Int J Leg Med (1991) 104:193–196

International Journal of
Legal Medicine
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Production and characterization of monoclonal antibodies against tissue specific epitopes on ABO blood group substances in saliva

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Received December 29, 1990 / Received in revised form April 24, 1991

Summary. Mouse monoclonal antibodies (P4-2F, P4-5C) against ABO blood group substances in saliva were produced by immunization with ABO blood group active-glycoprotein after ethanol precipitation from heated saliva. These antibodies bound to saliva, irrespective of the ABO blood group and secretor status. Saliva diluted at least 3.2×10^4-fold could be detected by ELISA using these antibodies. Tissue and species specificity of the antibodies was tested by ELISA and counterimmunoelectrophoresis and showed that the antibodies were specific for human saliva. By immunoblotting of the deglycosylated ABO blood group substances it was evident that the epitopes for the antibodies were localized on the core protein of blood group substances in saliva. These antibodies could be extremely suitable reagents for the identification of saliva in medico-legal examinations. Furthermore, they may be used as capture antibodies in sandwich methods for ABO blood grouping of saliva from mixtures of body fluids.

Key words: Monoclonal antibody – ABO blood group substances – Saliva

Zusammenfassung. Monoklonale Antikörper von Maus (P4-2F, P4-5C) gegen ABO-Blutgruppensubstanzen im Speichel wurden hergestellt durch Immunisierung mit ABO-aktivem Glykoprotein, welches durch Ethanol-Präzipitation aus erhitztem Speichel gewonnen wurde. Diese Antikörper fanden sich an Speichel, unabhängig von der ABO-Blutgruppe und vom Sekretorstatus. Speichel mit einer Verdünnung bis zu 3.2×10^4 konnte mit Hilfe der ELISA-Technik mit diesen Antikörpern nachgewiesen werden. Die Resultate der Untersuchungen zur Gewebs- und Spezies-Spezifität der Antikörper mit Hilfe der ELISA-Technik und der Kreuz-Elektrophorese zeigten, daß die Antikörper spezifisch für menschlichen Speichel sind. Durch Immunoblotting der deglycosylierten ABO-Blutgruppensubstanzen wurde es offensichtlich, daß die Epitope für die Antikörper auf dem Proteinkern der Blutgruppensubstanz im Speichel lokalisiert sind. Aufgrund der vorstehenden Befunde dürften die Antikörper extrem nützliche Reagenzien für die Identifizierung von Speichel darstellen. Weiterhin können sie benutzt werden zur Antikörper-Bindung in Sandwich-Methoden mit dem Ziel der ABO-Blutgruppenbestimmung des Speichels bei Vorliegen von gemischten Körperflüssigkeiten.

Schlüsselwörter: Monoklonale Antikörper – ABO-Blutgruppensubstanzen – Speichel

Introduction

It is well known that ABO blood group substances (ABO-BGS) are glycoproteins and are present in all body fluids. However, the biochemical and immunological properties of these glycoproteins, with the exception of the blood group activity expressed on the oligosugar moiety, have not yet been sufficiently resolved [1]. If there are epitopes expressing tissue specificity in ABO-BGS other than the oligosugar moiety mentioned above (Fig. 1), antibodies specific for tissue and the ABO blood groups should be produced after immunization of animals with ABO-BGS. We have attempted to produce monoclonal antibodies (mAbs) against tissue specific epitopes on ABO-BGS in saliva, in order to use the antibodies for the identification of saliva and for ABO blood grouping of saliva from mixed body fluids by sandwich ELISA.

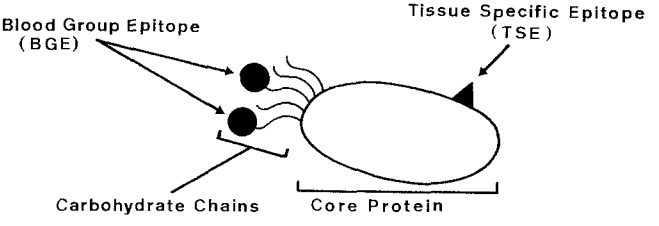

Fig. 1. Hypothetical structure of BGS in body fluid

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In this report, we describe the production and characterization of mAbs against the tissue specific epitope of ABO-BGS in saliva.

