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LOCALIZATION OF BLOOD-GROUP SUBSTANCES A AND B IN ALCOHOL-FIXED HUMAN GINGIVAE BY INDIRECT IMMUNOFLUORESCENCE TECHNIQUE

by

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Blood-group substances have been demonstrated by serologic methods to be present in many human secretions as well as in tissue extracts and cell suspensions. Recently the histologic distribution of the blood-group antigens has been studied by immunofluorescence techniques, which combine the specificity of serologic reactions with cytologic localization (*Glynn, Holborow & Johnson,* 1957; *Glynn & Holborow,* 1959; *Szulman,* 1960; *Szulman,* 1962). The antigens have been shown to be either alcohol-extractable or water-soluble.

The present investigation is an attempt to detect blood-group substances A and B in gingival epithelium, and to compare their distribution in the surface epithelium and the pocket epithelium.

Sections of frozen tissues have been employed in earlier immunofluorescence studies of blood-group substances. However, such sections were found unsatisfactory for the application of fluorescence techniques to human gingiva (*Brandtzaeg & Kraus,* 1965). Sections of alcohol-fixed gingivae, on the other hand, permitted precise localization of fluorescent material. In the present study, alcohol fixation was satisfactory also for the demonstration of epithelial blood-group substances, although it had been reported that these are alcohol-soluble (*Szulman,* 1960).

MATERIALS AND METHODS

Gingival specimens

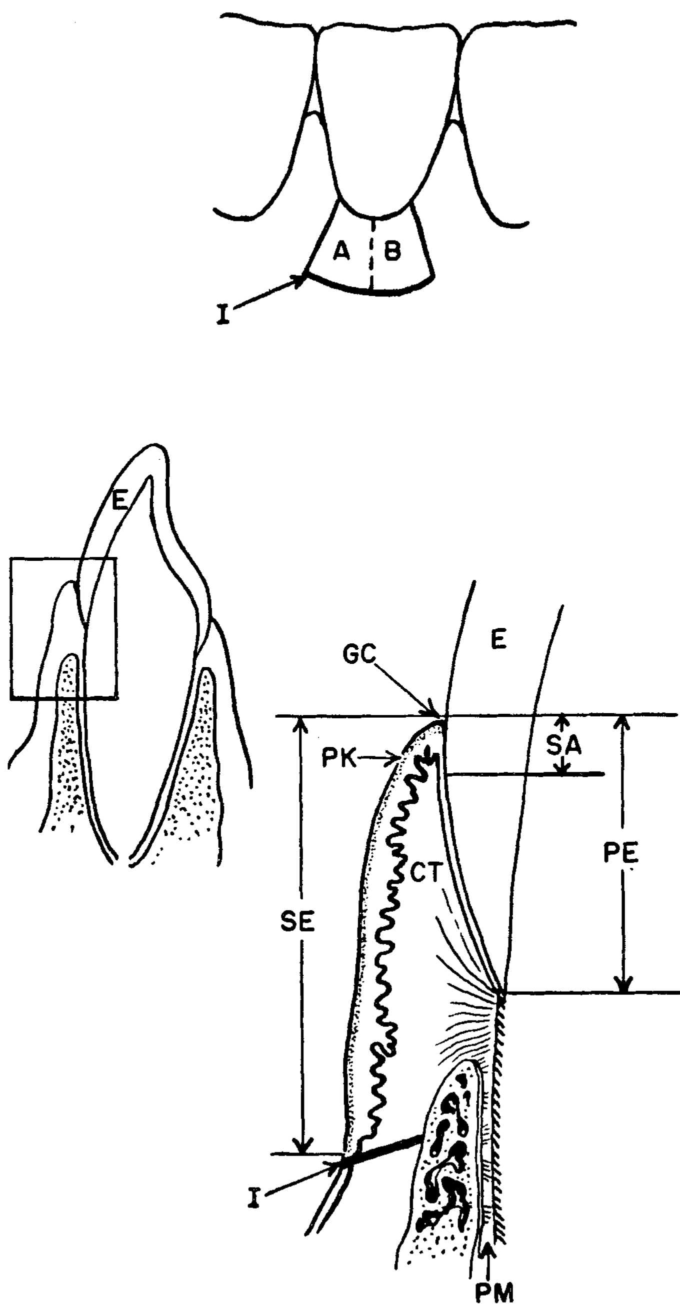
A gingival biopsy was obtained from each of five adult subjects as follows: After local infiltration with Xylocaine hydrochloride, the tissue specimen was removed with the aid of scalpel and periosteal elevator. It was 5 mm wide and extended vertically from the gingival crest to the alveolar mucosa (Fig. 1). Each specimen was immediately rinsed in isotonic saline to obtain an erythrocyte suspension for slide determination of the subject's blood group. Commercial anti-A and anti-B blood-grouping sera (Dade Reagents, Inc., Miami, Florida) were employed.

