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Immunohistochemical study on the localization of the epitope defined by a human saliva-specific mouse monoclonal antibody (P4-5C)

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Summary. A novel mouse monoclonal antibody (P4-5C) has been developed which recognizes the core portion of the protein carrying ABO(H) blood group antigens in human saliva. This proved to be specific for human saliva using immunochemical investigations such as enzyme-linked immunosorbent assay, Ouchterlony method and counter-immunoelectrophoresis. By light and electron microscope studies with immunohistochemical techniques using this human saliva-specific P4-5C as primary antibody, it was shown that P4-5C reacted specifically and exclusively with mucus from the mucous gland cells of human salivary glands. P4-5C reacted neither with the mucous gland cells of other primates (hamadryas baboon, Japanese monkey and Rhesus monkey) and four mammals (dog, cat, rabbit and mouse) nor with other human tissues. The epitope on the core portion of the ABO(H)-carrying protein was defined by P4-5C and could be discriminated from the epitope of ABO(H) blood group antigens using immunoelectronmicroscopy, although these 2 epitopes were localized relatively close to each other. The P4-5C monoclonal antibody can be also used for morphological species identification of tissue specimens from submandibular glands.

Key words: Immunohistochemistry – Salivary gland – Specific monoclonal antibody – ABO(H) blood group antigens – Species identification

Zusammenfassung. Ein neuartiger monoklonaler Antikörper von der Maus (P4-5C) wurde entwickelt, der den Kern des Proteins erkennt, welches die ABO-Blutgruppenantigene im menschlichen Speichel trägt. Dieser erwies sich als spezifisch für menschlichen Speichel über die Benutzung immunchemischer Methoden, wie enzymgekoppelter Immunabsorptions-Test, Ouchterlony-Methode, Kreuz-Elektrophorese. Durch licht- und elektronenmikroskopische Untersuchungen mit immunhistochemischen Techniken unter Verwendung dieses Antikörpers konnte gezeigt werden, daß eher spezifisch und exklusiv mit Schleim von den mukösen Zellen der menschlichen Speicheldrüsen reagiert. P4-5C reagierte weder mit mukösen Zellen anderer Primaten (Mantelpavian, japanischer Affe, Rhesus-Affe) und 4 Säugetieren (Hund, Katze, Ratte und Maus) noch mit anderen menschlichen Geweben. Das Epitop des Kernanteils des ABH-tragenden Proteins wurde definiert als P4-5C und konnte von dem Epitop der ABO-Blutgruppen-Antigene unterschieden werden mit Hilfe der Immun-Elektronenmikroskopie, obwohl diese beiden Epitope relativ nahe beieinander lokalisiert waren. Der P4-5C monoklonale Antikörper kann auch benutzt werden für die morphologische Spezies-Identifikation von Gewebsproben von den Glandulae submandibularis.

Schlüsselwörter: Immunhistochemie – Speicheldrüse – spezifischer monoklonaler Antikörper – ABH-Blutgruppenantigene – Speziesidentifikation

Introduction

The microscopical and ultramicroscopical localization of human-type ABO(H) blood group antigens has been reported by the authors in immunohistochemical investigations on tissue specimens of humans, other primates and bull frogs [1–4].

Kimura et al. [5] produced a human saliva-specific mouse monoclonal antibody after immunization with ABO(H) blood group active-glycoprotein precipitated with ethanol from heated human saliva of type B, secretor. This human saliva-specific monoclonal antibody specifically recognizes the core protein of blood group substances in human saliva, irrespective of ABO blood group and secretor status. This means that it discriminates the core protein from the epitope responsible for blood group activity. The antibody showed no cross-reaction with human urine, sweat, semen, vaginal fluid,

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supernatant of pancreatic tissue extract, canine saliva (2×) and feline saliva (4×) using enzyme-linked immunosorbent assay (ELISA), Ouchterlony method and counter-immunoelectrophoresis.

