馬血清トランスフェリン変異型間における成分比の相違

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Difference in Content Ratio of Components among Horse Serum Transferrin Variants

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ABSTRACT. Each of five genetic variants of horse serum transferrin (Tf), D, F, H, O, and R, was separated into two bands by polyacrylamide gel isoelectric focusing (PAGIEF). The more acidic band, termed component a, was more abundant than the other one, termed component b, in all variants. Components a and b of TFO variant were immunologically indistinguishable from each other by double immunodiffusion test. Determination of the content ratio of component a to component b in each variant revealed that the variants were classified into two groups: one group (D, F, and H) had a relatively high ratio within a range from 3.4 to 4.0 and the other group (O and R) had a relatively low ratio of 1.8 to 2.3. From these results and reference data on carbohydrate compositions of components a and b, it was proposed that there is a difference in glycosylation between the two groups.—KEY WORDS: genetic variant, glycosylation, heterogeneity, horse, transferrin.

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Serum transferrin (Tf) is a glycoprotein which has a molecular weight of about 80,000 and two iron binding sites [3, 7]. In 1964, Braend and Stormont [1] reported six genetic variants D, F, H, M, O, and R of horse Tf. So far, fifteen variants have been identified by starch gel electrophoresis and polyacrylamide gel isoelectric focusing (PAGIEF) [4, 15]. Chung and McKenzie [2] showed that there were at least two amino acid substitutions between variants D and R (Asp→Gly and Glu→Gly).

Horse Tf is heterogeneous, that is, each of the Tf variants gives two main bands in starch gel electrophoresis and PAGIEF [1, 15]. The faster migrating (or the more acidic) band is usually more thickly stained than the slower migrating (or the less acidic) one [1, 15]. In the present paper, the former is named component a and the latter component b. From carbohydrate analyses of these two components of TfO variant, Stratil *et al.* [13] proposed that horse Tf has the same diantennary carbohydrate chains as human Tf [12], and that component a has two identical chains, whereas component b has one.

We examined here the content ratio of component a to component b in each Tf variant to clarify whether there was a difference in glycosylation among them.

MATERIALS AND METHODS

Horse sera: Blood samples were collected from Thoroughbred and Tokara-native horses. Serum samples were stored at −20°C.

Protein determination: Protein was determined by the method of Lowry *et al.* [6] with bovine serum albumin as a standard.

Purification of Tf: All the procedures were carried out in a cold room (5°C) or in an ice bath. A). Horse Tf preparation containing several genetic variants (F, H, O and R) was purchased from Sigma Chemical Co. and was further purified by gel filtration on a column (2.0×100 cm) of Sephadex

G-100 Superfine (Pharmacia Fine Chemicals) equilibrated with 20 mM Tris/HCl buffer (pH 8.2) containing 100 mM NaCl and 5 mM NaN_3 (Buffer A). B). Components a and b of TfO variant were purified from Thoroughbred sera bearing phenotypes TfDO or TfFO. Serum (5 m*l*), to which 65 μl of an iron solution (Solution A) comprising 10 mM $Fe(NH_4)(SO_4)_2$, 100 mM sodium citrate, and 200 mM sodium bicarbonate was added to saturate iron-binding sites of Tf, was dialyzed overnight against 1 *l* of 10 mM Tris/HCl (pH 8.2). The dialyzed solution was centrifuged at 12,000 ×g for 15 min to remove insoluble materials. The clear supernatant was applied to a column (3.1 × 13 cm) of Cibacron Blue F3GA-Sepharose 4B, which had been prepared by the procedure of Travis *et al.* [14] and equilibrated with 10 mM Tris/HCl (pH 8.2), at a flow rate of 50 m*l*/hr. Whereas almost all serum albumins having affinities to the dye [14] bound to the column, Tf passed through the column without binding.

The eluate containing Tf was brought to 50% saturation by gradual addition of the calculated amount of solid ammonium sulfate. The mixture was left overnight and centrifuged at 27,000 ×g for 15 min. The supernatant obtained was brought to 75% saturation by gradual addition of the calculated amount of solid ammonium sulfate. The mixture was left for 8 hr and centrifuged at 27,000 ×g for 15 min.

