Z Rechtsmed (1988) 100:215–221

Zeitschrift für
Rechtsmedizin

Detection of AB0 Blood Group-active Glycolipids Extracted from Red Cell Membrane and Heat Hematoma Using TLC-Immunostaining*

M. Ota, H. Fukushima, I. Yonemura, and H. Hasekura

Department of Legal Medicine, Shinshu University School of Medicine, Asahi, Matsumoto, 390 Japan

Summary. Glycolipids extracted from groups A, B, and 0 erythrocytes were developed on thin-layer plates; their AB0 blood group antigenicities were detected by immunostaining method using avidin-biotin-complex (ABC). Among series of glycolipids of different flow rates, antigen-specific staining was observed in five bands from group A_1 erythrocytes, four bands from group B, and two bands from group O. Monoclonal anti-A, -B, and -H antibodies specifically stained glycolipids from A_1, B, and 0 erythrocytes, respectively.

AB0 blood grouping was possible from 5 g of epidural heat hematoma of a charred body by this method. ABC immunostaining on thin-layer chromatography is a useful and reliable method for AB0 blood grouping in forensic practice.

Key words: AB0 blood groups, immunostaining – Glycolipids, AB0 blood groups

Zusammenfassung. Glycolipide, welche von Erythrozyten der Blutgruppen A, B und 0 extrahiert wurden, wurden mittels der Dünnschichtchromatographie nachgewiesen; ihre AB0-Blutgruppenprägung wurde mit Hilfe der Immunfärbungsmethode unter Verwendung von Avidin-Biotin-Complexen nachgewiesen. Unter den Serien von Glycolipiden mit verschiedenen Fließgeschwindigkeiten wurden antigenspezifische Färbungen in fünf Banden von Erythrozyten der Blutgruppe A festgestellt, in vier Banden von Erythrozyten der Blutgruppe B und in 2 der Blutgruppe 0. Monoklonale Anti-A, B- und H-Antikörper färbten spezifisch Glycolipide der jeweiligen Erythrozyten.

* Supported in part by a scientific grant from the Ministry of Education, Science, and Culture, Japan

Offprint requests to: M. Ota

Im Falle einer verkohlten Leiche war die AB0-Blutgruppenbestimmung aus 5 g eines epiduraden Hitze-Hämatoms möglich. Die Immunfärbung mit Hilfe des Avidin-Biotin-Complexes auf Dünnschichtchromatographie-Platten ist eine nützliche und zuverlässige Methode für die AB0-Blutgruppenbestimmung in der forensischen Praxis.

Schlüsselwörter: AB0-Blutgruppen, Immunfärbung – Glycolipide, AB0-Blutgruppen

Introduction

Recently, immunostaining on thin-layer chromatography (TLC-Immunostaining) has been adopted to detect glycolipid antigens of colon carcinoma and cholera toxin using monoclonal antibodies [1, 2]. Hansson et al. [3] also performed immunostaining on thin-layer chromatography using the ^{125}I-labeled secondary antibody for the detection of blood group A- and B-active glycolipids.

In this study, various immunostaining methods on thin-layer plates using peroxidase-labeled antibodies were compared to analyze the AB0 antigen activities of glycolipids from erythrocytes. Moreover, we tried to detect AB0 blood group antigens from epidural heat hematoma by ABC method.

Materials and Methods

Preparation of AB0 Blood Group-active Glycolipids from Whole Blood

A, B, and 0(H)active glycolipids were prepared by the method of Folch et al. [4]. One hundred milliliters of each human red cell (A, B, and 0) from a bag of whole blood preserved in ACD (acid-citrate-dextrose) solution for 30 days were washed three times with saline and hemolyzed in 0.2% acetic acid. After being centrifuged and washed again with water, the stroma was stirred continuously in 200 ml acetone for 1 h. The dehydrated stroma was extracted with 200 ml chloroform/methanol (2:1, vol/vol) by constant stirring at room temperature for 1 h. The extracts were subsequently evaporated to dryness with a rotary evaporator. Total glycolipids containing AB0 glycosphingolipids were dissolved in 5 ml chloroform/methanol (2:1, vol/vol), followed by re-evaporation to dryness under a stream of nitrogen gas. The crude extracts were readjusted to a volume of 100 µl with chloroform/methanol (2:1, vol/vol), and were used for the detection of AB0 blood group antigens using immunostaining on thin-layer chromatography (TLC).

