

DETECTION OF BLOOD GROUP ANTIGENS IN
BENIGN AND MALIGNANT SQUAMOUS CELL
LESIONS USING SPECIFIC RED BLOOD CELL
ADHERENCE TEST (S.R.C.A.)

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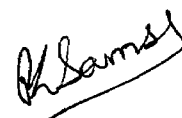
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(Dr.Miss Prabha K. Samsi)

CERTIFICATE

This is to certify that the work contained in this dissertation "Detection of Blood Group Antigens in Benign and Malignant Squamous Cell Lesions Using Specific Red Blood Cell Adherence Test (SRCA)" has been carried out by Dr. Miss Prabha K. Samsi under my guidance and to my satisfaction.



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I N D E X

	<u>Page</u>
1. INTRODUCTION	1
2. REVIEW OF LITERATURE	3
3. AIMS AND OBJECTS	27
4. MATERIAL AND METHODS	28
5. RESULTS	31
6. DISCUSSION	36
7. SUMMARY AND CONCLUSIONS	43
8. REFERENCES	45

INTRODUCTION

Today the foremost enigma for the medical world is malignancy. Since there is no complete cure for it, medical science is forever in search of methods of detecting it at an early stage, so that morbidity and mortality can be prevented or decreased by whatever means available at the present time.

At the present, diagnosis of malignancy on tissue sections is based on morphological criteria, behaviour and other circumstantial evidences. These are subjective and at times lead to differences of opinion. Therefore, a sensitive and specific test that would overcome these fallacies and/or detect the malignant change at a stage earlier than it is evident morphologically, would be welcome.

Yoshida, in 1928, studied for the first time the possible existence of A and B antigens in human epidermis; but the results were negative.

Witebsky in 1935, showed that the biological characteristics known as blood groups concern the entire organism and not only the blood. Group specific substances

have been found in alcoholic and watery extracts of organs of man and animals.

It has now been shown by many investigators^{6,7,8,9,12,13,14,16,17} that the blood group isoantigens A, B and H are present in epithelial and endothelial cells of some normal tissues. These antigens disappear when the tissues undergo a malignant change.

Kovarik et al⁶ used the 'specific red blood cell adherence test' also known as 'mixed cell agglutination reaction (MCAR)' for detecting the presence or absence of these antigens from the tissues. The test is highly sensitive and specific.

Davidsohn⁸ found that the loss of antigens was increasing progressively from Ca in situ to anaplastic, invasive and metastatic carcinoma, and interpreted this as evidence of 'immunologic dedifferentiation' analogous to morphologic dedifferentiation of anaplasia.

This test is now being increasingly used in the early diagnosis of cancer.

REVIEW OF LITERATURE

I) EXISTENCE OF BLOOD GROUP SUBSTANCES IN NORMAL TISSUES:

Witebsky²³ (1935) using hemolysis inhibition test on intestinal contents (in saline) from different sites, determined the presence of blood group property in upper intestinal tract and its absence in the lower intestinal tract. He also investigated the presence of blood group destroying factors in the intestinal tract possibly enzymes or microorganisms. He studied cases with blood group A.

Amos et al (1954) using special techniques detected circulating agglutinins for homologous blood cell following homologous skin transplantation. This showed that antigens common to skin and blood cells existed in mice.

In 1955, Coombs and Bedford⁴ applied the principle of mixed agglutination to confirm the presence of A and B antigens on platelets.

Coombs et al (1956)⁴ working on human epidermal cells found that isolated epidermal cells or aggregates of these cells prepared from small sheets of split skin do not give cellular suspensions suitable for agglutination tests.

The inhomogeneity of the suspension, the irregular shapes of the cells and the difficulty in obtaining suspensions in sufficient quantity militate against the use of this reaction.

On the other hand, if it were possible to obtain a specific link or attachment between antibodies combining with even a few epidermal cells and different but specially selected indicator cells, the interaction of the individual cells should be easy to observe under the microscope. Such specific mixed agglutination or combination between two cell types can occur if both cell types possess a particular antigen in common. For instance, Weiner and Herman (1939)⁴ showed that horse antipneumococcus type¹⁴ serum would form mixed agglutinates between type 14 pneumococci and human red cells because both these possess a common antigen.

If skin epidermal cells of a person of blood group A possess the antigen A - then accepting that ordinary agglutinating antibodies are at least bivalent, we would expect such epidermal cells to be capable of being linked by anti-A to group A red cells.

On these theoretical grounds, Coombs et al (1956) used the specific mixed agglutination as a means of revealing whether epidermal cells do in fact contain A and B blood group antigens on their membranes.

Suspensions of isolated epidermal cells were prepared from the split skin by tryptic digestion process and after treating with antisera and RBCs of different blood groups results were observed by phase contrast microscopy. They came to the conclusion that group A red cells showed a specific affinity for epidermal cells from group A persons only and only if these epidermal cells had been exposed previously to an anti-A serum.

They postulated its use and application in :

- 1) Skin grafting.
- 2) Forensic medicine - identification of ABO grouping of isolated piece of skin devoid of blood.
- 3) Could be used with undefined tissue antigen using antiglobulin.

In 1957, Nelken et al¹⁸ demonstrated presence of A and B blood group antigens corresponding with those of RBCs on human epidermis, by using absorption and elution methods.

II) HISTOLOGICAL DISTRIBUTION OF BLOOD GROUP SUBSTANCES A
AND B IN MAN :

Szulman (1960)¹⁹ investigated extensively by immunofluorescence the distribution of blood group substances in different tissues. The study included fetuses and senile individuals and gave similar results in all cases.

Before him, the distribution of A and B antigens in tissues had been investigated using serological tests on extracts of tissues. Previously workers had found relatively large amounts of water soluble, mainly mucus bound antigen in appropriate tissues of secretors, as contrasted with the small amounts in non-secretors.

On the other hand, antigen extractable by ethanol had been found in practically all tissues from individuals of appropriate blood group, regardless of their secretor status.

Szulman observed the following :-

(a) The intima of vessels of all calibres in all organs and tissues contained the antigen irrespective of secretor status. It stains more brightly in veins than in

arteries, in which the endothelial cells tend to be flat and are desquamated into the lumen. The sinusoids in liver, adrenal and pituitary are also well outlined.

(b) The stratified epithelia show antigens in the walls of the epithelial cells irrespective of secretor status. The distribution varies somewhat according to the type of membrane.

The stratified squamous non-cornifying epithelium for eg, Cervix, oesophagus and tongue give beautiful staining of Malpighian stratum with a strong suggestion that intercellular bridges participate in the staining. In the skin, the cells of the upper half of the Malpighian layer shows brightly staining walls. The granular layer also stains, often more prominently than the subjacent Malpighian stratum. In all these epithelia the basal layer remains invariably negative.

In the stratified transitional epithelium of the urinary tract, from calyces to urethra, the order is reversed. The cells of the basal and contiguous layers stain, while the surface layers tend to remain negative. In the pseudostratified epithelium of the respiratory tract,

the basal halves of the columnar cells ~~and~~ show antigen.

(c) Mucus secreting apparatus : The presence and distribution of antigens in mucus is of special interest since it reflects the secretor status of the subject. Being mucus bound the antigen apparently follows the latter's solubility in water and its staining is unaffected by methyl or ethyl alcohol. When found in mucus secreting cells, the antigen pervades the swollen homogeneous mucus blebs filling the cells, often obscuring the nucleus and overflowing across the cell membranes.

In goblet cells, the cytologic detail is apt to be clearer since the mucus is mainly confined to supranuclear portion.

While in secretors all the mucus produced (with the exception of distal colon) contains the antigen, the picture is somewhat more complex in the gastrointestinal tract of non-secretors.