The precipitate obtained was dissolved in 3 m*l* of Buffer A and the resulting solution was applied to a column (2.0 × 100 cm) of Sephacryl S-200 Superfine (Pharmacia Fine Chemicals) equilibrated with Buffer A at a flow rate of 20 m*l*/hr. The pooled eluate containing Tf was dialyzed against 1 *l* of 20 mM Tris/HCl (pH 8.2) overnight. The dialyzed solution was applied to a column (1.5 × 50 cm) of DE-52 cellulose (Whatman) equilibrated with 20 mM Tris/HCl (pH 8.2). Tf was eluted with 1 *l* linear gradient buffer of 20 mM to 120 mM Tris/HCl (pH 8.2) at a flow rate of 30 m*l*/hr. TfO was completely separated from TfD or TfF by this chromatographic step. Moreover, the two main components a and b of TfO were also well separated from each other.

Preparation of antisera: Each of the female rabbits obtained from Clea Japan, Inc., was immunized with 1.0 m*l* of an equal volume mixture of Freund's complete adjuvant and 1.0 mg of purified Tf in phosphate buffered saline (PBS: 20 mM sodium phosphate, 150 mM NaCl, pH 7.0). TfOa component purified from sera and a mixture of various Tf variants purified from commercial Tf preparation were used as immunogens. Emulsion was injected into several intradermal sites. At 3 and 4 weeks after the primary injection, each rabbit was injected subcutaneously with 0.5 m*l* of the same mixture. Rabbits were bled 7 days thereafter. Collected sera were stored at −20°C.

Immunological assays: Double immunodiffusion test and immunoelectrophoresis were carried out according to Ouchterlony and Nilsson [8].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE was carried out by the method of Laemmli [5].

PAGIEF: PAGIEF was performed with Bio-Rad Model 111 Mini IEF Cell according to the Bio-Rad instruction manual. Polyacrylamide slab gels which adhered to Gel Support Film consisted of 4.85% acrylamide, 0.15% N,N'-methylenebisacrylamide, 5% glycerol, 2% Bio-Lyte (pH 5–7), 0.0005% riboflavin, 0.05% N,N,N',N'-tetramethylenediamine, and 0.05% ammonium persulfate. Serum samples were mixed with 1/80 volume of Solution A, diluted appropriately wiht 0.15M NaCl, and applied to the gels.

After electrophoresis, the gels were immersed in a solution composed of 4%

5-sulfosalicylic acid, 12.5% trichloroacetic acid, and 30% methanol to fix the proteins. To detect Tf bands more sensitively, the gels were immersed in 1:30 diluted monospecific anti-Tf serum for 1 hr at 37°C to immunofix Tf bands, and then washed with three changes of 500 m*l* PBS each for 24 hr to remove non-precipitated proteins. The fixed protein bands were stained with 0.1% Coomassie Brilliant Blue G-250 in 45% methanol/9% acetic acid. After destained with 25% methanol/7% acetic acid, the gels were dried at room temperature.

Densitometric scan: The dried gels were scanned at 605 nm by Shimazu Dual-Wavelength Flying-Spot Scanner CS-9000 and the peak areas were computed.

RESULTS

PAGIEF patterns of horse Tf phenotypes: Fig. 1 shows horse Tf phenotypes identified by PAGIEF and immunofixation. The two-band patterns of each variant which had been reported by others [1, 15] were reproduced. The more acidic band (component a) was usually stained more thickly than the less acidic one (component b).

Immunolgical comparison of components a and b of TfO variant: The components a and b of TfO variant obtained by chromatography on DE-52 cellulose were homogeneous by PAGIEF (Fig. 2), double immunodiffusion test (Fig. 3), immunoelectrophoresis (data not shown), and SDS-PAGE (not shown). Molecular weight of TfOa and TfOb were estimated to be 79,200 and 77,300, respectively, by SDS-PAGE.

