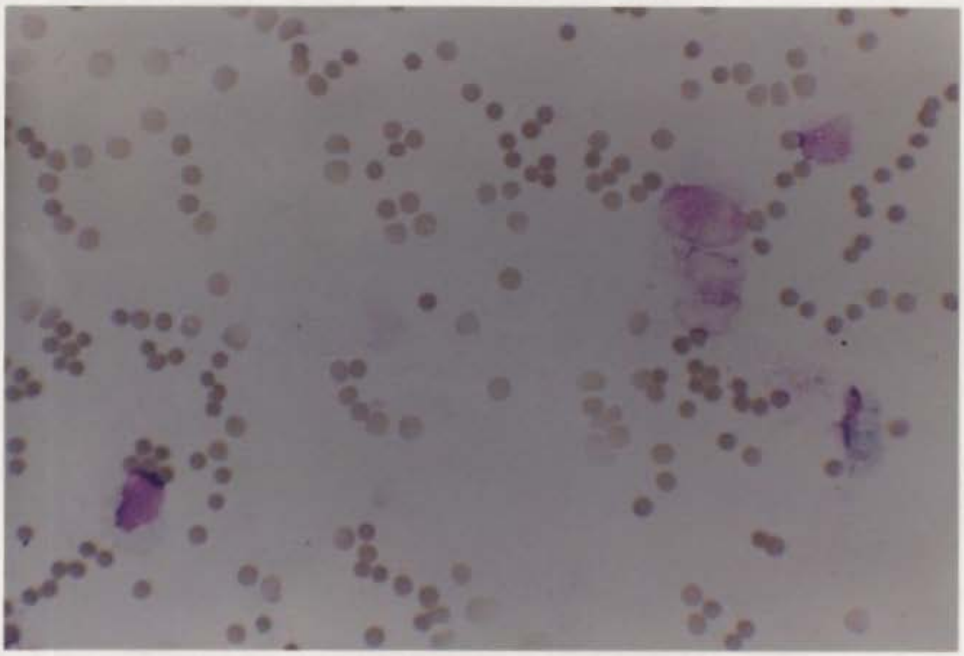


Figure 14

SRCA test slide from patient no. 3 stained with Wright's stain (x 400).

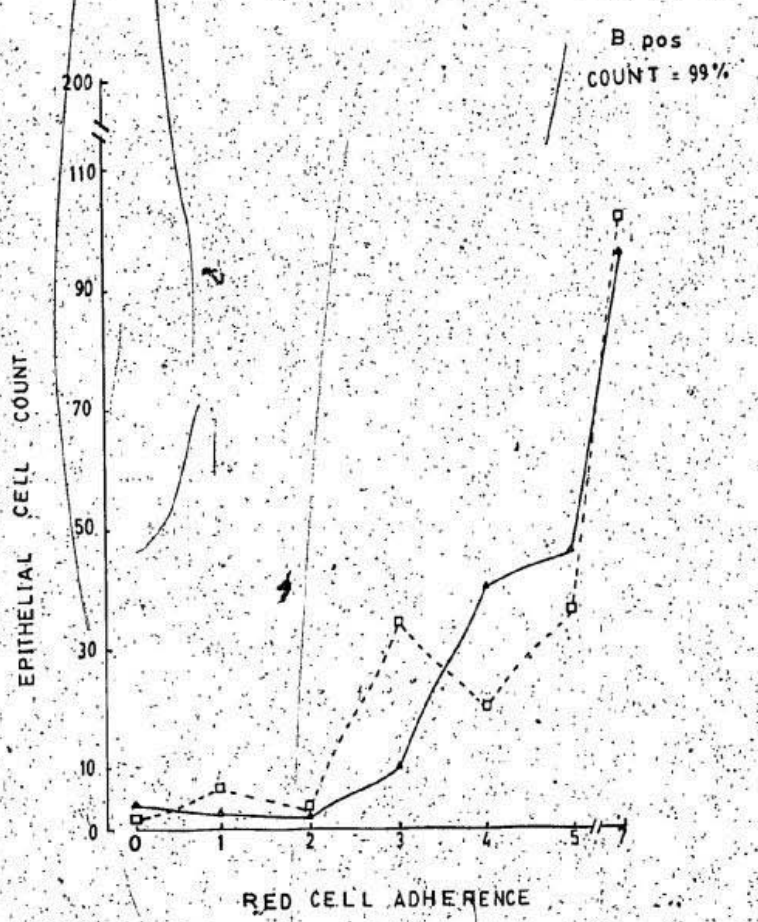


Figure 15

Graph of antigen positive cell count.

