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BLOOD GROUP SUBSTANCES A AND B ON THE ORAL EPITHELIUM OF AGING CAUCASIAN MALES

by

NOEL B. LAPP

A thesis Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirement for the Degree of Master of Science

June

1968

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To Doctor Joseph Jarabak for the opportunity and the concept of my Orthodontic graduate training.

LIFE

Noel B. Lapp was born in Chicago, Illinois on December 25, 1940. The south side of Chicago became his home and continued to be, even as it is today.

His secondary schooling was received at Mendal High School. In September of 1958 he matriculated at Loras College in Dubuque, Iowa. In September of 1960 his formal Dental training began at Marquette University from which he graduated in 1964 with a degree of Doctor of Dental Surgery. Two years followed serving with the United States Air Force, being separated in June of 1966. Also in June of that same year he entered a two year program in Orthodontics at the Chicago College of Dental Susgery, Loyola University, leading to a Master of Science Degree in Oral Biology.

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CHAPTER I

INTRODUCTION

Until recently, inherited characters were expected to remain unchanged during the life of an individual mammal, though botanists have long been familiar with plants in which a later developing part may differ genetically and in appearance from the original.

In man, presumedly acquired changes in the ABO blood group antigens have been reported in several cases, mostly in association with some type of malignancy. Any other changes or loses in the characters of blood groups, until this time, have not been documented.

The purpose of this thesis will be to examine the oral mucosal cells of men between the ages of ten and seventy for the presence of these blood group substances. Therefore, we may associate a change in the blood group substances of the mucosal epithelial cells with the normal aging process as well as the malignant process.

CHAPTER II

REVIEW OF LITERATURE

A. Oral Exfoliative Cytology:

It has been known for one hundred years that cells removed from a tumor could provide a clue to diagnosis. However, the cytological investigation of epithelial smears as a diagnostic aid for cancer detection was not understood or used until the classical studies of Dr. Papanicolaou which began in 1917. Papanicolaou's early work included an extensive investigation of the normal cellular pattern in epithelial smears prepared from vaginal secretion. His work subsequently involved the cytologic investigation of pathologic conditions and by 1928 he was able to demonstrate cancer cells in vaginal smears successfully. By 1940 his technique was sufficiently perfected to be put into practical use in the gynecology clinic of the New York Hospital where it has been in constant use since. In 1943 Papanicolaou and Trout published a monograph on the use of the vaginal

smear for the diagnosis of uterine cancer. The following a ten year study, a more complete presentation of the subject by these authors, together with Marchetti, was published in 1948.

With the increasing popularity of this method as an aid in diagnosing cervical and uterine cancer, the question of its value as an aid in diagnosing malignancies occuring at other cites was put forth. Fluid from the pleural and peritoneal cavities, sputum, urine and gastric washings have been studied with an equal degree of success.

There was, at first, no apparent urgency for exploration of cytodiagnosis of cancer of the mouth since the mouth is so readily assessible to direct examination and suspicious lesions could be biopsied with little difficulty. The first report describing the use of exfoliated cells in the study of cornification as related to the oral epithelium was undertaken by Weinmann in 1948. He used the Wright and Gram stains to differentiate degrees of cornification in various sites of the oral mucosa of normal males ranging in

age from twenty to thirty years and Montgomery followed up with an investigation of the normal human oral mucosa in 1951.

The usefulness of oral cytology as a diagnostic tool is predicated upon familiarity with normal and abnormal histology of the oral epithelium. The histologic structure of the normal oral epithelium is a stratified squamous type with an innermost basal layer (stratum germinativum) a prickle cell or acanthotic layer (stratum Malpighii) and a surface cornified layer (stratum corneum). A granular layer is present when there is overlying keratin and its thickness is in direct proportion to the thickness of the keratinized layer. Keratinization is not normal in the mouth except on these surfaces that are exposed to friction such as the palate, gingiva and dorsum of the tongue.

The life cycle of the oral epithelial cell begins with mitotic division usually in the stratum germinativum, or basal layer. There "mother cells" comprise one or two layers of columnar to cuboidal cells that contain ovoid

nuclei whose longer axes are more or less perpendicular to the basement membrane. The daughter cells separate, grow in size, and gradually are pushed or migrate away from the basal layer into the next layer above to form the prickle cell layer. This mutual push and migration gives the individual cell its shape and is responsible for the typical mosaic pattern of the prickle cell layer. A three dimensional view would approximate a honeycomb structure of cells with flattened sides approximately the ideal fourteen-sided tetradecapaihedron. At this stage intercellular fibrils or bridges are usually apparent in a histologic section.

As the cell matures it approaches the surface and cornifies so that it can ultimately assume its protective function. The cell continues to cornify and harden with the disappearance of the nucleus until in the normal course of abrasion and physiological function, the surface cells are constantly exfoliated and are continually replenished by a new crop from the underlying cells.

Direct scrapings of the surface oral epithelium may

dislodge not only the surface cornified cells but also any or all of the layers down to the corium as well as any other local cells. These other cells may include those of the nasopharynx, lungs, bronchi, larynx, as well as leukocytes, lymphocytes, histiocytes, plasma cells and erythrocytes. Most of these foreign cells can be readily identified, but some of their abnormal forms may sometimes resemble malignant epithelial cells and be a source of confusion.

B. Fluorescent Antibody Technique:

Before 1942 many advances had been made in the isolation and physiochemical characterization of proteins but little was known of their individual distribution in cells and tissues. Histochemical techniques were developed for a small group of enzymes, but a far greater number of proteins, including many interesting enzymes and hormones, could not be localized by existing techniques. Finally a method which, in principle, seemed applicable to the localization of any protein was reported by Coons, Creech, Jones and Berliner in 1942. These workers were able to localize foreign anti-

gens in tissues by using fluorescent antibody solutions as specific histochemical reagents and control experiments to show that the staining was a specific immunochemical reaction. Since those early experiments this technique has been utilized by Coons and his associates to further the investigation on antibody synthesis and the sites of reactions. It has been further adapted into the study of hypersensitivity reactions, staining of microorganisms such as viruses, bacteria, protozoa and fungi, staining of tissue antigens of the three groups, heteroantibodies, isoantibodies, and autoantibodies, demonstrating reticulin and basement membrane, blood group substances, muscle proteins and enzymes - all with excellent results.

