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Immunocytochemical Study on the Ultrastructural Localization of Human-Type ABO(H)-Blood Group Activities in a Macaque *(Macaca irus)* *

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Summary. The immunocytochemical study on the ultrastructural localization of human-type ABO(H)-activities in a crab-eating macaque *(Macaca irus)* was carried out by using postembedding and immuno-gold staining method. The tissue specimens examined were the esophagus, stomach (St), small intestine (Si), large intestine, liver, kidney, and pancreas. The specimens from these organs and submandibular gland (Sg) of a human (O-group) were used as staining reaction controls. Primary and secondary antibodies were commercially obtained mouse monoclonal anti-A, -B, -H (IgM), and goat anti-mouse IgM labeled with colloidal gold particles (∅ 20 nm), respectively. The results were as follows: (1) In macaque specimens, only A-activity could be observed as the location of gold particles on the peripheral rim of serous secretory granules (Sg) and of epithelial cells (esophagus), the mucous droplets in epithelial cells and brush border (St, Si), the intracellular secretory canaliculi [ISC (St)] and the zymogen granules and secretory ducts (pancreas). Gold particles could be also noted at the Golgi apparatus and nascent secretory granules. (2) By periodic acid-thiocarbohydrazide-osmium tetroxide (PA-TCH-OS) reaction, hexose-rich neutral mucopolysaccharides were noted on the peripheral rim of serous secretory granules (Sg), the mucous droplets (St, Si), the ISC (St), and the brush border (Si). Such a distributional pattern corresponded well with that of gold particles, indicating that the substances were responsible for ABO(H)-activities.

Key words: ABO(H)-activities, immuno-gold staining – Neutral mucopolysaccharides, ABO(H)-activities

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Zusammenfassung. Eine immunhistochemische Untersuchung von ultrastrukturellen Lokalisierungen der ABO(H)-Aktivitäten in den Zellen eines krabbenfressenden Affens *(Macaca irus)* wurde mittels der Post-Einbettung und Immun-Gold-Methode durchgeführt. Die Gewebeproben waren Oesophagus, Magen (Mag.), Dünndarm (Dün.), Dickdarm, Leber, Niere und Pankreas. Humane Proben von diesen Organen und der Glandula submandibularis (Gs) eines Mannes (O-Gruppe) wurden auch als Färbungskontrollen benutzt. Der primäre Antikörper war der käuflich erworbene monoklonale Antikörper gegen A, B oder H (IgM), und der sekundäre Antikörper war mit kolloidalem Gold markiertes Anti-Maus-IgM von der Ziege. Die Ergebnisse waren wie folgt: (1) In den Gewebeproben von *Macaca* wurde nur A-Aktivität am Rand der Sekretgranula der serösen Speicheldrüsenzellen (Gs) und der Epithelzellen des Oesophagus, in den mukoiden Sekretkörnchen der oberflächlichen Zellen und am Stäbchensaum (Mag., Dün.), an den intrazellulären Sekretkanälchen mit Mikrovilli [ISK (Mag.)] und in den Zymogengranula und den Sekretkanälen (Pankreas) beobachtet. Die Goldkörnchen wurden auch am Golgi-Apparat und an den neugebildeten Sekretkörnchen bemerkt. (2) Mit Hilfe der Perjodsäure-Thiokarbohydrazid-Osmiumtetroxyd (PS-TKH-OS)-Reaktion wurden Hexose-reiche, neutrale Mukopolysaccharide am Rand von Sekretgranula (Gs), in der mukoiden Sekretkörnchen (Mag., Dün.), und an der ISK und dem Stäbchensaum dargestellt. Ihre Lokalisation haben mit derjenigen von Goldkörnchen übereingestimmt. Diese Befunde zeigen, daß die ABO(H)-Aktivitäten diesen Substanzen entsprechen.

Schlüsselwörter: ABO(H)-Aktivitäten, Immun-Gold-Technik – Neutrale Mukopolysaccharide, ABO(H)-Aktivitäten

Introduction

Improved immunohistochemical techniques have made it possible to investigate the distribution of blood group activities (BGAs). Several light-microscopic studies using normal and/or pathologic tissues as well as stains (such as saliva, semen, vaginal fluids) have been reported [1–4]. We also reported that the distribution and alteration of BGAs, especially those distributed in epithelial mucins of human digestive tracts, were closely associated with those of neutral mucosubstances detected by periodic acid-Schiff (PAS) reaction [5].