Key: The two duplicate counts from slide 3 and slide 4 are shown by Δ——Δ and □- - -□

Patient no. 4

Slide no. 1

Solitary transitional epithelial cells stained with toluidine blue (Figure 16).

Slide no. 2

SRCA control slide showing no adherent red cells (Figure 17).

Slide no. 3

SRCA test slide showing red cell adhering to some of the epithelial cells (Figure 18).

Graph of antigen positive cell counts from slide no. 3 and slide no. 4 (Figure 19).

Mean antigen positive cell count - 67%

Blood group - A positive.

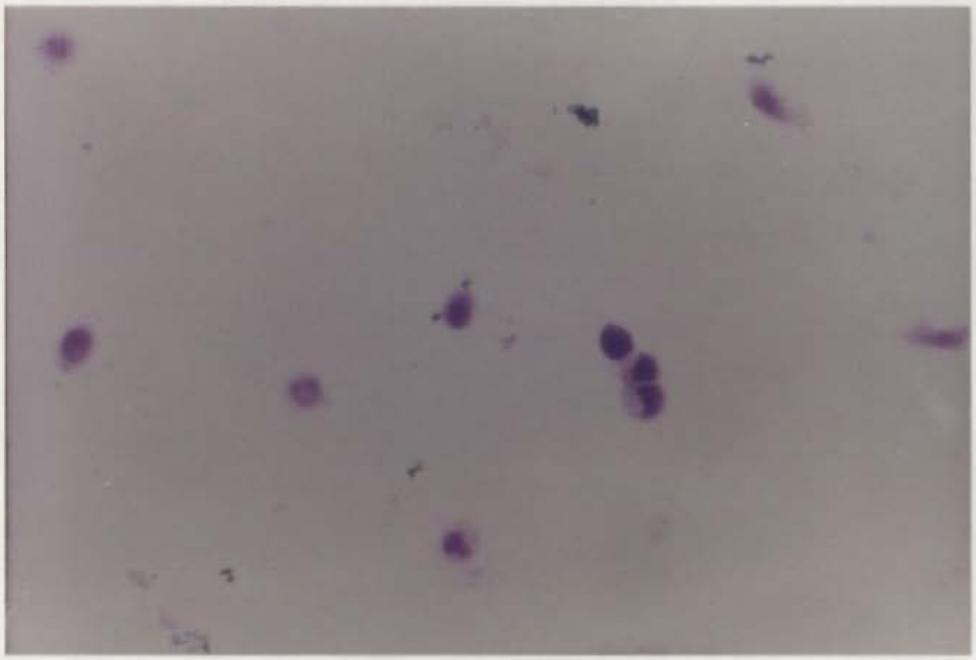


Figure 16

Solitary epithelial cells from patient no. 4 stained with toluidine blue (x 300).

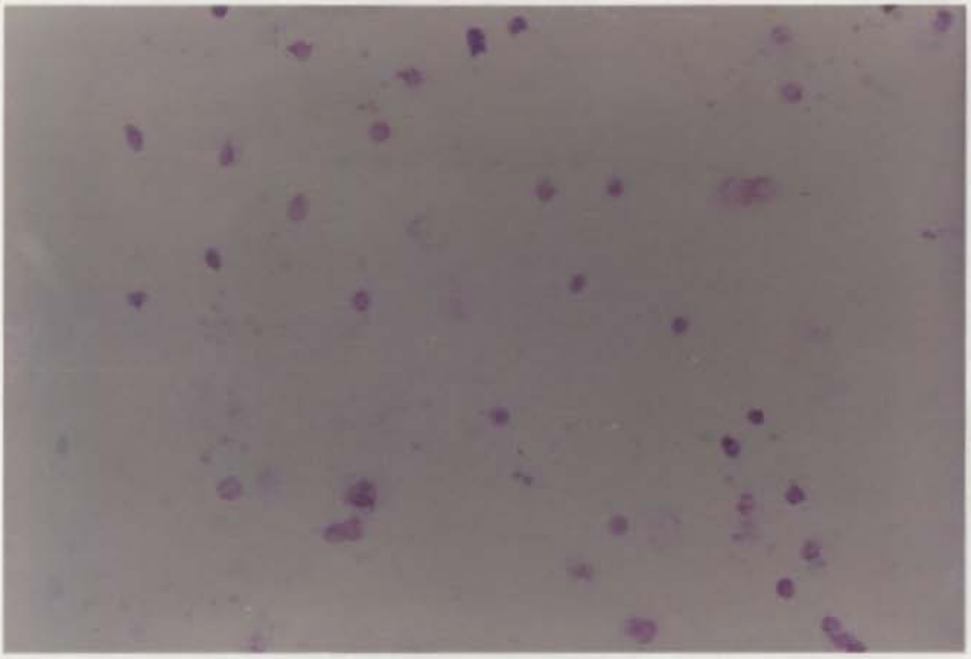


Figure 17

SRCA control slide from patient no. 4 stained with Wright's stain (x 250).