Preparation of AB0 Blood Group Glycolipids from Epidural Heat Hematoma of a Charred Body

Five grams of epidural heat hematoma was obtained from a body which was burned to death in a house-fire. The sample of hematoma was washed with water three times. Separation of blood group-active glycolipids was made essentially by the same way as from the whole blood. Finally, the crude extract was dissolved in 50 µl chloroform/methanol (2:1, vol/vol).

Procedures for AB0 Grouping by Immunostaining on TLC

1. The preparation (8 µl) was applied on an aluminium plate (6 × 8 cm) coated with Silicia Gel 60 (HPTLC, Merck, Darmstadt, FRG).

Table 1. Monoclonal antibodies employed for TLC-immunostaining

Monoclonal antibody	Immunogen
Anti-A	GalNAcα(1–3)(Fucα(1–2))Galβ-
Anti-B	Galα(1–3)(Fucα(1–2))Galβ-
Anti-H	Fucα(1–2)Galβ(1–4)GlcNacβ-

Fuc: fucose; Gal: galactose; GalNAc: N-acetylgalactosamine; GlcNAc: N-acetylglucosamine

2. Glycosphingolipids were developed on the plate in a chromatography tank containing chloroform, methanol, and water (60:35:8, vol/vol/vol).

3. The plate was air-dried, and then covered with phosphate-buffered saline containing 5% bovine serum albumin (BSA-PBS) for 30 min in a moist chamber.

4. BSA-PBS was then tipped off; as the primary antibody, blood grouping antibodies (anti-A, anti-B, and anti-H monoclonal antibodies; DAKO, Santa Barbara, Cal., USA) were overlaid on the plate and let react for 1 h in a moist chamber. These monoclonal antibodies were produced by immunization against the sugar groups listed in Table 1. Each antiserum used was diluted to 1/30 of the original volume in PBS containing 0.5% BSA. Agglutination titers of commercial DAKO anti-A, anti-B, and anti-H monoclonal antibodies with groups A, B, and 0 erythrocytes were 256, 256, and 64, respectively.

5. The antiserum was tipped off and the plate rinsed three times with BSA-PBS. For the determination of AB0 blood groups, the following three immunostaining methods were performed, (a) avidin-biotin-peroxidase complex method: ABC method, (b) unlabeled antibody enzyme method with peroxidase-anti-peroxidase complex: PAP method, and (c) indirect method.

(a) In the ABC method [5], a biotinylated anti-mouse IgM antiserum (Medical Biological Laboratory, Nagoya, Japan) was used at the dilution of 1/30 with BSA-PBS.

(b) In the PAP method [6], a rabbit anti-mouse immunoglobulin serum (DAKO, Santa Barbara, Cal., USA) and peroxidase-anti-peroxidase complex mouse serum (DAKO, Santa Barbara, USA) were used respectively diluted to 1/25 and 1/100 with BSA-PBS.

(c) In the indirect method, peroxidase-conjugated rabbit anti-mouse immunoglobulin serum (DAKO, Santa Barbara, Cal., USA) was used at the dilution of 1/50 in BSA-PBS. Reaction time of the secondary antibody or the peroxidase-conjugated antibody on the plate was 1 h each in a humid atmosphere. At the interval of each step, the plate was washed three times with BSA-PBS.

6. For staining of blood group-specific antibody binding, the plate was treated with 4-chloro-1-naphthol (10 mg/ml in ethanol) diluted 1:50 in PBS (1/15*M*, pH 7.2) to which fresh 30% H_2O_2 was added to a final concentration of 0.005%.

