Edward T. Blake,[1] *D.Crim.*

Progressive Desialidation of Human Transferrin

REFERENCE: Blake, E. T., **"Progressive Desialidation of Human Transferrin,"** *Journal of Forensic Sciences*, JFSCA, Vol. 32, No. 5, Sept. 1987, pp. 1342-1347.

ABSTRACT: Transferrin is a serum glycoprotein which contains four sialic acid residues located at the end of two branched carbohydrate structures. The presence of these four acidic residues influences the electrophoretic mobility of the transferrin molecule. Alterations in the electrophoretic mobility of transferrin may be encountered in forensic science case work, particularly in association with postmortem samples. These altered transferrins usually appear in a highly stylized "ladder" banding pattern. To determine whether these altered transferrins are the result of sialic acid removal, serum samples of known transferrin type were treated with neuraminidase. These experiments support the hypothesis that the "ladder" banding pattern of transferrin observed in some case samples is due to the removal of sialic acid residues by bacterial or endogenous neuraminidase. These studies also demonstrate that partially desialidated transferrin variants cannot be clearly typed until the sialic acid is completely stripped from the transferrin molecule. Reliable typing of partially desialidated samples can be accomplished by treating these samples with neuraminidase.

KEYWORDS: forensic science, genetic typing, transferrin, sialic acid

Genetic variation of human transferrin (Tf) has been known for over 20 years; during that time more than 18 transferrin variants have been described [*1*]. The most common transferrin variant phenotype, CD, occurs in approximately 10% of the black population; genetic variation of transferrin that is detectable by conventional electrophoretic procedures occurs in white populations with considerably less frequency. The limited genetic variation of transferrin in the general population has undoubtedly retarded the use of this protein as a genetic marker for the individualization of blood. More recently, it has been recognized that transferrin can be typed concurrently with the group specific component (Gc) proteins [*2*]; thus, information about the transferrin type of a sample can be obtained without additional analytical effort or consumption of sample. This development, in turn, has stimulated interest in the stability and survival of transferrin in forensic science case samples.

Since the levels of transferrin in normal blood are three to four times greater than the Gc

This work was supported, in part, by Grant 83-IJ-CX-0050 from the National Institute of Law Enforcement and Criminal Justice. Points of view or opinions expressed in this document are those of the author and do not necessarily represent the official position or policies of the Department of Justice. This paper has been presented at the combined meeting of the Interamerican Congress of Forensic Sciences and the California Association of Criminalists, Sacramento, CA, 2 Nov. 1982; the American Academy of Forensic Sciences, Cincinnati, OH, 15-19 Feb. 1983; and the joint meeting of the Canadian Society of Forensic Science and the Northwest Association of Forensic Scientists, Vancouver, B.C., Aug. 1983. Received for publication 5 Sept. 1986; revised manuscript received 29 Nov. 1986; accepted for publication 2 Dec. 1986.

[1]Forensic Science Associates, Emeryville, CA.

proteins [3], transferrin typing is frequently successful when Gc typing is not successful. Unfortunately, "peculiar" transferrin electrophoretic patterns are occasionally observed in older liquid reference samples such as autopsy specimens and in bloodstains found in "dirty" environments which might be expected to promote bacterial growth. The peculiar transferrin patterns usually take the form of a series of isomorphs that are cathodal to the normal transferrin band(s).

It is known that the common transferrin type C contains four sialic acid residues at the end of two branched carbohydrate structures [4]. It has been demonstrated that these four acid residues have a dramatic influence on the electrophoretic mobility of the transferrin molecule [5]. For example, the sequential removal of sialic acid from transferrin gives rise to five possible charge states where the most anodal isomorph contains four sialic acid residues and the most cathodal isomorph contains no sialic acid residues [5]. For each sialic acid residue that is removed, an additional cathodal isomorph should appear, revealing a cathodal "ladder" pattern in a partially desialidated sample. Since this is the type of phenomenon that is observed in the case samples with peculiar Tf patterns, it is possible that the phenomenon is caused by bacterial or endogenous neuraminidase activity.

