TECHNICAL NOTE

Rieko Uchihi,[1] *M.S.; Keiji Tamaki,*[1] *M.D.;*
Toshinori Kojima,[1] *B.S.; Toshimichi Yamamoto,*[1] *M.S.; and*
Yoshinao Katsumata,[1] *M.D.*

Deoxyribonucleic Acid (DNA) Typing of Human Leukocyte Antigen (HLA)-DQA1 from Single Hairs in Japanese

REFERENCE: Uchihi, R., Tamaki, K., Kojima, T., Yamamoto, T., and Katsumata, Y., "**Deoxyribonucleic Acid (DNA) Typing of Human Leukocyte Antigen (HLA)-DQA1 from Single Hairs in Japanese,**" *Journal of Forensic Sciences*, JFSCA, Vol. 37, No. 3, May 1992, pp. 853–859.

ABSTRACT: The deoxyribonucleic acid (DNA) typing of human leukocyte antigen (HLA)-DQA1 from single hairs is described. HLA-DQA1 genotypes could be determined from single plucked hair roots. However, it was not easy to type HLA-DQA1 with hair shaft portions. Increase in the specimens of hair shaft portions (over 10 cm in length) to get sufficient DNA caused inhibition of polymerase chain reaction (PCR). Synthetic melanin as well as the one extracted from hairs inhibited the PCR of the genomic DNA template when added to the PCR reaction at the concentrations over than 15 ng/100 μL. Therefore, typability of hair shaft portions seems to depend on the delicate balance of the concentrations of DNA and the contaminated melanin in the final DNA extracts.

KEYWORDS: pathology and biology, deoxyribonucleic acid, genetic typing, DNA polymorphism, HLA-DQA1 locus, polymerase chain reaction

The discrimination of human hairs is one of the most important aims of forensic science. Sometimes a single hair is the only remnant at the scene of a crime. However, since forensic science examination of human hair has been dominated by morphological methods and a few serological blood grouping tests, it has thus far been impossible to identify an individual from a human hair sample.

Recent advances in recombinant deoxyribonucleic acid (DNA) technique using variable number of tandem repeat (VNTR) probes or minisatellite probes have changed current forensic procedures and brought about new applications in forensic science practice. These include positive individual identification [*1–5*] and paternity testing [*5–8*], for example. In order to obtain accurate data in such investigations, high molecular weight and relatively large amounts of DNA must be used. This inevitable condition precludes the analysis of forensic science samples, such as an aged small bloodstain or single hairs, by these techniques.

In 1985, Saiki et al. reported that polymerase chain reaction (PCR) procedure allowed the enzymatic amplification of a specific segment of human genomic DNA in vitro [*9*]. The PCR procedure has significantly increased our ability to detect genetic variation,

[1]Assistant professors, research students, and professor, respectively, Department of Legal Medicine, Nagoya University School of Medicine, Nagoya, Japan.

and has been applied to forensic identification. Higuchi et al. [*10*] reported the DNA typing of single hairs by amplifying a polymorphic region of the human leukocyte antigen (HLA)-DQα locus and a genetically variable region of mitochondrial DNA. Westwood et al. also reported successful typing of HLA-DQα from single hairs [*11*]. However, they used the root portion of single hairs with sheath for the typing, and there have been no reports concerning HLA typing from the shaft portion of hairs.

We recently reported DNA typing of HLA-DQA1 from blood samples using nonradioisotopic probes and gene frequencies of HLA-DQA1 alleles in the Japanese population [*12*]. In the present study, we attempted to apply this technique to HLA-DQA1 typing of single hairs, especially from the shaft portion.