In the present study, the localization of the saliva-specific glycoprotein was investigated by immunohistochemical techniques using this novel antibody and compared to that of ABO(H) blood group antigens.

Materials and methods

Tissue specimens

The following formalin-fixed (10%) tissue specimens were investigated; human submandibular gland from 7 donors of secretor status [Se, Le(a−b+)] including 2 donors of types A, B, and O and 1 donor of type AB, and from 2 donors of non-secretor status [se, Le(a+b−)] phenotype including 1 donor of type B and 1 of type AB. Specimens of submandibular gland were also obtained from other primates (hamadryas baboon, Japanese monkey and Rhesus monkey), dog, cat, rabbit and mouse. Other human organ tissues (brain, lung, heart, liver, kidney, pancreas, spleen, adrenal gland, stomach, small intestine and ascending colon) were obtained from 1 donor, type A, Le(a−b+).

Light microscopical immunohistochemistry

1. Detection of P4-5C definied epitope. The avidin-biotin-peroxidase-complex (ABC) method [6] was applied to paraffin embedded tissue sections using the P4-5C antibody (mouse IgM, dilution 1:10,000) as primary antibody and biotinylated goat anti-mouse IgM (dilution 1:100; Tago, Burlingame, CA, USA) as secondary antibody. The reactions were carried out at room temperature, and the incubation times for primary and secondary antibody were 2 h and 1 h, respectively. For visualizing the reaction products the Vectastain ABC kit (Vector, Burlingame, CA, USA) and 3,3′-diaminobenzidine were used [2].

2. Detection of ABO(H) blood group antigens. For detecting ABO(H) blood group antigens, the monoclonal antibodies, anti-A, anti-B (mouse IgM, 1:50, Biotest, Frankfurt/Main, FRG) and anti-H (mouse IgM, 1:50, Chembiomed, Edmonton, Canada) were used as primary antibody and biotinylated goat anti-mouse IgM (1:100) as secondary antibody. The procedure for the ABC method was as previously described [2, 3].

3. Double immunohistochemical staining. In order to directly clarify the correlation of the P4-5C defined epitope to ABO(H) blood group antigens, double immunohistochemical staining [7] was performed on one tissue section. The first step was the detection of ABO(H) antigens as described above. The second step was the detection of P4-5C defined epitope by the avidin-biotin-alkaline phosphatase complex (ABC-AP) method using the Vectastain ABC-AP kit (Vector) and the same dilution of P4-5C as presented above [8]. In contrast to the ABC method, the reaction products of the ABC-AP method were red in color. This means that brown-red reaction products were seen in the cells where ABO(H) antigens and P4-5C defined epitope co-exist.

Ultramicroscopical immunohistochemistry

Human submandibular gland (donor B, Le(a−b+)) was formalin-fixed, embedded in Lowicryl K4M [9] and cut into ultrathin sections. The immunogold staining procedure for embedded sections was carried out as previously reported [1] and ultramicroscopical double staining was performed by applying the immunogold method to both surfaces of an ultrathin section [10, 11]. Firstly, the P4-5C defined epitope was labeled using P4-5C (dilution 1:1,000) and a colloidal gold (∅ 5 nm)-conjugated anti-mouse IgM (dilution 1:15 or 1:20, E.Y.Lab., San Mateo, CA, USA) on one face of an ultrathin section mounted on a nickel grid. B blood group antigen was detected using mouse monoclonal anti-B (dilution 1:100, Biotest) and a colloidal gold (∅ 20 nm)-conjugated anti-mouse IgM (dilution 1:15 or 1:20, E.Y.Lab.) on the opposite face. The order of immunostaining for these 2 epitopes did not make any difference to the results. For detection of P4-5C defined epitope only, anti-mouse IgM antibody conjugated with colloidal gold (20 nm diameter) was used as secondary antibody, instead of the anti-mouse IgM conjugated with smaller particles of 5 nm diameter.

