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Immunohistological Studies on ABH-Activities in Secretory Cells of Human Major Salivary Glands— Correlation between ABH-Activities in the Secretory Cells and Secretor-Nonsecretor

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Summary. The activities of A, B and H in serous cells (S-cells), mucous cells (M-cells) and excretory duct cells were examined in a large number of paraffin sections of three major salivary glands obtained from 91 corpses, using the immunofluorescence technique. The results are:

1. By taking H activity in S-cells of the submandibular gland or A, B and H activity in M-cells of the sublingual gland as an indicator, the salivary glands were classified as Type I showing activity and Type II showing no activity. No glands corresponding to the intermediate type, as seen in the case of saliva, were noted at all. Among 91 corpses, 70 cases were classified as Type I and 21 as Type II. The results matched well with those of Lewis type tested on blood. The frequencies of the typing (Type I; 76.9%, Type II; 23.1%) were approximately in concordance with those of secretor and nonsecretor in Japanese saliva. From these results, it was assessed that the former corresponded to the secretor type in the case of saliva, and the latter to the nonsecretor type.

2. Even in the same individual, both S-cells and M-cells exhibited different productivities of substances, depending on the glands to which they belonged. Namely, only S-cells in the submandibular gland belonging to Type I showed only H activity independent of the blood group of the individual, but the other S-cells in the other major glands did not show any activity for A, B and H. M-cells exhibited strong activity for H and/or A and/or B in the sublingual and submandibular gland and belonged to Type I, but little activity in the sublingual gland belonged to Type II. In the submandibular gland of Type II, some M-cells showed activity and others did not.

3. On the basis of the above results, we discuss the applicability of the present genetic theory concerning the secretor and nonsecretor type in saliva

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to salivary glands and cells, and further refer to the reasons for appearance of the weak secretor type or intermediate type in saliva.

Key words: ABH-activities, Salivary glands – Secretor – Nonsecretor

Zusammenfassung. Es wurde die A-, B- und H-Aktivität der serösen (S), der mukösen (M) und der Ausführungsgangszelle von aus 91 Leichen entnommen und in Paraffin eingebetteten Hauptspeicheldrüsen mittels des Immunofluoreszenzverfahrens untersucht.

Die Speicheldrüsen wurden in zwei Typen eingeteilt: in den aktiven Typ I und den inaktiven Typ II; die Submandibularis mit der H-Aktivität der S-Zellen und die Sublingualis mit der ABH-Aktivität der M-Zellen. Ein Zwischentyp wie beim Speichel wurde nicht beobachtet. Die Gruppierung und die Typisierung der Speicheldrüsen stimmten mit dem ABO- und Lewis-System der zugehörigen Blute überein. Unter den 91 Leichen gehörten 70 zum Typ I (76,9%) und 21 zum Typ II (23,1%). Diese Frequenzen waren in Einklang mit dem einschlägigen Befund (Ausscheider bzw. Nichtausscheider) bei den Japanern.

Unabhängig vom Individuum wiesen die S- und M-Zellen der zugehörigen Speicheldrüsen variable Aktivitäten auf. Die S-Zellen des Typs I der Submandibularis erzeugten nur H-Aktivität, die von der Blutgruppe unabhängig war, während die übrigen S-Zellen der Hauptspeicheldrüsen keine Aktivität zeigten. Die zum Typ I gehörenden M-Mandibularis- und Sublingualiszellen zeigten eine starke H- und A- und/oder B-Aktivität. Beim Typ II erzeugten nur ein Teil der M-Zellen der Submandibularis- und Sublingualisdrüse H- und A- und/oder B-Aktivität.

Aufgrund dieser Ergebnisse wurde die Wechselbeziehung zwischen der Typisierung der Speicheldrüsen und der des Speichels, die Gültigkeit der gegenwärtigen genetischen Theorie über Sekretor und Nichtsekretor im Speichel sowie die Ursache für das Auftreten des schwachen Sekretors oder des Zwischentyps erörtert.

Schlüsselwörter: ABH-Aktivität in Speicheldrüsen – Sekretor, Speichel – Nichtsekretor, Speichel

Many years have passed since the discovery of A, B, and O substances in exocrine fluids such as human saliva [1, 2].