A single precipitin line formed between TfOa and anti-TfOa serum completely fused with that formed between TfOb and anti-TfOa (Fig. 3). The result indicates the immunological identity of TfOa with TfOb. Although the components a and b of other Tf variants were not compared immunologically, it was assumed that there would be also no difference in antigenicity between the two components of any other variant.

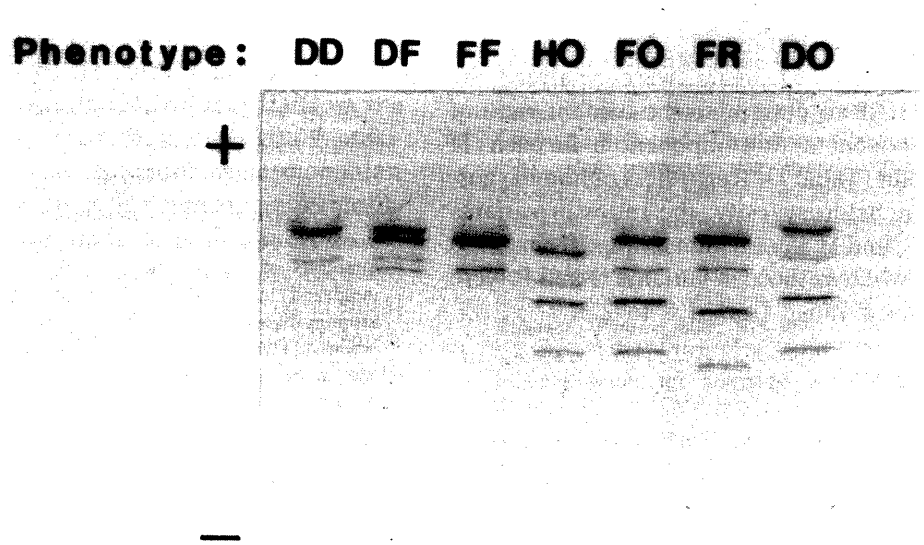

Fig. 1. PAGIEF patterns of horse Tf phenotypes. Different Tf phenotype sera were applied and Tf bands formed were immunofixed with antiserum to various Tf variants which were purified from commercial Tf preparation before staining with Coomassie Brilliant Blue G-250.

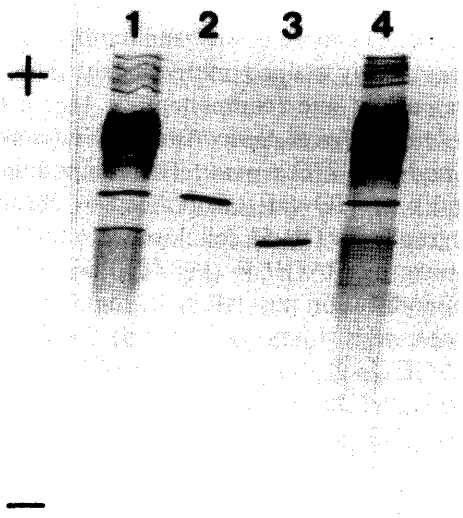

Fig. 2. PAGIEF of purified components a and b of TfO variant. Horse serum of TfFO phenotype (0.5 μl, lanes 1 and 4), TfOa (0.8 μg, lane 2), and TfOb (0.8 μg, lane 3) were applied and stained with Coomassie Blue after electrophoresis.

The ratio of component a to component b in each Tf variant: The amounts of TfOa component applied linearly correlated with the densitometric peak areas of the stained bands up to 100 ng (Fig. 4). Sera of horses aged 3 to 14 years were analyzed by PAGIEF to determine the content ratio of component a to component b in each Tf variant (Table 1). Regardless of homozygosity or heterozygosity, the ratios in variants D, F, and H were within a range from 3.4 to 4.0, whereas those in variants O and R were within a range from 1.8 to 2.3.