The basic manipulation of the Coons fluorescent method can be simply stated. An antibody solution of high titer is conjugated with fluorescein isocyanate. The resulting fluorescent antibody solution is employed as a specific histochemical stain on tissue sections or smears.

The antigenic material present on tissue cells and

more particularly in sections of them, will react specically with antibody complimentary to it. This immunological reaction results in the deposit of minute amounts of specific antibody over those areas of a tissue section where the antigen is present. When the antibody molecules have previously been chemically marked with fluorescein, the micro-deposit of fluorescent antibody is visible under the fluorescence microscope, because fluorescein emits a brillant yellow-green fluorescence under bombardment by ultraviolet and blue light, thereby permitting histologic localization of the antigen, while other structures are visualized by virtue of natural blue-gray autofluorescence. The specific step in the reaction is the selection by the antigen of its specific antibody from a solution of fluorecent proteins. Other serum protein molecules present in the fluorescent solution are washed away leaving the specific deposit in situ.

The two essentials in the preparation of tissue smears or sections for use with these conjugates are that the anti-

gen retain its immunological activity, and that the architecture and cytology of the tissue be sufficiently well preserved. i.e. The antigenic micromolecule being a polysaccharide-protein complex must be unchanged or unaffected. The preparation of tissue sections must therefore be carried through without chemical fixatives which would injure the immunological activity of the antigenic material. This limits the general methods available to the use of smears, or of freeze-dried sections, or of frozen sections of unfixed tissues. Clearly, methods involving tissue fixatives applicable to one antigen may not be suitable for another depending on the chemical characteristics of the antigen.

Until 1958, the only labeling substance of practical value was fluorescein isocyanate (Coons and Kaplan 1950; Coons et al 1942) chosen because of the brilliant apple-green fluorescence of its protein conjugates. The chemical procedures originally required for labeling with fluorescein isocyanate were too complicated to appeal to most workers and a welcome advance was made when Riggs et al (1958)

introduced a simpler conjugation method with fluorescein isothiocyanate (FITC).

At the present time the two fluorochromes used most widely are fluorescein and rhodamine. Fluorescein has a yellowish green fluorescence with a maximum at about 550 mu, and lissamine rhodamine B 200 or rhodamine B having a reddish orange fluorescence with a maximum at about 640 mu. These have been the fluorochromes of choice because the intensity or efficiency of fluorescence of these compounds is greater than those of other fluorochromes that have been studied. The green fluorescence of the fluoresceins offers two important advantages over the red fluorescence of rhodamine: (a) although fluorescein and rhodamine have about the same intensity of fluorescence, the eye is more sensitive to apple green than to reddish orange; (b) red autofluorescence is more common in nature than green autofluorescence.

C. Blood Group Substances and Their Distribution:

The first division of blood into groups were based on

differences among the antigenic substances on the surfaces of the red cells of a given species of animal. These antigens are distinguished by means of antibodies in serum that combine with the red cells. Blood group characters are inherited according to simple Mendelian laws, and the antigens, believed to be products of allelic or closely linked genes, are classified together in blood-group systems. Inherited variations in serum proteins and enzyme activities in both serum and red cells are other factors now known to differentiate bloods within a species.

The first human blood group system, the ABO system, was discovered by Landsteiner (1926) as a result of his attempts to determine whether specific serological differences existed between individuals of the same species, and the importance of a knowledge of the ABO groups for the safe practice of blood transfusion was promptly recognized.

The ABO classification of bloods is based on two red cell antigens, A and B and the two antibodies to these, respectively, anti-A and anti-B. These antibodies always occur

in the serum or plasma when the corresponding antigen is absent from the red cell.

Early reports concerning A and B antigens are few and lacking in detail. Yosida, in 1928, concluded that these antigens are absent from human skin epithelium. Then in 1955 Coombs and Bedford, applying the principle of mixed agglutination proved the existance of these antigens on platelets and then by using the same method in 1956 they confirmed A and B antigenic presence on skin epidermal cells of group-A and group-B individuals.

Attempts to follow the distribution of soluble antigens in the animal body began early in the history of immunology. Metchnikoff was apparently the first, (1897) using toxicity as a tracer. He studied the fixation of tetanus toxin in the tissues of chickens. Various other investigators continued in this vein until in 1939 Sabin introduced color for in vivo studies of antigenic distribution. The use of radioisotopes as tracers in such studies was introduced in 1947 by Libby and Madison. It was almost at this same time that

the use of fluorescein isocyonate labeled conjugates became a popular research too.

The distribution of blood group antigens in human tissues has been studied using the mixed agglutination technique, the agglutination inhibition technique, and more recently, the fluorescent antibody technique. By these methods the ABO antigens have been shown to be widely distributed in many human tissues and body fluids other than red blood cells and saliva.

D. Localization of Tissue Antigens:

Employing the specificity of the fluorescent antibody technique various investigators have sought to identify and localize the blood group antigens of certain tissues throughout the entire human body. Reports were published by Szulman in 1960, 1962 and Holborow, et. al. also in 1960. Szulman again in 1964 followed the same theme but using embryos at differing age levels to detect any change in the antigenic status. Then in 1965, Brandtzaeg demonstrated the A and B substances in alcohol-fixed human gingiva.

