

The Expression of the Epithelial Blood-group Substances: Normal and Malignant Tissues

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The blood-group isoantigens are macromolecules localized to the plasma membranes of certain epithelial tissues.^{2,11-15} These substances are not detectable on the epithelium once it has undergone malignant transformation.^{2,9,13} Results of this investigation have demonstrated that the loss of detectability of the blood-group isoantigens does not appear to be related to a "masking" effect by an increase in surface sialic acid. Using fluorescein-labeled lectins specific for sugar subunits which are components of the blood-group oligosaccharide chain, it was found that the malignant cells and cells of the parabasal layer of normal oral epithelium had high levels of N-acetyl-D-glucosamine (GlcNAc), the subterminal sugar residue of the blood-group chain. The basal cells of normal epithelium and a minority of the malignant cells demonstrated levels of D-galactose-N-acetyl-D-galactosamine, which are the most proximal blood-group sugar subunits, as well as subunits of other membrane antigens.

Our results indicate that malignant cells seem to be capable of synthesizing the blood-group oligosaccharide chains to the same level as the normal cells of the parabasal layer of stratified squamous epithelium. This level is just subjacent to the terminal D-galactose residue of the blood-group precursor chain. Increased or decreased differentiation characteristics of squamous cell carcinomas did not alter the level of blood-group synthesis. However, there may be a correlation between the level of synthesis of these antigens and the ability of the cells to demonstrate motility and to proliferate.

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Introduction.

Previous studies¹⁻⁴ have demonstrated that malignant oral squamous epithelial cells are non-reactive to antisera directed against the blood-group isoantigens. Normal epithelial cells above the parabasal layer of the strati-

fied squamous epithelium have the blood-group substances located on their plasma membranes and therefore react with antibodies directed against these isoantigens. Non-malignant lesions previously tested in our lab, such as epithelial hyperplasias and papillomas, also demonstrate plasma membrane blood-group isoantigens.¹ Previous investigators have reported that epithelium extending across oral wound margins fails to demonstrate reactivity for the blood-group substances.^{5,6} Therefore, it appears that cells which fail to demonstrate blood-group reactivity are cells which actively proliferate and demonstrate motility. These cells are the basal and parabasal cells of normal epithelium, normal cells proliferating across a wound margin, and malignant epithelial cells.

The present study sought to investigate the failure to demonstrate the "complete" blood-group oligosaccharide antigens on some normal cell populations, as well as on the malignant cell surfaces. This loss of antigen detectability was considered to be caused by one of two mechanisms. The first of these dealt with a possible increase in cell surface sialic acid which could "mask" the blood-group specific sugars.⁷ The second was that malignant cells and normal cells of the basal and parabasal cell layers may not synthesize the "complete" blood-group oligosaccharide chains. We hoped to determine any similarities between the malignant cells and the cells of the basal and parabasal layers of normal oral stratified squamous epithelium.

Materials and methods.

Specimens of fresh oral malignant and non-malignant tissue were obtained from the Otolaryngology Department at the University of Michigan Hospital, as well as from the

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Department of Oral Surgery, School of Dentistry. Consent of the patient was obtained for the use of the specimen after an explanation of its use was provided. The blood-type was recorded in almost all cases. Paraffin-embedded specimens were obtained from the Department of Oral Pathology, School of Dentistry, University of Michigan.

The fresh tissue specimens were initially placed in phosphate-buffered saline (PBS) at 10°C after surgical removal. Two methods for examining this fresh tissue were utilized. One group of specimens was removed from the PBS, mounted on a metal cryostat freezing stage with an embedding compound,* and frozen quickly in liquid nitrogen. Once frozen, the tissue was sectioned on a cryostat at 4 µm, transferred to pre-cleaned glass slides, and allowed to air dry for ten min. The second technique for utilizing the fresh surgical specimens was to fix them in 2% glutaraldehyde in cacodylate buffer, while stirring for 15 min at 4°C. The tissue was then rinsed in PBS, frozen, and sectioned as above. Hematoxylin- and eosin-stained sections were made of each sample in order to determine the exact content of the specimen. Paraffin-embedded tissue specimens were sectioned at 4 µm and deparaffinized in a xylene rinse for 30 min, followed by 100% alcohol and 70% alcohol for 15 min each. These were then rehydrated in PBS.