Materials and methods

Preparation of antigen. Fresh human saliva (B, Se) was boiled for 30 min and then centrifuged. Two vols of ethanol (99.5%) were added to the supernatant and the resulting precipitate was collected by centrifugation. The precipitate was dissolved in PBS to the same volume as the original saliva sample.

Production of mAbs. The antigen solution was emulsified in an equal volume of Complete Freund's Adjuvant. Fifty µl of the emulsion was injected subcutaneously into each hind foot pad of BALB/c mice (7 week, female). After eleven days the mice were killed, and draining popliteal lymphnodes were dissected out and used as the source of antibody producing cells [2]. The myeloma cell line P3U1 was used as the fusion partner. Fusion and cultures were essentially performed as described by Köhler and Milstein [3].

Buffers

Dilution buffer: 10 m*M Tris*-HCl, pH 7.4 containing 0.5 *M* NaCl, 0.2% Tween-20, 0.3% gelatin and 0.01% Thimerosal.

Washing buffer: 10 m*M Tris*-HCl, pH 7.4 containing 0.5 *M* NaCl and 0.2% Tween-20.

Blocking buffer: 10 m*M Tris*-HCl, pH 7.4 containing 0.15 *M* NaCl, 0.3% gelatin and 0.01% Thimerosal.

PBS: 10 m*M* phosphate buffered saline, pH 7.4.

Enzyme-linked immunosorbent assays (ELISA). Micro ELISA plates (Sumitomo Bakelite, Tokyo, Japan) were coated with 50 µl of antigen solution (10 µg/ml 0.05 *M* carbonate buffer, pH 9.6) and blocked with blocking buffer. Goat anti-mouse IgG + IgM-biotin conjugate (X200 dil.; Tago, Ca, USA) was used as the secondary antibody. Avidin-horseradish peroxidase (HRP) (X5,000 dil.; Vector, Ca, USA) and o-phenylenediamine were used as reagents for colour development. Absorbance at 492 nm (620 nm as reference) of each well was measured by an EIA reader (EASY READER 400 FW, SLT-Labinstruments GmbH, Salzburg, Austria).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE was performed on a 10% polyacrylamide gel according to Laemmli [4]. Protein was visualized with Coomassie brilliant blue (CBB) and carbohydrate with periodic acid-Schiff (PAS) reagent.

Immunoblotting. Immunoblotting was carried out by separating the proteins on a SDS-polyacrylamide gel and electroblotting onto a nitrocellulose membrane (Bio-Rad, Ca, USA) as described by Towbin et al. [5]. The membrane was incubated with the ascites of P4-5C or P4-1F (X1,000 dil.) for 2 h at room temperature and the antigen-antibody complexes were identified using goat anti-mouse IgG + IgM-biotin conjugate (X200 dil.) and avidin-HRP (X5,000 dil.). 4-chloro-1-naphthol was used as substrate for color development.

Deglycosylation of BGS. Deglycosylation of BGS was performed by trifluoromethanesulfonic acid (TFMS) treatment according to Edge et al. [6]. Briefly, 1 mg of dry BGS was dissolved in 1 ml of ice-cold TFMS solution (1:2 mixture of anisol and TFMS) and nitrogen gas was bubbled through the solution for 30 s followed by magnetic stirring at 0°C for 2.5 h. The reaction mixture was diluted with 2 ml of diethylether cooled to −40°C and 3 ml of ice-cold 50% (v/v) aqueous pyridine was added. The resulting precipitate was redissolved by vortexing the suspension and the ether phase was removed and discarded. After repeating the ether extraction of the aqueous phase, the combined aqueous portions were dialyzed against 2 m*M* pyridine acetate buffer, pH 5.5 and lyophilized.

Counterimmunoelectrophoresis. Counterimmunoelectrophoresis was carried out on a microscope slide by the procedure of Gocke et al. [7].