To clarify BGAs' biosynthesis and their biologic function, it is important to examine their ultrastructural localization and relation to the biosynthetic process of mucosubstances. However, few reports have been found concerning this fundamental theme [6, 7].

Thus, for revealing these problems, the ultrastructural localization of BGAs was investigated by the postembedding method using the immuno-gold staining (IGS) technique [8], and it was discussed in relation to neutral mucopolysaccharides which were detected by periodic acid-thiocarbohydrazide-osmium tetroxide (PA-TCH-OS) reaction [9] at the electron-microscopic level.

Materials and Methods

Preparation of Tissue Sections for Immunocytochemistry

The tissue samples were as follows: the esophagus, stomach, small intestine, large intestine, liver, kidney, and pancreas from a crab-eating macaque *(Macaca irus)*. In addition, the specimens from these organs and the submandibular gland of a 13-year-old boy (O-group) were used for ensuring the specificity of immunostaining. In the macaque, its type on red blood cells in human ABO-system could not be examined by hemagglutination method because of the lack of peripheral blood. However, by using the avidin-biotin-peroxidase complex (ABC) method, B-activity was light-microscopically detected in the endothelia of vessels in every specimen. On the other hand, A-activity was immunostained using monoclonal anti-A in the mucous epithelial cells or goblet cells of the stomach or small intestine, and in the acinar cells of the pancreas (data not shown) [5].

Small tissue specimens were fixed with 4% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.4) containing 8% sucrose at 4°C for 4–5 h, dehydrated in increasing concentration of ethanol, and embedded in Epon 812 cured at 55°C for 48 h.

The ultrathin sections (about 70 nm) were cut with a Sorvall MT-6000 ultramicrotome and mounted on nickel grids coated with 0.5% (w/v) solution of chloroprene rubber in toluene [10].

Antisera

Primary Antibody. Mouse monoclonal anti-A, anti-B (IgM class, Biotest, FRG) and anti-H (IgM class, Chembiomed, Canada) were used after the dilution of 1:100, 1:100, and 1:50, respectively, with 5% (v/v) normal goat serum (Vector, USA) in 0.05 *M* Tris-buffered saline containing 0.2% bovine serum albumin (TBS/BSA) (Ortho, USA) and 0.01% sodium azide.

Secondary Antibody. Goat anti-mouse IgM labeled with colloidal gold particles (∅ 20 nm, E · Y Lab., USA) was used after diluted 1:20 with TBS/BSA.

Postembedding Method by the IGS Technique

The procedures for IGS were carried out in the wells of a plastic microtitration plate as used by Batten [11].

1) Etching in 10% hydrogen peroxide (15 min) for partially removing resin and exposing the determinants of antigens.
2) Washing the section in double-distilled water (10 min) and in TBS/BSA (10 min).
3) Preincubation in 5% normal goat serum diluted with TBS/BSA (30 min).
4) Incubation with primary antibodies at 4°C for 18–20 h.
5) Washing 3 × 10 min in TBS/BSA.
6) Incubation with secondary antibody at 20°C for 30 min.
7) Washing 3 × 10 min in TBS/BSA, 15 min in 1% glutaraldehyde for fixation of gold particles, 5 min in double distilled water and blotting dry.

After these procedures, the sections were stained lightly with saturated solution of uranyl acetate in 50% ethanol (2 min) and with 0.1% solution of lead citrate in 0.1 *M* NaOH (7 min), and were examined with a JEOL 100SX electron microscope.

Specificity Controls for the IGS

To ensure the specificity of the IGS technique, the following control procedures were employed: omission of primary or secondary antibody, and substitution of normal mouse serum for primary antibody.

Ultrastructural Visualization of Neutral Mucopolysaccharides by PA-TCH-OS Reaction [9]

To detect vicinal glycols appearing in mucopolysaccharide chains, PA-TCH-OS reaction was performed at room temperature as follows: the specimens fixed in the same solution as for IGS were cut (about 40 μm thickness) with a Microslicer DTK-2000 (D.S.K. Ltd., Japan).

After washing in 0.1 *M* phosphate buffer (pH 7.4), the sections were incubated in 1% PA for oxidation of vicinal glycols (60 min). The aldehyde groups, which had been formed through oxidation, were revealed by 0.5% TCH solution in 5% acetic acid (2 h) because the thio-radicals could be made electron-dense and visible by 1% OS (90 min).

Then sections were dehydrated, embedded in Epon 812, and examined by electron microscopy without electron-dense stain. Specificity of the reaction was certified by omission of PA or TCH.