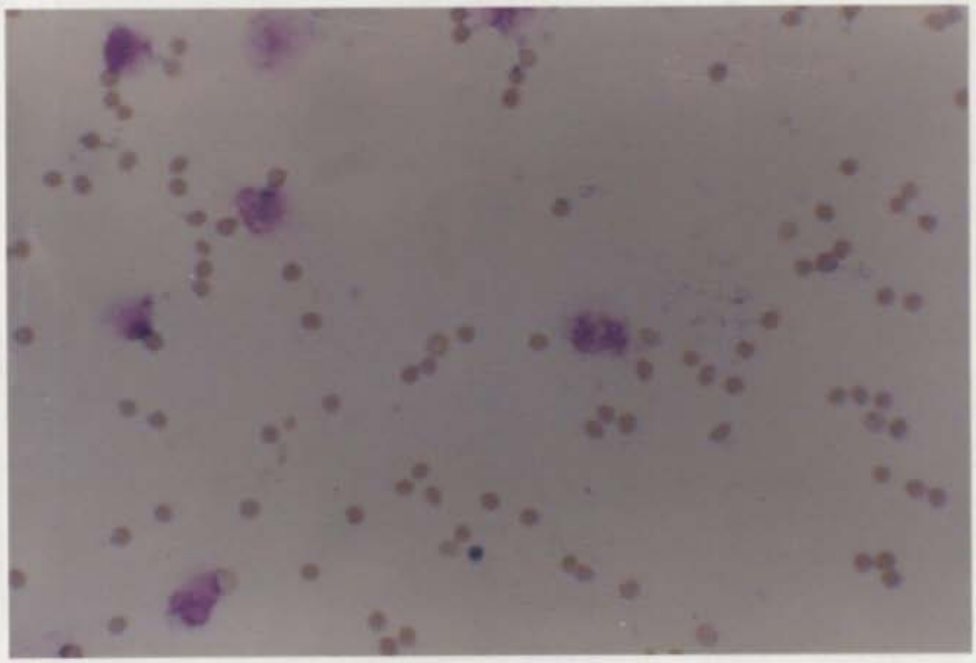


Figure 18

SRCA test slide from patient no. 4 stained with Wright's stain (x 400).