Results

Monoclonal antibody production

After injection of saliva ABO-BGS (blood group B, Se) into the hind foot pads of mice, clones against tissue specific epitopes (anti-saliva) and blood group epitopes (anti-H and -B) were activated. These clones were present in almost identical proportions. The clones against tissue specific epitopes were selected by screening with ELISA and the hemagglutination test and established as a single clone by the limiting dilution method. Finally two clones (P4-2F, P4-5C) against tissue specific epitopes were established. The isotype of both clones was IgM.

The binding of 10^6-fold diluted ascites of these clones to plates coated with 10^3-fold diluted native saliva were detected by ELISA (Fig. 2). These results were not affected by blood group and secretor status of the saliva (data not shown). At least 3.2×10^4-fold dilutions of saliva could be detected by ELISA with 10^3-fold dilutions of ascites of these clones. These results were also not affected by blood group and secretor status of the saliva (Fig. 3).

Tissue specificity

The tissue specificities of P4-2F and P4-5C were investigated using two methods. Firstly, counterimmunoelectrophoresis was used to test the antibodies against saliva,

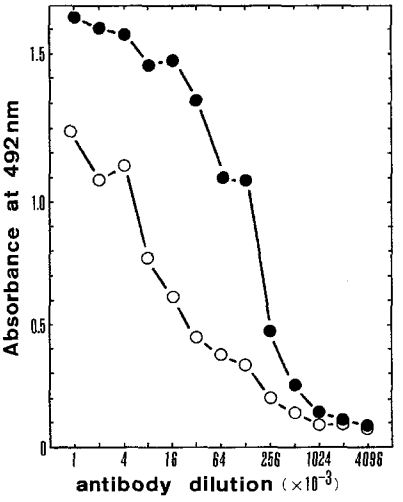

Fig. 2. Binding properties of ascites of P4-2F and P4-5C to saliva-coated plate. 1000-fold dilution of native saliva (blood group B Se) used as the solid phase antigen. ELISA was performed as described in Materials and methods. ○: P4-2F, ●: P4-5C

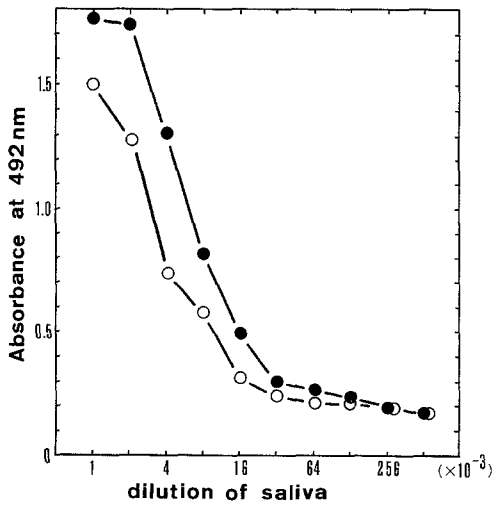

Fig. 3. Detection limits of saliva with P4-2F and P4-5C by ELISA. 1000-fold dilution of ascites were used as primary antibodies. ELISA was performed as described in Materials and methods. ○: P4-2F, ●: P4-5C

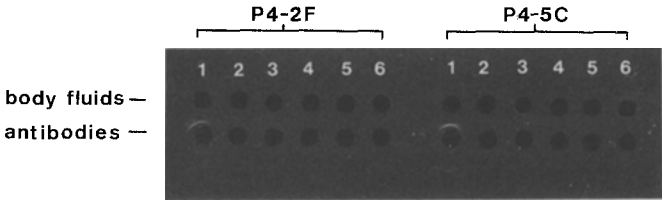

Fig. 4. Counterimmunoelectrophoresis of P4-2F and P4-5C against body fluids. 10-fold dilution of ascites and diluted body fluids (1. saliva: ×50, 2. semen: ×1, 3. vaginal secretion: ×1, 4. urine: ×1, 5. sweat: ×1, 6. serum: ×1) were electrophoresed as described in Materials and methods

semen, vaginal secretion, urine, sweat and blood serum. Only saliva showed a single-sharp precipitin line (Fig. 4). Secondly, ELISA was used to test their reactions to saliva, semen and vaginal secretions. Table 1 shows that a positive reaction was detected only in saliva. These results show that P4-2F and P4-5C are specific for saliva.