Schiff & Sasaki [3] divided saliva into secretor type and nonsecretor type according to the quantities of the substances secreted in saliva and disclosed that this secretory ability was a "genetic marker" controlled by Mendel's law of dominant inheritance. Since then, the studies of typing have been performed mainly using the agglutination-inhibition test in which the whole saliva was used as a sample, and a large number of papers have been written [4].

As Schiff & Sasaki have stated the typing should be interpreted by ignoring obvious small differences. In practice the so called intermediate or weak secretor type is present in the examination of the whole saliva, so that it has been said that the typing of secretor or nonsecretor should be performed by refering

not only to salivary agglutination inhibition titers, but also to the Lewis blood groups of erythrocytes [5, 6, 7]. On the other hand, Fotino & Aloman noticed that persons of secretor type secrete H substance, and recommended the use of extract of Evonymus europaea as anti-H [8].

Since then studies using a variety of anti-H lectins have been added [9, 10, 11], but the typing of secretor or nonsecretor has not been completely clarified.

In this paper, we investigate the activities of blood group substances by the immunofluorescence technique in salivating cells and present a classifying method of the secretor or nonsecretor type in the salivary gland tissue, and finally comment about the problem of genes in salivating cells and about the reason for the presence of the intermediate type in the whole saliva.

Materials and Methods

1. Preparations of Tissue Sections

The tissue samples were 215 salivary glands (91 submandibular glands, 70 sublingual glands and 45 parotid glands) collected within 10 hours postmortem from 91 human corpses (70 males and 21 females) after sudden death. The average age of the dead was 49.2 ± 18.3 years (2 to 82 years old).

These salivary glands were embedded in paraffin after fixing in 10% formalin solution, cut thinly consecutively, fixed on glass slides and subjected to the experiment. Some of them were stained with H&E. For reference to the experiment, the blood groups of ABO and Lewis system were determined on erythrocytes.

2. Anti-Sera

2.1 Anti-A and anti-B sera (human source, Ortho Diagnostic Systems, USA)
2.2 Anti-Human-immunoglobulin goat sera conjugated with fluorescein isothiocyanate (anti-human Ig conj. FITC; Medical Biological Laboratory, Japan)
2.3 Preparation of anti-H chicken globulin conjugated with FITC (anti-H conj. FITC)

One ml of human saliva of type OSe (inhibition titer; 1:1024) together with an equal volume of Freund's complete adjuvant was immunized intraperitoneally in each of 8 chickens (white leghorn, female adult). After three weeks the blood was collected and crude anti-H sera were obtained. The antisera of 4 of the 8 chickens showed agglutination titers of 1024 to 2048 against human O-cells and of 128 to 256 against human A-, B- and AB-cells. These crude anti-H sera were salted out with ammonium sulfate, adjusted to a concentration of 12% crude γ-globulin after dialysis and preserved at 4°C after adding 0.1% of $NaNO_3$. After absorption with human OSe-saliva, the agglutinating ability of the anti-H against human red cells disappeared, but the absorption with an equal volume of human O-se saliva only reduced the titer to a half. This globulin was conjugated with FITC (BBL Co., USA) by partly modifying the routine method [12]. Namely, 17 μg of FITC per mg of globulin was mixed, stirred slowly at 4°C for 6 hours, passed finally through a Sephadex G-200 column and only FITC conjugated IgG was collected. This IgG showed agglutination titers of 8 to 16 against human O-cells but it is not yet confirmed whether the agglutination ability is derived from IgG itself or from contamination by IgM. The anti-H conj. FITC was treated once or twice before use by absorption at 4°C for 60 to 90 min with mouse liver-acetone powder (50 to 100 mg/ml) [13].

3. Staining of A and B Blood Group Substances

Before staining with antiserum, each section was pretreated with PBS with 10% bovine serum albumin (BSA). The treatment was effective in preventing non-specific staining.

Anit-A and anti-B were dropped separately onto a set of two tissue sections. After reaction at room temperature for 30 min, both were washed three times for 15 min each with PBS (pH; 7.2) while vibrating and the excess antiserum was removed. Then the sections were covered with anti-human Ig conj. FITC (IgG conc; 1.5 to 2 mg/ml) and stained at 37°C for 30 min. More than 200 ml of chilled PBS was passed over the sections so that the antibody in excess was removed. A carbonate buffer solution (pH; 9.5) was dropped onto the stained section; they were covered with a cover glass; the surrounding were sealed with paraffin, and the presence of A and B activity was microscopically examined using a fluorescence microscope (Nikon Co., type FL) with the interference filter [14] for FITC (Nikon Co.). H activity was also observed in the same way.