Results

Stomach. Only A-activity was detected as the densely localized gold particles in the secretory granules of surface mucous cells and of mucous neck cells. In these cells, gold particles were found localized at the Golgi apparatus and nascent secretory granules (Figs. 1, 2). A-activity was also positive on the surface of intracellular secretory canaliculi around the nucleus in the parietal cells (Fig. 3). By PA-TCH-OS reaction, neutral mucopolysaccharides could be noted on the secretory canaliculi (Fig. 4).

Small Intestine. Through PA-TCH-OS reaction, neutral mucopolysaccharides were closely detected in the secretory granules, at the Golgi apparatus of goblet cells, and further on the brush border of absorptive epithelial cells (Figs. 5, 6). The cisternae of the innermost Golgi lamella were occasionally observed as continuous with nascent mucous granules. Gold particles were densely distributed in the PA-TCH-OS positive structures as described above only when immunostained with anti-A (Figs. 7, 8a, b).

Pancreas. In the acinar cells, some gold particles could be seen at the Golgi apparatus and nascent granules because of the relatively well preserved structure when compared with human tissue specimens (Fig. 9). The particles were also noted on the surface of secretory zymogen granules, and in the luminal surface and secreted fluids in the ductules.

Esophagus. Gold particles were apparently observed in the peripheral rim of the cytoplasm and on the surface of mucosal cells in the stratum spinosum and corneum when anti-A was used for a primary antibody. These findings showed that the location of A-activity agreed with that obtained by the avidin-biotin-peroxidase complex (ABC) method at the light-microscopic level (data not shown) [12].

Kidney. In the luminal surface of collecting tubules, gold particles were located when anti-A was applied, but the number of particles was smaller than in the stomach and small intestine.

Liver and Large Intestine. In these organs, apparent localization of the gold particles could not be seen under the condition employed in this study.

Tissue Specimens of O-Group Human. In all of these specimens only O(H)-activity was localized at the same portion as that in a macaque, and neither A- nor B-activity could be observed.

For example, in the submandibular gland, gold particles were densely distributed over the peripheral rim in the serous granules of acinar cells and in the

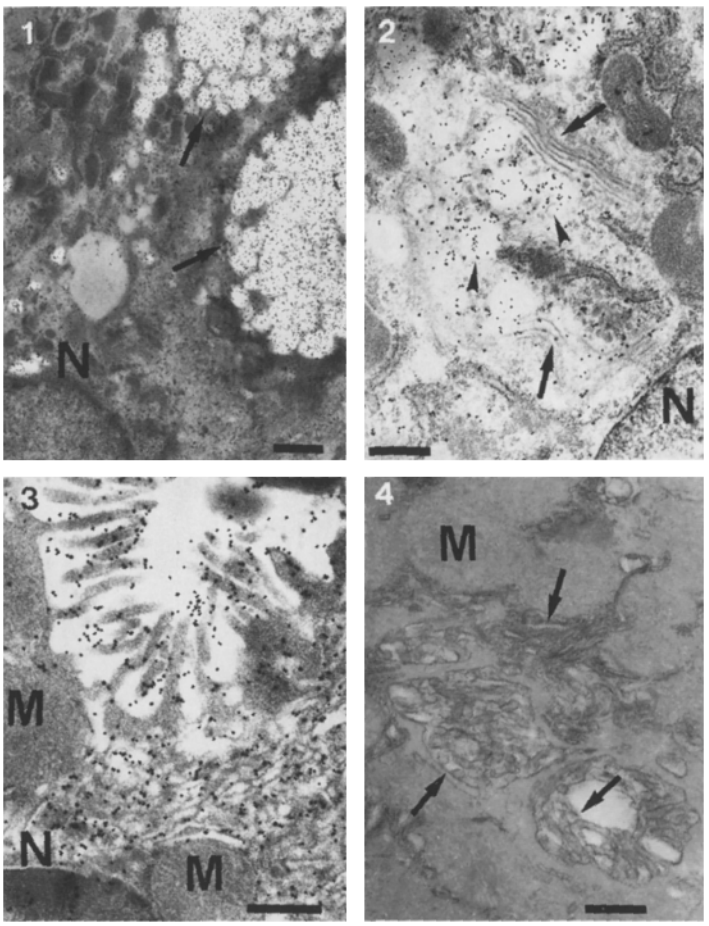

Fig. 1. Surface mucous cell of the stomach (macaque). Gold particles are densely located on the secretory droplets *(arrows). N,* nucleus. Immuno-gold staining (IGS) method. Anti-A. Bar: 1 μm. × 6,400

