Jpn. J. Human Genet. **29**, 415–419, 1984

AN IMMUNOBLOTTING TECHNIQUE FOR COMPLEMENT C6 TYPING: THREE NEW VARIANTS

Katsushi TOKUNAGA, Noriko YAMAMURA, and Keiichi OMOTO

Department of Anthropology, Faculty of Science, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

Summary An immunoblotting method for C6 typing was developed. After isoelectric focusing in thin-layer polyacrylamide gel, C6 proteins were transferred passively to nitrocellulose and then detected by a two-step enzyme immunoassay. A population sample of northeastern Japanese was investigated using this method. Three common and four rare allotypes were observed. The allele frequencies estimated from 495 blood donors were as follows: *C6*A* 0.423, *C6*B* 0.510, *C6*B2* 0.062, and rare alleles (*91*, *M11*, *B4*, and *B5*) 0.005. Three variants, *91*, *M11*, and *B5*, were considered to be newly found. This method could be applicable to many other protein systems.

INTRODUCTION

Genetic polymorphism of the sixth component of human complement (C6) was first described by Hobart *et al.* (1975). The polymorphism has been shown to be controlled by codominant alleles at a single autosomal locus. Two predominant alleles, *C6*A* and *C6*B*, and many rare variants have been described so far (Hobart and Lachmann, 1976; Mauff *et al.*, 1980; Ranford *et al.*, 1982; Tokunaga *et al.*, 1983). The reason why such a number of variants occur at C6 locus is still unknown. The C6 system should become a valuable marker in the fields of human population genetics and forensic medicine, because of a high degree of polymorphism and a marked variation in allele frequencies among populations.

In the conventional procedure for C6 typing, C6 bands were visualized by a specific hemolytic overlay containing sensitized sheep erythrocytes and C6 deficient serum. Recently, Whitehouse and Putt (1983) described a new detection method for C6 which involves an electrophoretic transfer of proteins from an isoelectric focusing gel to a nitrocellulose sheet and an enzyme immunoassay.

In the present study, we developed an alternative immunoblotting method with a passive transfer and applied the procedure to a population study in northeastern Japanese.

Received August 23, 1984

MATERIALS AND METHODS

Population material. ACD-plasma were obtained from 495 healthy blood donors living in Iwate Prefecture, northeastern part of Japan, and stored at −30°C for one year until used.

Isoelectric focusing. Isoelectric focusing in thin-layer polyacrylamide gel (pH 5–8) was performed as described previously (Tokunaga *et al.*, 1980) except that the thickness of the gel was 0.5 mm. Plasma samples of approximately 10 μl were applied with rectangular pieces of filter paper on the gel near the anode.

Transfer to nitrocellulose. The polyacrylamide gel was overlayed with 1% agarose gel (thickness 1 mm) in phosphate buffered saline at pH 7.2 (PBS) in order to avoid sticking of nitrocellulose on the polyacrylamide gel. Then a nitrocellulose sheet (Schleicher & Schuell, West Germany), two sheets of filter paper (Whatman, 3MM, England), paper towels, a glass plate, and finally a 2 kg weight were placed on the gel.

Immunochemical detection. After one hour, the nitrocellulose sheet was immersed in 3% bovine serum albumin in PBS (BSA-PBS) overnight at 4°C to saturate remaining protein binding sites. The sheet was washed with 3 changes of PBS and incubated with goat anti-human C6 antiserum (Cappel, USA) diluted 1/300 in BSA-PBS for 30 min at 37°C. After five washes with PBS, the blot was incubated with peroxidase-conjugated anti-goat immunoglobulins (Dako, Denmark) diluted 1/800 in BSA-PBS for 30 min at 37°C. The nitrocellulose sheet was then washed with 5 changes of PBS and incubated with a substrate solution of *o*-dianisidine/H_2O_2/phosphate buffer (pH 7.3) for 30 min at room temperature. The sheet was washed with distilled water, dried, and stored in the dark.

RESULTS

The immunoblotting procedure on nitrocellulose described here gave the same C6 patterns as those obtained by hemolytic overlay method (Fig. 1). Five common and five rare phenotypes were observed, in which seven allotypes were distinguishable. Among these allotypes, four were identified as *A*, *B*, *B2*, and *B4*, respectively, by direct comparisons with our reference samples (Tokunaga *et al.*, 1983).

The remaining three allotypes were considered to be different from any variants so far reported and designated based on the terminology proposed by Mauff *et al.* (1980). A basic type was designated *B5*, because it showed still more basic bands than *B4* bands. By careful comparisons, two 'intermediate' types between *A* and *B* were confirmed to be different from other 'intermediate' types described previously (Fig. 2, a and b). The intermediate type between *M1* and *M2* was designated *M11*, and that very close to *A* was designated *91* on the suggestion by Dr. G. Mauff, Köln.