1) Secretors :

Stomach - surface epithelium and gastric pits pyloric

glands, small bowel - goblet cells of villi, crypts of Lieberkuhn and Brunner's glands of duodenum show presence of antigen.

Parietal cells and stomach : The parietal cells stain well irrespective of secretor status. The antigen appears as granular material filling the cytoplasm. Staining is abolished by prior exposure for 10-20 minutes to ethanol.

Large bowel - goblet cells of surface epithelium and crypts in appendix and proximal half of colon show presence of antigen, whereas antigen is absent in distal colon.

Salivary gland - Mucus acini of submandibular, sublingual and minor glands in mouth and upper oesophagus show antigen whereas no antigen is found in parotid.

Larynx, trachea and bronchus - goblet cells of surface epithelium and mucus glands or mucus components of mixed glands show presence of antigens in the wall.

Gall bladder - Blood group iso-antigen is seen in tall columnar mucus secreting epithelium.

Uterine cervix - In the tall columnar mucus secreting epithelium of surface and glands and in pseudomucinous ovarian cysts, abundant mucus bound antigen is seen.

2) Non Secretors :

In non-secretors there is no detectable mucus bound antigen except in the stomach and small bowel. Stomach - The surface and most of the foveolar epithelium show no antigen except for the bottom of the foveolae - small amounts of mucus bound antigen are visible in the foveolar lumina and some of it may trickle down into the lumina of the gastric glands proper. The status of the mucus neck glands is difficult to determine but is likely that they are active in producing blood group antigen.

In the pylorus there is secretion from the deep parts of the glands and occasionally at higher levels.

Small bowel - the goblet cells of the villi are invariably free of antigen. In the crypts of Lieberkuhn, the goblet cells secrete variable amounts of antigen. Most of the active cells are in the lower half of the crypts. In the duodenum this picture is often obscured by antigen loaded mucus welling up from the Brunners glands.

(d) Other organs of Secretion and Excretion :

Pancreas - Plentiful antigens often in the shape of fine granules are demonstrated in the cytoplasm of acinar cells and lumina. No difference is seen between secretors and non-secretors. The ductal epithelium is free from staining.

Kidney : There is remarkable staining of the luminal aspect of epithelial cells of collecting tubules in the medulla, much less frequent and less intense in the cortex and similar staining of the superficial cells of the calyces. The antigen seems to be attached to the luminal and upper lateral borders of the cells with granules in the cytoplasm and others spilled into the lumina. The rest of the cell envelope is often delicately outlined, but this outlining disappears after pre-exposure to alcohol; on the other hand the luminal accretions of antigens are alcohol fast. Since they are present in secretors only, their nature is probably that of tubular concentrates of excreted mucus bound antigen.

Sweat-glands - fine outlining of cells of sweat glands with frequent blebs of well stained antigens in the lumina of coils and ducts is present in both secretors and non-secretors.

Breast - Only occasional cells or groups of acinar cells show staining at the luminal tips usually accompanied by luminal droplets of secretions carrying the antigen.

Endometrium - All phases of the menstrual cycle were investigated. The epithelial cells of the glands disclose antigen primarily during the late proliferative phase. It is confined to the luminal portions of the cells, with much granular material in the luminal extremity of cytoplasm and cell membrane and similar glandular material in the gland lumen. The appearance reaches its height at the late proliferative phase and fades out rapidly in the early secretory phase.

An identical picture is seen in the epithelium of the fallopian tube, in which the appearance and fading of antigen parallels that of endometrium.

Specimens from non secretors reveal no antigen; but occasional proliferative endometria from secretors also fail to disclose it. The tubal epithelium is also negative in these cases.

Epididymus - Very occasional staining of single cells or small groups of cells at the luminal tips is seen in secretors.

Seminal Vesicles - In constant specific outlining of epithelial cell membranes irrespective of secretor status with occasional luminal capping in secretors.

Prostate : Specific staining at luminal tips is seen in single or small groups of glandular epithelial cells representing 10-20% of cells in sections from secretor subjects only. Areas from benign hypertrophy and corpora amylacea disclose no antigen.

Testis : In the tubules the precursors of spermatozoa with the exception of the basal layer of spermatogonia give occasionally feeble outlining of cell envelopes irrespective of secretor status. Spermatozoa are not stained.

Differences between Secretors and Non-secretors :

Whereas the antigen in endothelia and stratified epithelia and that of sweat and pancreatic secretions are independent of the secretor (S,s) genes, the antigen found in mucus, and milk remain under their control. In the case

of mucus borne antigen this control is incomplete as A and B antigens are regularly found in deeper parts of the gastrointestinal mucosa of non-secretors. Thus the ability to make and secrete the mucus bound antigen is retained in non-secretor individuals but its exercise remains restricted. The simplest explanation will be that the absence of secretion is due to suppression of production of this form of antigen due to the action of the non-secretor gene. While the suppression is more or less complete in the salivary glands and the rest of the mucus secreting apparatus, it stops short in the depths of the gastrointestinal mucosa.

An alternative possibility however suggested by Hartmann is that the absence of the antigen in non-secretors may be due to its enzymatic degradation beyond serologic recognition.

Szulman (1962)²⁰ in another study demonstrated that there is a consistent parallel in the occurrence of antigen H and A and B in their respective forms and locations, including the behaviour of the water soluble forms in secretors and non-secretors. While in group O tissues H constitutes the sole antigen of the ABH system, it generally

appears also in non-O tissues in amounts varying from zero to those apparently equivalent to or exceeding A or B.

The problem of origin of the antigens in cell membranes of epithelium and endothelium is complicated by the fact that the adsorption of antigens from the surrounding fluids onto cell surfaces can be accomplished experimentally. It would seem doubtful whether the concentration of group substances in the plasma and tissue fluids is sufficient to be the responsible factor although in the salivary glands and in the breast the secreted antigen may contribute to the outlining of the walls of the glandular cells. The more convincing circumstance arguing for the generally autochthonous character of the cell-wall antigens however, is their appearance in embryos, long antedating that of water soluble forms and their presence in non-secretors in locations devoid of the water soluble substance. Under this are included - vascular endothelia, skin, buccal, oesophageal, ectocervical, respiratory, calyceal and vesical epithelium.

In 1968 Kovarik, Davidsohn et al⁶ used the MCAR (a modification of Coomb's MCAR) for detection of isoantigens in paraffin sections of normal tissues and of benign and malignant neoplasms. Their findings were as follows :

MCAR in normal tissues:(Reproduced from Arch Path 86,12,1968)

Location	MCAR Positive	MCAR Negative
1.Skin	Sweat glands, epithelial cells surrounding hair, Horny layer of stratified epithelium.	Cells of Basal and Malphigian layers.
2.Tongue Larynx Oesophagus	Mucus glands, stratified squamous epithelium intensity increasing towards superficial layers.	Basal layer
3.Bronchus Trachea	Glands and surface epithelial cells	Basal layer
4.Cervix Vagina	Endocervical columnar epithelium, mucus gland stratified cervical epithelium.	Basal layer
Ureter Urinary Bladder	Transitional epithelium	

III) LOSS OF ISOANTIGENS IN MALIGNANCY :

Weiler in 1952²¹ had shown that organ specific antigen of rat liver was lost in primary hepatoma induced by 4-dimethylaminoazobenzene.

Later in 1956 he also showed by using fluorescing antibodies in the hamster kidney that the kidney specific antigen that is seen in normal hamster is lost in stilbesterol induced kidney carcinoma.

Key et al in 1957¹⁴ using the MCAR test (reinforced in a few cases by a simple antibody inhibition technique) demonstrated the loss of antigens in tumors arising from the human urinary tract epithelium.

The finding by Kent¹⁵ that both water soluble and alcohol soluble isoantigens are demonstrable in formalin fixed and paraffin embedded tissues opened a vast source of readily available material for study.