Two of the subjects had blood group A, two had blood group B, and one had blood group 0. Four of the gingival specimens were clinically inflamed, whereas the fifth (blood group B) was clinically healthy.

Washing, fixation, and sectioning

Each tissue specimen was divided vertically into two halves (2.5 mm $\times$ 4 mm $\times$ 2 mm) (Fig. 1), which were processed immediately. Part A was placed in 95 per cent ethanol precooled to 4° C, passed through cold absolute ethanol and xylol, and then embedded in paraffin according to *Sainte-Marie* (1962). Sections were cut serially at 5 μ. Part B was placed in a bag of cheese cloth, and was washed with gentle agitation in 800 ml of isotonic

Fig. 1. Schematic Drawings
(Top) Biopsy technique
(Left) Anatomic orientation
(Right) Gingival topography
A, B: Parts of biopsied tissue
CT: Connective tissue
E: Dental enamel
GC: Gingival crest
I: Incision
PE: Pocket epithelium
PK: Parakeratotic or keratinized surface layer
PM: Periodontal membrane
SA: Sulcus area
SE: Surface epithelium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phosphatebuffered (pH 7.2) saline, in order to extract water-soluble materials. The washing took place at 4° C for 48 hours, the saline being changed three times. This procedure sufficed to extract diffusible plasma proteins (*Brandtzaeg & Kraus*, 1965). After being washed, piece B was processed in the same way as portion A.

Antisera

To remove the blue and yellow dyes from the anti-A and anti-B sera (human) (Dade Reagents), the globulin fractions were precipitated with ammonium sulphate, redissolved in buffered saline, dialyzed, and then concentrated by evaporation at 4° C. Approximately 2 ml of clear antiserum solution was thus obtained from 5 ml of blood-grouping serum.

The following antisera were used both unlabeled and conjugated with fluorescein isothiocyanate: anti-human γG-globulin* (rabbit) (Antibodies, Inc., Davis, Calif.), anti-human γA-globulin* (goat), anti-human γM-globulin* (goat), anti-human albumin (rabbit), and anti-human fibrinogen (rabbit) (all from Hyland Laboratories, Los Angeles, Calif.). The specificities of these antisera were established by micro-double-diffusion and immunoelectrophoresis as previously described (*Brandtzaeg & Kraus*, 1965).

Nonconjugated and dissociated fluorescein isothiocyanate was removed from the antisera by gel filtration (Sephadex G-25, Medium; Pharmacia Ltd., Uppsala, Sweden). The sera were then adsorbed with mouse-liver powder (Baltimore Biological Laboratory, Baltimore, Md.) to eliminate proteins producing nonspecific staining of tissue sections (*Nairn*, 1962).

Indirect immunofluorescence technique

The "sandwich" or multiple layer method employed was evaluated in preliminary experiments with blood-group A substance of human submandibular or sublingual glands. The specimens were fixed and embedded as described above. After deparaffini-

* The terminology is that recommended by the WHO (1964: Nomenclature for human immunoglobulins. Bull. Wld Hlth Org. *30*: 447–450).

zation, the sections were washed for 1 minute in each of three baths of cold buffered (pH 7.2) saline (*Sainte-Marie,* 1962). The slides were wiped dry, except for the areas containing the sections. These were covered with drops of the anti-A solution. The slides were immediately placed in a moist container and incubated for 30 minutes at room temperature. The antiserum was decanted, the slides were rinsed twice in buffered saline, and then washed with gentle agitation in two changes of buffered saline — each time for 5 minutes. The slides were next wiped dry, except for the areas with the sections. Each tissue section was covered with one or the other of the labeled antisera for 20 minutes, was then rinsed, washed, and dried as described. A drop of semipermanent mounting medium (*Rodriquez & Deinhardt,* 1960) was placed on each section, and a cover slip was applied.