Results and discussion

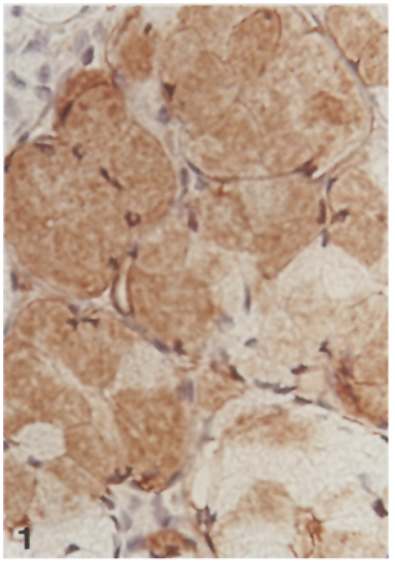
The mouse monoclonal antibody P4-5C reacted specifically with the mucous gland cells, especially with mucus in the cytoplasm, irrespective of the ABO blood group and the secretor status of the donors (Fig. 1). P4-5C antibody did not react with mucous gland cells of other primates (hamadryas baboon, Japanese monkey and Rhesus monkey) and four different species of mammals (dog, cat, rabbit and mouse) (Fig. 2). P4-5C also did not react with the serous gland cells and the secretory duct epithelial cells of human salivary glands (Figs. 3, 4).

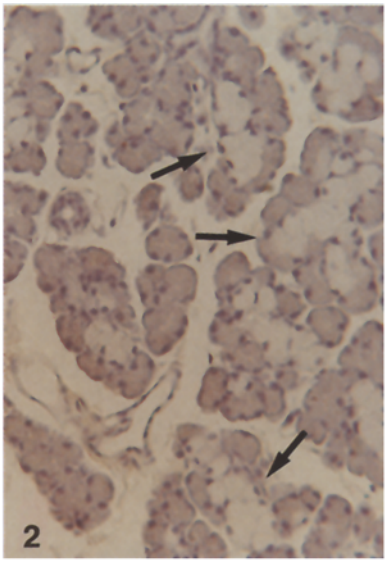
These findings show that P4-5C is specific for *human mucous* gland cells of the salivary gland. The staining of mucous gland cells was not, however, homogenous, but varied from cell to cell (Fig. 1). This seems to result from the intercellular quantitative variability of the epitope defined by P4-5C. Bunai et al. [12] found a dissociation in the conversion to the A and B antigens in human tissues, including the salivary gland, from AB-type donors, which also suggests an intercellular difference among mucous gland cells of physiological status and/or gene expression.

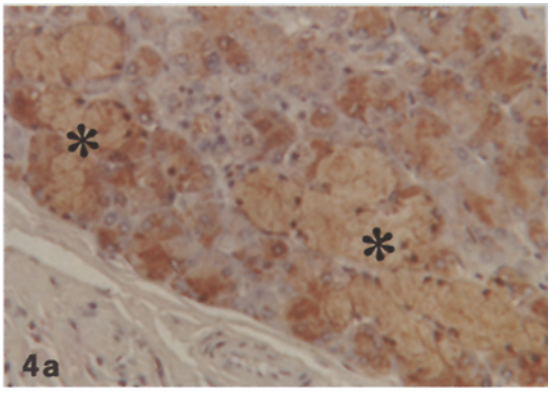
Moreover, P4-5C gave positive results only with the mucous gland cells of salivary glands and showed no positive reactions with tissues and cells of other human organs including pancreatic exocrine glands. This result corresponds with the findings of Sagisaka et al. [13] in which the carrier protein of ABO(H) blood group antigen in human saliva was revealed not to be amylase by investigation with a monoclonal anti-human amylase antibody. It can be concluded, therefore, that P4-5C is specific only to human salivary glands.