The ABO blood antigens occur in tissues in two forms; the alcohol soluble form found in red blood cells, vascular endothelium, etc., and the more abundant water soluble form found in epithelial mucins. Employing fresh frozen sections the alcohol soluble as well as the water soluble forms of blood group antigens have been identified.

The mapping out of the A and B antigens in the tissues of embryos and fetuses six to fourteen and one-half weeks ovulation age was undertaken in 1964 by Szulman. He found that: The endothelial cell wall antigens throughout the cardiovascular system are permanent and persist from prenatal into postnatal life, the epithelial cell wall antigens are found in the epithelia of the integument, the pharynx, the alimentary, respiratory, and lower urinary tracts. They present a pattern of maximal antigenic distribution since continuation of these antigens into adult life is seen only in the epithelia forming stratified squamous, transitional or simple confining membranes: i.e., those of the skin, the pharynx, the mouth and esophagus, and the lower urinary

tract from the collecting tubules to the urethra. On the contrary, the cell wall antigens of the epithelia of the gastrointestional tract, including the pancreas, of the thyroid and pituitary (anterior lobe), of the respiratory and upper female genital tracts undergo a gradual recession upon further development of the fetus. Cronologically, the waning of the antigens can be said to coincide generally with the morphologic differentiation and functional maturation of the organ concerned.

Certain body elements and organs are completely free of antigens at all times and others have them in the embryonic stage only to lose them with maturity or further growth. The liver, the posterior lobe of the pituitary, adrenal, peripheral and central nervous systems are free of antigens. The gonads, adrenal, pancreas, and parathyroid glands of the adult demonstrate no antigens as they did previously. The epithelia of the gastrointestinal tract, primitive and resembling that of the esophagus, are rich in antigens. The latter however, are to wane and disappear upon further de-

velopment of the mucosa of the various segments, each proceeding at its own pace. Thus, the mucosa of the body of the stomach, of the pylorus and of the small intestive rapidly lose their cell wall antigen with the colon and rectum following. In each case the antigen loss coincides with the assumption of the specialized function of mucous secretion.

The water soluble antigens borne in the secretion of the salivary glands and of the mucosa of the gastrointestinal tract are demonstrable in the submaxillary, sublingual, and parotid glands as soon as the lumens of the primordia become discernible with the pylorus and stomach body secretion active at the same time. The duodenum, large bowel, retro-sigmoid, respiratory system and pancreas commence in the above order with growth and development of the fetus. These water soluble antigens continue to be secreted during subsequent fetal development and during extrauterine life by all the organs described above.

The antigens of the stratified epithelia (and of the

simple epithelia of the renal collecting tubules), together with the endothelial antigens, are permanent and persist into and throughout adult life. All other cell wall antigens disappear at a time characteristic for each organ. The antigenic recession coincides with recognizable steps of morphological advancement and often with assumption of function by the organ concerned; it is completed at about the end of the first trimester of pregnancy.

E. Effects of Aging on Human Tissues:

Much study has been dedicated to the alterations affecting the various organs and tissues of the body in relation to the aging process, both in man and in various other animal species. The majority of such studies deal with the morphological aspects, a lesser number are of a qualitative nature, while those comprising quantitative statistics are particularly rare.

Meriggi and Zavanella completed an extensive study in 1963 of body organs, tissues and systems and concluded from their work that there is a numerical reduction in the cells

of practically all the organs involved in the aging process. This phenomenon had already been generally recognized for some time. What they proved to be statistically significant was that senility involves a reduction in the thickness of the epidermis, cellular layers, depth of the epithelial introfelxions, and of nuclear surfaces.

Thuringer and Katzberg (1959) found that: as the individual passes from birth to old age, the epidermis undergoes further differentiations and modifications which adjust it morphologically to meet its requirements as a protective covering of the external body surface in environmental extremes. Throughout the aging process many generations of epidermal cells continually pass through the phases of micosis, differentiation, maturation, senescense, death cornification, and desquamation. As the process of growth, maturation and senescence make varying demands on the body as a whole, similiar demands are placed on the epidermis in the various periods of life while it continues its functions.

The process is a continuous one of cellular exhaustion,

decline and death at the upper surface level which must be balanced by an equal intensive replacement of cellular units by the surviving cells with whatever materials are available and whatever capacities they may retain. It may be speculated that these intrinsic capacities become increasingly limited with the aging of the individual. However, while these decline, the process of cellular replacement is maintained by the deeper cells which are closer to the circulatory system and still retain the capacity to undergo mitotic division. Further yet, it has been indicated in studies by Cooper, Thuringer, and Katzberg (1951) that with increase in age, the rate of loss and regeneration of epidermal cells is accelerated along with a gradual increase in the rate of desquamation until the rate of desquamation slightly exceeds the regeneration of epidermis. Thuringer and Katzberg (1959) also showed that the mitotic index increased from birth through about the fifth decade and then remained relatively stationary. The mitotic activity of the basal layers showed a sustained increase from birth to old age while that of

the spinous layer showed a decrease.

Meriggi and Zavanella (1963) attempted to explain why the mitotic index is higher in old people than in the young. There is a possibility that this phenomenon may be merely apparent inasmuch as it is connected with the longer duration of mitosis or perhaps the discrepancy is due to the reduction in the average life of the cell demonstrated in the abdominal skin by Katzberg, who pointed out that from the first to the eighth decade of life there is a diminution in the average life of the cell from one hundred one to forty six days.

Lorincz compiled a short index of senile changes of human epidermis in 1960:

(a) widespread involution of sebaceous glands with deficient sebum production occurs post-menopausally in women and around the age of seventy in men.

(b) sweat secretions diminish gradually until around the age of seventy it is practi-

cally stopped completely.