Fig. 2. Surface mucous cell of the stomach (macaque). Golgi apparatus *(arrows)* and nascent mucous granules *(arrowheads)* can be noted as labeled with gold particles revealing A-activity. *N,* nucleus. IGS. Anti-A. Bar: 0.5 μm. × 16,000

Fig. 3. Parietal cell of the stomach (macaque). Numerous gold particles are seen on the surface of intracellular secretory canaliculi (ISC), showing the localization of A-activity. *M,* mitochondria; *N,* nucleus. IGS. Anti-A. Bar: 0.5 μm. × 20,000

Fig. 4. Parietal cell of the stomach (macaque). Neutral mucopolysaccharides are covering the surface of ISC and are observed as electron-dense thin layers *(arrows). M,* mitochondria. PA-TCH-OS reaction. Bar: 0.5 μm. × 16,000

secretory ductule system only when anti-H was used (Fig. 10a, b). They were, however, hardly located at the central core. PA-TCH-OS reaction showed that hexose-rich neutral mucopolysaccharides were densely located over the peripheral rim of the serous secretory granules, which corresponded with the localization of gold particles and thus indicated that O(H)-activity was localized on the mucosubstances (Fig. 11).

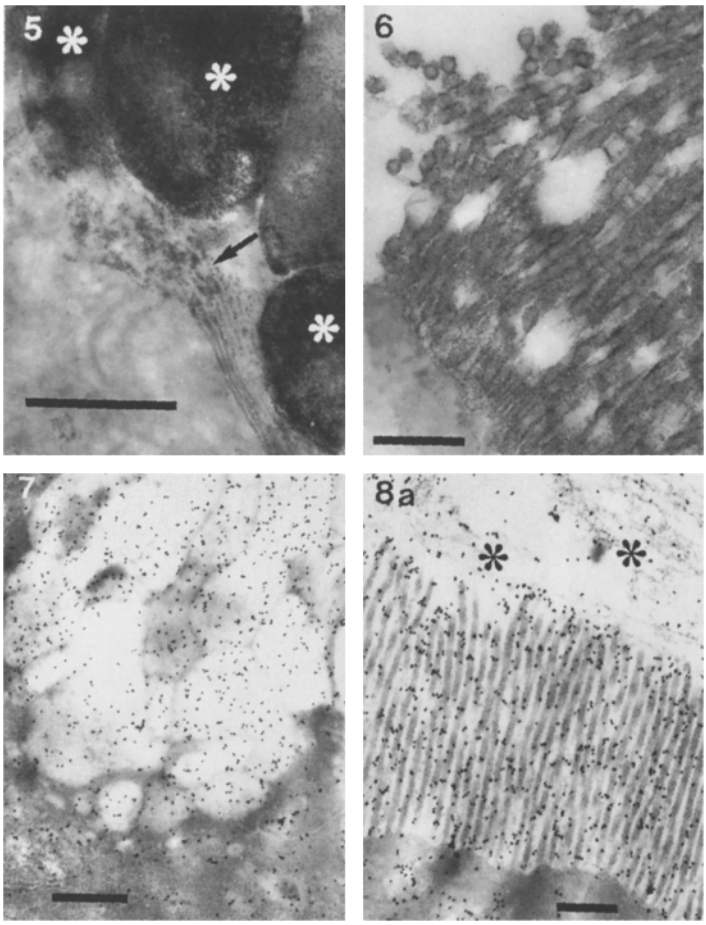

Fig. 5. Goblet cell of the small intestine (macaque). The innermost or mature surface of the Golgi apparatus is noted as electron-dense *(arrow),* showing that it contained neutral mucopolysaccharides. The mature surface is enlarged and continuous with nascent secretory granules (*). PA-TCH-OS reaction. Bar: 0.5 μm. × 40,000

Fig. 6. Brush borders of the absorptive epithelial cell in the small intestine (macaque). Electron-dense neutral mucopolysaccharides are seen on the surface of brush borders. PA-TCH-OS reaction. Bar: 0.5 μm. × 24,000

Fig. 7. Goblet cell of the small intestine (macaque). Gold particles are located on the secretory mucous droplets of different degrees of maturation. IGS. Anti-A. Bar: 0.5 μm. × 20,000