Fluorescence microscopy was carried out with an ultraviolet light source (Osram HBO) and BG38 and UG1 primary filters, with a Euphos-glass secondary filter. The sections were examined for specific, yellow-green fluorescing features. Photographs were taken with 35 mm daylight color reversal film (High Speed Ektachrome), the exposure time varying from 60 to 90 seconds.

The anti-γG-, anti-γA- and anti-γM-globulin sera produced staining of glandular acini (Fig. 2). The anti-A antibodies (human globulins) reacted with the A substance in the mucous acini; the anti-globulin sera, in turn, combined with the bound human globulins. The labeled anti-albumin and anti-fibrinogen sera did not stain glandular sections exposed to anti-A antibodies. Moreover, the anti-globulin sera did not stain the sections except when there was an intervening layer of bound anti-A antibodies. These antibodies apparently were present in the γG-, the γA- and the γM-fractions of the blood-grouping serum since staining was obtained with all of the three corresponding antisera. This was in accord with the recent findings of *Kunkel & Rockey* (1963).

Since the anti-γG- and the anti-γA-globulin sera produced the most intensive fluorescence of mucous-bound anti-A antibodies, these sera were chosen for the final antibody layer in the study of gingival tissues. Sections of each of the five specimens were first stained with anti-γG and anti-γA by a direct technique, be-

cause it was known that certain areas of gingival epithelia may contain the corresponding antigens (*Brandtzaeg & Kraus*, 1965). Nonreactive areas were selected for the localization of blood-group substances. Sections adjoining those examined by the direct technique, were first exposed to anti-A or anti-B solution, and then to anti-γG- or anti-γA-globulin serum, in the manner described for the glandular sections.

Controls

The following findings served to insure that the yellow-green staining patterns were due to specific antigen-antibody reactions:

(1) The areas under investigation did not fluoresce when treated with the labeled antiserum alone (conf. above).

(2) The areas did not fluoresce when treated with heterologous blood-grouping serum and the labeled antiserum. Thus, when in the first antibody layer anti-A was applied to B tissue, or anti-B to A tissue, no fluorescence occurred (Fig. 4). Epi-

Fig. 2. Human (Blood Group A) Submandibular Gland Acini.

Specific green fluorescence after exposure to anti-A antibodies (human) and fluorescein-labeled anti-human γA-globulin serum. × 410.

Fig. 3. Distribution of Blood-Group Substance in Surface Epithelium of Human Gingiva (Washed Specimen; Blood Group A).

Specific green fluorescence of cell membranes in stratum spinosum after exposure to anti-A antibodies (human) and fluorescein-labeled anti-human γA-globulin serum. Arrows indicate basement membrane. × 410.

Fig. 4. Negative Control; Section Adjoining Fig. 3.

No specific fluorescence after exposure to anti-B antibodies (human) and fluorescein-labeled anti-human γA-globulin serum. Arrows indicate basement membrane. × 410.

Fig. 5. Pocket Epithelium of Human Gingiva (Washed Specimen; Clinically Healthy; Blood Group B).

The section was cut at an angle to the tooth surface. No appreciable specific fluorescence of epithelial cell membranes after exposure to anti-B antibodies (human) and anti-human γG-globulin serum. Some plasma cells, and a couple of epithelial cells (lower right corner), fluoresce because of their cytoplasmic γG-globulin which was not removed by the washing. Also the fluorescence of a few vessel walls is probably due to the presence of non-diffusible γG-globulin, but possibly in part due to blood-group substance B. Arrows indicate basement membrane. × 410.