Materials and Methods

Extraction of DNA from Single Hairs and Fresh Blood

Freshly plucked hair samples were obtained from 12 healthy Japanese. They were black, straight, without sign of aging, 72 to 127 μm in diameter, and round in cross section. Hairs were separated into root portions (0.5 cm in length from the root end) and shaft portions. DNA was extracted according to the protocol described by Higuchi et al. [*10*] with minor modifications. Shaft portions (10 cm in length) cut into ten pieces or root portions were rinsed in distilled water followed by absolute ethanol. After drying completely, they were digested in 0.4 mL of a solution containing 0.01*M* Tris-hydrochloric acid (HCl) (pH 8.0), 0.005*M* ethylenediaminetetraacetate (EDTA) (pH 8.0), 0.1*M* sodium chloride (NaCl), 2% (w/v) sodium dodecylsulfate (SDS), 0.039*M* dithiothreitol (DTT) and 200-μg/mL proteinase K (Sigma Chemical Company, St. Louis, MO). The final suspension was incubated overnight at 56°C. DNA in the specimens was extracted twice with phenol saturated by TE buffer (0.01*M* Tris-HCl [pH 8.0] and 0.001*M* EDTA [pH 8.0]) and once with chloroform. The aqueous phase was desalted by a Centricon 30 ultrafiltration device (Amicon, Grace Japan K.K., Tokyo, Japan) using TE buffer and recovered in less than 30 μL. DNA was extracted from fresh blood of the same individuals as described previously [*13*]. The concentration of DNA was determined fluorometrically using bisbenzimidazole (Hoechst 33258) with TKO 100 Dedicated Mini Fluorometer (Hoefer Scientific Instruments, San Francisco, CA) as described by Brunk et al. [*14*] and Labarca and Paigen [*15*].

PCR Amplification of HLA-DQA1 Gene

The specific region of HLA-DQA1 gene (242 bp) was amplified by PCR using the primers, GH26 and GH27 [*16*]. Twenty pmols of each primer and 2.5 units of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) were added to PCR buffer (100 μL) containing 10m*M* Tris-HCl (pH 8.4), 50m*M* potassium chloride (KCl), 1.5m*M* magnesium chloride ($MgCl_2$), gelatin (100 μg/mL), 0.02% (v/v) NP-40 (Sigma), and 200 μ*M* each of the four deoxyribonucleoside 5′-triphosphates (dATP, dCTP, dGTP, and TTP). Thirty-five cycles of PCR were carried out using an automated device (DNA Thermal Cycler, Perkin-Elmer Cetus), and each cycle was performed for 2 min at 95, 55, and 72°C. After the last cycle, the samples were incubated for an additional 5 min at 72°C. The amplified DNA was checked using a gel of 3% (w/v) NuSieve (FMC Corp., Rockland, ME) stained with ethidium bromide.

Determination of HLA-DQA1 Genotype

Determination of the HLA-DQA1 genotype was performed according to the method described previously [*12*] as follows: After amplification, the PCR product was dot-blotted

onto nylon membranes (Hybond N^+, Amersham International plc., Amersham, United Kingdom). Nonradioactive hybridization method (Boehringer Mannheim, Mannheim, Germany) with digoxigenin-tailed sequence specific oligonucleotide (SSO) probes [*17*] was used, and samples were typed as a combination of blue spots after exposure to enzyme substrates.

Measurement of Melanin in Hair Shaft

The melanin content in a hair shaft was assayed colorimetrically as described by Whittaker [*18*] with slight modifications as follows. A hair shaft (10 cm in length) was digested in the solution used for the extraction of DNA. The suspension was centrifuged followed by two washes with ether-ethanol (1:3) and one wash with absolute ether. The dried residue was digested in 1.0 mL of 0.85*N* potassium hydroxide (KOH) heated to 100°C for 10 min. The optical density was measured at 400 nm with a Hitachi 557 spectrophotometer. Pure synthetic melanin (Sigma) was used as a quantitative standard.

In some experiments, DNA was extracted from 0.5 g of hairs containing 7660 μg of melanin, and the final extract (150 μL) was obtained. The absorption spectrum of the extract was compared with synthetic melanin and human genomic DNA. Part of the extract was digested by adding excess volume of 0.85*N* KOH, and the melanin amount was determined as well.

Results and Discussion

Figure 1 shows the results of electrophoresis of the PCR amplification products of HLA-DQA1 gene from various specimens. The specific region (242 bp) was clearly observed in PCR products using DNA prepared from fresh blood (100 ng of DNA, equivalent to 5 μL whole blood) or half of a plucked hair root (80 ng of DNA). A heterozygous type as DQA1* 0103/DQA1* 0301 was determined completely from these