Additionally, when the section of A-type salivary gland was sensitized with anti-A absorbed by boiled human ASe-saliva, staining with anti-human Ig conj. FITC was made impossible. The same was observed in B-type salivary gland.

4. Staining of H Blood Group Substance

After pretreatment with 10% BSA the tissue section was covered with anti-H conj. FITC and stained at 37°C for one hour. Then more than 200 ml of chilled PBS (pH; 7.2) was poured onto the section. The nonreacted anti-H was removed and immediately the section was microscopically examined.

The pretreatment of the section of O-type salivary gland with ulex-extract anti-H made staining with anti-H conj. FITC impossible.

Results

I. Activity A, B and H in Cytoplasm of Secretory Cells in the Three Major Salivary Glands

1. Submandibular Gland. Independently of individual blood groups, glands in which cells showed H activity, and other glands in which cells showed no H activity, were observed in serous cells (S-cells) consisting mostly of the submandibular gland. The fluorescent pictures of the two groups exhibited a distinct contrast and there were no findings corresponding to an intermediate of the two. Therefore, we termed the former Type I and the latter Type II. Figure 1a shows a H-positive finding of S-cells in the B-type gland and Fig. 3 H-negative finding in the O-type gland. The S-cells in both types showed no A and/or B activity at all.

On the other hand, although some of dispersing mucous cells (M-cells) showed strong H and A and/or B activity, no findings that strictly distinguished both types were obtained, differing from the case of S-cells. Type II, however, exhibited a tendency to have more M-cells showing neither activity. Activity A or B corresponding to blood groups was noted in erythrocytes and hemangioendothelial cells as well as some M-cells. Figure 1b shows activity B in the corresponding section shown in Fig. 1a.

Table 1 summarizes the A, B, and H activities in both salivating cells on each submandibular gland of Type I and Type II. Here, the signs of + and ++ in the table represent positive degrees and ++ is stronger than + in degree. The positive picture of M-cells is always superior to that of S-cells. The sign of – represents negative activity (These are the same in Table 2).

2. Sublingual Gland. As to M-cells, consisting mostly the sublingual gland, there were observed glands in which all M-cells exhibited H and/or A and/or B activ-