The distribution of C6 phenotype and allele frequencies in 495 healthy donors

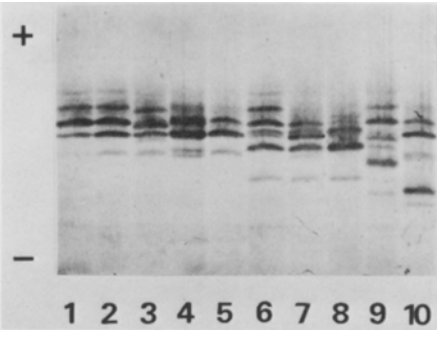

Fig. 1. Photograph showing C6 phenotypes obtained by polyacrylamide gel isoelectric focusing and immunoblotting. (1) C6 A, (2) C6 AB, (3) C6 91B, (4) C6 M11B, (5) C6 B, (6) C6 AB2, (7) C6 BB2, (8) C6 B2, (9) C6 AB4, (10) C6 BB5.

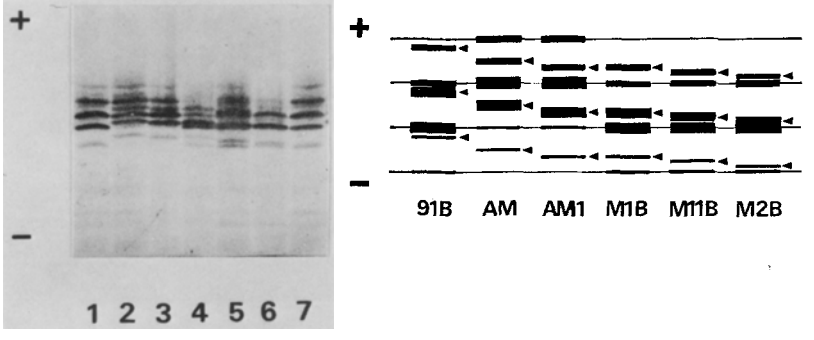

Fig. 2a. Photograph showing intermediate types between C6 A and C6 B. (1) and (7) C6 AB, (2) C6 AM (provided by Dr. G. Mauff, Köln), (3) C6 AM1, (4) C6 M1B, (5) C6 M11B, (6) C6 M2B.

Fig. 2b. Diagram for intermediate types between C6 A and C6 B. Wedges indicate the variant bands.

Table 1. Distribution of C6 phenotype and allele frequencies.

Phenotypes	Obs. No.	(%)	Exp. No.	Allele frequencies
A	86	(17.4)	88.67	
AB	227	(45.9)	213.73	*C6*A*: 0.423±0.016
B	117	(23.6)	128.80	
AB2	19	(3.8)	25.82	*C6*B*: 0.510±0.016
BB2	40	(8.1)	31.12	
B2	1	(0.2)	1.88	*C6*B2*: 0.062±0.008
AR*	1	(0.2)	2.12	
BR*	4	(0.8)	2.55	*C6*R*: 0.005±0.002
B2R	0	(0)	0.31	
R	0	(0)	0.01	
Total	495	(100.0)	495.01	1.000

(χ^2=8.469, 6d.f., 0.2<p<0.3) * Incidence of rare phenotypes: 91B 2, M11B 1, AB4 1, BB5 1.

from a northeastern area of Japan (Iwate Pref.) is demonstrated in Table 1. The allele frequencies for *C6*A*, *C6*B*, *C6*B2*, and rare variants were estimated to be 0.423, 0.510, 0.062, and 0.005, respectively. The deviation of the observed numbers of phenotypes from those expected on the Hardy-Weinberg equilibrium was statistically non-significant ($\chi^2=8.469$, 6d.f., $0.2<p<0.3$).

DISCUSSION

We previously investigated genetic polymorphism of C6 using a hemolytic overlay technique (Tokunaga *et al.*, 1983). In the present study, we used an immunoblotting procedure on nitrocellulose for the detection of C6 bands. The procedure has some advantages, as follows. (1) No C6 deficient serum and sensitized erythrocytes are required. (2) Immunoblotting gave more reliable results. In the hemolytic assay, the strength of bands is much influenced by the qualities of sensitized erythrocytes. (3) Only a small amount of antiserum is needed. Usually we use 20 μl of first antiserum and 7 μl of second antiserum for each nitrocellulose sheet. (4) The passive transfer described here is simple and easy to handle. (5) Because the diffusion of protein bands hardly occurs on nitrocellulose, the fine resolution of polyacrylamide gel isoelectric focusing is successfully applicable. (6) And finally, the procedure can potentially be applied to the investigations of many other protein systems.