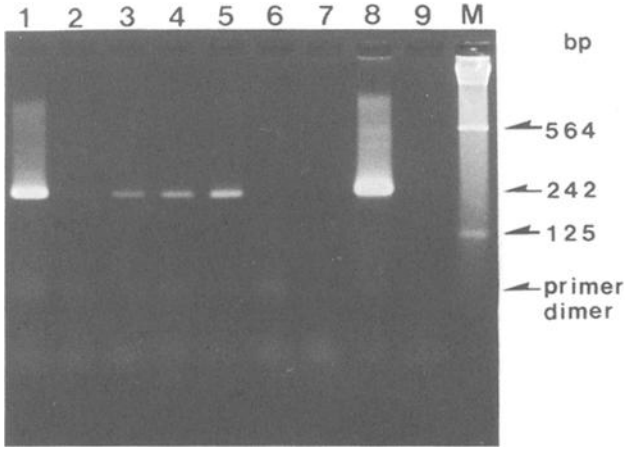

FIG. 1—*Amplification of HLA-DQA1 sequences from DNA samples prepared from half of a freshly plucked hair root (lane 1), hair shaft (lanes 2 to 7), and fresh blood (100 ng of DNA, lane 8). Amounts of DNA template from hair shaft are equivalent to DNA prepared from hair shaft of 1, 2, 5, 10, 20, and 50 cm in length (lanes 2 to 7, respectively). As a negative control, only TE was added to the PCR system (lane 9). Five microliters of each sample was electrophoresed on a 3% (w/v) NuSieve gel (FMC), and visualized by staining with ethidium bromide. M: λ DNA cleaved by HindIII.*

PCR products. In DNA samples prepared from hair shaft portion, PCR products seemed to increase gradually with an increase of samples up to 10 cm in length. However, no PCR product was observed in samples from 20 and 50 cm in length. Moreover, even "primer-dimer," which is an amplification artifact often observed in PCR product when many cycles of amplification were performed on a sample containing very few initial copies of template [*19*], was not shown in the sample from 50 cm (lane 7). These post-PCR samples from the hair shaft portion gave very weak signals in HLA typing, and the correct typing was difficult.

The DNA extract (150 μL) from 0.5-g hairs contained 10.1-μg melanin, the recovery of which was 0.13%. This extract showed a broad absorption band having a small shoulder at near 260 nm, and the absorbance sharply increased with the decrease in the wavelength from 230 nm (Fig. 2*a*). The synthetic melanin gave essentially the same spectrum between 500 and 230 nm, although it did not show any shoulder at near 260 nm (Fig. 2*b*). DNA showed the peak at 260 nm, and the absorbance sharply increased with the wavelength from 225 nm (Fig. 2*c*). Thus, the spectrum of the DNA extract appears to contain melanin in addition to DNA and some other substrates having absorbance at wavelengths lower than 230 nm.

Figure 3 shows the changes in the amount of PCR product of HLA-DQA1 gene from DNA template (100 ng) prepared from fresh blood in a 100 μL reaction system when various amounts of synthetic melanin (Fig. 3*a*) or hair shaft extract (Fig. 3*b*) were added to the system. PCR product decreased gradually with an increase of hair shaft extract or synthetic melanin. In the present experiment, more than 15 ng of synthetic melanin per 100 μL of PCR amplification system completely inhibited the PCR of the genomic DNA template (Fig. 3*a*). The same amount of melanin extracted from hair shaft portion also inhibited the reaction (Fig 3*b*).

DNA content in a plucked hair root and melanin concentration in a hair shaft portion of 12 individuals are shown in Table 1. HLA-DQA1 genotypes could be typed correctly with all of the DNA samples prepared from single plucked hair roots. However, with extracts of hair shaft portions, only 3 out of 12 samples could be typed correctly (Nos. 3, 7, and 12). A hair shaft contains melanin ranging from 7.6 to 16.5 μg/10 cm in length, and mean value and standard deviation (SD) are 12.2 ± 2.8 μg/10-cm hair shaft.

We reported that frequencies of HLA-DQA1 in the Japanese population and HLA-DQA1 is very polymorphic [*12*] as in other populations in [*10,11,20*]. The heterozygosity

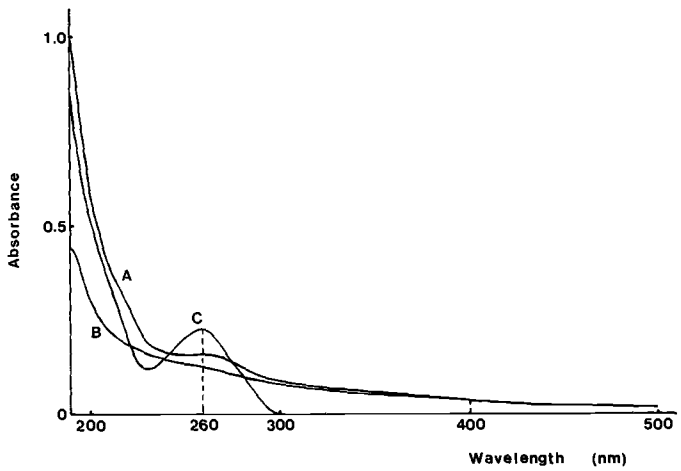

FIG. 2—*Comparison of absorption spectra:* (a) *DNA extract from hair shaft containing 5.0-μg melanin/mL of distilled water,* (b) *synthetic melanin (5.0 μg/mL of distilled water), and* (c) *human genomic DNA (10 μg/mL of distilled water).*