Davidsohn et al⁵ in 1966 studied the ABO substances in gastrointestinal carcinoma using immunofluorescent technique. Blood group substances were found in normal cells, adenomas and in cancer cells that have preserved the

abilities to produce mucus. Blood group substances could not be detected in all anaplastic cells that ceased to secrete mucus nor in some that continued to secrete.

Davidsohn et al⁶ in 1968 using the MCAR test studied 56 cases of malignant lesions from different sites.

Intensity of MCAR of Cancerous Tissue as compared with normal epithelium. (Reproduced from Arch Path. 86, 12, 1968).

Carcinoma		Intensity of reaction				Total
Cell type	Location	+++	++	+	-	
Squamous	Skin	0	0	3	11	14
Basal	Skin	0	0	0	15	15
Squamous	Oral Cavity	0	2	0	3	5
Squamous	Larynx Bronchi	0	1	2	6	9
Squamous	Cervix Vagina	0	1	0	6	7
Transi- tional	Urinary bladder	0	1	4	1	6

Key : +++ = Stronger than normal.
 ++ = Same as normal
 + = Weaker than normal
 - = No reaction.

Davidsohn et al in 1969 using MCAR studied 82 cases of varied cervical lesions. The density of the indicator RBCs was related to the number of epithelial cells present in a microscopic field; it was greatest where the epithelial cells were crowded.

Squamous Metaplasia and Dysplasia :

Isoantigens were demonstrated in all these cases. The distribution of isoantigens was slightly different in metaplastic and dysplastic epithelium and was related to the degree of stratification and maturation. In metaplastic epithelium (closely resembling normal squamous epithelium) positive MCAR was present in all layers except the basal, as is the rule in normal epithelium. When the normal stratification was lost and basal layer was no longer discernable, MCAR was positive throughout the thickness. The indicator RBCs showed decreased density of varying degrees over dysplastic epithelium.

CARCINOMA IN SITU:

Out of 21 cases MCAR was negative or patchy in 18 and positive in 3.

SQUAMOUS CELL CARCINOMA WITHOUT METASTASIS :

Out of 35 cases 26 were negative for MCAR and the rest showed patchy and irregularly distributed MCAR; this was interpreted as reduction of the isoantigens.

SQUAMOUS CELL CARCINOMA WITH METASTASIS :

MCAR was negative in all cases.

In 1969 Davidsohn and Lolusa et al used MCAR to study Carcinoma lung. Their observations were as follows :

MCAR in non neoplastic conditions :

Normal Tissue : The massive accumulations of indicator erythrocytes on the mucus glands of the normal submucosa of the bronchus and their absence on the serous glands were good examples of a positive and a negative MCAR in normal tissues.

Inflammation : The MCAR was positive in the surface epithelium and on the endothelial cells of the richly vascularised subepithelial connective tissue. The positive reaction in the vascular spaces of the connective tissue was a built-in natural positive control.

Metaplasia : The metaplastic transformation involving the squamous epithelium on the surface and extending into the submucosal connective tissue did not influence the MCAR, indicating preservation of the tissue isoantigens.

Squamous cell Carcinoma : In the squamous cell carcinoma, the MCAR was negative. The positive MCAR was limited to a few streaks in the necrotic core in one area of the metastatic mass. The positive MCAR in the vascular spaces of the connective tissue was the built in control permitting the inference that the negative MCAR in the carcinoma was due to loss of the isoantigen.

Anaplastic carcinoma : The isoantigen was absent in the oat cell carcinoma studied. The negative MCAR in the thick wall of the blood vessel was accentuated by the positive MCAR in other blood vessels.

Metastatic Carcinoma : The MCAR was negative in all distant metastasis.

In the case of adenocarcinoma in which necropsy material was available, positive and negative MCAR were present side by side in the primary carcinoma and in the

regional lymph nodes whereas the MCAR was negative in all distant metastasis.

The prostate was studied in 1973 by Gupta et al. They found that isoantigens were absent in primary and metastatic carcinoma whereas there was an increase of isoantigens in cases of benign prostatic hypertrophy. In the group of younger normal cases, isoantigens were only seen in traces. Thus the traces of antigens in normal prostates below 30 years of age showed a significant increase as the prostate undergoes changes of benign hypertrophy. This finding supported the work of others, who have shown that in other tissues the intensity of MCAR in benign lesions is comparable to or of a higher intensity than the normal tissue of origin, while in malignancies of the same organ the intensity of MCAR is considerably reduced or absent. Szulman¹⁹ studying the histological distribution in different tissues found that in the normal prostate antigen was present but was lost in benign hypertrophy.

Isoantigens A, B and H in benign and malignant lesions of the breast were studied by Gupta et al (1973). They found that in benign lesions - fibroadenoma, fibroadenosis,

and gynaecomastia MCAR was positive but in primary and metastatic carcinoma it was negative.

They also observed that in tumors with malignant glandular differentiation, some differences exist when compared to squamous differentiation. One of the differences appears to be a greater loss of isoantigens in glandular malignancies;

Biopsy specimens of 24 patients with squamous cell carcinoma of the larynx were studied by Fritzlin et al in 1977. Isoantigens were absent in 15 cases, partially lost in 8 and completely retained in one case.

IV) PATHOGENESIS OF THE LOSS OF ISOANTIGENS:

A) In Malignant tissues :

The inconstant loss of isoantigens in neoplastic transformation suggests that this immunologic alteration is 'tumor associated' rather than 'tumor specific'¹⁷.

Many postulations have been made. The decrease and loss of antigens in malignancy may be due to :

- 1) decreased ability or inability by cancer cells to produce the isoantigens^{6,7}.

- 2) Inability of Cancer cells to store them if produced elsewhere^{6,7}.
- 3) Destruction of antigens by the cancerous transformation⁶.
- 4) Masking of the antigen^{6,14}.
- 5) Possibly changes in the cellular membranes⁷.
- 6) A change in their demonstrability by the MCAR⁷.
- 7) Other as yet unknown factor or a combination of these⁷.

B) In Normal Tissues :

Witebsky²³ and Szulman²⁰ observed that in adults the blood group substances were absent in the colon, distal to the hepatic flexure whereas in fetuses and newborns blood groups isoantigens were present along the whole length of the colon.

The change to the adult status supervenes during the neonatal period with the establishment of bacterial flora. They therefore postulated that this change was due to degradation of the blood group substances by the microorganisms and their enzymes in the distal colon.

V) THE MIXED CELL AGGLUTINATION REACTION SPECIFIC RED CELL OR ADHERENCE TEST :

A) Demonstration of presence of isoantigens :

Coombs et al (1956)⁴ at first made use of MCAR to

- 25 -

demonstrate A and B blood group isoantigens on human epidermal cells. They observed their results under phase contrast microscopy.

B) Demonstration of loss of isoantigens in malignancy :

Davidsohn et al (1966)⁵ and others initially used immunofluorescent technique to demonstrate loss of isoantigen in malignancy. In 1968 Davidsohn⁶ used a modification of the original technique of Coombs, for study on paraffin embedded tissue sections. They made observations on wet unstained preparation using the low power through the thickness of the inverted slide with the tissue section remaining on the lower surface.

Alroy et al¹, discovered a method for demonstrating blood group isoantigens in permanent tissue sections. They made use of this technique in studying primary bladder carcinoma.

C) MCAR in Malignant Tissue following Radiotherapy :

Isoantigens in carcinoma of urinary bladder following radiotherapy were investigated using MCAR in 1978², Clinical histories in their cases revealed that all patients with invasive carcinoma who had strongly positive SRCA had received

prior radiotherapy to the bladder region. None of the patients with invasive bladder carcinoma with negative or weakly positive SRCA had been radiated. Histopathology of tumors in both groups was similar. These results supported the hypothesis that radiation may induce differentiation in tumors, possibly through an enhancement of golgi apparatus function. The SRCA should therefore not be used as a predictor of the biological behaviour of future recurrences in patients who have received therapeutic radiation, since radiation may produce false positive SRCA test results.