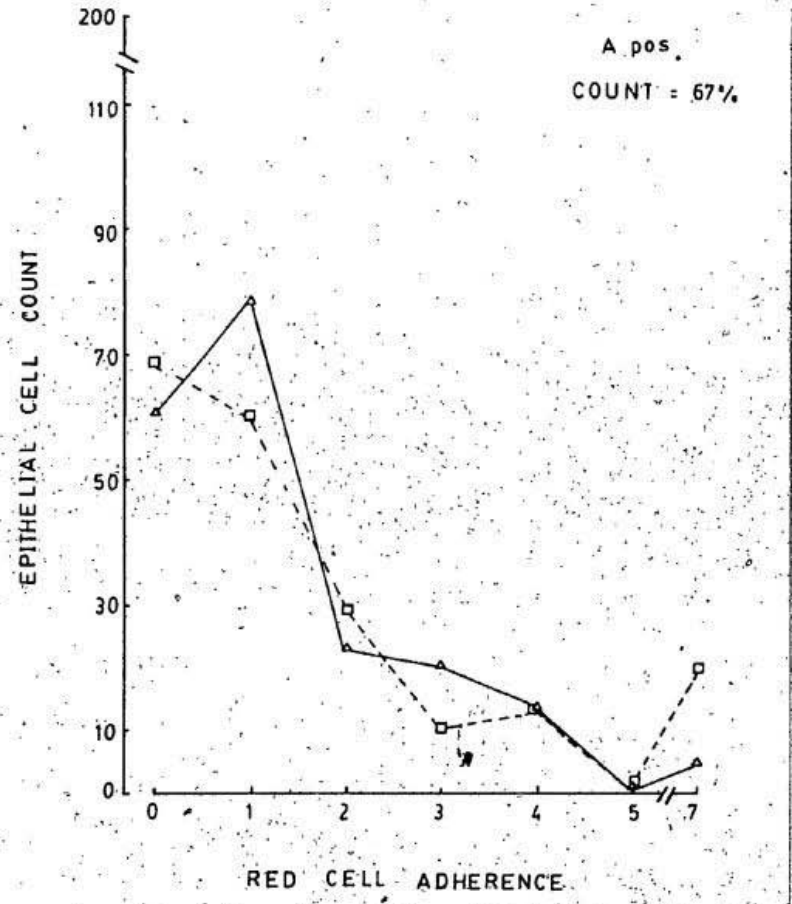


Figure 19

Graph of antigen positive cell count.

Key: The two duplicate counts from slide 3 and slide 4 are shown by Δ——Δ and □- - - -□

Patient no. 5

Slide no. 1

Solitary epithelial cells stained with toluidine blue (Figure 20).

Slide no. 3

SRCA test slide showing no adherent red cells (Figure 21).

Graph of antigen positive counts from test slide no. 3 and slide no. 4.

Antigen positive count - 6%

Blood group - O positive (Figure 22).

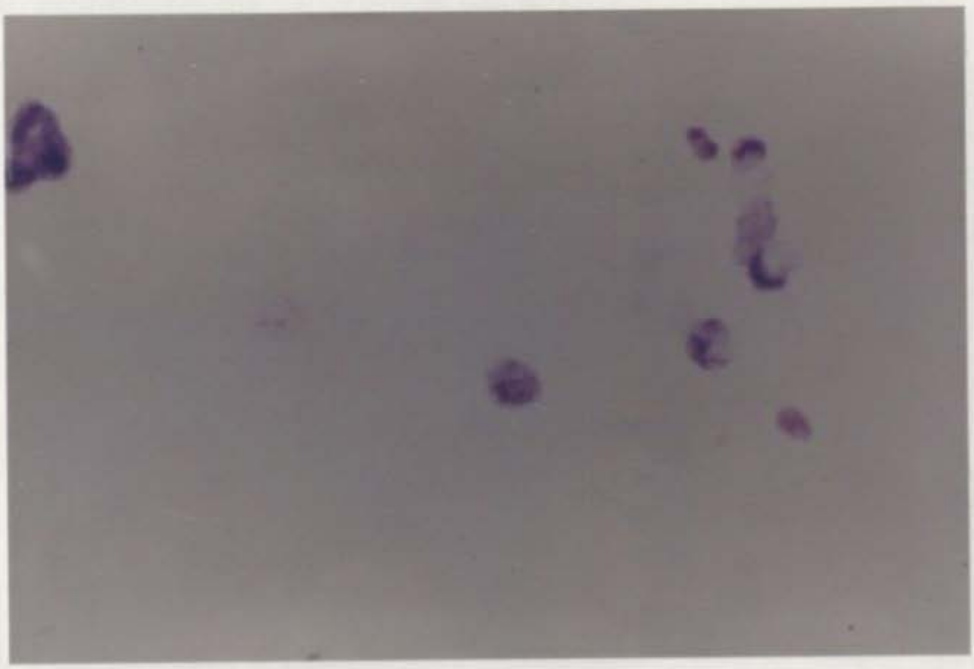


Figure 20

Solitary epithelial cells from patient no. 5 stained with toluidine blue (x 300).