(c) diminution of body and scalp hair

(d) nail growth rates diminish

(e) graying of hair

(f) small blood vessels become fragile

(g) sluggish vasomotor responses to temperature changes

(h) tensile strength of skin increases

(i) water content increases

(j) sulphur content decreases

(k) calcium content increases

(l) elastic fibers undergo fragmentation, clumping and acquire an affinity for basic stains as confirmed by Banfield (1954)

In summary, senile skin can be viewed physiologically as having generally reduced homeostatic faculties and greater vulnerability to injury.

F. Effects of Aging on Human Oral Tissues:

While working with normal oral tissues, Miller, Sober-

man and Stahl (1951) noted that the highest incidence of cornification was found in the gingiva followed by the dorsum of the tongue; next, the parietal surface of the cheek and lastly, the ventral surface of the tongue.

Then in contrast, Zimmerman and Zimmerman (1965) discovered that the mean cell count for keratinized cells in the gingiva and hard palate decreased with age while that in the tongue and buccal mucosa remained relatively unchanged.

The lips, the oral mucosa and the tongue in senescence present changes which are comparable with those observed in other tissues. They became atrophic, there is a loss of elasticity, an increased coarseness in fibrous substance, a decrease in the number of connective tissue cells and a decrease in the tunica propria (Wentz, Maier, and Orban, 1952 Muller, 1959). The characteristic stippling of the healthy gingival and oral tissue seen in adult life is absent in the aged. The mucosa now has a satiny, wax-like or edematous appearance. The tissues frequently lack a protective layer

of heratinized cells, due to a dimished gingival kerati-
nization occuring with increased age, which renders them
more susceptible to mechanical chemical or bacterial irri-
tation.

G. Demonstration of Antigenic Alteration:

It must be considered an established fact that the capa-
city to produce specific blood group antigens is geneti-
cally determined, (Hogman, 1960). He found that in normal
surroundings (in situ) the macroorganisms' genetic consti-
tution seems to be able to express itself in a variety of
cell types. When, after explanation of cells, this ability
seems to be lost, the explanation could be either a defi-
ciency of the growth medium, a differentiation of the cell
making it incapable of synthesizing antigenic substance, or
a mort fundamental change of the cell equivalent to a muta-
tion.

The most likely explanation of the findings seems to be
that, during cell division, the capacity to produce antigenic
substance is lost. Some cells divide more frequently, and

lose their antigens faster than those dividing slowly. This would explain the successive decrease in the number of positive cells, while those remaining still give quite strong reactions.

In 1956 the work of Weiler suggested that loss of antigens occurs in some forms of neoplasia. This phenomenon was explained by Kay in 1957 as being due to inability of the malignant cells to absorb antibody rather than to a failure at a later stage of the agglutination procedure, e.g., inhibition of absorbed antibody or detachment of loosely bound antibody during washing. He proposed as an alternative that there occurs some general change in the surface of the cell which masks the A and B antigens. In support of this view one may quote a variety of related phenomena. Irregularity of the surface as viewed by electron microscopy (Coman and Anderson, 1955), a deficiency in the capacity to bind calcium (Dunham, Nichols and Brunschwig, 1946), decreased adherence (McCutchem, Coman and Moore, 1948), absence of contact inhibition (Abercrombie

and Heays, 1953) and accelerated electrophoretic mobility (Ambrose, James and Lowich, 1956) have all been demonstrated.

The blood group antigens have been demonstrated in many different types of cancer regardless of the degree of their histologic differentiation. Glynn et. al. (1957) reported specific immunofluorescent staining of blood group substances in cancer cells of the stomach. This finding was confirmed in a later paper by Glynn and Holborow (1959). From here it became apparent that these antigens, demonstrable in tissues, may serve as natural labels for the study of antigenic changes in various disease processes, including cancer. The results of some research along this vein has shown that in the absence of cancer, the blood group substances were present abundantly in the epithelial cells and in secreted mucus; however, in cancer, the antigenic substance was absent in the secreted mucus. The failure to secrete the blood group antigen is interpreted as a manifestation of dedifferentiation of the cancerous process. In

a previous article of Glynn and Holborow it was noted that, whereas A and B positive individuals always exhibited the antigens on all cells of his epithelium, tumors arising in the same tissue often appeared partially or completely deficient in antigen. Furthermore, there seemed to be some correlation between the extent of tumor spread, the loss of structural and cytological differentiation and, the degree of antigenic loss. But the correlation was not close and there were some exceptions.

It is apparent that tumors may totally lose their antigens while remaining relatively benign and conversely, that anaplastic, pleomorphic tumors with metastases may have normal A and B antigens.

Hogman (1960) has reported that cultures of kidney cells regularly lose their AB antigens, as detected by mixed cell aggultination, after a number of subcultures. On the other hand, the normal transitional epithelium is rather constant in its preservation of antigen.

Green and Anthony (1963) and Nairn (1962) also have

some normal tissues lose antigens when converted to neoplastic growths by carcinogens. However, Corruthers and Baumler (1966) have introduced the possibility that some carcinogen-induced tumors acquire new antigens.

In the malignant transformation of mouse epidermis into squamous cell carcinomas, previous studies showed that:

(a) normal and hyperplastic mouse epidermis had antigens not detectable in squamous cell carcinomas, and

(b) these carcinomas contained antigens not apparent in the epidermis (Carruthers and Baumler, 1965)

There have been reported cases of a previously AB patient who, after the onset of some form of cancer acquired an anti B antigen in their serum. Family studies of these patients strongly suggested that they all lacked a B gene and most of them proved to be AA or AO. In 1958-59 Richards presented a case of a forty year old woman who had chronic lymphatic leukemia and was undergoing whole body radiation,

transfusions and steroid therapy. After ten months treatment on the last day of radiation therapy her red cells grouped A without a trace of B. This phenomenon was explained as being the result of the sematic mutation of a modified gene.