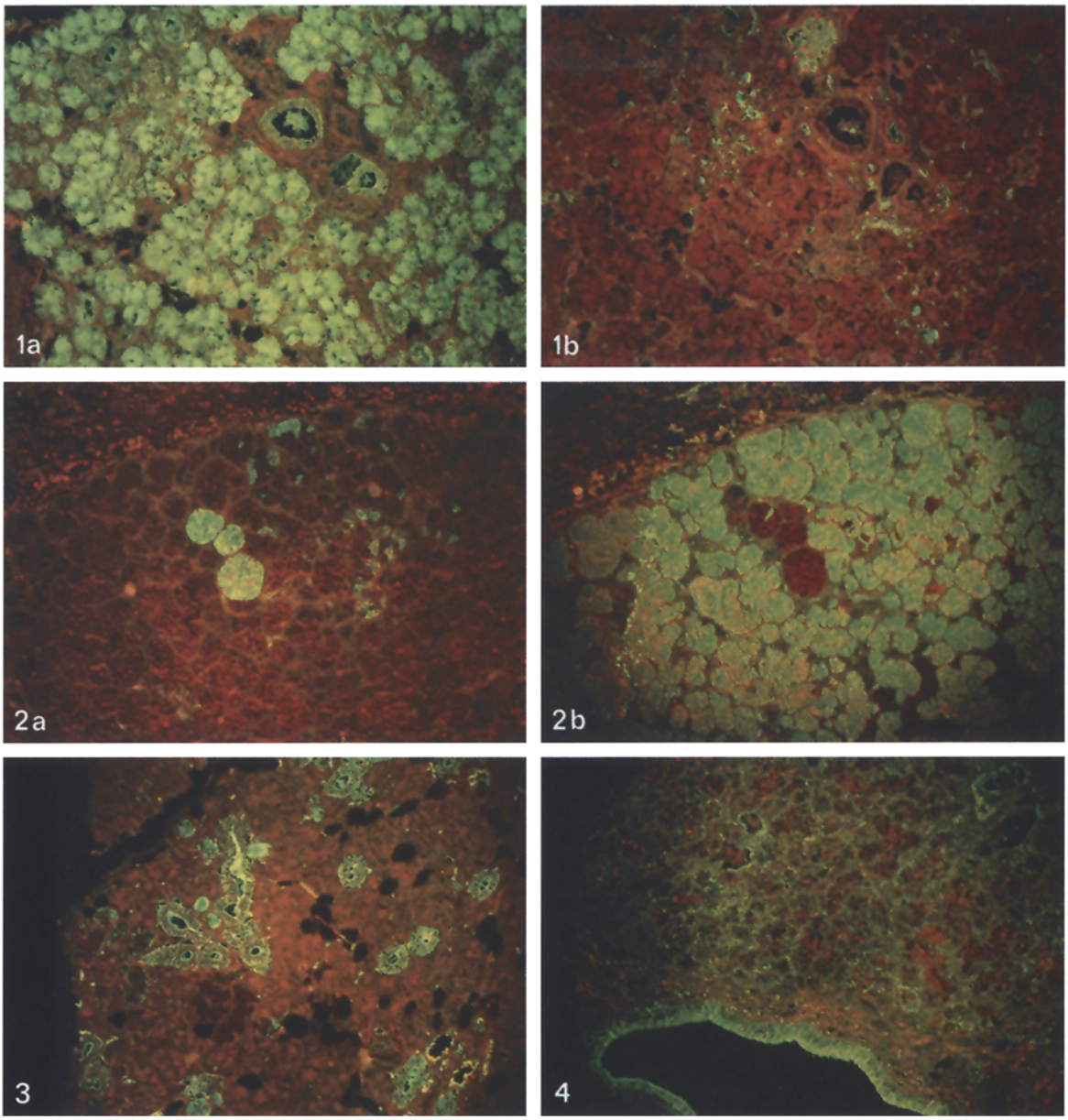

Fig. 1a, b. Immunofluorescent views (× 50) of H- and B-activities in the sections of a submandibular gland (Type I) obtained from a 70-year-old male, whose blood type was B, Le(a−b+). **a** Positive findings of H-activity in all of the serous cells and ducts. **b** Positive views of B-activity in scattered mucous cells, blood vessels and red blood cells, but not in serous cells

Fig. 2a, b. Immunofluorescent views (× 50) of H- and A-activities in the sections of a sublingual gland (Type I) from a 35-year-old male, whose blood type was A, Le(a−b+). **a** Positive findings of H-activity seen in 3 acini of the mucous cells. **b** Positive findings of A-activity in the other acini of the mucous cells except for the 3 acini, which showed H-activity, as seen in **a**

Fig. 3. Immunofluorescent view of H-activity (× 50) in the section of a submandibular gland (Type II) obtained from a 43-year old male, whose blood type was 0, Le(a+b−). Negative findings of H-activity in serous cells, but positive in some mucous cells and all ducts

Fig. 4. Immunofluorescent views (× 50) of H-activity in the section of a sublingual gland (Type II) from a 44 year-old male, whose blood type was B, Le(a−b−). Negative findings in all of the mucous cells, but positive in ducts. In another section B-activity was observed only in blood vessels and red blood cells

Table 1. Difference of ABH-activity of secretory cells in Type I and Type II of human submandibular glands. The Type I could be discriminated from the Type II in having H-activity in the serous cells, as shown in Fig. 1a (Type I) and Fig. 3 (Type II)

Blood type	Type I		Type II	
	S-cells	M-cells	S-cells	M-cells
O	H(+)	H(++)	H(−)	H(++ or −)
A, B, AB	H(+) A, B(−)	H(++) or/and A, B(++)	H(−) A, B(−)	H(++ or −) or/and A, B(++ or −)

Table 2. Difference of ABH-activity of secretory cells in Type I and Type II of human sublingualglands. The Type I could be discriminated from the type II in possessing strong ABH-activity in the mucous cells, as shown in Fig. 2 (Type I)

Blood type	Type I		Type II	
	S-cells	M-cells	S-cells	M-cells
O	H(−)	H(++)	H(−)	H(−) partly(++)
A, B, AB	H(−) A, B(−)	H(++) or/and A, B(++)	H(−) A, B(−)	H, A, B(−) partly(++)

ity, and other glands in which most M-cells exhibited no activity, also, no gland corresponded to the intermediate of the two. Therefore, we termed the former Type I and the latter Type II in contrast with the case of S-cells of the submandibular gland. Figure 2 a shows a H-positive picture in the A-type section and Fig. 2b a A-positive picture in the adjacent section. The M-cells in the glands of types A, B and AB belonging to Type I exhibited most frequently, A and/or B activity alone and most infrequently H activity alone. M-cells in the O-type section belonging to Type I showed a very strong H activity. Comparison of this with H activity in the A-type gland seen in Fig. 2a indicated a distinct contrast.