Species specificity

Determination of species specificity of the monoclonal antibodies was performed by counterimmunoelectrophoresis and by ELISA. Figure 5 shows the results of counterimmunoelectrophoresis of P4-2F against human, dog and cat saliva. The P4-2F gave single-sharp precipitin line only with human saliva. In ELISA, a positive reaction was also seen only in human saliva (Fig. 6). Identical results were obtained for P4-5C (data not shown) and show that P4-2F and P4-5C are specific for human saliva.

Antigens of P4-2F and P4-5C

Identification of the antigens recognized by P4-2F and P4-5C, was performed by immunoblotting. Saliva was electrophoresed in a 10% SDS-PAGE, blotted on to nitrocellulose membrane and stained by P4-2F and P4-5C.

Table 1. Tissue specifity of P4-2F and P4-5C

Antibodies	Body fluids		
	Saliva (1/1,000)	Semen (1/1,000)	Vaginal secretion (1/50)
P4-2F	1.516*	0.076*	0.098*
P4-5C	1.586*	0.072*	0.112*

* Absorbance value at 492 nm

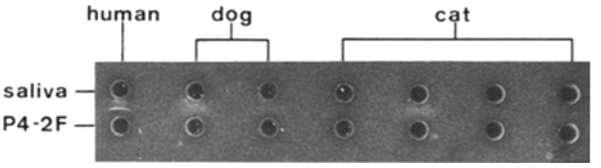

Fig. 5. Counterimmunoelectrophoresis of P4-2F against stain extracts of human saliva and saliva of animals

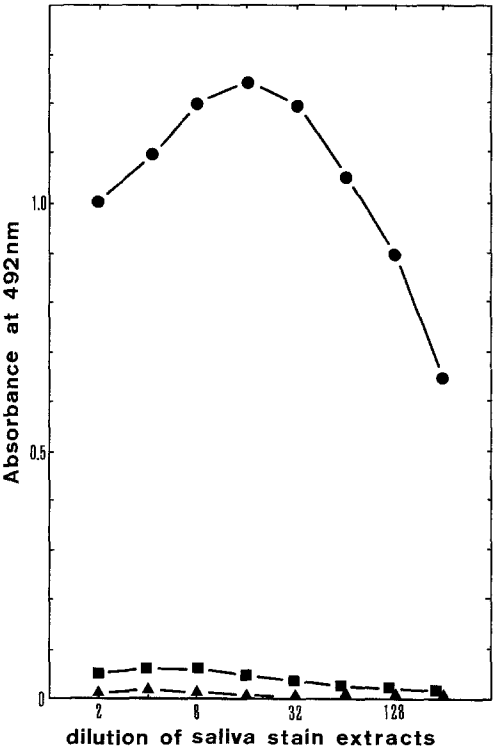

Fig. 6. Binding properties of P4-5C to human and animal saliva. 1000-fold dilution of ascites of P4-5C was used as the primary antibody. ELISA plates were coated with serial dilutions of extracts from saliva stains. ELISA was performed as described in Materials and methods. ●: Human saliva, ■: cat saliva, ▲: dog saliva

The position of the area stained immunologically by both P4-2F and P4-5C was demonstrated by PAS staining directly on the gel (Fig. 7). This position was also stained by anti-ABO blood group specific antibodies. Therefore, these two mAbs recognize the high molecular weight glycoprotein of saliva (ABO-BGS).