Kay and Wallace (1961) found a partial or complete loss of A and B antigen associated with tumors of the urinary epithelium. There was also a correlation with other properties of the tumors revealing an increased frequency of antigen loss particularly among the more pleomorphic, anaplastic, infiltrating and rapidly fatal tumors. There appeared to be no connection between the age of the patient and loss of antigen.

CHAPTER III

MATERIALS AND METHODS

The experimental sample in this study consisted of one hundred male subjects between the ages of ten and seventy years. The intervening span was broken up into four age groups; 10-20, 25-35, 45-55, 60-70 and upward. Each group was in turn subdivided into an equal representative number of A and B blood types.

A. Blood Typing:

All subjects were first screened by testing for blood type using anti-A and anti-B blood grouping sera in the slide agglutination test. Only subjects with type A or B blood were scheduled for cytological examination. The reasoning here being that the type A and B individual demonstrate the corresponding antigen on their red cells as well as in various tissue cells and body fluids while the type O individual has no detectable antigens and the AB type had both, which was felt could possibly produce erroneous identification pro-

blems later in the microscopical examination.

B. Oral Exfoliative Cytology:

The objective of exfoliative smears is to obtain a representative sample of epithelial cells from the oral buccal mucosa by scraping off the superficial layer of cells and spreading on a microscopic slike. The basic technique is as follows:

1. Cleanse the surface of the area if it is covered by debris, mucous or slough, by wiping the area with gauze moistened in normal saline. The surface was never completely dried since moisture is essential.
2. The area of the sample is then scraped with between ten to twenty strokes using moderate pressure on a #7 wax spatula.
3. The scrapings picked up on the spatula were then spread on the glass slide as evenly as possible and without delay were

immersed in the fixative (ethyl alcohol 95 per cent. Fixation was carried out at 27 degree centigrade for thirty minutes. This fixation technique with ethanol removes the alcohol soluble antigens if any were present but does not interfere with the demonstration of the water soluble antigens according to Glynn et. al. 1957.

C. Staining Technique:

Immediately following fixation the slides are removed from the coplin jars and are allowed to dry. Then they are washed in three consecutive one minute baths of normal saline, buffered at pH 7.2 by adding ten ml. of Sorensen's phosphate buffer to each titer of salt solution (1000 ml). The slides were dried and then flooded with *homologous antisera, placed in a petri dish with some moist filter paper thus creating a high humidity enviroment to prevent evapo-

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* Human typing sera without coloring agent purchased from Hyland Laboratories, Los Angeles, California.

ration, covered and incubated at 37 degrees centigrade for twelve hours. The slides were again washed in three saline baths but of five minute durations and then dried. Next application was that of the **conjugated fluorochrome globulin and another twelve hour incubation period. After thorough washing in the three saline baths and drying, a drop of reagent glycerol was applied to the smear and a clean coverglass dropped over it. Now the preparation is ready for examination under the fluorescence microscope.

The special staining employed was the sandwich method which has been assessed by Coons to have ten times the sensitivity over single layer tracing. This extra sensitivity is presumed to be due to the additional combining sites which are made available by the antibody molecules of the middle layer acting as antigen for the fluorescent anti-globulin. Refer to figure I and II for explanation of reaction.

Specific staining denotes the process of coating anti-

** Fluorescein isothiocyanate conjugated to goat anti-human globulin; Hyland Laboratories, Los Angeles, Calif.

gen with fluorescent antibody and of providing by suitable tests that the reaction is specific. Therefore, as in any serologic method, control tests constitute an integral part of the experimental procedure.

In this particular experiment the specificity of the reactions was shown by the lack of staining with labelled normal serum or by the blocking technique, in which unlabelled heterologous antiserum was applied prior to treatment with labelled antiserum, figure III. Thus no staining was obtained or very weakly observed with conjugated antisera applied alone to the antigens or when an anti-A serum was reacted with the B antigen before the conjugate layer or an anti-B serum reacted with the A antigen.

Prior to attaining a specific reaction the main complication of fluorescent studies, namely, unreacted fluorescent material and non-specific staining, had to be removed. Firtually all unconjugated dye was removed from the antisera by gel filtration *or by shaking with powdered activated

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* Sephadex G-25, Medium, Pharmacia Ltd., Uppsala, Swedan.

charcoal. These prepared serum conjugates were further absorbed with mouse-liver powder ** to remove unwanted antibodies or other proteins responsible for non-specific staining reactions.

D. Fluorescence Microscopy:

All slide smears were examined under an ultra-violet light source through a Reichert Zetopan research microscope with a HBO high pressure mercury vapor lamp. The specimens were examined for specific yellow-green fluorescing features at one hundred and four hundred magnifications.

Photography was accomplished immediately upon visualization of the fluorescent cellular configurations at exposures varying between sixty and ninety secons using outdoor high speed exctachrome film with an ASA of 160.

** Baltimore Biological Laboratory, Baltimore, Maryland.

CHAPTER IV

FINDINGS

Oral cytological smears were taken from the "normal" buccal mucosal of one hundred individuals ranging in age from ten to seventy-seven years. The total number taken was two hundred forty, having been one from each side of the oral cavity. Each smear was immediately fixed in a solution of ethanol with fluorochrome staining to be processed within two hours and then immediately examined by fluorescent microscopy.

A. Group 1: Ages 10-20

Twenty-five males in this catagory were examined and provided fifty specimens equally divided into type A and type B. Upon microscopic examination all twenty-five that were treated with their corresponding antisera and conjugated fluorochrome produced a positive reaction. (slides 1 and 2). The remaining twenty-five were treated with either conjugate alone or heterologous anti-sera and then conjugate

to determine specificity of the reaction. Added to this group as well as the other three, were five specimens from type O phenotypes which were examined with both A and B anti-sera and fluorochrome for positive fluorescence. These reactions in that the brilliant green cellular fluorescence of the positive reaction was missing with only some light background fluorescence visible, (slide 5) under high illumination.