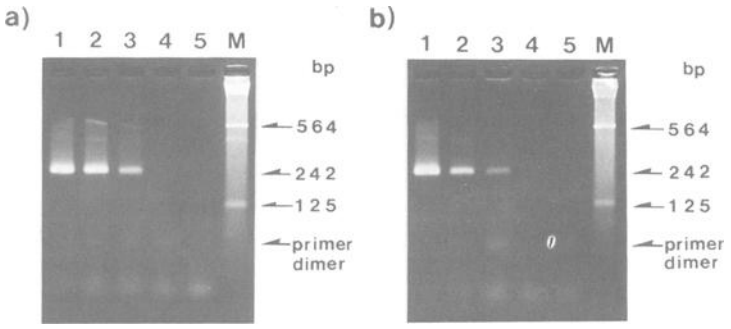

FIG. 3—*Changes in the amount of PCR product of HLA-DQA1 sequences from DNA prepared from fresh blood (100 ng) in a 100-μL amplification system when various amounts of synthetic melanin or DNA extracts prepared from hair shaft were added to the system:* (a) *Synthetic melanin of 0, 5, 10, 15, and 20 ng (lanes 1–5, respectively) was added.* (b) *DNA extract from hair shaft samples containing 0, 5, 10, 15, and 20 ng of melanin (lanes 1–5, respectively) was added. M: λ DNA cleaved by HindIII.*

TABLE 1—*Hair samples used in the present study. All samples were freshly plucked from head, and general characteristics are black, straight, and round in cross section.*

Number	HLA-DQA1 Genotype	Root DNA[a], ng	Shaft Diameter[b], μm	Shaft Melanin[c], μg
1	*0103/*0301	24	127	15.0
2	*0301/*0301	90	98	16.5
3	*0102/*0301	820	109	14.0
4	*0301/*0301	400	88	12.2
5	*0101/*0103	520	93	10.2
6	*0101/*0301	40	111	15.3
7	*0103/*0301	1280	99	12.0
8	*0101/*0301	140	102	11.5
9	*0301/*0301	280	105	13.5
10	*0103/*0103	102	82	7.6
11	*0103/*0301	160	72	8.5
12	*0101/*0301	480	110	9.7

[a]DNA in a freshly plucked hair root.
[b]Mean value of three measurements in a hair shaft portion.
[c]Melanin contained in hair shaft (10 cm in length).

and the discriminating power of this genetic marker in Japanese are 0.75 and 0.91, respectively (calculated from our population study; see Table 2). Thus, if HLA-DQA1 can be completely typed from hair samples, it would be very helpful to perform individualization.

The present results showed that HLA-DQA1 could be typed with only three samples out of twelve hairs when the shaft portion was analyzed, although all twelve hairs could be typed when the root portion was analyzed. Interestingly, the DNA extract prepared from hairs itself inhibited the PCR amplification of HLA-DQA1 gene. The spectrum of DNA extract from hair shaft showed the presence of melanin (Fig. 2). Furthermore, the hair shaft extract inhibited PCR amplification of HLA-DQA1 gene, and the reaction was also inhibited by synthetic melanin equal in amount to the melanin in the hair shaft extract (Fig. 3). Since 50-cm-long hair shaft weighs approximately 5 mg and the contamination of melanin in the DNA extract from 0.5 g of hair shaft was 0.13%, the 15 ng of melanin that can completely inhibit PCR was calculated to be derived from hair shaft 5