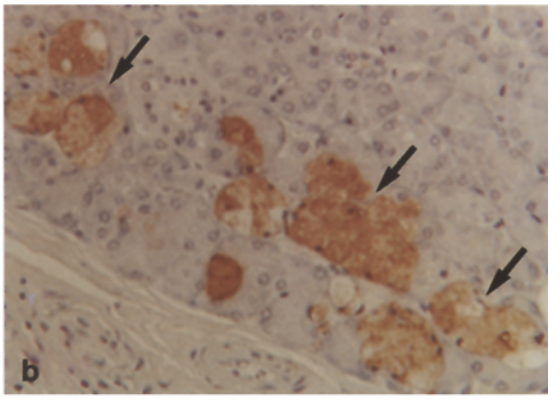
The distribution patterns of the epitope defined by P4-5C were not necessarily the same as those of ABO(H) blood group antigens. Some mucous gland cells had only ABO(H) antigens and gave negative staining for the P4-5C defined epitope (Fig. 3). The mucous gland cells which could be stained with P4-5C generally gave positive staining for ABO(H) antigens, although there were some differences in the intensity of immunostaining (Figs. 3, 4).

The heterogeneity of blood group substances carrying an identical blood group antigen and the effect of formalin fixation should be considered as a reason why some mucous gland cells are positive for ABO(H) antigens and negative for the P4-5C epitope of the core protein. This means that P4-5C antibody may not recognize a different core protein of a different blood group substance. Formalin fixation may result in stable preserva-









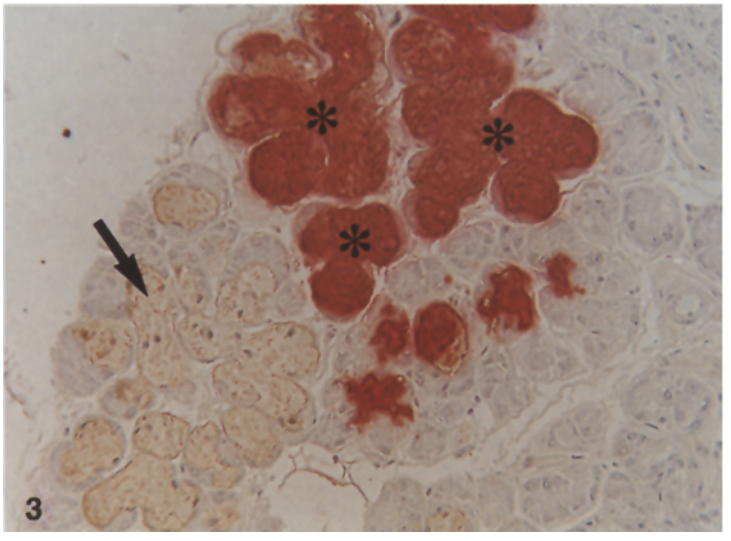


Fig. 1. Human submandibular gland, B-Se (secretor). The mucous substance of the mucous gland cells, which was positively stained brown, is the glycoprotein carrying the epitope defined by monoclonal P4-5C antibody. Differences in the stain intensity with P4-5C among mucous gland cells could be seen, which seems to result from intercellular quantitative changes of the P4-5C defined epitope. ABC method, × 360

Fig. 2. The submandibular gland of a hamadryas baboon. P4-5C defined epitopes were never observed in the mucous gland cells (*arrows*) nor the serous gland cells and secretory ductules. ABC method, × 180

Fig. 3. Double staining of A blood group activity and the P4-5C defined epitope in human submandibular gland, A-Se. Brown reaction products by the ABC method indicate the location of A blood group activity in the mucus of mucous gland cells and red reaction products by avidin-biotin-alkaline phosphatase-complex (ABC-AP) method show the location of the P4-5C defined epitope. The reaction products in brown-red color (*asterisks*) indicate the co-existence of these 2 epitopes and the P4-5C defined epitope could be seen on the A antigen positive cells of mucous glands. Mucous gland cells negative for P4-5C defined epitope and positive for A antigen were also observed (*arrow*). × 180

Fig. 4a, b. Human submandibular gland, O-Se. **a** shows O(H) blood group antigen in the mucous gland cells (*asterisks*), as well as serous gland cells and secretory duct epithelia. **b** indicates the localization of P4-5C defined epitope (*arrows*) only in the mucous gland cells with positive O(H) activity as shown in **a**. ABC method, × 180

tion of the antigens determined by sugar residues (such as ABO(H) antigens) and in the destruction of the epitope of the core protein defined by P4-5C, as formalin solution does not react with sugar residues but with amino groups of glycoproteins [14].