B. Group II: Ages 25-35

This group produced another fifty oral smears which were treated exactly as in the first group. The results upon microscopic examinations were identical to those found in the younger age group.

C. Groups III and IV: Ages 45-77

Within these two groups one hundred specimens were fixed, stained, and examined for fluorescence. Again the finding paralleled the results of the younger individuals in that the smears treated with homologous antisera followed by conjugate and incubation were all positive and that the

blocking reaction, whether they were simply conjugate were or heterologous antisera and conjugate negative. However, there was detected a slight diminution in the degree of fluorescence of the positively reacting specimens. This discrepancy was noticed at various times and no definite age could be determined. Furthermore, due to many variables in fixing, staining, washing, and incubation, this phenomenon could be discarded as due to chance instead of a diminution with age, since it was not a constant finding among the sixty and seventy year olds as you would have then suspected. If this phenomenon had been a constant finding we could have postulated that it's cause was due to the fact that as the mitotic index increases with advancing age the cells capacity for antigenic substance production is lost or reduced and would therefore account for decreased reactivity with fluorescent antibodies. We must assume that in our technique the cells were obtained from the cellular layers of the stratum spinosum following the documented evidence provided by Szulman (1960) and Brandtzaeg (1965). Also, another con-

stant finding which corroborates the work of Prendergast (1967) was that the intensity of the reaction in the type A specimen was always greater than that seen for the type B. Refer to slides 1 through 4. This also confirms the previous findings of Swinburne (1961) and Glynn and Holborrow (1959).

CHAPTER V

DISCUSSION

The blood group substances of man are individual characteristics inherited by allelic genes and clasified according to systems based on the antigenic differences on the surfaces of the red blood cells of each particular person. These differences plus other inherited variations serve to differentiate individuals within a species.

Midway during the twentieth century these antigenic differences were found to be present on not only the blood cells of a subject but on certain cells throughout his entire body. Further study led to the fact that some of these antigens are present in prenatal life but absent in post-natal life while other areas persisted with their antigens into adulthood. This quantitative change is explained as a morphologic differentiation and functional maturation of the organs concerned as the fetus passes from pre-natal to post-natal environment. Still, other body elements have no anti-

genic presence whatsoever at any time.

The human A and B antigens in foetal red cells are weaker than those in erythrocytes of adults and undergo marked quantitative changes during the early years of Life. Morville, in 1929, found that in most instances the A and B antigens seemed to have reached maximal strength by the time the child was four months of age. However, according to Thomson and Kettel (1929) the antigenic strength increased more slowly and did not reach a maximum until fifteen to twenty years of age. The intervening years before 1953 produced no new related concepts until Lodenkamper and Steiner provided data indicating that the A and B antigens reached a maximum value at one or two years then remained constant until thirty to forty years after which their strength decreased to zero. Obviously, there was some discrepancy in the previous investigations.

The latest research on this problem was undertaken in 1964 by Grundbacker who showed that the antigenic strength of A and B antigens remains constant and does not decrease,

even in old age.

Here it may be stated that all these investigations have been performed on red blood cells without any connection or influence upon epithelial cell antigens being mentioned.

This paper concerns itself with certain human blood group substances that appear on the surfaces of the cells of the normal epithelium. These substances have been extensively followed through disease and health of the various body organs with certain definite observations and conclusions, but no absolute explanations. For example, there is the fact that normal cells react positively to the presence of their antigenic substances while neoplastic cells can react either positively or negatively. Could this negative reaction be due to a lack of antigenic development, a complete absence of the antigens, or a change in cellular surface electrical charges? Louis (1958) has attributed this reaction failure with a cytoplasmic loss of a basic protein in malignancy. But the question still remains unanswered.

Could it be that as a person grows older his cellular capacity to produce these antigens is decreased or lost. If so, then there could be a correlation between again and carcinoma.

Proven facts concerning aging and epidermis are that there is a numerical reduction in cell number, cell life is reduced, etc., with a general view of having reduced homeostatic faculties and greater potential for serious injury and prolonged repair. The oral epithelial tissues compare and parallel the epidermis and its related changes, but of course to different degrees, and it must be from here, in the oral cavity, that this study was conducted.

Our only concern was with normal cells and their antigenic properties through the aging process. Positive results were obtained from age ten up to age seventy-seven with an occasional qualitative dimunition of fluorescence which by no means was significant enough to suspect an antigenic loss. Specificity of reactions was accomplished therefore, a safe assumption would be that qualitatively with age the cellular

antigenicity remains unchanged within the epithelial lining of the buccal mucosa.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Oral cytological specimens were obtained from one hundred individuals exhibiting either type A or type B blood. The smears were removed from the normal epithelium of their buccal mucosa, immediately fixed in 95 per cent ethanol, reacted with their homologous antisera followed by the sandwich staining technique with a conjugated flourochrome. Blocking reactions were examined in all cases to verify specificity of staining.

The results of this examination parallel previous studies with normal oral epithelium and complete that done on human red blood cells and their antigenic components. A statement of findings is as follows:

1) Type A and B tissue smears fixed in ethanol flouresced positively when reacted with corresponding antisera and conjugated flourochrome.

2) Type A tissue produced a stronger degree of fluorescence than type B tissue.

3) Blocking reactions were always negative.

4) Type O tissues produced a response identical to an A or B block.

5) It was concluded that the aging process does not influence the antigenic reactivity or the cellular productivity of it's blood group substances.