Results

Total glycolipids extracted from human blood were separated by TLC using the solvent chloroform-methanol-water. Immunochemical staining of erythrocyte membrane glycolipids on TLC was performed to detect AB0 blood group activity by using anti-A, B, and H monoclonal antibodies. Table 2 compares the sensitivity among the immunostaining techniques employed in this study, ABC,

Table 2. Comparison of immunostaining methods on TLC

Method	Original red cell volume (ml)			
	10	20	50	100
ABC	±	+	++	++
PAP	−	±	+	++
ID	−	±	±	+

(−): a negative staining; (±): an equivocal staining; (+): a moderate staining; (++): a good staining. ABC: avidin-biotin-peroxidase complex method; PAP: unlabeled antibody enzyme method with peroxidase-anti-peroxidase complex; ID: Indirect method

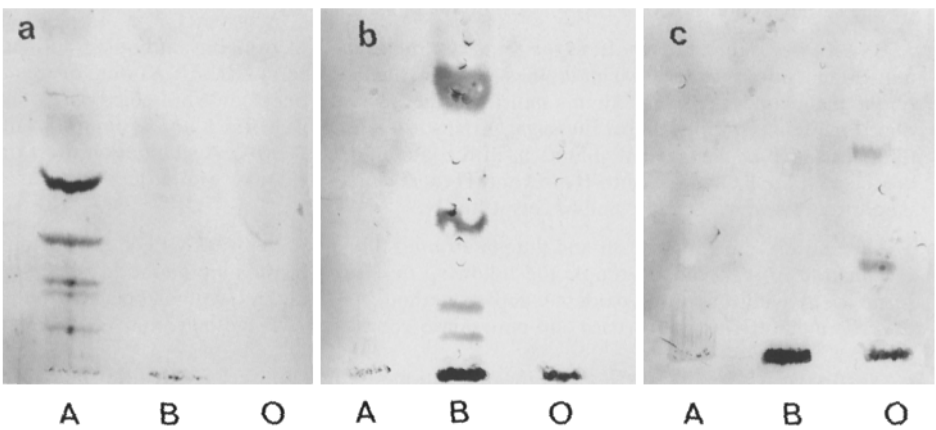

Fig. 1a–c. Glycolipids extracted from erythrocytes of different blood groups immunostaining by the avidin-biotin-complex (ABC) method on thin-layer chromatography. Lanes A, B, and 0 were applied with samples from A, B, and 0 erythrocytes, respectively. The chromatography was developed with chloroform/methanol/water (60:35:8) and (**a**) stained by anti-A monoclonal antibody (MoA), (**b**) by anti-B MoA, and (**c**) by anti-H MoA

PAP, and ID. The ABC method was the most sensitive and reliable for determination of AB0 blood grouping.

As shown in Fig. 1, prominent bands observed in glycolipid preparations of A_1, B, and 0 erythrocytes differed from each another. Group A_1 (Fig. 1a) and group B (Fig. 1b) bloods revealed five bands and four bands with monoclonal antibodies, respectively, whereas group 0 blood revealed only two bands with an anti-H monoclonal antibody (Fig. 1c). Figure 2 shows the result of AB0 grouping of glycolipid preparation obtained from markedly heat-coagulated epidural blood mass using the ABC method. The glycolipids extracted from epidural heat hematoma reacted with only anti-B monoclonal antibody (Fig. 2b). Lane P on the thin-layer plates showed reactivity neither with anti-A nor anti-H