TABLE 2—*Distribution (%) of HLA-DQA1 alleles.*

	Population				
	Japanese		Caucasian	United States	United Kingdom
DQA1 Allele	[12] (n = 580)	[20] (n = 184)	[20] (n = 826)	[10] (n = 410)	[11] (n = 156)
*0101	11.0	8.7	13.7	13.9	. . .
*0102	16.2	12.0	19.7	20.7	39.0
*0103	19.5	22.8	8.5	3.4	
*0201	0.4	0.5	10.9	10.5	9.0
*0301	40.7	44.6	20.1	21.2	35.0
*0401	4.0				
*0501	6.6	11.4	27.1	30.2	18.0
*0601	1.7				
HZ	0.75	0.71	0.81	0.79	0.68
DP	0.91	0.88	0.94	0.92	0.85

NOTE: n = number of alleles in samples. HZ = heterozygosity. DP = discriminating power.

to 10 cm in length. Thus, the DNA extract from 20 or 50 cm of hair shaft (Fig. 1) should have contained over 15 ng of melanin, and that is why PCR was completely inhibited.

Given the present protocol for extracting DNA from hair, it is inevitable that the extract be contaminated by melanin. Too much hair extract should not be used as a template for PCR, even if contains a fairly large amount of DNA, since the melanin contamination may also increase and inhibit PCR. Therefore, typability of hair shaft portions seems to depend on the delicate balance of the concentrations of DNA and the contaminated melanin in the final DNA extracts. The DNA extracts of three typable specimens may have contained larger amounts of DNA or smaller amounts of melanin, or both, than those of the other nine hair shafts in the present study.

In these experiments, only Japanese hair was used. Microscopically, hairs show black, brown, and yellow pigment granules, the shade depending on the amounts of pigment present. The pigment of most types of hair, for example, that of Japanese hair, is melanin (eumelanin), while that of true blond and red hair is phaeomelanin [21]. So it will be necessary to examine whether the PCR amplification is inhibited by phaeomelanin as well as eumelanin.

HLA is a very complicated system including many genotypes, and HLA-DRB1 or HLA-DPB1 have many more alleles (34 and 19, respectively) [22] than HLA-DQA1 (8 alleles). The typing of such genetic markers is extremely promising for personal identification. However, further study is needed to perform such typing with hair shaft samples containing a fairly large amount of melanin.

Acknowledgments

We gratefully acknowledge the assistance of members of Criminal Science Laboratory, Aichi Prefectural Police Headquarters, for supplying the "freshly plucked" hairs and kind advice in the course of this study.

References

[1] Jeffreys, A. J., Wilson, V., and Thein, S. L., "Individual-Specific 'Fingerprints' of Human DNA," *Nature* (London), Vol. 316, No. 6023, 4 July 1985, pp. 76–79.

[2] Jeffreys, A. J., Brookfield, J. F. Y., and Semeonoff, R., "Positive Identification of an Immigration Test-Case Using Human DNA Fingerprints," *Nature* (London), Vol. 317, No. 6040, 31 Oct. 1985, pp. 818–819.

[3] Gill P., Jeffreys, A. J., and Werrett, D. J., "Forensic Application of DNA 'Fingerprints'," *Nature* (London), Vol. 318, No. 6046, 12 Dec. 1985, pp. 577–579.

[4] Honma, M., Yoshii, T., Ishiyama, I., Mitani, K., Kominami, R., and Muramatsu, M., "Individual Identification from Semen by the Deoxyribonucleic Acid (DNA) Fingerprint Technique," *Journal of Forensic Sciences*, Vol. 34, No. 1, Jan. 1989, pp. 222–227.

[5] Smith, J. C., Newton, C. R., Alves, A., Anwar, R., Jenner, D., and Markham, A. F., "Highly Polymorphic Minisatellite DNA Probes. Further Evaluation for Individual Identification and Paternity Testing," *Journal of the Forensic Science Society*, Vol. 30, No. 1, Jan. 1990, pp. 3–18.

[6] Odelberg, S. J., Demers, D. B., Westin, E. H., and Hossaini, A. A., "Establishing Paternity Using Minisatellite DNA Probes When the Putative Father is Unavailable for Testing," *Journal of Forensic Sciences*, Vol. 33, No. 4, July 1988, pp. 921–928.

[7] Wells, R. A., Wonke, B., and Thein, S. L., "Prediction of Consanguinity Using Human DNA Fingerprints," *Journal of Medical Genetics*, Vol. 25, 1988, pp. 660–662.

[8] Akane, A., Matsubara, K., Shiono, H., Yuasa, I., Yokota, S.-I., Yamada, M., and Nakagome, Y., "Paternity Testing: Blood Group Systems and DNA Analysis by Variable Number of Tandem Repeat Markers," *Journal of Forensic Sciences*, Vol. 35, No. 5, Sept. 1990, pp. 1217–1225.