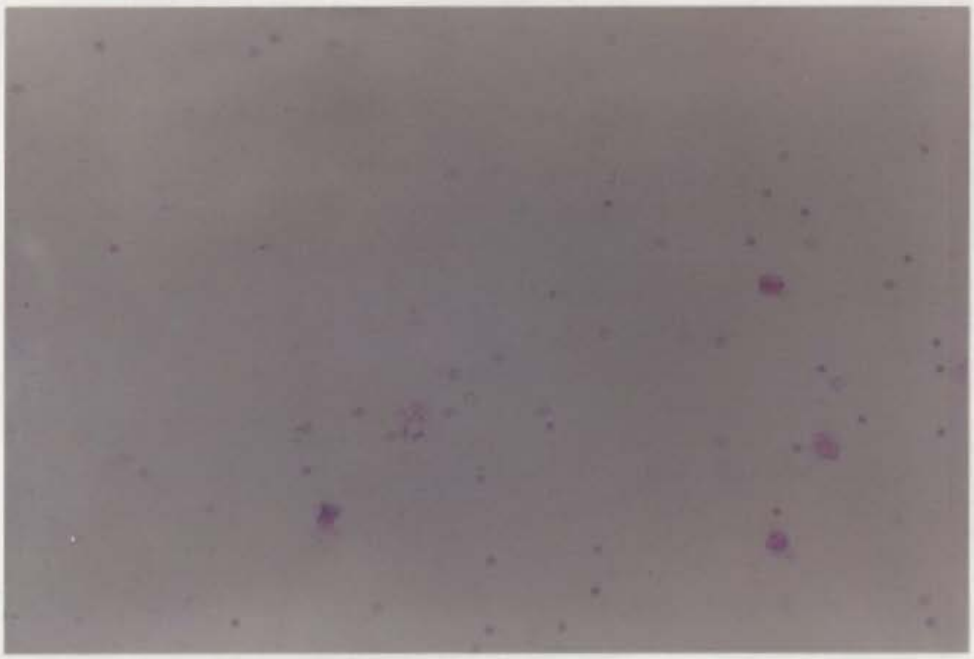


Figure 21

SRCA test slide from patient no. 5 stained with Wright's stain (x 250).

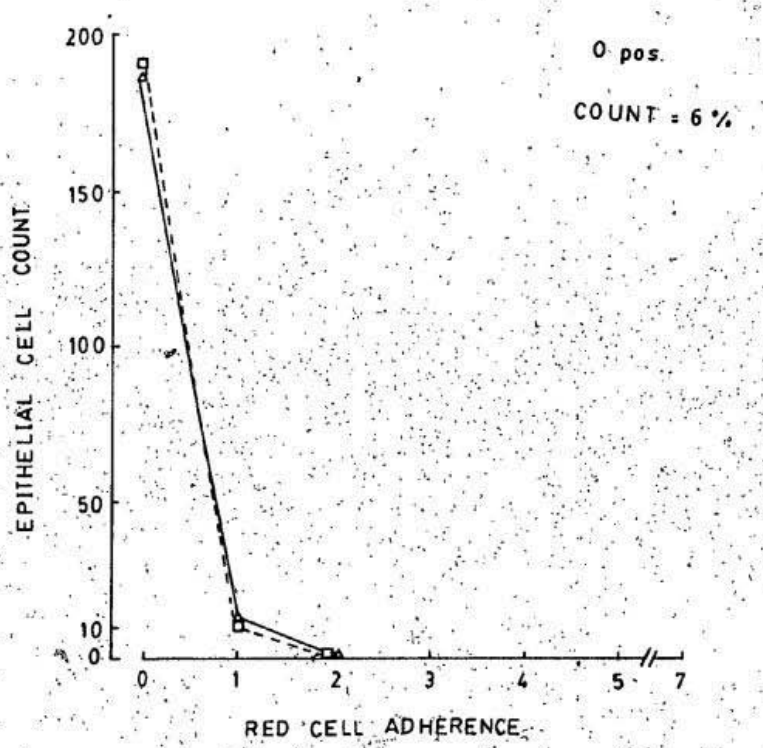


Figure 22

Graph of antigen positive cell count.

Key: The two duplicate counts from slide 3 and slide 4 are shown by Δ——Δ and □-----□

On comparing the antigen positive cell count of the 15 patients with the histology it was found that there were 8 normal bladders and 7 bladder tumours.

In the 8 normal bladders the antigen positive cell count ranged from 75% to 99% with a mean of 88% (SD $\pm$ 9.31). In the 7 bladder tumours the antigen positive cell count ranged from 0% to 77% with a mean of 33% (SD $\pm$ 32.54) (Figure 23).

The mean antigen positive counts for the normal bladder group and the bladder tumour group were compared using a two tailed t-test. This showed that the mean antigen positive counts of the two groups were significantly different at the $p < .001$ level.