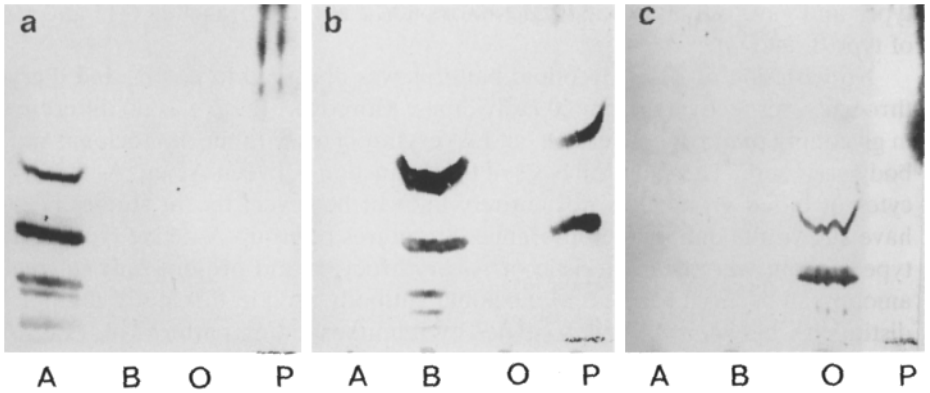

Fig. 2a–c. Immunostaining by the ABC method on thin-layer chromatography. Glycolipids extracted from an epidural hematoma of a charred body. Lane P shows the immunostaining pattern of glycolipid antigens extracted from 5 g heated epidural hematoma stained with monoclonal antibodies. Samples in lanes A, B, and 0 are the same as in Fig. 1

monoclonal antibody (Fig. 2a, c). These results clearly demonstrated that the AB0 phenotype of the charred body was B.

Discussion

Immunostaining on thin layer plates has been used to identify components of glycolipid fractions from blood [7, 8, 9]. In these studies, blood group active substance was purified to neutral glycolipids; but crude glycolipids was sufficient for identification of blood groups in forensic practice.

Immunostaining of the erythrocyte glycolipids was performed using other polyclonal anti-A (Ortho) and anti-B (Ortho) antibodies. The results (data not shown) were essentially identical with the patterns shown in Fig. 1. However, most sensitive and consistent results were obtained by using commercially prepared anti-A, anti-B, and anti-H monoclonal antibodies.

It is known that the blood group A and H determinant is carried mainly by types 2 and 3 chains in red cells, by type 1 chain in gastrointestinal tract, and by type 4 chain in kidney; the blood group B determinant, however, lacks type 3 chain structure [10]. A, B, and H-active type 2 chain structures have four subtypes, respectively [11–16].

The five bands observed in the group A chromatogram in this study would indicate that the monoclonal anti-A antibody cross-reacted with all the varieties of group A chain structures defining the common epitope of type 3 chains and the four bands probably representing the four subtypes of type 2 chain.

In contrast, only two bands were shown in the group H chromatogram. Probably, the monoclonal anti-H used might have been more chain-specific and reacted with only two subtypes of resembling structures, such as the unbranched chains (H1 and H2 of type 2) and failed to react with the branched. Another possibility would be that the solvent used could not separate the sub-

types and gave only two bands: the unbranched and the branched (H3 and H4 of type 2, and type 3).

No variation of TLC glycolipid patterns was observed in A_1, B, and 0 erythrocytes, respectively, from 20 individuals. Moreover, there was no difference in glycolipid patterns between A_1 and A_2 erythrocytes with the monoclonal antibody used here. The chemical basis of the distinction between A_1 and A_2 erythrocytes of blood group A is not entirely known; however, recent studies [7, 8] have shown the qualitative difference. Structures of group A-active type 3 and type 4 chain were characteristic of A_1 erythrocytes and present only in trace amounts in A_2 erythrocytes. Monoclonal antibody used in this study could not distinguish between A_1 and A_2 types by immunostaining patterns of TLC. A further study of immunostaining patterns on TLC would be required using erythrocyte glycolipids from other AB0 variants.

The ELISA method is, though accurate and simple, sometimes disturbed by false positive reactions due to endogenous peroxidase activity of hemoglobin. The ABC method used here was free from this problem, as hemoglobin was eliminated from the TLC plate by its faster flow rate than glycolipids enabling specific staining.

Nagano et al. [17] reported that A, B, and 0(H) blood group glycolipids retained the blood group activities after being heated in the solid state at 120°C for 1 h. We succeeded in typing the AB0 blood groups of markedly heated epidural hematoma by using immunostaining on TLC.