Meanwhile, S-cells in the sublingual gland had no cases showing any activity in both Type I and Type II. The fact that S-cells of Type I in the submandibular gland showed activity H, whereas S-cells in the sublingual gland did not, demonstrated and apparent difference in secretory function of blood group substances, although both of them were morphologically the same S-cells. As a matter of course, the Types of the submandibular and sublingual gland from the same individual corresponded without exception.

3. Parotid Gland. S-cells and excretory duct cells in the parotid gland showed little A, B or H activity. The determination of the type of the gland itself, however, was possible according to the activity in hemangioendothelial cells or erythrocytes.

4. Excretory Duct Cells. Excretory duct cells in the submandibular and sublingual glands showed frequently H activity regardless of the blood groups of the ABO

Table 3. Frequencies of Type I and Type II, tested in salivary gland sections, in respect to ABO blood type

Blood type	Type I	Type II	Total (%)
O	22	5	27 (29.67%)
A	28	8	36 (39.56%)
B	14	6	20 (21.98%)
AB	6	2	8 (8.79%)
Total %	70 (76.9%)	21 (23.1%)	91 (100%)

Table 4. Frequenies of Type I and Type II, tested in salivary gland sections, in respect to Lewis Type of red cells

Lewis blood type	Type I	Type II	Total (%)
Le(a−b+)	45	0	45 (61.6%)
Le(a−b−)	11	5	16 (22.0%)
Le(a+b−)	0	12	12 (16.4%)
Total (%)	56 (76.7%)	17 (23.3%)	73 (100%)

system (Figs. 1a, 3 and 4). A and/or B activity indicated a tendency to localize in the cell membrane rather than in the cytoplasm (Fig. 4).

II. Relationship between Frequency of Type I or Type II and Secretor or Nonsecretor Types in Saliva

As a result of the examination for salivary glands of the 91 corpses, Type I was assessed for 70 cases (76.9%) and Type II, for 21 cases (23.1%). No samples showed disagreement with the blood groups tested on the individual blood-samples. Table 3 shows the incidences of both types classified by the blood groups of the ABO system.

Of 73 cases in which erythrocytes were examined according to the Lewis system, 45 cases (61.6%) belonged to Le(a−b+) were assessed as Type I, 12 cases (16.4%) belonged to Le(a+b−) were as Type II without exception; and among 16 cases (22.0%) belonged to Le(a−b−), 11 cases were classified to Type I and 5 cases to Type II as shown in Table 4. Furthermore, the incidence (76.9%:23.1%) was also nearly in concordance with that in Japanese saliva (75.7%:24.3%) [15]. From these facts, it was estimated that the salivary gland of Type I corresponds to the secretor type in saliva and the gland of Type II to the nonsecretor type.

Discussion

Little has been reported on A, B and H substances in the salivary glands at the cellular level. Glynn & Holborow [16]. Holborow et al. [17] and Szulmann [18, 19] investigated blood group activity in frozen sections of human systemic tissues

using the immunofluorescence technique and mentioned little about the salivary glands. Glynn & Holborow and Holborow et al. have proved A activity alone merely in M-cells of the salivary gland and as to H substance they have noted weak activity only in one case. Szulmann has also noted blood group activity in M-cells of secretors. H substance, which was invariably present in saliva of secretors was not proved sufficiently in the tissues of the salivary gland. The most important reason for this, as Glynn & Holborow have pointed out as one of the reasons, is probably that the substance was removed from the frozen section. Together with the development of a improved immunofluorescence technique, the use of the formalin-fixed thin sections (3 μ in thickness) enabled us not only to prevent, as much as possible, disappearance of substances in tissues, but also to observe each secretory cell and the activity of substances more clearly.