RESULTS

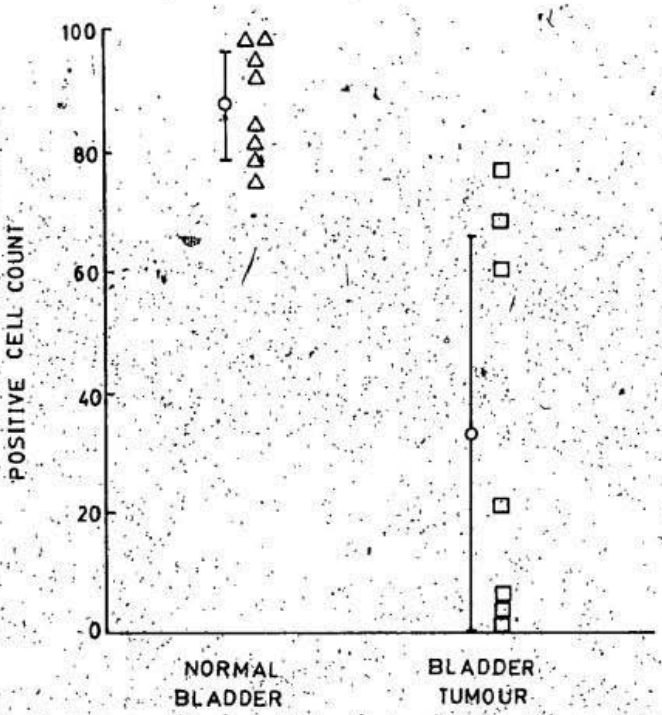


Figure 23

Graph of final results comparing the antigen positive counts in normal bladder and bladder tumour.

Key: Each biopsy of normal bladder is denoted by Δ; each of bladder tumour by □ . Means and standard deviation are shown for each group.

DISCUSSION

Single cell suspensions

The use of solitary bladder transitional epithelial cells appeared logical for performing the test since:

1. it was the epithelial cells which were being tested for isoantigen
2. solitary cells are free from their attachment to adjoining cells and thus have an extra surface for antigen-antibody reaction
3. solitary cells can be counted to determine the number of antigen positive cells.

A technique had to be developed to obtain single-cell suspensions from biopsies with preservation of ABO(H) isoantigens. A modification of the technique used in the toad bladder (42) was adopted to obtain a single cell suspension of transitional cells from bladder mucosal biopsy specimens.

Techniques of dissociating human bladder epithelial cells mechanically or with trypsin have been used in the past to initiate culture cell lines from transitional and squamous cell tumours (43). Trypsin which had been used in these previous techniques could not be used for this experiment since it would very likely digest the glycoproteins constituting the ABO(H) isoantigens. It has been shown that exposure of the luminal bladder surface to trypsin resulted in conversion of the normal specialized luminal plasma membrane into an undifferentiated membrane with loss of asymmetric unit membrane specialization (20). It has also been shown that trypsin digestion removes a large amount of surface sialic acid and glycoprotein surface coating from cells (44).

The mixed cell agglutination has been performed with solitary cell suspensions obtained by mechanical scraping from invasive tumours. However it gave a large number of false positive results since the cell suspensions included a variable amount of cells from adjacent antigen positive bladder mucosa and vascular endothelium (45).

Solitary transitional cells obtained from bladder washings have also been used to perform the SRCA test (46), however, the exact anatomical locations of these cells cannot be determined and the number of cells obtained is small.

The technique developed in this project can be completed in 3 hours and overcomes these problems. It is a relatively simple procedure of obtaining a high yield of viable transitional cells from known anatomical locations in the bladder. Biopsies can be taken from different parts of the bladder and each group of epithelial cells can be studied separately for the presence of surface isoantigen. The surface isoantigen can be preserved indefinitely if the cell suspension is air dried on the slides immediately thus allowing measurement at a later date.

Collagenase which has a high specificity for collagen was used for obtaining the cell harvest. Collagen which comprises 30% of the body proteins in mammals has a molecular weight of 300,000 and the amino acids proline, hydroxyproline and glycine are its main constituents. All collagens or collagen-like proteins are susceptible to proteolysis by the collagenase enzyme obtained from clostridium

histolyticum (47). It has been shown in the mammary gland that purified collagenase makes it possible to separate ducts, acini and cells from the surrounding adipose tissue (48). Commerically available collagenase used in these experiments is contaminated with very small concentrations of trypsin and this could cause minimal digestion of cell wall isoantigens. It would be ideal to use purified collagenase which is free of any contaminants.

During cell separation some of the cells become damaged and form mucinous clumps. Much of the mucinous material is DNA from these damaged cells, and small amounts of DNAse is required to disperse this mucinous material.

Since the technique followed (42) had previously been used only on toad bladders, it was decided to try it first on human autopsy specimens. In the initial six experiments carried out on human autopsy specimens cell separation was not achieved. There was no improvement on altering the concentration of reagents or incubation times and it was concluded that the epithelial cells had autolysed in the interval from death.