In contrast to the mucous gland cells of human submandibular glands, the serous gland cells and the secretory duct epithelial cells with ABO(H) antigens do not possess P4-5C defined epitopes as shown in Figs. 3 and 4. Negative staining of these cells is considered to be due to the qualitative difference of ABO(H) antigen-carrying substances between mucous cells and the negative cells (serous gland and duct epithelial cells). The immunogen in the saliva used for the P4-5C production

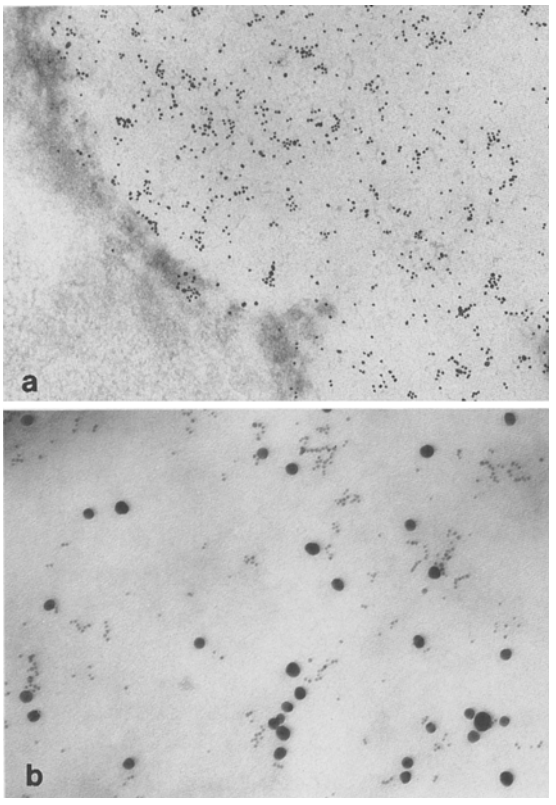


Fig. 5a, b. Human submandibular gland, B-Se. In **a**, colloidal gold particles of 20 nm diameter show the localization of P4-5C defined epitope on mucous substances in the cytoplasm of mucous gland cells (Immunogold method, × 25,000). In **b**, electronmicroscopical double staining reveals the close spatial relationship between P4-5C defined epitope, indicated by small colloidal gold particles (∅ 5 nm) and B blood group antigen shown by large particles (∅ 20 nm) (Immunogold method, × 90,000)

(heat-stable and ethanol-precipitated glycoprotein) seems to be secreted from the mucous gland cells of the submandibular gland of the donor (type B-Se).

From the results described above, it can be concluded that human submandibular salivary gland can be distinguished histochemically from that of other primates and mammals and from other human organ tissues. The P4-5C antibody can therefore be used for species identification of tissue specimens from submandibular glands.

In immuno-electronmicroscopical studies (Fig. 5a, b), the larger colloidal gold particles (20 nm in diameter) were found near to the smaller particles of 5 nm diameter (Fig. 5b). These particles were not, however, aggregated at identical sites. Morphologically, this seems to suggest the existence of 2 different epitopes and to show the close spatial relationship between the epitopes of B blood group antigen and the epitope defined by P4-5C antibody. This result corresponds well with the immunochemical quality of P4-5C, in that it recognizes the core portion of the carrier glycoprotein of ABO(H) blood group antigens [5].

By immunohistochemical staining methods, the quality of the monoclonal P4-5C antibody was also defined morphologically, thus demonstrating the usefulness of immunostaining for quality control of monoclonal antibodies [15].

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