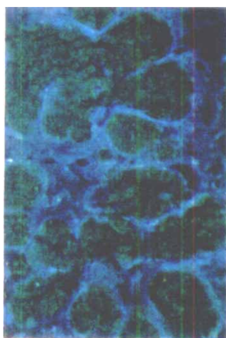

Fig. 2

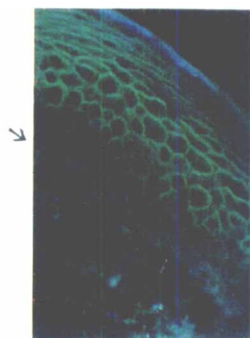

Fig. 3

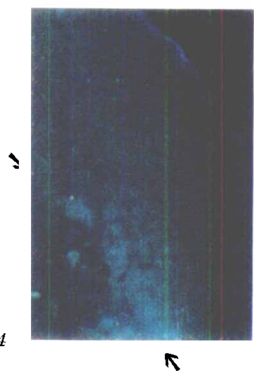

Fig. 4

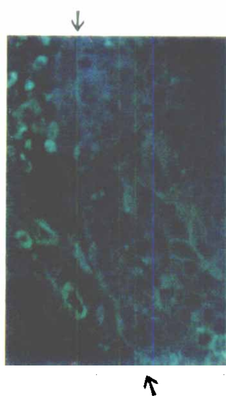

Fig. 5

thelium of blood-group 0 was unstained both when anti-A or anti-B was used as the first antibody layer.

(3) To 0.5 ml of the anti-A solution was added 0.5 ml of a commercial solution of blood-group substance A (Knickerbocker Biologicals Inc., New York, N. Y.). Also, 0.5 ml of the antiserum was mixed with 0.5 ml of saline. The mixtures were incubated for an hour at 37° C and for 12 hours at 4° C. They were then centrifuged at 700 g for 30 minutes. When the antiserum adsorbed with the A antigen was used in the first antibody layer on tissue of blood group A, the epithelium showed no fluorescence. Thus a specific blocking of the combining properties of the antiserum had occurred. When the antiserum mixed with saline was used, the staining reaction was similar to that produced by nonadsorbed serum.

(4) When tissue sections of blood group A — after being exposed to anti-A serum — were treated with unlabeled anti-γG for 30 minutes, the staining reactions with labeled anti-γG were more or less completely inhibited. Normal rabbit serum, as a substitute for the unlabeled anti-γG, did not interfere with the staining reactions. Specific blocking of the staining was also obtained by applying unlabeled anti-γA prior to the incubation with labeled anti-γA-globulin serum. Blocking reactions were carried out with similar results on sections of B tissue pretreated with anti-B antibodies.

RESULTS

Blood-group substances A and B were disclosed in gingival surface epithelium of subjects with the corresponding blood groups. No difference was observed in the distributions of the two antigens. Specific fluorescence, indicating the presence of blood-group substance, intensively outlined the cell membranes of the stratum spinosum (Fig. 3). In a few specimens with wide interstices in the epithelium, the intercellular bridges appeared stained as well. The basal layer, of from one to five cells in thickness, was invariably unstained as was the parakeratotic or keratinized layer (Fig. 3).

The staining intensity did not become reduced by washing of the tissue specimens for 48 hours in saline. Furthermore, the

specific fluorescence was not abolished by washing the sections in 95 per cent ethanol for 20 minutes prior to the staining; only occasional ethanol-treated sections showed a slightly reduced fluorescence.

The superficial layers of the pocket epithelium in the sulcus area (Fig. 1) fluoresced similarly to the stratum spinosum of the surface epithelium; the remainder of the pocket epithelium showed no appreciable specific staining (Fig. 5).

There was no apparent difference between staining patterns of clinically healthy and inflamed gingivae.

DISCUSSION

The distribution of blood-group substances A and B in gingival surface epithelium and in pocket epithelium of the sulcus area, matched the distribution observed in other stratified epithelia (*Szulman*, 1960). Blood-group substance could not be detected in the pocket epithelium apical to the sulcus area.

The biosynthetic capacity of producing blood-group antigens seems to depend on a certain maturity of the epithelial cell, since the basal layer of the surface epithelium invariably was devoid of these antigens. The turnover of the pocket epithelium is much shorter than that of the surface epithelium (*Beagrie & Skougaard*, 1962; *Greulich*, 1961; *Skougaard & Beagrie*, 1962), indicating that the pocket epithelium at all times consists of young cells. This may be supported by the observation that most of these cells lack the biosynthetic capacity of producing blood-group substances.

Water extraction did not affect the epithelial blood-group antigens, in accord with earlier reports (*Szulman*, 1960). Nor did treatment of the sections with 95 per cent ethanol appreciably influence the concentrations of the antigens. *Szulman* (1960) found that such treatment completely extracted the epithelial blood-group substances; he consequently concluded that they are alcohol-soluble. The explanation of the controversial results might be that *Szulman* (1960) used acetone-fixed sections of frozen tissue, whereas sections of alcohol-fixed tissue were employed in the present study. The alcohol fixation seemed to render the blood-group antigens resistant to alcohol extraction, and

may therefore be recommended for studies of epithelial as well as of watersoluble blood-group substances. The method permitted excellent localization of fluorescent material.