AIMS AND OBJECTS

- 1) To demonstrate presence of blood group isoantigens in normal tissues and benign lesions using the most inexpensive and easiest technique available - the MCAR.
- 2) To demonstrate loss of isoantigens in tissues undergoing malignant change using MCAR.
- 3) To investigate the state of antigenicity in metaplastic and dysplastic tissues.
- 4) To see if MCAR can be used in early detection of cancer when morphologically the evidence is equivocal.
- 5) To test its efficacy in identification of blood group from tissue sections.

MATERIAL AND METHODS

Tissue sections of benign and malignant lesions of the squamous epithelium from cases with blood groups A, B and AB were studied. 5-6 microne thick sections of formalin fixed paraffin embedded tissue were mounted on slides. Antisera A and B (Span Diagnostics) were used. Fresh RBCs (not more than 24 hours after collection) of group A and B collected in citrate phosphate dextrose were obtained from the blood bank for use as indicator cells. A 5% suspension of these in normal saline was made after washing the cells twice in normal saline.

- 1) The mounted sections were deparaffinised and brought to water.
- 2) They were washed in PBS (Phosphate Buffered Saline pH 7.4)
- 3) They were then stained with Haemotoxylin for 5-7 minutes.
- 4) Nuclear differentiation was achieved with 1% acid alcohol.
- 5) They were then kept for blueing in water.
- 6) Excess water was mopped off by blotting on Whatman paper.
- 7) Antiserum corresponding to the blood group in each case was placed on the tissue and allowed to react for 10 minutes.
- 8) Slides were washed in PBS with constant agitation for 30 minutes.

- 9) The 5% RBC suspension in saline was placed over tissue sections and allowed to react for 10 minutes.
- 10) Two matchsticks were placed in a petridish and the slide was inverted quickly.
- 11) Using a pasteur pipette PBS was poured in the petridish until it touched the undersurface, of the slide. It was allowed to stand for 20 minutes.
- 12) The slide was then transferred to another petridish and kept inverted. Using a pasteur pipette 4% glutraldehyde in PBS was poured into the petridish until it touched undersurface of the slide. It was allowed to stand for 30 minutes.
- 13) The slide was washed with water.
- 14) It was then immediately stained with eosin.
- 15) Dehydrated with absolute alcohol.
- 16) Mounted in DPX.

Study of the Slides : The sections were studied under low and high power magnification. The distribution of the indicator RBCs in the tissue was observed. This was compared with the distribution of RBCs in the endothelium of the blood cell vessels in the section (built-in positive control). Absence of RBCs on connective tissue fibres served as the negative control.

The results were interpreted as :

- +++ - Stronger than normal.
- ++ - Same as normal
- + - Weaker than normal
- - No reaction.
- ± - Equivocal, when the distribution was patchy.

RESULTS

Total number of cases studied 58.

Table No.1 : Giving details of results and observations in all cases studied.

Tissue	Histopathological diagnosis	No. of cases	Blood group	MCAR
Skin	Moderately differentiated squamous cell carcinoma	1	B	-ve
	Well differentiated squamous cell carcinoma	1	A	±
	Verrucous carcinoma	1	A	+
	Keratoacanthoma	2	a) AB	+
			b) A	+
	Squamous Papilloma	3	a) A	++
			b) A	++
			c) B	++
	Pseudoepitheliomatous hyperplasia	4	a) A	++
			b) A	++
			c) A	++
			d) B	+
Penis	Moderately differentiated squamous cell carcinoma	2	a) A	-ve
			b) B	-ve
	Well differentiated squamous cell carcinoma	2	a) A	+
			b) B	-ve

Tissue	Histopathological diagnosis	No. of cases	Blood group	MCAR
	Well differentiated squamous cell carcinoma with secondaries	4	a) A b) A c) B d) B	-ve + ± -ve
	Verrucous Carcinoma	3	a) A b) A c) B	+ + ±
	Pseudoepitheliomatous hyperplasia	3	a) A b) A c) B	+++ ++ +
Tongue	Moderately differentiated squamous cell carcinoma	2	a) A b) B	-ve -ve
	Moderately differentiated squamous cell with secondaries	4	a) A b) A c) A d) B	-ve -ve -ve -ve
	Well differentiated squamous cell carcinoma	5	a) AB b) A c) A d) B e) B	+ ± ± -ve -ve

(preop.
Radiated)

Tissue	Histopathological diagnosis	No. of cases	Blood group	MCAH
Cheek	Well differentiated squamous cell carcinoma	4	a) A b) A c) A d) B	-ve -ve -ve -ve
Floor of mouth	Well differentiated with secondaries	2	a) A b) A	+ -ve
Pyriform fossa	Poorly differentiated with secondaries	4	a) A b) A c) B d) B	-ve -ve -ve -ve
	Moderately differentiated with secondaries	2	a) A b) B	-ve -ve
	Moderately differentiated	4	a) A b) A c) A d) B	-ve + -ve -ve
Pyriform fossa	Dysplasia	1	A	+
	Leukoplakia Dysplasia	1	A	+
Tonsil	Moderately differentiated squamous cell carcinoma	2	a) A b) B	+ -ve
Ear	Well differentiated squamous cell carcinoma with secondaries	1	A	±

Table No.2 : Summarising MCAR positivity in different lesions studied.

Histopathological diagnosis	No. of cases	MCAR				
		+++	++	+	±	-
PD - S.C.C.	4					4
MD - S.C.C.	17				1	16
WD - S.C.C.	19			4	5	10
Verrucous carcinoma	4			3	1	
Dysplasia	1			1		
Leu ^{ko} plakia with dysplasia	1			1		
Keratocanthoma	2			2		
Papilloma	3		3			
Pseudoepitheliomatous hyperplasia	7	1	4	2		
Total	58 cases					

Key to MCAR intensity :

- (i) +++ Stronger than normal
- (ii) ++ Same as normal
- (iii) + Weaker than normal
- (iv) - No reaction
- (v) ± Equivocal

PD - Poorly differentiated

MD - Moderately differentiated

WD - Well differentiated

SCC- Squamous cell carcinoma.

DISCUSSION

The specific red blood cell adherence test is based on the principle of the three-layer sandwich reaction. The bottom layer contains the isoantigen in the cells of the tissue, the unknown. The middle layer is the reagent, antiserum, that combines with the isoantigen in the tissue on one hand and with the top layer, the indicator erythrocytes, on the other. To give a positive reaction the tissue cells must contain the same antigen as is present in the indicator erythrocytes. The simplicity and specificity of this test make it a valuable experimental tool.

Studies on permanent tissue sections were preferred because the contrast in adherence of indicator cells on normal tissue cells and anaplastic cells could be well appreciated. The loss of antigen and the deranged morphology could be studied in the same section and co-related.

Previous workers²⁰ have found relatively large amounts of water soluble mainly mucus bound antigen in appropriate tissues of secretors, as contrasted with the small amounts in non-secretors, as On the other hand, antigen extractable by ethanol had been found in practically all tissues from

individuals of appropriate blood group regardless of their secretor status. Kent¹⁵ found that both water soluble and alcohol-soluble isoantigens could be demonstrated in formalin fixed and paraffin embedded tissues. Therefore the readily available formalin fixed and paraffin embedded tissues were used in our study.

Red cell isoantigens vary in strength²² and specific antisera vary in titre. Antigen A is stronger and anti-A antibodies are usually of high titre so that the results have been reproducible and reliable. B antigen is of moderate strength, B antisera of moderate titre; yet results have been almost as good. H antigen, however, is weak and specific antisera are not available. The plant lectin extract of *Ulex europaeus* nanus seeds is used as a substitute. This extract is difficult to make in high titre, requires special equipment and produces variable results. It has been shown by Szulman²⁰ that there is a consistent parallel in the occurrence of antigen H and antigens A and B in their respective forms and locations. Therefore we studied only cases belonging to A, B and AB groups.