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TABLE I

CAUCASIAN MALE AGES 10-20

SEX	AGE	BLOOD TYPE	BLOCKED REACTION	NORMAL * REACTION	FIXATIVE ETHANOL
M	15	A	-	x	x
M	15	A	-	x	x
M	14	A	-	x	x
M	14	B	-	x	x
M	13	B	-	x	x
M	11	A	-	x	x
M	11	A	-	x	x
M	13	A	-	x	x
M	12	B	-	x	x
M	10	A	-	x	x
M	10	A	-	x	x
M	13	A	-	x	x
M	17	A	-	x	x
M	13	B	-	x	x
M	11	B	-	x	x
M	13	B	-	x	x
M	11	A	-	x	x
M	11	B	-	x	x
M	14	B	-	x	x
M	10	B	-	x	x
M	10	B	-	x	x
M	13	B	-	x	x
M	12	B	-	x	x
M	11	A	-	x	x
M	10	B	-	x	x
M	14	O	-	x	x
M	12	O	-	x	x
M	13	O	-	x	x
M	10	O	-	x	x
M	15	O	-	x	x

* The reaction was determined as either positive or negative.

TABLE II

CAUCASIAN MALE AGES 25-35

SEX	AGE	BLOOD TYPE	BLOCKED REACTION	NORMAL * REACTION	FIXATIVE ETHANOL
M	27	A	-	x	x
M	30	A	-	x	x
M	26	A	-	x	x
M	25	B	-	x	x
M	27	A	-	x	x
M	29	A	-	x	x
M	28	A	-	x	x
M	29	A	-	x	x
M	26	A	-	x	x
M	25	A	-	x	x
M	27	A	-	x	x
M	25	A	-	x	x
M	27	A	-	x	x
M	25	A	-	x	x
M	25	B	-	x	x
M	25	B	-	x	x
M	26	B	-	x	x
M	28	B	-	x	x
M	29	B	-	x	x
M	25	B	-	x	x
M	25	A	-	x	x
M	31	B	-	x	x
M	28	B	-	x	x
M	31	B	-	x	x
M	32	B	-	x	x
M	27	O	-	x	x
M	25	O	-	x	x
M	26	O	-	x	x
M	29	O	-	x	x
M	27	O	-	x	x

* The reaction was determined as either positive or negative.

TABLE III

CAUCASIAN MALE AGES 40-55

SEX	AGE	BLOOD TYPE	BLOCKED REACTION	NORMAL REACTION *	FIXATIVE ETHANOL
M	41	A	-	x	x
M	42	B	-	x	x
M	45	AB	-	x	x
M	49	A	-	x	x
M	43	A	-	x	x
M	50	A	-	x	x
M	54	AB	-	x	x
M	48	B	-	x	x
M	43	B	-	x	x
M	51	B	-	x	x
M	46	B	-	x	x
M	47	B	-	x	x
M	50	B	-	x	x
M	50	AB	-	x	x
M	49	A	-	x	x
M	53	B	-	x	x
M	55	B	-	x	x
M	47	A	-	x	x
M	47	A	-	x	x
M	51	A	-	x	x
M	49	AB	-	x	x
M	40	A	-	x	x
M	46	B	-	x	x
M	49	B	-	x	x
M	54	B	-	x	x
M	52	O	-	x	x
M	47	O	-	x	x
M	43	O	-	x	x
M	46	O	-	x	x
M	41	O	-	x	x

* The reaction was determined as either positive or negative.

TABLE IV

CAUCASIAN MALES AGES 60-70

SEX	AGE	BLOOD TYPE	BLOCKED REACTION	NORMAL * REACTION	FIXATIVE ETHANOL
M	66	B	-	x	x
M	74	B	-	x	x
M	64	B	-	x	x
M	63	B	-	x	x
M	75	A	-	x	x
M	74	B	-	x	x
M	69	B	-	x	x
M	67	A	-	x	x
M	69	A	-	x	x
M	64	B	-	x	x
M	77	B	-	x	x
M	76	B	-	x	x
M	67	A	-	x	x
M	62	B	-	x	x
M	65	A	-	x	x
M	63	B	-	x	x
M	60	B	-	x	x
M	66	B	-	x	x
M	63	A	-	x	x
M	76	B	-	x	x
M	67	A	-	x	x
M	67	B	-	x	x
M	76	B	-	x	x
M	64	B	-	x	x
M	72	B	-	x	x
M	68	O	-	x	x
M	64	O	-	x	x
M	67	O	-	x	x
M	67	O	-	x	x
M	61	O	-	x	x

* The reaction was determined as either positive or negative.

FIGURE I

I Parallel preparations (a and b) which contain the antigen (solid black) are treated respectively with specific antiserum (hatched) and non-immune serum (strippled).

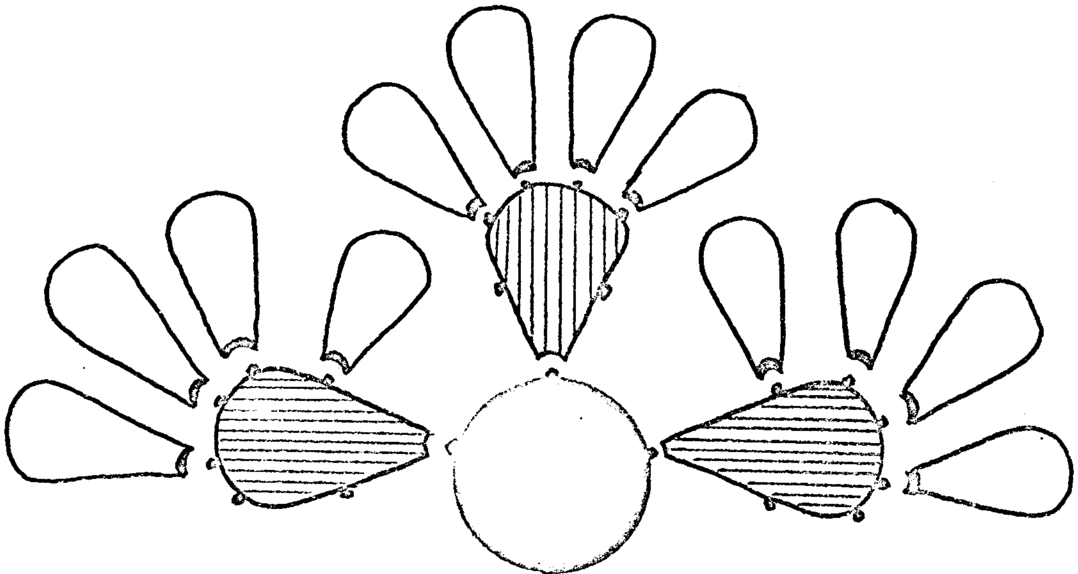
II The non-immune serum is removed from b by washing while the antibody in the immune serum has combined with the antigen in a.