DISCUSSION

Human Tf is composed of one component [10, 11] and contains two branched oligosaccharide chains which attach to the 413th and 611th asparagine residues from the N-terminal [7, 12]. On the other hand, horse Tf is composed of two main components (Fig. 1) [1, 15]. It was proposed that component a has two identical carbohydrate

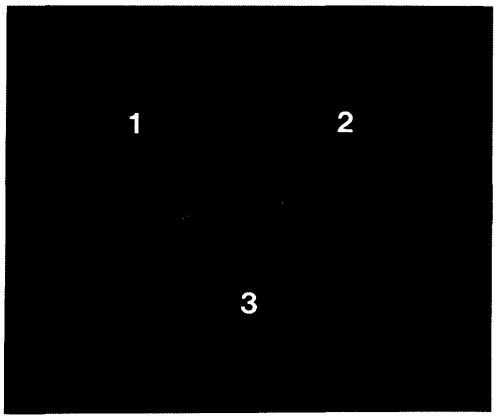

Fig. 3. Double immunodiffusion test. TfOa (2 μg in 10 μl, well 1), TfOb (2 μg in 10 μl, well 2), and anti-TfOa serum (20 μl, well 3) were diffused in 1% agarose gel for 2 days.

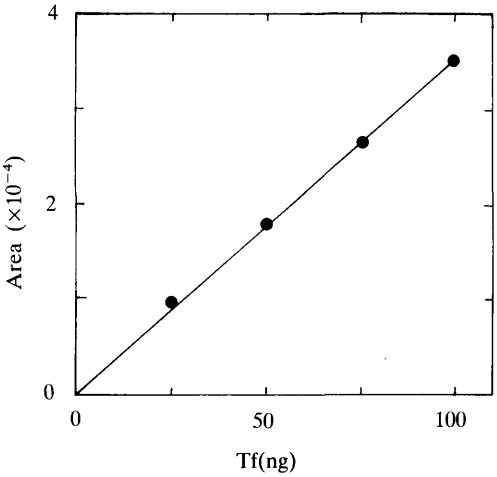

Fig. 4. Relation between amounts of Tf and densitometric peak areas of stained bands. Various amounts of component a of TfO variant were analyzed in PAGIEF. Tf bands formed were immunofixed with anti-TfOa serum, and then stained. The dried gel was scanned at 605 nm and the peak areas were determined. Each point represents the mean of duplicate determinations.

chains, whereas component b has one [13]. The difference in the number of glycosylated sites between the two Tf components may be due to incomplete glycosylation [9]. In the present study, we found that five horse Tf variants (D, F, H, O and R) were classified into a group of more acidic

Table 1. The content ratio of component a to component b in each Tf variant detected in different phenotype sera

Variant	Phenotype								
	DD[a](10)	DF[a](8)	DO[a](2)	DR[a](4)	FF[a](8)	FH[a](2)	FO[a](3)	FR[a](3)	RR[b](6)
D	3.60 ±0.13	3.63 ±0.17	3.98 ±0.31	3.97 ±0.27					
F		3.57 ±0.12			3.40 ±0.17	3.62 ±0.06	3.93 ±0.39	3.55 ±0.17	
H						3.78 ±0.41			
O			1.87 ±0.01				1.91 ±0.15		
R				2.26 ±0.08				1.83 ±0.08	2.20 ±0.06

Each Tf variant and component was separated by PAGIEF, immunofixed, and stained as shown in Fig. 1. The ratio of component a to component b was calculated from the densitometric peak areas of the stained bands. Each value represents mean±S.E.
( ): Number of animals.
a : Thoroughbred.
b : Tokara-native horse.

variants D, F, and H having relatively high content ratios of component a to component b and a group of less acidic variants O and R having relatively low content ratios, which indicated that there was a difference in glycosylation between two groups.

Kaminski *et al.* [4] divided horse Tf variants into the "faster-migrating" group (D, D_2, F, F_2, H, and J) and the "slower-migrating" group (M, O, and R) from the results of their immunological cross-reactivities and electrophoretic mobilities. The variants in each group are antigenically identical with each other, but those in the former are antigenically partially identical with those in the latter. Kaminski *et al.*[4] suggested that the immunological difference between the two groups may be due to structural differences other than substitutions or deletions of a single amino acid. From the present results, we suspect that there may be significant primary or tertiary structural differences in the vicinities of glycosylation sites between the two groups.