Findings in normal tissues :

In the squamous epithelia, studied, we found that in

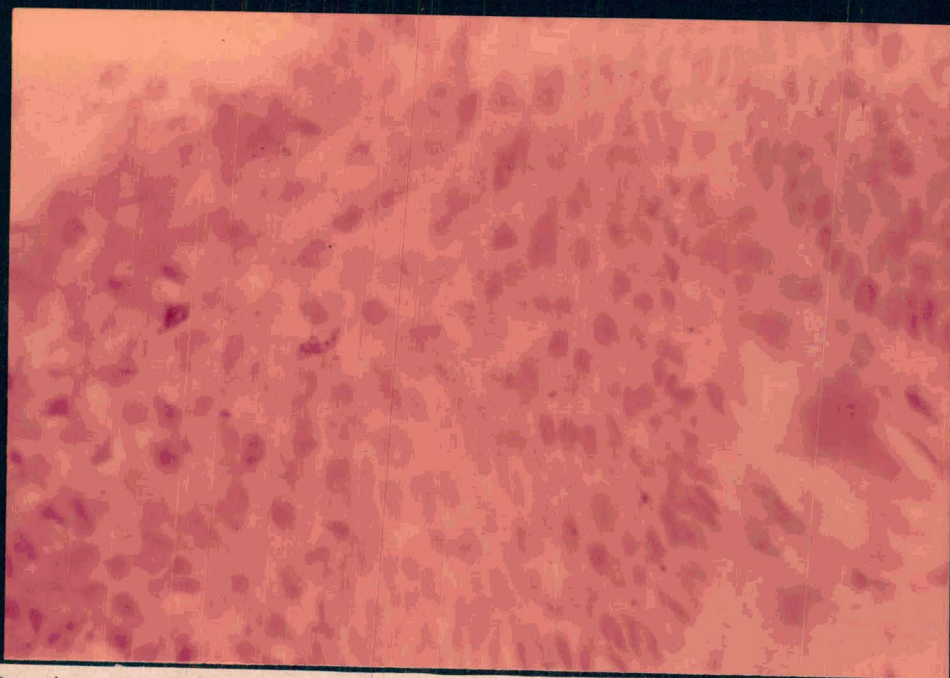
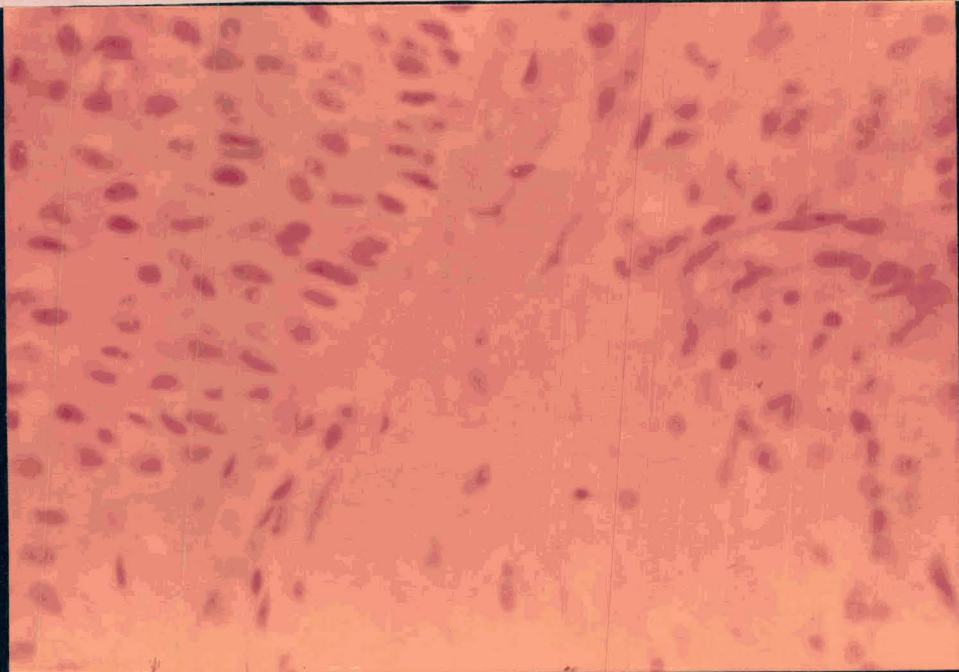


Fig.1.: Normal uterine cervix : upper layers of the squamous epithelium and the endothelium of the blood vessel show a positive (++) SRCA. Basal layer of the squamous epithelium shows a negative (-ve) SRCA.

Fig. 2: Dysplastic tissue from pyriform fossa : SRCA weakly positive (+) in a patchy distribution.



the morphologically normal tissues the SRCA was strongly positive in the keratin and parakeratin layers, weakly positive or negative in the Malphigian layer and negative in basal layer. This finding corresponds with that of Davidsohn et al⁶, whereas Szulman¹⁹ had observed presence of iscantigens in all the layers except the basal layer.

Positive and negative control :

In all the sections studied the endothelia of blood vessels were strongly positive and served as built in positive control whereas connective tissue fibres were negative and served as negative control.

Also in the cases of unknown blood groups, the blood group could be deduced by the positivity in the endothelium of blood vessels.

In a few blood vessels adjacent to malignant tissue, the MCAR was reduced or absent. This has been described by Davidsohn (1969)⁷.

Benign lesions : In the benign lesions studied, MCAR was positive. Gupta et al (1973) in their study on prostate, found traces of antigens in normal prostates below 30 years

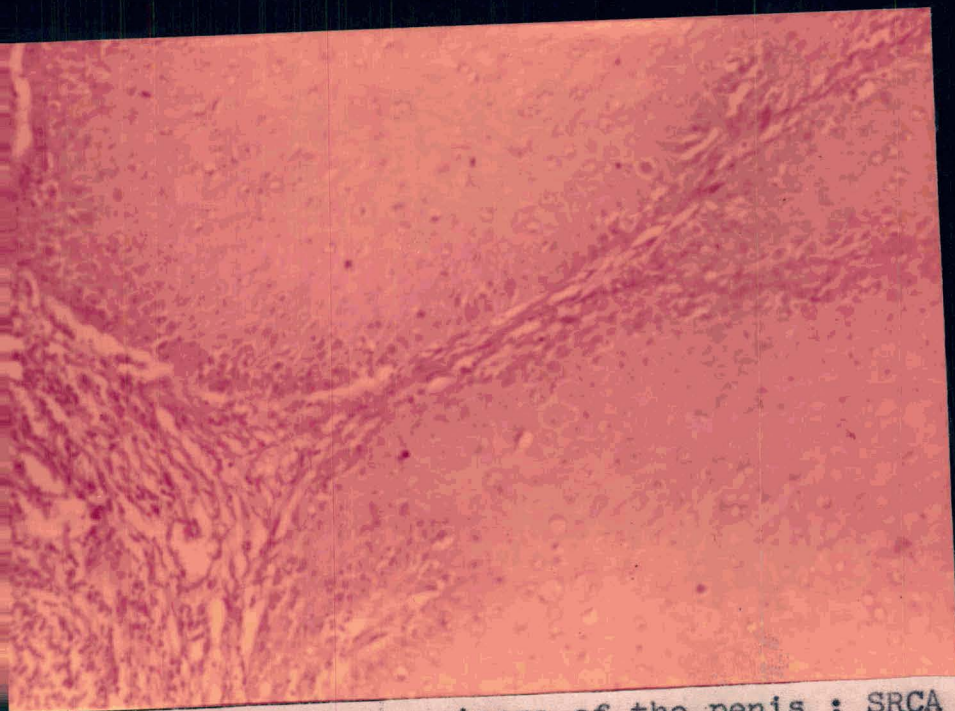


Fig.3 : Verrucous carcinoma of the penis : SRCA weakly positive and patchy ($\pm$).