III The conjugated antiglobulin serum (chequered) is applied.

IV The fluorescent antiglobulin has combined with the antibody attached to the antigen in a and is washed away from b.

Sandwich method of antigenic staining

FIGURE 2



Fourfold Sensitivity of Sandwich Staining

Only three sites in the antigen (black) are available for the unconjugated antibody (hatched), which behaves as an antigen for the conjugated antiglobulin. Thus reactive sites for fluorescent staining are increased fourfold.

FIGURE 3

I Parallel preparations a and b which contain antigen (black disks) are treated respectively with meterorogous antiserum (strippled) and specific homologous antiserum (hatched).

II The antiserum is removed from a by washing and does not stain, while the antibody in the immune serum combines with the corresponding antigen in b.

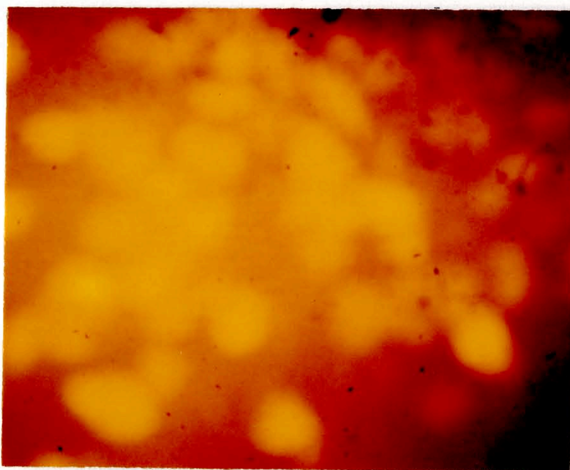
III The conjugated specific antiserum (chequered) is applied to the combined antigen-antisera.

IV The fluorescent antibody combines with the antigen antibody complex in b only, the combining sites in a remain unoccupied by the labeled antibody and do not stain.

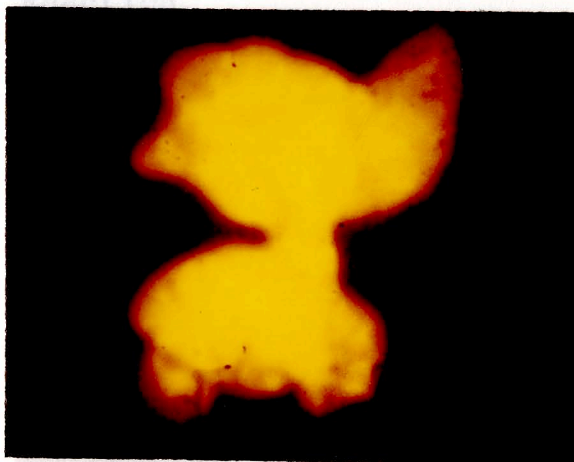
Blocking Reaction

PHOTOMICROGRAPHS

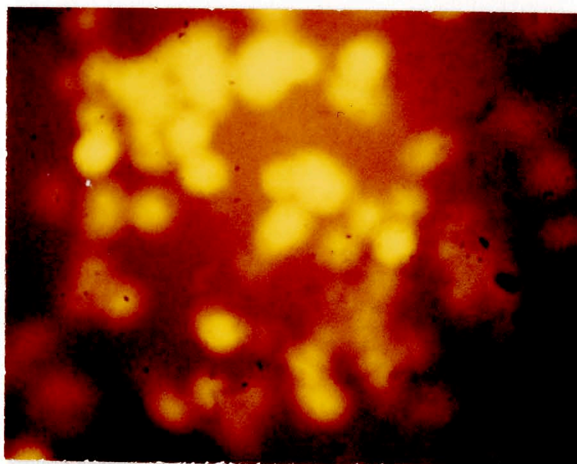
Slide 1. 100X magnification of positive reacting type A epithelial cells.



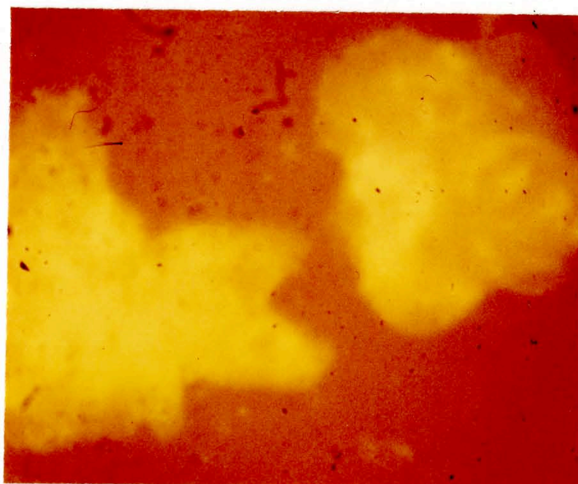
Slide 2. 400X magnification of positive type A Reaction.



Slide 3. 100X magnification of positive reacting type B epithelial cells.



Slide 4. 400X magnification of positive type B Reaction.



Slide 5. 400X magnification of well-illuminated blocking reaction.

APPROVAL PAGE

The thesis submitted by Noel B. Lapp has been read and approved by three members of the faculty of the Graduate School.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 15, 1968
DATE

SIGNATURE OF ADVISOR