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REFERENCES

1. Braend, M. and Stormont, C. 1964. Studies on hemoglobin and transferrin types of horses. *Nord. Vet.-Med.* 16: 31–37.
2. Chung, M. C.-M. and McKenzie, H. A. 1985. Studies on equine tansferrin. I. The isolation and partial characterization of the D and R variants. *Comp. Biochem. Physiol.* 80B: 287–297.
3. Crichton, R. R. and Charloteaux-Wauters, M. 1987. Iron transport and storage. *Eur. J. Biochem.* 164: 485–506.
4. Kaminski, M., Didkowski, S., and Sykiotis, M. 1981. Polymorphism of transferrin locus in horses: immunochemical evidence of two structurally different subgroups of the allelic proteins. *Comp. Biochem. Physiol.* 68B: 505–507.
5. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227: 680–685.
6. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265–275.
7. MacGillivray, R. T. A., Mendez, E., Shewale, J. G., Sinha, S. K., Lineback-Zins, J., and Brew, K. 1983. The primary structure of human serum transferrin: the structures of seven cyanogen bromide fragments and the assembly of the complete structure. *J. Biol. Chem.* 258:

3543–3553.
8. Ouchterlony, Ö. and Nilsson, L.-A . 1978. Immunodiffusion and immunoelectrophoresis. pp. 19.1–19.44. *In*: Handbook of Experimental Immunology, 3rd ed. (Weir, D. M. ed.), Blackwell Scientific Publications, Oxford.
9. Powell, J. R. and Castellino, F. J. 1983. Amino acid sequence analysis of the asparagine-288 region of the carbohydrate variants of human plasminogen. *Biochemistry* 22: 923–927.
10. Smithies, O. 1957. Variations in human serum β-globulins. *Nature (Lond.)* 180: 1482–1483.
11. Smithies, O. and Hiller, O. 1959. The genetic control of transferrins in humans. *Biochem. J.* 72: 121–126.
12. Spik, G., Bayard, B., Fournet, B., Strecker, G., Bouquelet, S., and Montreuil, J. 1975. Studies on glycoconjugates. LXIV. Complete structure of two carbohydrate units of human serotransferrin. *FEBS Lett.* 50: 296–299.
13. Stratil, A., Tomášek, V., Bobák, P., and Glasnák, V. 1984. Heterogeneity of horse transferrin: the role of carbohydrate moiety. *Anim. Blood Grps. Biochem. Genet.* 15: 89–101.
14. Travis, J., Bowen, J., Tewksbury, D., Johnson, D., and Pannell, R. 1976. Isolation of albumin from whole human plasma and fractionation of albumin-depleted plasma. *Biochem. J.* 157: 301–306.
15. Yokohama, M., Watanabe, Y., and Mogi, K. 1985. A new equine transferrin phenotype detected by iso-electric focusing. *Jpn. J. Zootech. Sci.* 56: 116–121.

要　約

馬血清トランスフェリン変異型間における成分比の相違：渡辺清隆・蘇原　正・武田美和・上野　究・鈴村直広・六朗田　靖[1)]・六朗田　巌[1)]・山本晋二(北里大学獣医畜産学部獣医生理化学教室，[1)]株式会社タイヘイ牧場)——馬血清トランスフェリン (Tf) の5つの遺伝的変異型 (D, F, H, O, R)は，ポリアクリルアミドゲル等電点電気泳動 (PAGIEF) においてそれれぞれ主要な2本のバンドを与え，より陽極側に泳動するバンド(成分a)は，より陰極側に泳動するバンド(成分b)より濃く染色された．TfO変異型の成分a及びbは，二重免疫拡散法により免疫学的に互いに区別できなかった．それぞれの変異型の成分a/成分b比を測定した結果，変異型は3.4~4.0の範囲の比較的高い成分比を有する変異型D, F, Hのグループと1.8~2.3の比較的低い成分比を有する変異型O, Rのグループに分類された．以上の結果及びすでに報告されている成分a及びbの糖質組成のデータから，これら2つのグループの間には糖鎖形成の相違があると考えられる．