Earlier investigations concerned with the histologic distribution of blood-group substances were based on direct immunofluorescence techniques, employing one antiserum layer of labeled anti-A or anti-B of high titers. In the study here reported an indirect technique was chosen for two reasons. First, multiple layer or "sandwich" staining is substantially more sensitive than single layer tracing (*Beutner,* 1961; *Nairn,* 1962). Second, there is no need for the production of strong and fluorescein-labeled anti-A and anti-B sera. Commercially available blood-grouping sera were used in the first antibody layer, and either anti-γG- or anti-γA-globulin serum in the second layer. One has to bear in mind, however, that the antigens corresponding to the latter antisera are present throughout the gingival connective tissue (*Brandtzaeg & Kraus,* 1965). Connective tissue fluorescence due to blood-group antigens may therefore be obscured by fluorescence caused by reactions between the second layer of antibodies and the corresponding plasma proteins (Fig. 5). This drawback may be avoided by using antiserum produced in rabbit as the first layer, and anti-rabbit-globulin serum as the second layer. Such a combination of antisera may reveal whether or not the alcohol fixation affects the blood-group antigens of blood vessels which — like the epithelial antigens — are alcohol-extractable (*Szulman,* 1960).

SUMMARY

Blood-group substances A and B were disclosed in gingival epithelium by an indirect immunofluorescence technique. The antigens were resistant to water extraction, and were not affected by alcohol fixation. They were present in the stratum spinosum of the surface epithelium, and in the superficial layers of the epithelium of the sulcus area — but not farther apically in the pocket epithelium. This may support the concept that the pocket epithelium at all times consists of young cells, since the biosynthetic capacity of producing blood-group antigens seems to depend on a certain maturity of the epithelial cell.

Acknowledgment

The advice and criticism of Dr. *S. D. Schultz-Haudt* in the preparation of the manuscript are gratefully acknowledged.

RÉSUMÉ

REPÉRAGE DES AGGLUTINOGÈNES A ET B DANS UN TISSU GINGIVAL HUMAIN FIXÉ À L'ALCOOL, AU MOYEN D'UNE MÉTHODE D'IMMUNO-FLUORESCENCE INDIRECTE

Les agglutinogènes A et B ont été mis en évidence dans l'épithélium gingival par une méthode d'immunofluorescence indirecte. Les antigènes étaient résistants envers la déshydratation et la fixation à l'alcool restait sans influence sur eux. Ils étaient présents dans la couche épineuse de l'épithélium de surface et dans les couches superficielles de l'épithélium de la région du sillon gingivo-dentaire, mais ne s'étendaient pas plus loin en direction apicale dans l'épithélium du cul-de-sac. Ce fait peut venir à l'appui de la théorie selon laquelle l'épithélium du-cul-de-sac est toujours constitué de cellules jeunes, puisque la capacité biosynthétique de production d'agglutinogènes sanguins semble dépendre d'une certaine maturité de la cellule épithéliale.

ZUSAMMENFASSUNG

LOKALIZATION VON BLUTGRUPPENSUBSTANZEN A UND B IN ALKOHOL-FIXIERTEN HUMANEN GINGIVALGEWEBEN BEI ANWENDUNG VON INDIREKTER IMMUNOFLUORESZENSTECHNIK

Das Vorhandensein von Blutgruppensubstanzen A und B sind mittels einer indirekten Immunofluoreszenstechnik in Epithelien des Gingivalbereiches festgelegt worden. Die Antigene waren resistent gegenüber Wasserextraktion und liessen sich durch Alkohol fixieren. Sie waren vorhanden in Stratum spinosum des Oberflächenepithels und weiterhin in den oberflächlichen Schichten des Sulcusepithels — doch nicht in den mehr apikal belegenen Teile des Taschenepithels. Das Ergebnis kann die Auffassung unterstützen, dass Taschenepithel immer aus jungen Zellen besteht, da die biosynthetische Kapazität Blutgruppenantigene zu bilden anscheinend von einer gewissen Reife der Epithelzellen abhängig ist.

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