Blood-group reactivity. — In order to test for the “complete” blood-group glycoproteins, the tissue sections were first rinsed with PBS for 15 min, patted dry with absorbent paper, and then incubated in a drop of blood-group antisera in a humidity chamber for one h at 20°C. This antiserum consisted of human anti-A and anti-B† at a dilution of 1:16. The specificity of the antiserum to be used was determined by the patient’s blood type or by trial and error, until positive results were obtained on areas of normal epithelium or on the endothelial cells of capillaries.¹

Following the antisera, the slides were rinsed in PBS for 30 min at 20°C to remove any non-binding antiserum from the tissue section. The slides were patted dry and covered with a drop of fluorescein isothiocyanate-labeled (FITC) rabbit anti-human immunoglobulin.‡ This fluorescent anti-immunoglobulin was reacted with the tissue-bound antisera for one h. Non-binding rabbit anti-immunoglobulin was rinsed off in PBS for 30 min, and the slides were counterstained with a 40X dilution of eriochrome black stain for 15 sec. A “thin-O” coverslip was then sealed over the tissue section with clear fingernail polish, and the slide was viewed on a Leitz fluorescent microscope.

Blood-group O antigen was tested with the fluorescein-labeled lectin *Lotus tetragonobolus*.¶ This lectin, diluted 1:80, was applied to the tissue sections in a humidity chamber for one h. Since the lectin itself was fluorescent, a sandwich technique needed for the human antiserum was not used. The slides were counterstained with eriochrome black and coverslipped, as described above.

Sialic acid removal. — Tissue sections were reacted with *Vibrio cholerae* neuraminidase (VCN, 500 U/ml)[§] for one h at 37°C, pH 5.5, following initial rinsing in Medium 199. The neuraminidase was diluted to concentrations of 25 U/ml, 100 U/ml, and 250 U/ml with Medium 199, pH 5.5. The tissue sections were then rinsed three times in PBS to remove the enzyme. Following removal of the VCN, the tissue sections were tested for the “complete” blood-group substances (described previously), as well as for the blood-group subunits as described below. Periodic acid-Schiff staining was also performed on sections treated with VCN and on controls which were not reacted with the enzyme. This histochemical technique is useful in determining the presence or absence of sialic acid on tissue sections.¹⁸

Lectins. — Several plant lectins which are specific for certain sugars on the cell membrane surface and labeled with FITC were

*Lipshaw M-I Embedding Matrix, Lipshaw Manufacturing Co., Detroit, MI
†Hyland, Costa Mesa, CA

‡Bio-Rad Laboratories, Richmond, CA
¶E-Y Laboratories, San Mateo, CA
§GIBCO, Grand Island, NY

purchased from E-Y Laboratories. These lectins were titrated 1:80 in order to obtain acceptable fluorescence.

The sialic acid content of both normal and malignant tissue specimens was investigated. The lectin *Limulin polyhemus agglutinin*, with specific cross-reactivity for the sugar N-acetylneuraminic acid, was used to study sialic acid distribution. Also used were *Dolichos biflorus agglutinin*, specific for N-acetyl-D-galactosamine (blood-group A substance), and *Arachis hypogae agglutinin* (Peanut agglutinin), specific for the sugars D-galactose- β -(1-3)-N-acetyl-D-galactosamine (a blood-group subunit). *Triticum vulgare agglutinin* (wheat germ agglutinin), specific for N-acetyl-D-glucosamine, and *Bandeirea simplicifolia* II, specific for only terminally positioned N-acetyl-D-glucosamine,¹⁷ were tested as well.

Controls for these lectins were conducted consisting of immunocompetition between the lectin and its specific sugar prior to the application to the tissue section. This resulted in a complete absence of tissue fluorescence. As negative controls, the lectin was mixed with an incorrect sugar which caused no reduction in fluorescence. Antibody controls were built into many of the sections, due to the presence of areas of non-malignant epithelium or endothelial cells of blood vessels. The authors have previously found that these cells demonstrate normal blood-group reactivity.¹

Results.