In this report, evidence is presented which supports the hypothesis that the ladder banding pattern of transferrin observed in some case samples is due to the action of neuraminidase. These studies demonstrate that partially desialidated transferrin variants cannot be typed clearly until the sialic acid is completely removed from the transferrin molecule.

Methods and Materials

Electrophoresis was conducted in 1% agarose gels (Sigma, type V) using a Tris/glycine buffer system (Group III) [6]. The buffer contained 0.29*M* glycine and 0.037*M* Tris base (tris[hydroxymethyl]aminomethane); the buffer pH is about 8.2 and is not further adjusted. The same buffer is used for the tank and gel. The gels were poured onto the hydrophilic surface of Gelbond (Marine Colloids) and were 20 cm in length and 1 to 2 mm thick. Electrophoresis was conducted for approximately 3 h at 20 V/cm on the surface of cooling plates at 3 to 4°C. Samples were inserted into the gel on thin strips of cellulose acetate membrane; more dilute samples can be pipetted into slots cut in the gel with a slot cutter. Under these conditions the transferrin C isomorph comigrates with hemoglobin A; hemoglobin is added to one sample slot as a reference marker.

Transferrin was detected using the conventional immunofixation procedure [7]. Antihuman transferrin (Atlantic Antibodies) was diluted with isoTris (0.14*M* NaCl, 10m*M* Tris, pH 7.4, one part antisera to four parts isoTris) and soaked into cellulose acetate membranes. The membrane was then placed on the surface of the gel in the transferrin region and allowed to incubate for at least 3 to 4 h. Following immunoprecipitation, the membrane was removed and the gel was pressed between several thicknesses of blotter paper and an appropriate weight for 20 to 30 min and then washed overnight in isoTris. The gel was pressed again and then washed in water for 10 min to remove salt, then pressed again, and dried in a 50°C circulating oven. The dried gel was stained with 0.2% Coomassie Brilliant Blue in methanol (5): acetic acid (1): water (5) and destained in the same solvent. Weak samples can be stained subsequently with a silver stain [8].

Serum samples were treated with neuraminidase in the following manner. Neuraminidase (Sigma, type VI, *C. perfringens*) was obtained as a lyophilized powder. This preparation was dissolved in a minimum amount of water and suspended in a known volume of 3.2*M* ammonium sulfate such that the enzyme activity per unit volume could be calculated from the supplier's specifications. To one volume of serum was added 1/2 volume of 0.1*M* acetate buffer, pH 5.5, and a measured amount of neuraminidase; sample specimens contained either 0.003 or 0.03 units of neuraminidase/mL. The samples were allowed to incubate at 37°C and aliquots were removed periodically and frozen before electrophoretic analysis.

Results and Discussion

The progressive desialidation of the common transferrin C phenotype is illustrated in Fig. 1. It can be seen that transferrin moves through five different charge states as predicted by the sialic acid content of the protein. In normal serum there is one primary isomorph where the protein contains four sialic acid residues on all molecules. In the completely desialidated sample there is again a single primary isomorph where sialic acid is absent on all molecules. In the various intermediate stages of desialidation there are multiple isomorphs indicative of a discrete number of charge states. As desialidation progresses, the ladder pattern gradually shifts to the more desialidated forms.

The progressive desialidation of the transferrin CD phenotype is illustrated in Fig. 2. It is apparent from the pattern of desialidation that the charge difference between the C and D isomorphs is the same as the charge difference between the native C isomorph and the C isomorph with one sialic acid residue removed. Thus, the D4 isomorph has the same mobility as the C3 isomorph. In a similar way the D3 isomorph has the same mobility as the C2 isomorph; and this relationship continues through the ladder banding pattern until all of the sialic acid is removed. As a result, genetic typing of partially desialidated samples is not possible. This situation is illustrated in Fig. 3 where partially desialidated transferrin C and CD phenotypes are compared directly. While this comparison shows that partially desialidated samples cannot be reliably typed, it also demonstrates that the phenotypic patterns are clarified when all of the sialic acid is removed.