[9] Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., and Arnheim, N., "Enzymatic Amplification of β-Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia," *Science*, Vol. 230, No. 4732, 20 Dec. 1985, pp. 1350–1354.

[10] Higuchi, R., von Beroldingen, C. H., Sensabaugh, G. F., and Erlich, H. A., "DNA Typing from Single Hairs," *Nature* (London), Vol. 332, No. 6164, 7 April 1988, pp. 543–546.

[11] Westwood, S. A. and Werrett, D. J., "An Evaluation of the Polymerase Chain Reaction Method for Forensic Applications," *Forensic Science International*, Vol. 45, No. 3, April 1990, pp. 201–215.

[12] Tamaki, K., Yamamoto, T., Uchihi, R., Katsumata, Y., Kondo, K., Mizuno, S., Kimura, A., and Sasazuki, T., "Frequency of HLA-DQA1 Alleles in the Japanese Population," *Human Heredity*, Vol. 41, 1991, pp. 209–214.

[13] Tamaki, K., Yamamoto, T., Sato, K., Okajima, H., and Katsumata, Y., "The Evaluation of Species Identification in Blood by Dot Hybridization Using a Recombinant DNA Probe," *Acta Criminologiae et Medicinae Legalis Japonica*, Vol. 56, No. 3, June 1990, pp. 99–106.

[14] Brunk, C. F., Jones, K. C., and James, T. W., "Assay for Nanogram Quantities of DNA in Cellular Homogenates," *Analytical Biochemistry*, Vol. 92, No. 2, 15 Jan. 1979, pp. 497–500.

[15] Labarca, C. and Paigen, K., "A Simple, Rapid, and Sensitive DNA Assay Procedure," *Analytical Biochemistry*, Vol. 102, No. 2, 1 March 1980, pp. 344–352.

[16] Scharf, S. J., Horn, G. T., and Erlich, H. A., "Direct Cloning and Sequence Analysis of Enzymatically Amplified Genomic Sequences," *Science*, Vol. 233, No. 4768, 5 Sept. 1986, pp. 1076–1078.

[17] Fukui, Y., Nishimura, Y., Iwanaga, T., Kimura, A., Inamitsu, T., Hanaoka, Y., Kitagawa, T., and Sasazuki, T., "Analysis of Glycosuria and Insulitis in NOD Mice Expressing HLA-DQw6 Molecule," *Journal of Immunogenetics*, Vol. 16, 1989, pp. 445–453.

[18] Whittaker, J. R., "Changes in Melanogenesis during the Dedifferentiation of Chick Retinal Pigment Cells in Cell Culture," *Developmental Biology*, Vol. 8, 1963, pp. 99–127.

[19] Saiki, R. K., "The Design and Optimization of the PCR," in *PCR Technology*, H. A. Erlich, Ed., Stockton Press, New York, 1989, pp. 7–16.

[20] Helmuth, R., Fildes, N., Blake, E., Luce, M. C., Chimera, J., Madej, R., Gorodezky, C., Stoneking, M., Schmill, N., Klitz, W., Higuchi, R., and Erlich, H. A., "HLA-DQα Allele and Genotype Frequenices in Various Human Populations, Determined by Using Enzymatic Amplification and Oligonucleotide Probes," *American Journal of Human Genetics*, Vol. 47, 1990, pp. 515–523.

[21] Bisbing, R. E., "The Forensic Identification and Association of Human Hair," *Forensic Science Handbook*, R. Saferstein, Ed., Prentice-Hall Inc., Englewood Cliffs, N.J., 1982, pp. 184–221.

[22] Bodmer, J. G., Marsh, S. G. E., Parham, P. Erlich, H. A., Albert, E., Bobmer, W. F., Dupont, B., Mach, B., Mayr, W. R., Sasazuki, T., Schreuder, G. M. Th., Strominger, J. L., Svejgaard, A., and Terasaki, P. I., "Nomenclature for Factors of the HLA System, 1989," *Tissue Antigens*, Vol. 35, No. 1, 1990, pp. 1–8.

Address requests for reprints or additional information to
Rieko Uchihi, M.S.
Department of Legal Medicine
Nagoya University School of Medicine
65 Tsuruma-cho,
Showa-ku, Nagoya 466, Japan