Fig. 4 : Well differentiated squamous cell carcinoma of the tongue : SRCA negative.

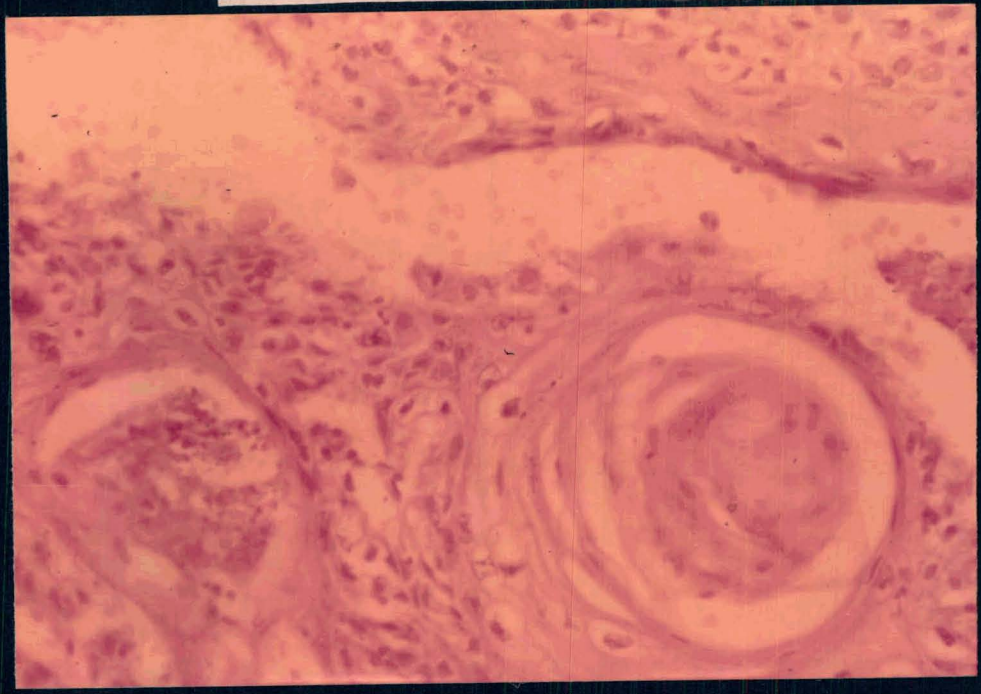
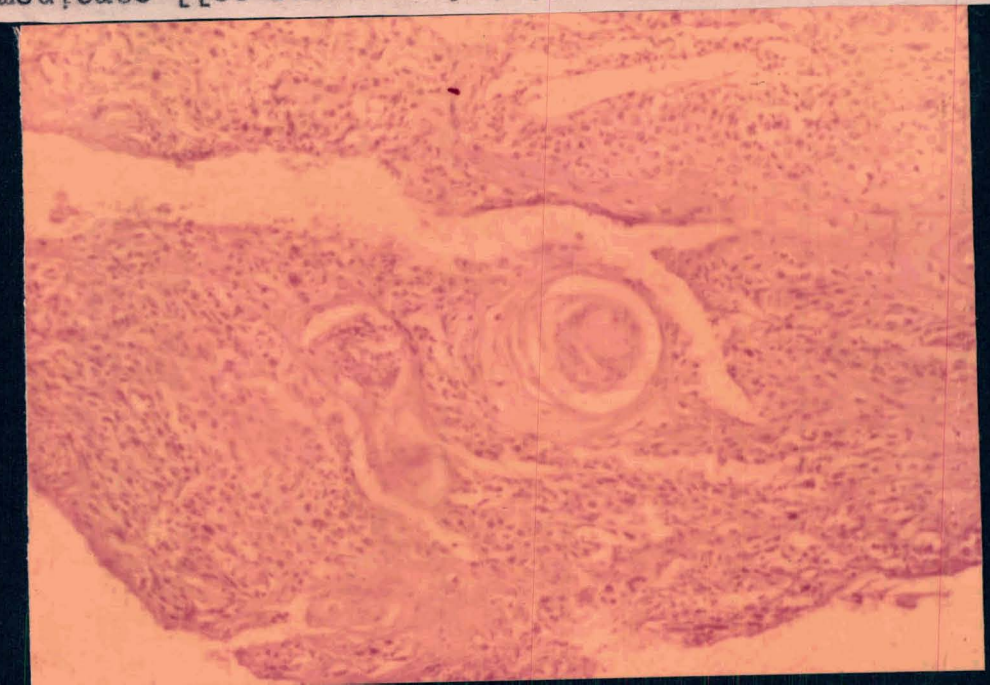


Fig. 5 : Higher magnification.

of age, showed a significant increase as the prostate underwent changes of benign hypertrophy, while in malignancy the intensity of MCAR was considerably reduced or absent. A similar increase in intensity of the MCAR in benign lesions has been described by other workers.

Dysplasia : In dysplasia, in the area which appeared morphologically normal, SRCA was positive whereas in the dysplastic area it was weakly positive and irregularly distributed. Thus there is evidence of gradual loss of isoantigens corresponding to morphologic transition from normal squamous mucosa to various stages of intraepithelial alterations and finally to overt carcinoma¹⁷.

Well-differentiated carcinoma : of the 19 cases studied, 4 were weakly positive and 5 showed weakly positive and irregularly distributed MCAR. In all these 9 cases the positive areas were very well differentiated and the preservation of isoantigens was confined to the keratinised centres of epithelial pearls. This has been described by Fritzlin et al (1977) in their study on cases of carcinoma of larynx. They found that out of 8 cases where epithelial pearls with keratin formation were present, 4 had preservation

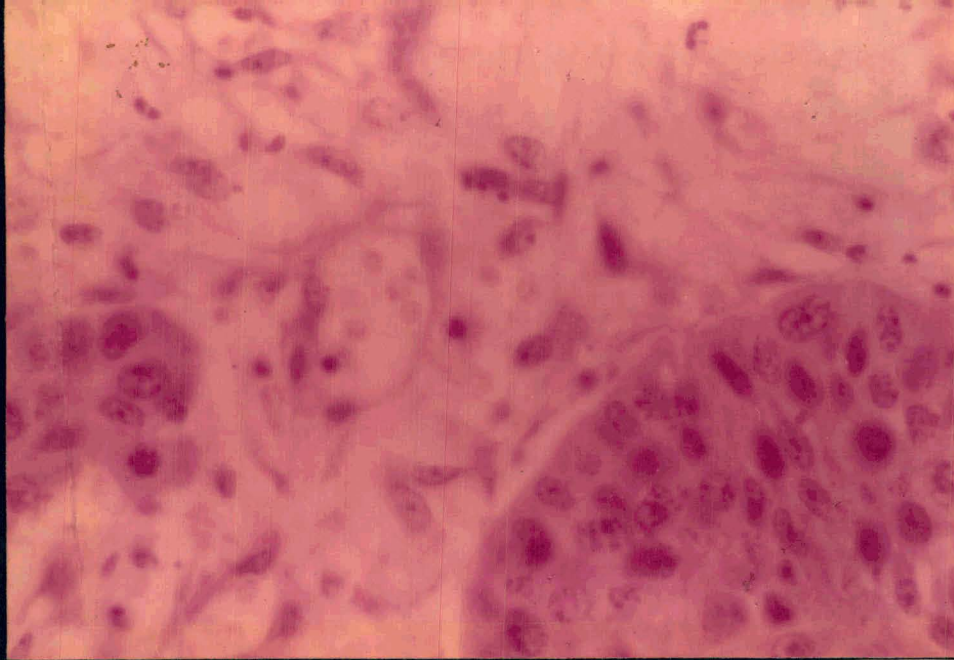


Fig.6 : Moderately differentiated squamous cell carcinoma of the tongue : SRCA negative (-ve).