A similar pattern of desialidation exists for the transferrin CB phenotype. The transferrin C isomorph has the same electrophoretic mobility as the B isomorph with one sialic acid residue removed; and, this relationship continues through the ladder banding pattern until all sialic acid residues are removed. Just as the C and CD phenotypes cannot be distin-

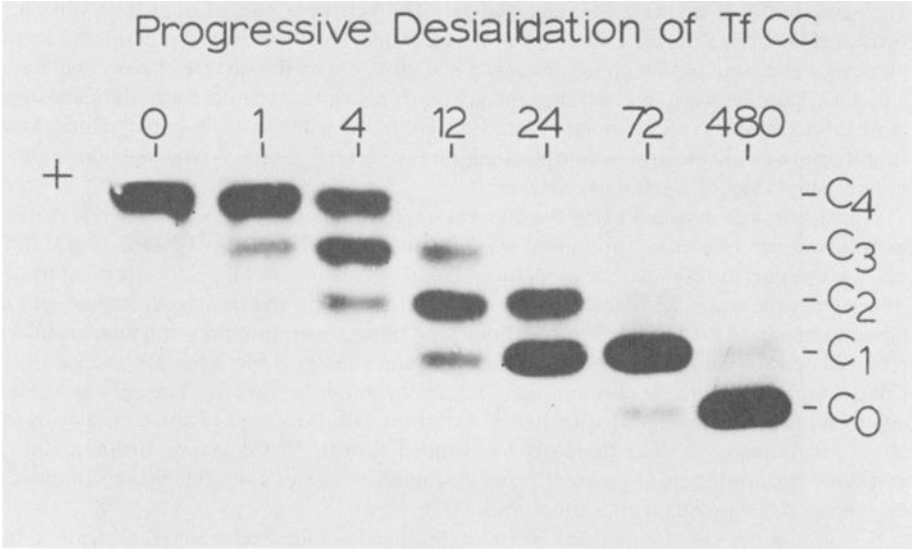

FIG. 1—*The progressive desialidation of Tf type C. The numbers associated with each sample are a relative indication of the length of incubation and amount of neuraminidase. For numbers up to 96, the number indicates the hours the sample was incubated with neuraminidase at 0.003 IU/mL. For numbers greater than 96 the sample was incubated for one tenth the amount of time indicated by the number with 10 times the amount of neuraminidase (0.03 IU/mL). The numbers along the right margin indicate the sialic acid residues associated with each charge state.*