Three fresh dog bladder specimens were used and the techniques worked well confirming that fresh bladder specimens were essential to obtain a transitional cell suspension.

The technique was then tried on fresh human bladder biopsy specimens. In the initial experiment the yield of cells was very low but increased considerably during the later experiments when the volume

of all reagents was reduced. This was important since the amount of tissue available from human biopsies obtained with a Storz biopsy forceps is roughly 100 mg. whereas in the dog it was about 5 gm.

It was also found that if the biopsies were stored in cold Hanks' balanced salt solution overnight, the viability of cells dropped to 40%. Thus it was essential to perform the cell separation as soon as the biopsies were taken.

Immunological technique for quantitative analysis

Bombay blood which has a high titre of anti-H antisera is superior to Ulex europeus extract (49) and it was decided to use this to perform the test in 'O' group patients. It worked well in these cases.

A modified specific red cell adherence test (SRCA) was performed on the single cell suspension. The biopsies were subjected to cell separation as developed in phase 1 and air dried on the slides immediately. Duplicate slides from these patients were also preserved for 2 months, to test whether the surface isoantigen was destroyed after storage at room temperature. It was found that the isoantigen did not get destroyed.

The practical significance of this finding is that if this test has to be done on a regular clinical basis, air dried single-cell suspension slides can be stored, without damage to the surface isoantigen, until it is convenient to perform the test.

After completion of the SRCA test eight different fixation and staining techniques were tried in an attempt to develop the best technique for maintaining permanent records and enabling cell counts to be carried out. The major problem encountered in the first seven techniques was that the red cells were either haemolysed or washed off. Thus the red cell adherence to the antigen-antibody complexes on the epithelial cells could not be maintained. In previous descriptions of the SRCA test, the slides were being examined while still inverted over

saline and photographed if necessary to maintain records. This appeared to be a suboptimal method since the slides had to be read immediately, while unstained, and could not be cross-checked by independent investigators. However, the same technical problem of maintaining red cell adherence, could have influenced investigators to continue with the old method.

The 8th. fixation technique used consisted of immediately heating the slide, fixing in methanol and carrying out Wright's staining procedure. This was suitable for fixing the red cells on to the slide and maintaining the red cell adherence to the antigen-antibody complexes on the epithelial cells. This allowed a cell count to be carried out at a convenient time and also maintenance of permanent records. The only drawback to this technique was that the histological morphology of some of the epithelial cells was destroyed -- possibly by the heat. However since heating was essential to fix the red cells on to the slide and since it did not interfere with the cell surface isoantigen, as evidenced by red cells adhering tightly to epithelial cells which had lost their intrinsic cellular morphology, it was decided to continue with this technique.

The final Wright's stain differentially stained the epithelial and red cells. Thus solitary epithelial cells could be counted and the number of red cells adherent to each of these solitary epithelial cells could also be recorded easily.

Double-blind clinical study

A double-blind clinical study utilizing this new technique of quantitative analysis was carried out on a total of 15 patients with normal bladder and bladder tumour.

The bladder biopsies were all subjected to cell separation as developed in phase 1. Four slides were smeared with this cell suspension and the protocol was followed in each case. After completion of the SRCA test the staining technique developed in phase 2 was used.

In each case 200 epithelial cells were counted and the number of red cells adherent to each of these epithelial cells was recorded.

At present there are conflicting reports as to what constitutes a positive rosette test for antigen-antibody complexes. While performing tests on human peripheral blood lymphocytes and sheep red blood cells, previous investigators felt that a minimum of three red blood cells adherent to a lymphocyte were necessary to classify it as a positive rosette (50). However some investigators now feel that even if one red cell is adherent, it constitutes a positive rosette (51, 52).

Since this research technique of quantitating the isoantigens on bladder epithelial cells was being done for the first time, it was decided to differentially count the number of red cells attached to each of the 200 epithelial cells. Graphs, utilizing these counts,

were plotted in each case. For this project it was decided to use the criterion of regarding all epithelial cells with one or more red cells adherent to their surface as antigen positive and thus obtain antigen positive cell counts in each case. The mean antigen positive count from the two test slides was thus calculated and expressed as a percent.

If this technique of differentially counting the red cell adherence to 200 epithelial cells is followed each time, graphs can be drawn for each case in all prospective studies. At a later date when a large number of bladder tumours have been followed for a sufficient period of time, these graphs can be examined critically. This may help to correctly determine the number of red cells that are required to adhere to a bladder epithelial cell to classify it as an 'antigen positive' cell.