Fifty samples examined in the early stage of the present study were also investigated with the mixed agglutination reaction technique of Davidsohn [20] and the restult were confirmed to be well in concordance with those by immunofluorescence. However, this technique has not been used any more, because positive cells were covered with the indicator red cells, so that they could not be identified and the localization of intracellular activities could not be recognized.

We classified active glands to Type I and inactive glands to Type II by observing blood group activity in S-cells of the submandibular gland or in M-cells of the sublingual gland by means of immunofluorescence. The typing (also) did not contradict the Lewis blood groups of red cells and the frequency of the typing agreed well with that of the secretor type and nonsecretor type in Japanese salvia. On the basis of these results, the gland of Type I was assessed as secretor type and that of Type II as nonsecretor type. Furthermore, it was found that although S-cells and M-cells in the submandibular and sublingual glands were mixed in different quantities under the same respective name, both cells showed different productivity depending on the glands to which they belonged even in the same individual. Se, a regulator gene, is at present supposed to be the gene that determines typing of secretor or nonsecretor type. It has hitherto been interpreted that in the secretor type gland the catalytic action of L-fucosyltransferase [21, 22] combines L-fucose with precursor sugar and produces H substance and further on this basis A and/or B substances are produced, whereas in the nonsecretor type gland L-fucose cannot combine with the precursor because of the absence of L-fucosyltransferase, so that A and/or B substances are also not synthesized [23, 24].

However, the results of our study indicated that M-cells in the sublingual gland were the only secretroy cells agreeing with the present genetic theory and the other cells did not agree with it. In other words, S-cells of the submandibular gland belonging to Type I produced merely H substance but not A and/or B substances. This fact cannot be explained by the present theory that non-productivity of A and/or B substances attributed to non-productivity of H substance. In addition, S-cells in the sublingual and parotid gland exhibited non-productivity of A, B and H substances in all of the samples examined. Moreover, the majority of M-cells in the submandibular gland showed H, A and/or B activity, even though the gland belonged to Type II (nonsecretor type). The presence of these S-cells and M-cells not conforming to the present

genetic theory suggested that the gene Se did not necessarily control all secretory cells in the gland.

It has been said that the ratios of salivary amounts derived from three major salivary glands are 69% for the submandibular gland, 26% for the parotid gland and 5% for the sublingual gland [25]; After stimulation, the ratio of saliva from the parotid gland increases to a maximum of 66.6% [26]. The ratios of saliva derived from other minor salivary glands are not so much different after stimulation, occupying 7 to 8% of the whole saliva [27].

Meanwhile, Milne & Dawes [28] reported that more than 70% of A substance in saliva was secreted from the sublingual gland and minor salivary glands in the palate and lip, a little from the submandibular gland and very little from the parotid gland. Hensten-Pettersen & Kornstad [29] also showed that ABH substances in saliva were derived from the minor salivary glands at a high rate. Namely, when the inhibition titer of the whole saliva is termed 1.00, those of the separate saliva were: palate gland saliva (6.66) > lip gland saliva (2.27) > whole saliva (1.00) > sublingual and submandibular gland saliva (0.29) > submandibular gland saliva (0.07) > parotid gland saliva (0.01 or less). The average ratios shown in the parentheses are calculated by us from their report.

These two reports agreed with each other in the point that A and/or B substances were derived from glands with many M-cells, although the numbers of cases examined were a very few, probably because of the difficulty in collecting of samples, and further H substance was not examined.

As mentioned above, our present investigation on the major salivary glands gave no findings for intermediate type. Our results together with the reports of Milne & Dawes and Hensten-Petterson & Kornstad suggests sufficently that appearance of weak secretor type of intermediate type is merely a phenomenon observed only when the whole saliva is taken as a sample. Two cases are considered; in saliva from weak secretors the greater portion of A and/or B substances and a portion of H substance originated from the sublingual gland an minor salivary glands secreting small amounts of saliva, are always diluted with saliva from the parotid gland scarcely secreting substances and with saliva from the submandibular gland mainly secreting H substance. The rate of dilution increases after stimulation, mainly due to an increase of saliva from the parotid gland. On the other hand, even in saliva from nonsecretors, the majority of M-cells in the submandibular and minor salivary glands secrete more or less the ABH substances, and excretory cells in the submandibular and sublingual gland, secrete H substance. Both cases are estimated to contribute to the appearance of the weak secretor type or intermediate type in saliva.

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