Previous studies^{1,2} have shown that "complete" blood-group oligosaccharide chains (A, B, O) are present and reactive in the strata spinosum and corneum layers (middle and upper layers) of normal oral squamous epithelium (Fig. 1). These findings are in agreement with the results of our current investigation as can be seen in the Table. Fluorescein-labeled immunoglobulins against the blood-groups A and B were found to be reactive to cells above the parabasal layer of normal epithelium (Fig. 2). Malignant epithelium showed no reactivity to these "complete" blood-group antibodies. *Limulin polyhemus agglutinin*, specific for sialic acid, failed to demonstrate reactivity on the normal or malignant epithelial cells (Table), and treatment with neuraminidase did not alter blood-group reactivity. Results

NORMAL ANTI-BLOOD GROUPS

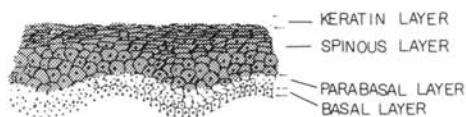


Fig. 1 - Immunofluorescent blood-group reactivity of normal epithelium. Shaded area represents reactive cell layers.

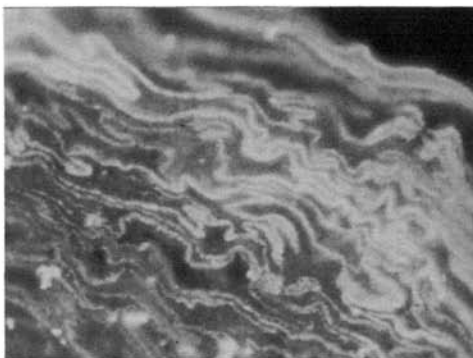


Fig. 2 - Fluorescein-labeled anti-A reactivity of the normal stratum corneum and keratin epithelial layers. 200x

of the PAS histochemical analysis before and after neuraminidase digestion of the tissue sections also failed to demonstrate the presence of sialic acid on the normal or malignant epithelial cell plasma membranes. *Lotus tetragonobolus* (Lotus A), used to test for L-fucose or the H substance, demonstrated a weak and randomly isolated reactivity on the plasma membranes of normal stratum spinosum cells in non-type O individuals. No reactivity to L-fucose was detected on the malignant cells. N-acetyl-D-glucosamine (GLcNAC) was found to be the most reactive sugar on the plasma membranes of the parabasal and lower spinous cell layers of normal epithelium using *Triticum vulgare agglutinin* (wheat germ agglutinin, WGA) and the lectin *Bandeirea simplicifolia* II, which is specific for only the terminally located N-acetylglucosamine (Figs. 3, 4, and Table).¹⁷ Malignant epithelial cells demonstrate high levels of N-acetyl-D-glucosamine on their cell surfaces (Fig. 5 and Table). Similar

TABLE
RESULTS OF THE IMMUNOFLOUORESCENT STUDIES ON NORMAL AND MALIGNANT ORAL EPITHELIUM. (+) INDICATES REACTIVITY OF THE LABELED ANTISERUM OR LECTIN WITH ITS CONJUGATE ON THE TISSUE. (-) INDICATES LACK OF REACTIVITY.

	NORMAL EPITHELIUM			MALIGNANT
	Basal Cells	Parabasal Cells	Spinous Cells	
Antiserum-A	-	-	+++	-
Antiserum-B	-	-	+++	-
Anti-H Lectin (<i>Lotus tetragonobolus</i>)	-	-	+++	-
Anti-Sialic Acid (<i>Limulin polyphemus</i>)	-	-	-	-
Anti-L fucose	-	-	±	-
Anti-GlcNAC (<i>Triticum vulgare</i>)	-	+++	+	+++
Anti-Gal-GALNAC (<i>Arachis hypogae</i>)	+++	-	-	+

results of cellular membrane reactivity were obtained with both of the above lectins. D-galactose-N-acetyl-D-galactosamine reactivity was limited solely to the basal cell layer of normal epithelium (Figs. 6, 7, and Table) and was only mildly present on the cell membranes of malignant epithelial cells as determined by the fluorescein-labeled lectin *Arachis hypogae agglutinin* (Peanut agglutinin, PNA) (Table).

Fig. 8 illustrates a schematic of the oligosaccharide sequence specific for the blood-group B.¹⁶ The group-specific sugar for the B blood-group is the final galactose (a)

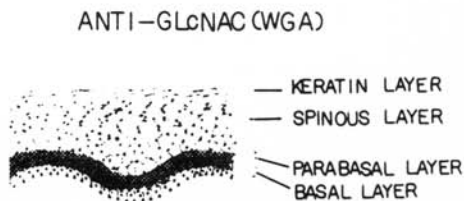


Fig. 3 - Immunofluorescent reactivity of parabasal cell layers to anti-N-acetyl-D-glucosamine (GlcNAC). Shaded area represents reactive cell layer.