Fig. 8a. Brush borders of the absorptive epithelial cell in the small intestine (macaque). Gold particles are apparently located on the surface of brush borders and filamentous mucous substances (*) which are both positive for PA-TCH-OS reaction, and on the lateral cell membrane. IGS. Anti-A. Bar: 0.5 μm. × 16,000

Discussion

Several immunohistochemical studies on BGAs have been reported, but their biosynthetic process and relationship to mucopolysaccharides have not been fully investigated. In addition, an electron-microscopic study is necessary to

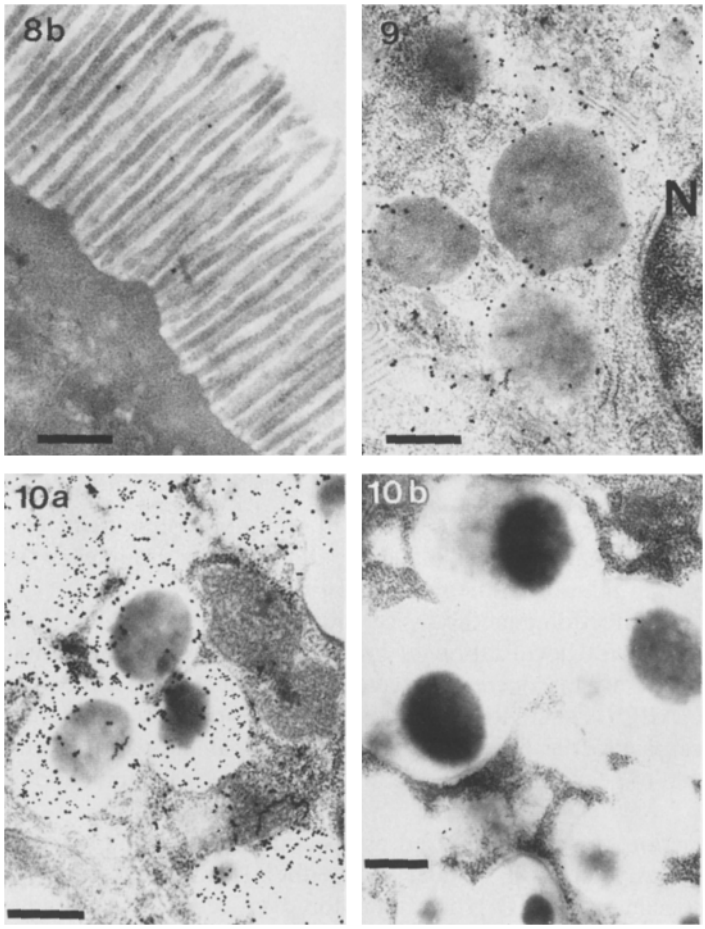

Fig. 8b. Brush borders of the absorptive epithelial cell in the small intestine (macaque). As shown here, gold particles are hardly noted on the brush borders, thus indicating that there is not B-activity. IGS. Anti-B. Bar: 0.5 μm. × 20,000

Fig. 9. Supranuclear region of the exocrine acinar cell in the pancreas (macaque). Zymogen granules and the Golgi apparatus are found labeled with gold particles. *N*, nucleus. Anti-A. Bar: 0.5 μm. × 20,000

Fig. 10. a Submandibular gland (human, O-group). Gold particles, which indicate the localization of O(H)-activity, are densely distributed over the peripheral rim of the serous secretory granules of acinar cells, while they are scarcely located at the central core. IGS. Anti-H. Bar: 0.5 μm. × 20,000. **b** Submandibular gland (human, O-group). Gold particles are scarcely observed on the serous secretory granules of acinar cells when immunostained with anti-A. IGS. Anti-A. Bar: 0.5 μm. × 16,000

demonstrate their localization and synthetic site in consideration of cellular ultrastructures.

From this point of view, the present study by the postembedding method using IGS technique has been performed, revealing that this method together with mouse monoclonal antibodies is available for detecting BGAs in ultrathin sections, especially for the activities in such mucoproteins as epithelial mucins

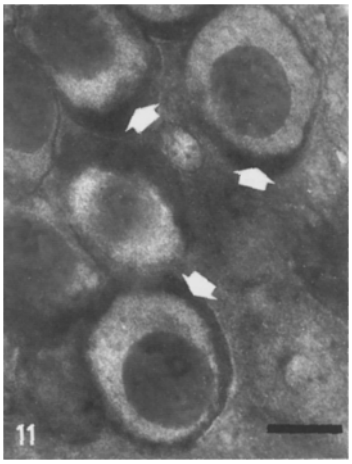

Fig. 11. Submandibular gland (human, O-group). Hexose-rich neutral mucopolysaccharides are noted as electron-dense substances over the peripheral rim of serous secretory granules *(arrows)*. PA-TCH-OS reaction. Bar: 0.5 μm. × 20,000

or secretory mucous granules. Moreover, BGAs in secretory components (ex. mucous droplets of the stomach or small intestine, and zymogen granules of the pancreas) were shown in relation to the maturation process.