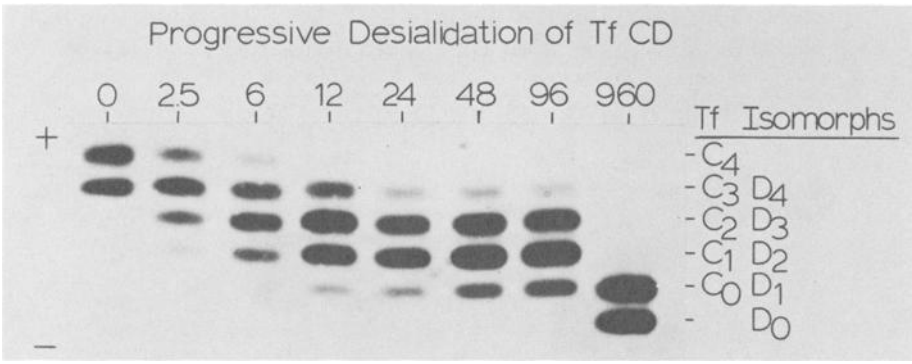

FIG. 2—*The progressive desialidation of Tf type CD. See text for discussion.*

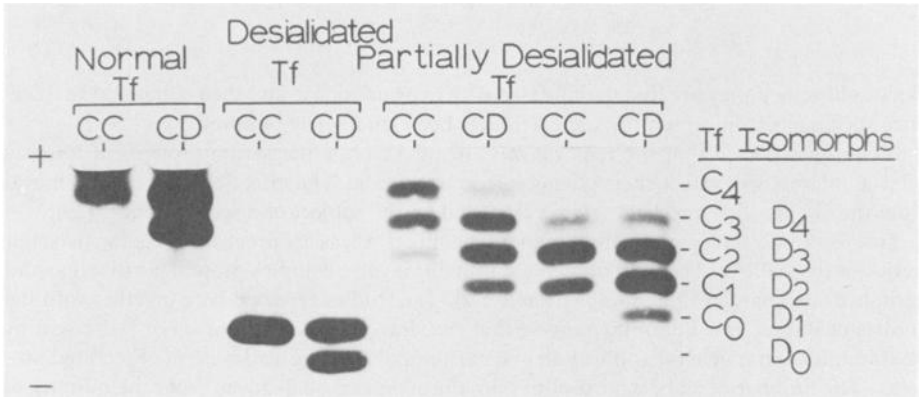

FIG. 3—*Comparison of partially desialidated Tf C and Tf CD. The partially desialidated samples cannot be clearly typed until the sialic acid is completely stripped from the transferrin molecules.*

guished in partially desialidated samples, the C and CB phenotypes cannot be readily distinguished once desialidation has commenced (Fig. 4). After the samples have been completely desialidated, typing again becomes possible.

These studies provide support for the hypothesis that the ladder banding pattern observed in the transferrin analysis of some case samples is the result of a desialidation reaction. The cause of the desialidation may not always be clear. In postmortem specimens endogenous neuraminidase may result from tissue leakage [*9*]. The presence of tissue enzyme in postmortem samples is illustrated by the high levels of PGM activity which are routinely observed in autopsy specimens as the result of enzyme leakage from dead or dying muscle and organ cells. Neuraminidase activity may also originate from bacteria that are adventitiously cultured in liquid blood specimens or in specimens that dry very slowly. Since bacteria will not grow in dried specimens, one can predict that the desialidation of transferrin and other serum glycoproteins will be a greater problem in humid warm environments than in dry ones.

While the desialidation of transferrin is a potential source of typing error, recognition of the highly characteristic isomorph pattern that is produced should act as a warning or diagnostic indicator that this phenomenon is operative. Problem samples can be reliably typed by

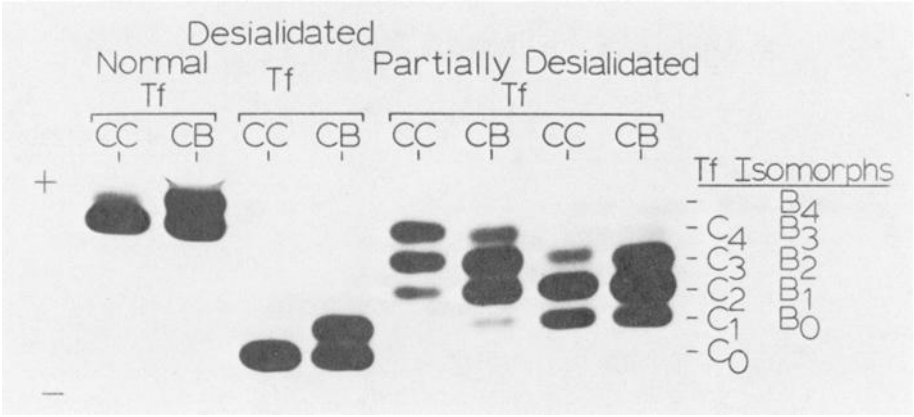

FIG. 4—*Comparison of partially desialidated Tf C and Tf CB. The partially desialidated samples cannot be clearly typed until the sialic acid is completely stripped from the transferrin molecules.*

electrophoresis if they are first desialidated with neuraminidase and then compared to reference specimens from which the sialic acid has been completely removed.