If an adequate numbers of samples is available, this technique would also be applicable to blood grouping of heated blood, bone tissues, and dental tissues.

Acknowledgement. The authors wish to thank Professor T. Nagano of Kanazawa University for his helpful advice.

References

1. Magnani JL, Brockhaus M, Smith DF, Ginsburg V (1981) Monosialoganglioside is a monoclonal antibody-defined antigen of colon carcinoma. Science 212:55–56
2. Magnani JL, Brockhaus M, Smith DF, Ginsburg V (1982) Detection of glycolipid ligands by direct binding of carbohydrate-binding proteins to thin-layer chromatograms. Methods Enzymol 83:235–241
3. Hansson GC, Karlsson K-A, Larson G, Samuelsson BE, Thurin J, Bjursten LM (1985) Detection of blood group type glycosphingolipid antigens on thin-layer plates using polyclonal antisera. J Immunol Methods 83:37–42
4. Folch J, Lees M, Sloane-Stanley GH (1957) A simple method for isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509
5. Guesdon J-L, Ternynck T, Avrameas S (1979) The use of avidin-biotin interaction in immunoenzymatic techniques. J Histochem Cytochem 27:1131–1139
6. Sternberger LA, Hardy PH, Cuculis JJ, Meyer HG (1970) The unlabelled antibody enzyme method of immunohistochemistry: Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antiperoxidase) and its use in identification of spirochaetes. J Histochem Cytochem 18:315–333
7. Clausen H, Watanabe K, Kannagi R, Levery SB, Nudelman E, Arano-Tomono Y, Halomori S (1984) Blood group A glycolipid (A^x) with globo-series structures which is specific for blood group A_1 erythrocytes: One of the chemical bases for A_1 and A_2 distinction. Biochem Biophys Res Commun 124:523–529

8. Clausen H, Levery SB, Nudelman E, Tsuchiya S, Hakomori S (1985) Repetitive A epitope (type 2 chain A) defined by blood group A_1-specific monoclonal antibody TH-1: chemical basis of qualitative A_1 and A_2 distinction. Proc Natl Acad Sci USA 82:1199–1203
9. Breimer M, Samuelsson BE (1986) The specific distribution of glycolipid-based blood group A antigen in human kidney related to A_1/A_2, Lewis, and secretor status of single individuals. Transplantation 42:88–91
10. Hakomori S (1986) Chemistry of blood groups: Structural polymorphism and oncodevelopmental changes. JJSBT 32:544–556
11. Hakomori S (1981) Blood group ABH and Ii antigens of human erythrocytes. Semin Hematol 18:39–62
12. Hakomori S, Stellner K, Watanabe K (1972) Four antigenic variants of blood group A glycolipid: Examples of highly complex, branched chain glycolipid of animal cell membrane. Biochem Biophys Res Commun 49:1061–1068
13. Watanabe K, Laine RA, Hakomori S (1975) On neutral fucoglycolipids having long branch carbohydrate chains: H-active glycosphingolipids in human erythrocyte membranes. Biochemistry 14:2725–2733
14. Fukuda MN, Hakomori S (1982) Structure of branched blood group A-active glycosphingolipids in human erythrocytes and polymorphism of A- and H-glycolipids in A_1 and A_2 subgroups. J Biol Chem 257:446–455
15. Hanfland P (1975) Characterization of B and H blood group active glycosphingolipids from human B erythrocyte membranes. Chem Phys Lipids 15:105–124
16. Hanfland P, Kordowicz M, Niermann H, Egge H, Dabrowski U, Peter-Katalinic J, Dabrowski J (1984) Purification and structures of branched and blood-group-B-active glycosphingolipids from human erythrocyte membranes. Eur J Biochem 145:531–542
17. Nagano T, Tsuji K, Ieda K (1976) Blood group determination of severely charred bodies – The effects of heating on the blood group activity of A, B, AB and 0(H) active glycolipids and A and B active glycoproteins. J Forens Sci Soc 16:163–168

Received September 28, 1987