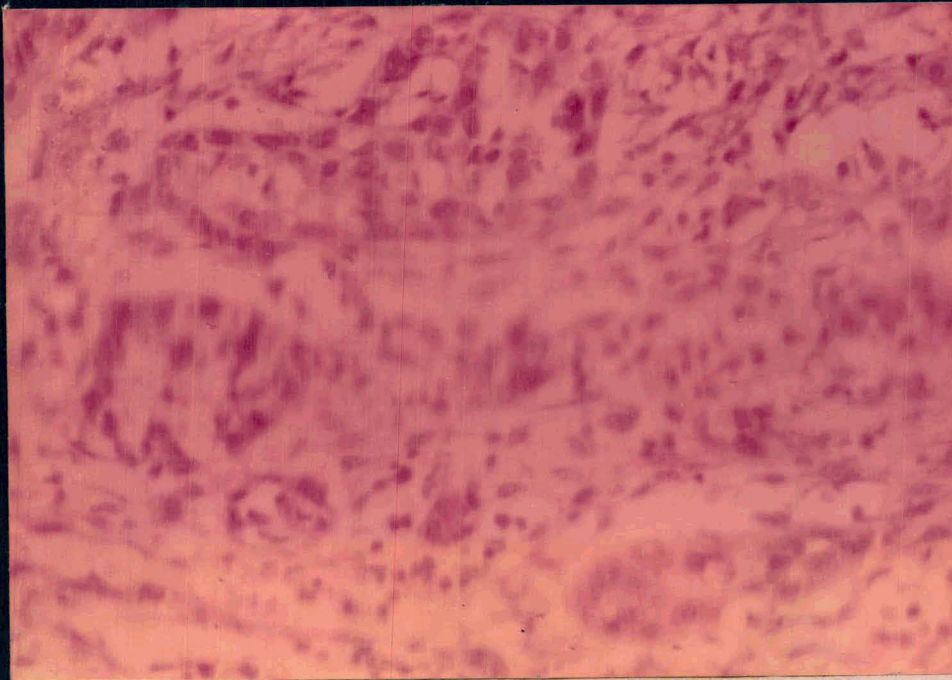


Fig.7 : Lymph node : Secondaries from moderately to poorly differentiated squamous cell carcinoma of the pyriform fossa : SRCA negative (-ve).

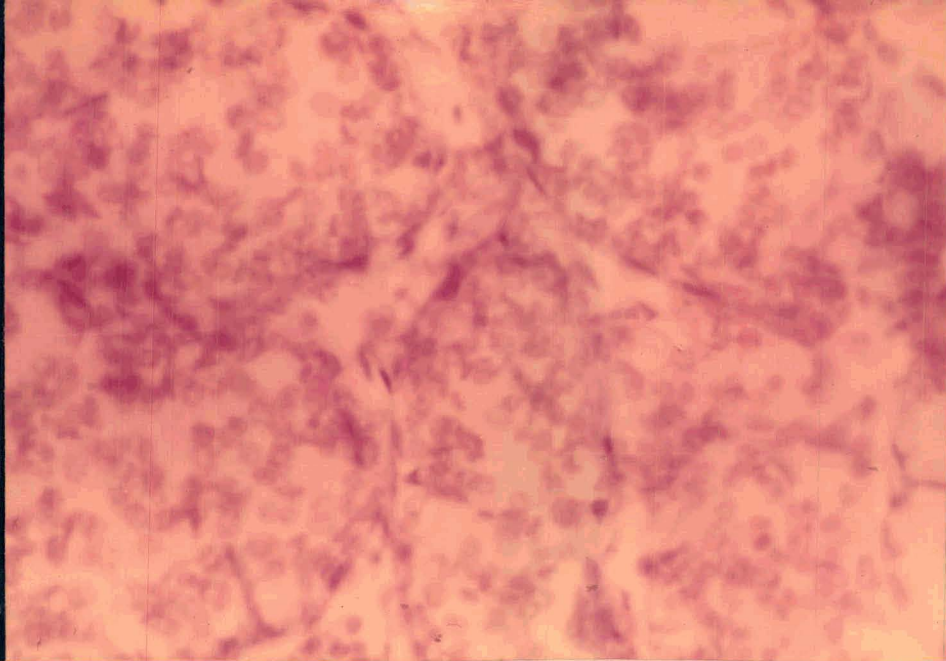


Fig.10: Higher magnification. Mucus salivary glands show strongly positive SRCA (+++).

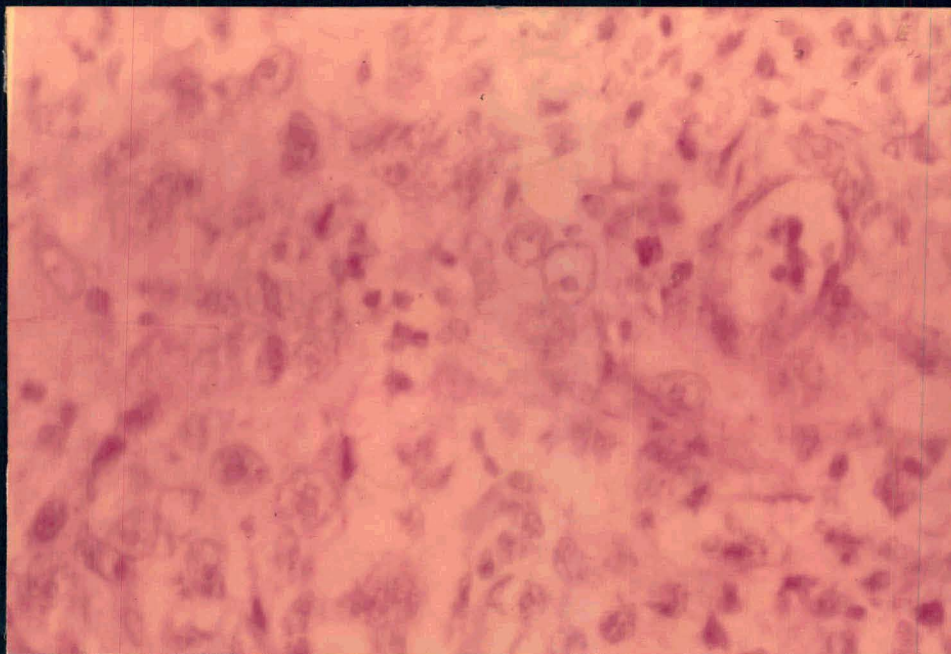


Fig.11 : Higher magnification. Secondaries from poorly differentiated carcinoma show negative SRCA (-ve).