Furthermore, we investigated whether the epitopes defined by these mAbs exist on saccharide chains or in the glycoprotein core. Firstly, saliva glycoproteins were

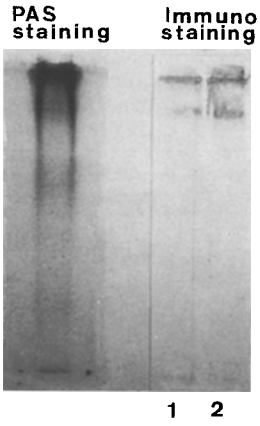

Fig. 7. Identification of antigen of P4-2F and P4-5C by immunoblotting. Proteins in native saliva were separated by electrophoresis in 10% PGA and transferred to a nitrocellulose membrane. The gel was stained with PAS and the nitrocellulose membrane was stained immunologically with mAbs (1, P4-2F; 2, P4-5C)

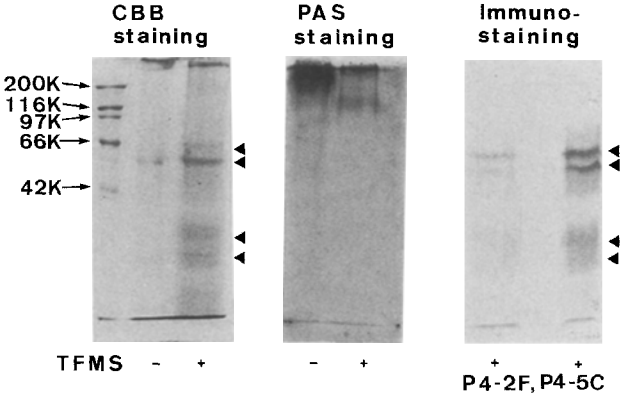

Fig. 8. Effects of deglycosylation of BGS on its staining pattern in electrophoresed gel and immunoblot. BGS of saliva was deglycosylated by TFMS and electrophoresed in 10% PAG. The gels were stained with CBB and PAS and blotted on to nitrocellulose membrane. The nitrocellulose membranes were stained immunologically with P4-2F and P4-5C. The arrows indicate the positions of new bands after TFMS treatment of saliva in PAS staining and on the blot

deglycosylated by treatment with TFMS and examined by immunoblotting in the same way as for native saliva. After treatment with TFMS the PAS positive bands appeared to be fainter and new CBB staining positive bands (66 K, 56 K, 34 K) were observed. These new bands were stained by P4-2F and P4-5C immunoblotting (Fig. 8). These results suggest that P4-2F and P4-5C recognize the core of a saliva glycoprotein.

Discussion

Two groups of clones were activated by immunization of mice with the ABO blood group substances of saliva. One group was ABO blood group specific and another group saliva (tissue) specific. These results supported our hypothesis concerning the structure of ABO blood group substances in saliva. Interestingly, multiple immunization of ABO blood group substances from saliva predominantly activated clones against ABO blood group epitopes.

The established clones P4-2F and P4-5C, showed high binding activity to saliva and furthermore, were specific for human saliva. It was extraordinary that these mAbs produced a precipitin line against saliva in counterimmunoelectrophoresis and immunodifusion. Presumably, multiple epitopes exist on single molecules of saliva ABO-BGS. Another possibility is the aggregation of BGS in solution.

These mAbs are extremely suitable for the identification of saliva using ELISA method or counterimmunoelectrophoresis. As both methods are highly sensitive, tissue specific and human specific, it is possible that they could replace the conventional blue starch method which is very sensitive but neither tissue nor species specific [8]. The two mAbs recognize an identical antigen which is a high molecular weight glycoprotein (> 200 K). This glycoprotein forms a broad band in SDS-PAGE due to heterogeneity of the oligosugar moiety of the molecule. The epitopes defined by these mAbs exist on the core protein of the glycoprotein. This is shown by deglycosylation and subsequent immunoblotting with mAbs (Fig. 8). After deglycosylation with TFMS, PAS negative and new CBB positive bands (66 K, 56 K, 34 K) were produced and were stained by both mAbs. These bands correspond to the core protein and fragments of the glycoprotein. Presumably the 66 K peptide is the core protein and the lower molecular weight peptides (56 K, 34 K) are its degredation products. These results also support our hypothesis about the structure of BGS.

In this study, two mAbs (P4-2F, P4-5C) against BGS of saliva have been produced and they are the first examples of antibodies which can completely distinguish saliva from other body fluids. Furthermore, these mAbs enabled the identification of ABO blood groups of saliva from mixed body fluids by sandwich methods. The detail of these methods are described in a subsequent article.

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