Another problem encountered was that in the positive slides, numerous red cells adhered onto the slide in locations where there were no apparent epithelial cells. The slides were left inverted over saline for longer periods of time (15 minutes) to allow these excess red cells to settle off the slide but this did not alter the picture. It seems likely that some antigen positive constituent, possibly lysed portions of epithelial cell wall, was present in these areas and caused the red cells to adhrere to the slides. Thus while counting the cells, it is essential to count only those red cells which are actually adherent to the epithelial cells and not those red cells which are lying in close proximity to the epithelial cells since these will give false positive counts.

In most cases where the red cells were actually adherent to the epithelial cells, a distinct colour change (dark blue) occurred at the interface between these two cells. More studies into this phenomena, possibly with electron microscopy, would be useful and later on this might prove to be a criterion for determining a positive red cell adherence.

After completion of the trial on 15 patients, the histology was examined independently by a pathologist. It was found that there were eight normal bladders and seven bladder tumours and the results of the antigen positive counts are shown in Figure 23 (page 64).

The mean antigen positive counts between the two groups were significantly different at the $P < .001$ level. The different blood groups were well distributed in both groups.

The normal bladders showed a high count and this is encouraging since normal epithelial cells should express the ABO(H) isoantigens. Since about 20% of the epithelial cells are damaged during the preparation of a single-cell suspension, antigen positive counts of 100% in all normal bladders cannot be expected. Thus antigen positive counts ranging from 80 to 100% in this group are acceptable since the cells damaged during cell separation, tend to lose their surface isoantigens.

The bladder tumour group showed a wide range and this is acceptable since there are some low grade, superficial tumours that are going to remain superficial and thus retain their ABO(H) isoantigens. There are other tumours which are either high grade at present or are low grade superficial but with a potential to become invasive and have

already lost their isoantigens and thus have low counts. These tumours, especially those with low counts, if followed up for 3 - 5 years may indicate the accuracy and usefulness of this test.

Quantitative analysis of ABO(H) isoantigens in bladder tumours would be of immense value in standardizing the test and incorporating it in different clinical prospective trials. The quantitative test described here can be performed on bladder tumour patients who are in different prospective trials. After 3 - 5 years' follow up of a large number of patients, the natural course of the tumour in each patient will be known. The antigen positive count for each patient at the time of initial diagnosis can then be reviewed. This will indicate the low antigen positive counts which result in ultimate tumour invasiveness and thus it should be possible for an antigen positive "cut-off point" to be identified - below which most superficial bladder tumours are likely to become invasive.

At a later date all patients with bladder tumours could have an antigen positive cell count performed as a routine test on admission and this should help the clinician in determining what the possibilities are of a particular tumour ultimately becoming invasive. All patients potentially at risk of invasiveness could undergo 'prophylactic cystectomy' at this early stage and this should markedly improve the prognosis in the management of bladder tumours.

CONCLUSION

Efforts to control bladder cancer in the past have emphasized the role of environmental carcinogens, especially in industries, in the aetiology. Thus, numerous known chemical carcinogens have been removed from use and large-scale screening of those exposed has been undertaken. While both procedures should be encouraged, it is not likely that they will, in fact, eliminate bladder cancer. Most superficial tumours can be effectively treated, however, some initially superficial tumours become invasive. A screening test with the potential to detect which superficial tumour will ultimately become invasive would be of immense clinical value and the measurement of ABO(H) isoantigens appears most promising.

A new technique of quantitatively analysing ABO(H) isoantigens in bladder tumours has been developed. This technique has practical applicability since it consists of three sections: (1) cell separation; (2) modified SRCA with staining; (3) cell count, each, of which can be performed at different convenient times. The staining technique developed, although not perfect, enables permanent records to be maintained. Graphs drawn of individual counts will help in establishing a 'normogram' which in future will help in predicting the course of bladder tumours in individual patients.



The technique of obtaining single cell suspensions from bladder biopsies will also be useful if more sophisticated and sensitive tests are used in future to measure the ABO(H) isoantigens quantitatively. The enzyme-linked immunosorbent assay (ELISA) is a very sensitive

immunological procedure which can produce quantitative results. In future it might be possible to perform the ELISA test on the single cell suspension to quantitatively measure the ABO(H) isoantigens.

At present, however, the double-blind study carried out in this project has indicated that this new quantitative test developed can be performed, has practical applicability and produces meaningful results. This quantitative test if incorporated in a prospective clinical trial, may, in a few years time, prove its value in the management of bladder tumours.

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