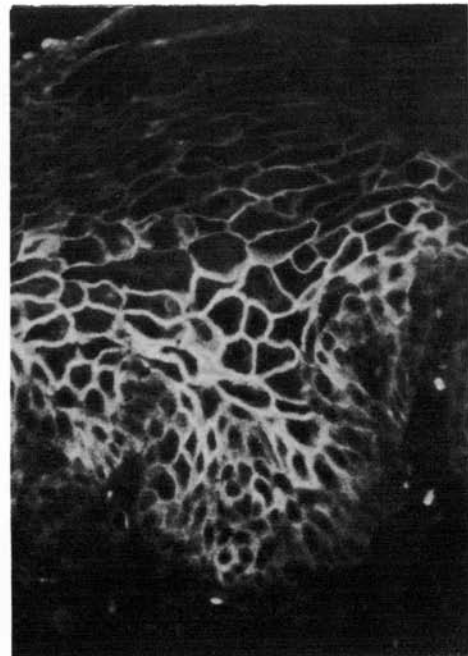


Fig. 4 - N-acetyl-D-glucosamine reactivity of the parabasal and lower spinous cell layers of normal oral epithelium. FITC-labeled *Triticum vulgare agglutinin*, 100x

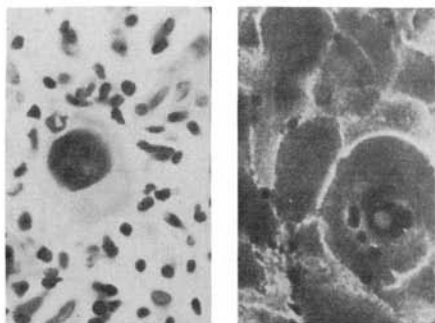


Fig. 5 - Left Side—Malignant epithelial cell (H&E, 100x). Right Side—Fluorescence of cancer cell membrane with FITC-labeled *Triticum vulgaris* agglutinin against N-acetyl-D-glucosamine. 400x

ANTI-GAL-GALNAC (PNA)

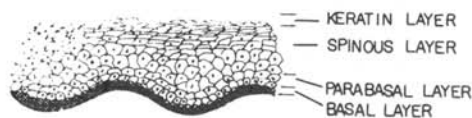


Fig. 6 - Immunofluorescent reactivity of basal cell layer to anti-galactose-N-acetyl-D-galactosamine (GALNAC). Shaded area represents reactive cell layer.

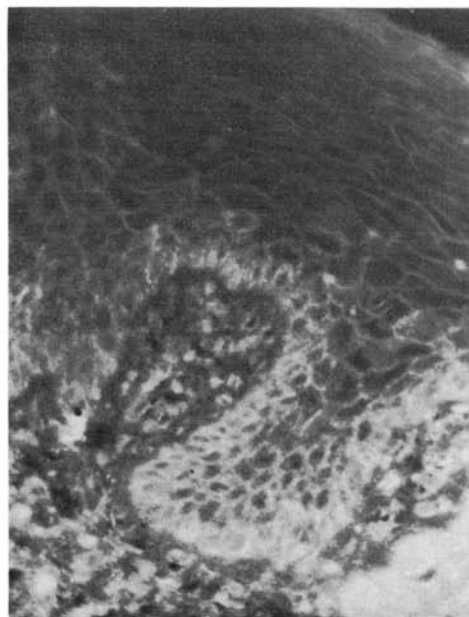


Fig. 7 - Immunofluorescence of basal cell layer of normal oral epithelium with *Arachis hypogae* agglutinin specific for N-acetyl-D-galactosamine. FITC. 100x

on the chain. The terminal galactose of the precursor chain is (b) and is theoretically the residue which is not present on malignant epithelial cells. An L-fucose (c) of the H specificity is attached to this terminal galactose. The subterminal N-acetyl-D-glucosamine (d) is theoretically the terminal sugar residue on normal parabasal and malignant epithelial cells. The L-fucose (e) attached to the subterminal N-acetyl-D-glucosamine (GlcNAC) controls Le^a (Lewis) specificity. The D-galactose-N-acetyl-D galactosamine (f) are the most proximal blood-group sugars, found on epithelial cells of the basal cell layer and in small amounts on the malignant epithelial cell plasma membranes.