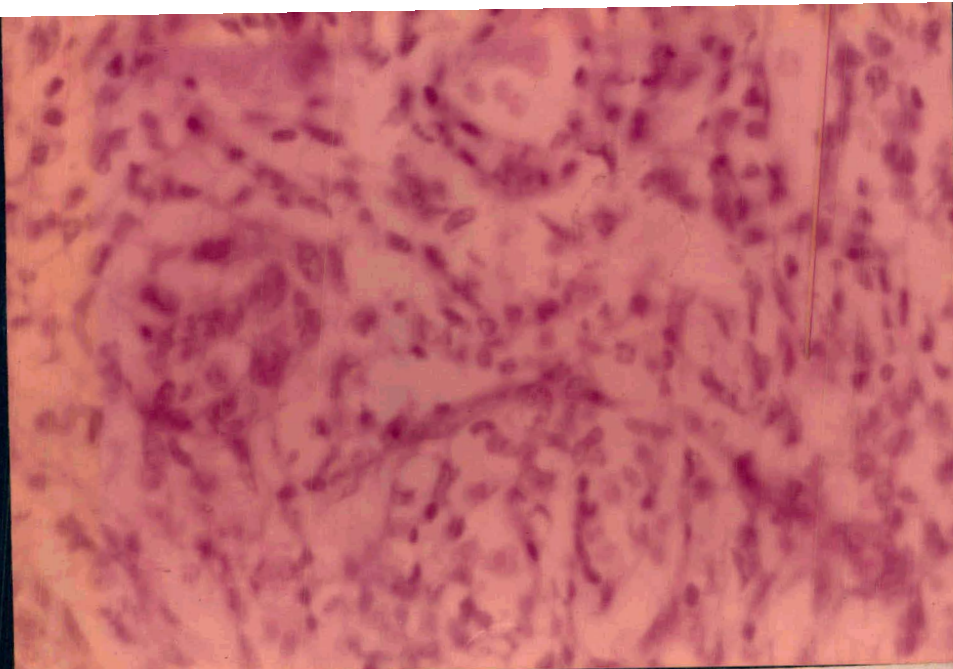


Fig.8 : Higher magnification from another area of the same lymph node.

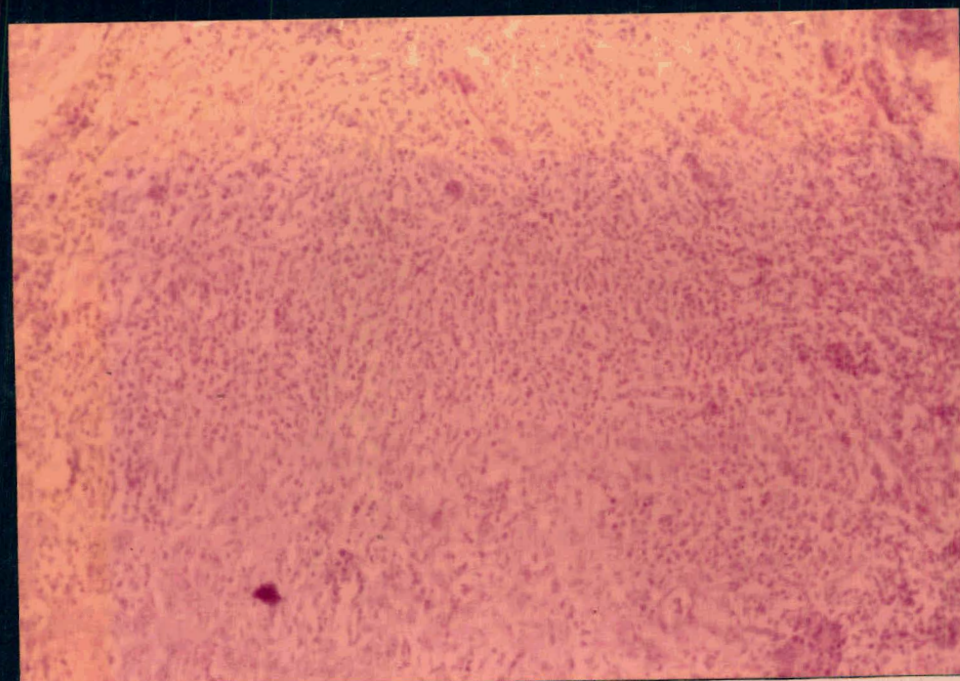


Fig.9 : Lymph node : Secondaries from poorly differentiated squamous cell carcinoma of the pyriform fossa. Upper right hand corner - mucus salivary glands show strongly positive SRCA (+++). Lower half - secondaries from poorly differentiated squamous cell carcinoma show negative SRCA (-ve).

of isoantigens within the keratinised centres.

Moderately differentiated and poorly differentiated carcinoma:

MCAR was negative in all the cases studied.

Metastatic Carcinomas :

a) In all the cases of secondaries where the primary lesions were moderately differentiated or poorly differentiated, the MCAR was negative.

b) In a few cases of secondaries, the MCAR was positive. But in all these cases the primary lesions were well differentiated squamous cell carcinomas with abundant epithelial pearls. The positive areas in the secondaries were contiguous to the primary site. Davidsohn et al (1969)⁷ studying isoantigens in cases of carcinoma of the lung have reported similar findings in a few cases with secondaries. They explained that the findings in the primary carcinoma suggested that the loss of the isoantigens need not necessarily take place in the entire carcinoma at the same time, the loss of isoantigens expressed in the negative MCAR need not be a 'all or none phenomenon'. If the concept is correct that the change from a positive to a negative MCAR is the result of an immunologic dedifferentiation then it would not be surprising to find different stages of dedifferentiation side

by side. The finding of a positive MCAR in a regional lymph node attached to or near the primary may be explained by continuity of growth from the primary carcinoma of both positively and negatively reacting portions of the cancer.

In the malignant lesions in our study, areas of haemorrhage had strongly positive MCAR. Davidsohn et al⁷ found that in cases of secondaries in adrenals, the rich vascular supply of the adrenal was responsible for positive MCAR.

Fritz Lin et al (1977) studied isoantigen in morphologically normal mucosa and carcinoma larynx. They found in a few cases that the initial biopsy specimens were histologically benign yet negative for isoantigens. In subsequent biopsies these showed the presence of carcinoma. This suggested that isoantigenic alteration as detected by MCAR was a sensitive sign, since it occurred in squamous mucosa prior to recognisable morphologic change for malignancy. In a case of dysplasia, in our study we found a few areas devoid of antigen indicated by a negative MCAR. These could be early carcinomatous areas that could not be morphologically identified as carcinoma.

Recently, analysis of bladder cancers have identified marker chromosomes. These are specific and distinct structural alterations of the chromosomes in the A group. Preliminary reports suggest that these abnormalities identify patients destined to develop invasive cancers. The correlation is apparently even stronger when abnormal chromosomes are combined with absence of surface isoantigens. Perhaps these are the cases requiring follow up at frequent intervals whereas those without chromosomal abnormality and showing presence of isoantigens need not be followed up as frequently.

SUMMARY AND CONCLUSIONS

In interpreting the results of SRCA, the normal distribution of the isoantigens in the different tissues has to be kept in mind. Even in normal adults the isoantigens are absent in the distal colon^{20,23}. In the prostate the antigen is observed only in traces in younger normal cases, increased in benign hypertrophy and markedly reduced or absent in malignancy¹².

Patients with blood group O cannot be brought under surveillance of SRCA because of the difficulty of obtaining anti-H in high enough titres and the variable results produced using it.

In our study of squamous epithelia from different tissues we were able to demonstrate presence of isoantigen in normal tissues and in benign lesions by a positive MCAR.

In the malignant lesions studied, we had a negative SRCA in most cases. In a few cases where it was weakly positive, the lesions were very well differentiated carcinomas with positivity confined to the keratinised centres of epithelial pearls. Thus, although in these cases MCAR may

not be helpful, in some cases of early carcinomas where the opinion, on morphological grounds is divided, a negative reaction favours the less auspicious interpretation.

In dysplastic tissues, the MCAR was weakly positive over scattered areas indicating reduced antigenicity.

The isoantigenic alteration as detected by MCAR is a sensitive sign since in some instance it occurs prior to recognisable morphologic change to malignancy.

MCAR being a specific test could be used in identification of blood group from tissue sections as indicated by our study. In a few cases where blood groups of patients were not known we submitted the tissue to MCAR using anti A and also anti-B. The blood group was decided by positive MCAR in endothelia of blood vessels in the sections.

The MCAR can be used in corroboration with chromosomal studies in investigation and follow up of cases of invasive carcinomas.

The MCAR is a sensitive, reproducible and inexpensive test.

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