It can be expected that the removal of sialic acid from other serum proteins of forensic science interest will affect their phenotypic presentation. The effect of sialic acid removal from the Gc proteins and haptoglobin (Hp) will be the subject of a second report [*10*].

This work may have significance in another context. Classical procedures for the determination of the sialic acid content of secreted proteins involve complex protein purification and carbohydrate analyses (for example, see Ref *4*). The studies reported here together with the studies of Parker and Bearn [*5*] suggest that the slow progressive removal of sialic acid by neuraminidase has general application in determining the sialic acid content of secreted proteins. The number of sialic acid residues on a protein can be deduced from the number of charge states produced during sialic acid removal where the number of sialic acid residues is equal to the number of charge states minus 1. This procedure would require that the protein of interest be relatively homogenous in charge in its native state.

Furthermore, while it has been demonstrated that all genetic variants of human transferrin that have been studied contain four sialic acid residues, the transferrin of several primates, including chimpanzee, rhesus, and long-tailed macaque, contains two sialic acid residues [*5*]. Thus, progressive desialidation of homologous proteins may be useful in distinguishing proteins of closely related species. Such a procedure would require only antibodies or a histochemical stain for the protein of interest.

Acknowledgment

The transferrin CB sample used in these studies was a gift from Fran Gdowski and Gayle McCormack of the New Jersey State Police Laboratory, West Trenton, New Jersey.

References

[*1*] Giblett, E. R., "The Plasma Transferrins," in *Progress in Medical Genetics*, Vol. II, Arthur G. Steinberg and Alexander G. Bearn, eds., 1962, pp. 34-63.
[*2*] Blake, E. T., "Concurrent Typing of Gc and Tf on Agarose Gels," presented to the Fifty-Fourth Semiannual Seminar of the California Association of Criminalists, Oct. 1979.
[*3*] Giblett, E. R., *Genetic Markers in Human Blood.* F. A. Davis, Philadelphia, 1969.
[*4*] Jamieson, G. A., Jett, M., and DeBernardo, S. L., "The Carbohydrate Sequence of the Glycopep-

tide Chains of Human Transferrin," *The Journal of Biological Chemistry*, Vol. 246, No. 11, June 1971, pp. 3686-3693.

[5] Parker, W. C. and Bearn, A. G., "Studies on the Transferrins of Adult Serum, Cord Serum, and Cerebrospinal Fluid," *The Journal of Experimental Medicine*, Vol. 115, 1962, pp. 83-105.

[6] Wraxall, B., et al., "Final Report: Bloodstain Analysis System," The Aerospace Corporation, Washington, DC, July 1978.

[7] Alper, C. A. and Johnson, A. M., "Immunofixation Electrophoresis: A Technique for the Study of Protein Polymorphism," *Vox Sanguinis*, Vol. 17, 1969, pp. 445-452.

[8] Dykes, D. D., Miller, S. A., and Polesky, H. F., "Transferrin and Plasminogen Phenotyping of Aging Blood Stains Using Isoelectric Focusing and Silver Stain," *Electrophoresis*, Vol. 6, No. 2, 1985, pp. 90-93.

[9] Tallman, J. F. and Brady, R. O., "The Purification and Properties of a Mammalian Neuraminidase (Sialidase)," *Biochimica et Biophysica Acta*, Vol. 293, 1973, pp. 434-443.

[10] Blake, E. T., "Transferrin: A Potential Diagnostic Indicator of Desialidation Reactions in Blood," presented at the combined Meeting of the Interamerican Congress of Forensic Sciences and the California Association of Criminalists, Sacramento, CA, 2 Nov. 1982, and the American Academy of Forensic Sciences, Cincinnati, OH, 15-19 Feb. 1983, in preparation for publication.

Address requests for reprints or additional information to
Edward T. Blake
Forensic Science Associates
1400 53rd St.
Emeryville, CA 94608