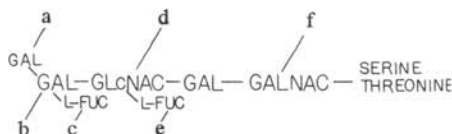


Fig. 8 - Type B oligosaccharide sequence. (a) - Group-specific galactose for blood-group B. (b) - Terminal galactose of blood-group precursor chain. (c) - L-fucose responsible for blood-group O(H) activity tested for with *Lotus tetragonobolus* (from Watkins, 1966).

Discussion.

Most investigators consider malignant cells to be less differentiated than normal cells, *i.e.*, they resemble fetal undifferentiated cells more than normal mature cells which produce end products.⁸ We therefore hypothesized that the oral malignancies may be composed of cells which were similar to the basal cells of normal epithelium. Previously the complete blood-group substances (A, B, O) were shown to be undetectable on malignant epithelial cells.^{2,3,5,6,9} Using the lectin *Lotus tetragonobolus* with a binding specificity for L-fucose, or the H substance (Fig. 8), we showed that the malignant cells did not possess the complete blood-group chain to this point. However, N-acetyl-D-glucosamine (GlcNAC), as detected with *Triticum vulgaris* agglutinin (wheat germ agglutinin) and *Bandeirea simplifolia* II, the next most proximal sugar (Fig. 8), was present in high quantities on the malignant cell membranes. This

level of blood-group differentiation correlates with the parabasal cell layers of normal epithelium. Another similarity exists between these cell types in that both can actively divide and both have the capacity of cell motility.⁶ We tested for the final sugar residues of Galactose-N-acetyl-D-galactosamine (Gal-GALNAC) (Fig. 8) using the lectin *Arachis hypogae* (Peanut agglutinin) on the malignant cells. These were found to be present, but in smaller amounts than that of the N-acetyl-D-glucosamine (GlcNAC) subterminal residue. Similar results have been obtained by others.⁶ This may indicate that malignant cells are able to synthesize the blood-group oligosaccharides to the level of N-acetyl-D-glucosamine (GlcNAC) but no further. It was found from our investigation that the degree of histologic "differentiation" of the squamous cell carcinoma cells did not influence the level at which the blood-group oligosaccharides were terminated. Even in neoplasms which formed large amounts of keratin, indicating a high degree of cellular differentiation similar to the cells of the cornifying layers of the normal epithelium, the blood-group reactivity was found to be terminated at the level of N-acetyl-D-glucosamine.

From our results it appeared that the incomplete synthesis of the blood-group substances on the surface of epithelial cells may be related to the ability of the cell to move through adjacent tissues rather than to divide mitotically. This idea has also been proposed by Dabelsteen.⁶ Several examples illustrate this hypothesis. The basal and parabasal cells of normal stratified squamous epithelium are cells which actively migrate toward the surface, while the remaining superficial layers are probably pushed passively toward the surface by the expanding bottom layers. Our study has shown that incomplete blood-group substances may be present in these lower cell layers. In epithelium adjacent to wound margins, Dabelsteen and Mackenzie have shown that the complete blood-group substances are not detectable.^{5,6} These cells are actively dividing in order to heal the wound, but are also migrating across and through the adjacent connective tissue in order to form a new surface epithelium. Once the opposing edges of the migrating epithelium contact each other, it has been

found that the blood-group substances appear on the cell membranes.^{5,6}

Several benign oral epithelial neoplasms such as the papilloma were tested previously¹ and found to demonstrate complete blood-group antigens. These benign neoplasms often exhibit a rapid mitotic rate; however, this does not appear to affect the cell surface antigenicity. Benign neoplasms, unlike their malignant counterparts, do not actively migrate through adjacent tissues. Therefore, it is possible that they do not have to demonstrate altered cell surface antigens, such as the blood-group antigens.

The findings of our study suggest that cell migration is related to the degree of development of the plasma membrane. Other cell functions such as keratin production do not appear to be related to the degree of membrane antigenicity, since many of the malignant neoplasms tested in our current study showed keratin production, but not complete blood-group oligosaccharides.

Sialic acid did not appear to be involved in a "masking" of the blood-group substances, since the antigens could not be detected following neuraminidase enzyme treatment. No sialic acid was detected on the surface of malignant cells using FITC lectins or histochemical staining techniques.

Therefore, it appears that malignant oral squamous epithelial cells may terminate synthesis of the blood-group oligosaccharide chains at a particular level of completeness, regardless of the degree of differentiation of the neoplasms. Further investigations regarding the mechanism involved in this specific termination are being conducted in our lab. It is possible that the incomplete blood-group phenotype is secondary to abnormally produced glycosyltransferase enzymes responsible for the construction of the sugar chains.¹⁰

Conclusions.