In general, the postembedding method is considered to be more suitable for analyzing the ultrastructural localization of antigens than the preembedding method [13], but some activities decreased or were almost lost more easily during processing [14]. ABO(H)-activities are, however, considerably resistant to such a physical adverse effect as heat [1, 15, 16], and such a characteristic may have made it possible to relatively clearly detect them even by postembedding method.

The staining reaction can be considered as specific because all specificity control tests gave negative staining results, and neither A- nor B-activity was found in the tissue specimens of an O-group boy. Moreover, results of a macaque by IGS method corresponded well with those at the light-microscopic level, thus showing the specificity of immunostaining.

In the macaque specimens, ABO(H)-activity in the endothelia (i.e., B-activity) was different from that in the mucosae or secretory droplets of the stomach or small intestine and in the zymogen granules of pancreas (A-activity). This phenomenon has not been found in the human cases examined by us where the type of activity in the endothelia was the same as that of red blood cells [12]. The vascular endothelia of mesodermal origin expressed ABH antigens independent of *Se* and *Le* gene products, but the digestive and respiratory mucosae of endodermal origin revealed them under the control of these genes [17]. Thus, it seems reasonable to speculate that the reason of this dissociation on ABO(H) expression may be due to the functional difference of sugar residue-transferase between these cells.

In this study, hexose-rich neutral mucopolysaccharides were clearly detected as electron-dense by PA-TCH-OS reaction [9] which corresponds to periodic acid-Schiff (PAS) reaction at the light-microscopic level. As shown, PA-TCH-OS positive mucosubstances were noted in the serous secretory granules of the submandibular gland, mucous secretions or droplets in the epithelia of stomach

and small intestine, and on the intracellular secretory canaliculi of parietal cells. The acinar cells in the pancreas are reported to possess PAS-positive cell coat (glycocalyx) on the plasma membrane, and zymogen granules contain many digestive enzymes composed of glycoproteins [18]. All of these structures were engaged in secretory or digestive function, and the mucosubstances seem to play a role for protection of surface layer from mechanical stimuli. For example, the intracellular secretory canaliculi have a role for hydrochloric acid secretion, so the coating mucosubstances over microvilli may have protected plasma membranes from such a strong acid [19].

It is very interesting that the Golgi apparatus of a macaque was positive for PA-TCH-OS reaction, especially at the maturing or inner face, and that the enlarged inner surface was continuous with nascent secretory or zymogen granules, thus showing that the apparatus produced hexose-rich neutral mucosubstances [20]. Moreover, colloidal gold particles were characteristically localized in these PA-TCH-OS positive substances, showing that mucopolysaccharides carried ABO(H)-activities in the cells with secretory function.

On the other hand, in the human tissues collected postmortem after 5 h, Golgi apparatus became unclear because of autolysis and poor fixation, and it became impossible to discuss the gold particle localization. However, considering that a macaque is phylogenetically near to human, it can be considered that the results from observation on a macaque are applicable to human.

These findings agree with the light-microscopic data which demonstrated that BGAs were immunostained by the ABC method in the supranuclear region (probably at the Golgi apparatus) where PAS-positive substances were found [5], indicating that the apparatus is closely associated with the biosynthesis of glycoproteins, which some BGAs [ABO(H)- or Lewis-activities] consisted of [21, 22].

In the rough endoplasmic reticulum and the mitochondria, however, ABO(H)-activity was not found although the former is a site for polypeptide chain synthesis. The reason why they had no ABO(H)-activity seems due to lack or little amount of sugar residues. In addition, such a limitation of IGS method as decrease and diffusion of BGAs during tissue processing can also be considered.

Hence, to improve the sensitivity of IGS, further investigations are necessary, e.g., by using the freeze-drying technique [23] or hydrophilic low-temperature embedding material [24] for preserving BGAs. The immunoelectron microscopy using IGS technique is, however, undoubtedly a powerful tool for forensic serologic studies and for analyzing the biosynthetic pathway of BGAs.

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