Table 2 demonstrates the C6 allele frequencies of three Japanese populations so far studied. We previously reported in the Tokyo population that *C6*B* is commoner than *C6*A*, contrary to many other populations (Hobart and Lachmann, 1976; Kühnl and Kreckel, 1980; Kunstmann *et al.*, 1980; Olving *et al.*, 1980; Ranford *et al.*, 1982), and that *C6*B2* was the third common allele in Japanese (Tokunaga *et al.*, 1983). These have been confirmed by the surveys on western Japanese (Nishimukai *et al.*, 1985) and northeastern Japanese (present study). Moreover, six rare variants (*A3*, *A21*, *M1*, *M2*, *B3*, and *B4*) were observed in the Tokyo population and four (*91*, *M11*, *B4*, and *B5*) in the present study. *B3* was reported also in the western population. Although inheritance has been confirmed only for *A3* and *M1*, the other rare types are also very likely to be controlled by codominant

Table 2. Distribution of C6 allele frequencies in three Japanese populations.

Populations	N	Alleles				Authors
		A	B	B2	Others	
Iwate	495	.423	.510	.062	.005 (*91*, *M11*, *B4*, *B5*)	Present study
Tokyo	288	.427	.483	.076	.014 (*A3*, *A21*, *M1*, *M2*, *M3*, *B4*)	Tokunaga *et al.*, 1983
Western Japan	135	.467	.481	.037	.015 (*B3*)	Nishimukai *et al.*, 1985

alleles for the reasons discussed previously (Tokunaga *et al.*, 1983). Our *B3* was confirmed to be indistinguishable from the *B3* in Whitehouse and Putt (1983) by the International Reference Laboratory (Dr. G. Mauff, Köln). Consequently three common and nine rare variants have been observed in Japanese, of which seven (*A21*, *91*, *M1*, *M11*, *M2*, *B4*, and *B5*) were newly described.

The frequencies of three common alleles (*A*, *B* and *B2*) in the Iwate population are close to those in the Tokyo population. On the other hand, there seem to be some differences in the frequencies of *A*, *B2*, and *B3* between the western population and the other two populations, although the differences are statistically non-significant because of the relatively small sample size of the western Japanese.

Acknowledgements We are grateful to Prof. S. Katsura, Iwate Medical University, and Dr. S. Shoji, Red Cross Blood Center, Iwate Prefecture, for making the blood samples available to the present study. We also wish to thank Prof. G. Mauff, Universität Köln, for reference samples and valuable discussion. This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

Hobart, M.J., Lachmann, P.J., and Alper, C.A. 1975. Polymorphism of human C6. In *Protides of the Biological Fluids*, Peeters, H., ed., 22nd Colloquim Brugge 1974, Pergamon Press, Oxford, pp. 575–580.

Hobart, M.J. and Lachmann, P.J. 1976. Allotypes of complement components in man. *Transplant. Rev.* **32**: 26–42.

Kühnl, P. and Kreckel, P. 1980. C6 phenotypes and allele frequencies in a German population. *Immunobiology* **158**: 50–54.

Kunstmann, G., Mauff, G., and Pulverer, G. 1980. C6 polymorphism and rare alleles in Western Germany. *Immunobiology* **158**: 55–59.

Mauff, G., Alper, Ch. A., Hobart, M., Kühnl, P., Kunstmann, G., Meo, T., Olving, J.H., and Rittner, Ch. 1980. Statement on the nomenclature of human C6 polymorphism. *Immunobiology* **158**: 139–143.

Nishimukai, H., Kitamura, H., Kishida, T., and Tamaki, Y. 1985. C6 polymorphism in Japanese: Typing by agarose gel isoelectric focusing-immunofixation. *Hum. Hered.* **35**: 30–33.

Olving, J.H., Teisberg, P., and Olaisen, B. 1980. Polymorphism of the sixth component of complement (C6) in Norwegian Lapps. *Hum. Hered.* **30**: 211–214.

Ranford, P.R., Kirk, R.L., and Zimmet, P. 1982. Distribution of complement factors BF, C2 and C6 in the Western Pacific. *Acta Anthropogenetica* **6**: 23–32.

Tokunaga, K., Omoto, K., Araki, C., and Juji, T. 1980. Genetic polymorphism of the second component of human complement (C2) in Japanese. *Jpn. J. Human Genet.* **25**: 287–293.

Tokunaga, K., Yukiyama, Y., and Omoto, K. 1983. Polymorphism of the complement component C6 in Japanese. *J. Immunogenet.* **10**: 419–424.

Whitehouse, D.B. and Putt, W. 1983. Immunological detection of the sixth complement component (C6) following flat bed polyacrylamide gel isoelectric focusing and electrophoretic transfer to nitrocellulose filters. *Ann. Hum. Genet.* **47**: 1–8.