1. The blood-group oligosaccharide chains normally reactive on the spinous cell layers of normal stratified squamous epithelium are not detectable on malignant epithelial cells.

2. Normal basal and parabasal cells of stratified squamous epithelium do not

demonstrate "complete" blood-group antigens on their cell membranes.

3. The blood-group substances on the above-mentioned cells do not appear to be "masked" by a surface coating of sialic acid.

4. The synthesis of the blood-group oligosaccharide chains is incomplete, possibly terminated at the level of the sub-terminal N-acetyl-D-glucosamine sugar residue on the parabasal cells of normal stratified squamous epithelium and on malignant epithelial cells.

5. The synthesis of the blood-group oligosaccharide chains may be incomplete at the level of the proximal N-acetyl-D-galactosamine on the basal cells of normal epithelium.

6. The degree of "differentiation" of the malignant cells did not alter the level of termination of the blood-group oligosaccharide chain.

REFERENCES

1. GEORGE, D.I.; BURZYNSKI, N.J.; MILLER, R.L.: Reactive Properties of Oral Lesions to the Specific Red Cell Adherence Test, *Oral Surg* 47(1):51, 1979.
2. GEORGE, D.I.; HANKS, C.T.; COURTNEY, R.M.; LOPATIN, D.E.: The Relationship Between the Expression of the Epithelial Blood-group Substances and Malignancy; A Preliminary Report, *J Dent Res* 59:831, 1980.
3. DABELSTEEN, E. and FULLING, H.J.: A Preliminary Study of Blood-group Substances A and B in Oral Epithelium Exhibiting Atypia, *Scand J Dent Res* 79:387, 1971.
4. DABELSTEEN, E. and ROAD-PETERSON, B.: Loss of Epithelial Blood-group Antigens A and B in Oral Premalignant Lesions, *Acta Path Microbiol Scand*, Sect A, 83:292 1975.
5. DABELSTEEN, E. and MACKENZIE, I.: Selective Loss of Blood-group Antigens During Wound Healing, *Acta Path Microbiol Scand*, Sect A 84:445, 1976.
6. DABELSTEEN, E. and MACKENZIE, I.: Expression of *Ricinus Communis* Receptors on Epithelial Cells in Oral Carcinomas and Oral Wounds, *Cancer Res*, 38:4676, 1978.
7. MABRY, A.: Sialic Acid in Human Cancer, *Cancer Res* 35:1520, 1975.
8. WEISER, M.M.: Intestinal Epithelial Cell Surface Changes Related to Mitosis, Differentiation, and Neoplastic Transformation, *University of Michigan Med Centr J* 40(4):168, 1974.
9. MACKENZIE, I.C.; DABELSTEEN, E.; and ZIMMERMAN, K.: The Relationship Between Expression of Epithelial B Blood-group Antigen, Cell Movement, and Proliferation, *Acta Path Microbiol Scand*, Sect A 85:49, 1977.
10. PODOSKY, D.K.; WEISER, M.M.; and ISSELBACHER, R.J.: A Cancer-associated Galactosyltransferase Isoenzyme, *N Engl J Med* 29(B):703, 1978.
11. DAVIDSOHN, I.: A, B, and O Substances in Gastrointestinal Carcinoma, *Arch Path* 81:381, 1966.
12. DAVIDSOHN, I. and LOUISA, Y.: Loss of Isoantigens A, B, and H in Carcinoma of the Lung, *Am J Path* 57:307, 1969.
13. DAVIDSOHN, I.: Early Immunologic Diagnosis and Prognosis of Carcinoma, *Am J Path* 57:715, 1972.
14. LIU, P.I.: Carcinoma of the Oral Cavity Evaluated by the Specific Red Cell Adherence Test, *Oral Surg* 38:56, 1974.
15. DABELSTEEN, E. and PINDBORG, J.J.: Loss of Epithelial Blood-group Substance A in Oral Carcinomas, *Acta Path Microbiol Scand*, Sect A 81:435, 1973.
16. WATKINS: The Blood-group Substances, *Science* 152, 1966.
17. PETERS, B., Personal communication.
18. Manual of Histologic Staining (AFIP), L. G. Luna, Ed., McGraw-Hill Co., 1968.