HLA CLASS I AND CLASS II TYPING OF CULTURED FIBROBLASTS DERIVED FROM CADAVERIC TISSUE USING SEROLOGICAL AND DNA TYPING METHOD

AKIKO ISHITANI
Department of Legal Medicine, Nara Medical University
Received March 12, 1989

Summary: A method for HLA typing of cultured fibroblasts derived from abdominal skin was developed and applied for personal identification of cadavers. Successful results were obtained using the conventional NIH HLA class I typing method for lymphocytes after a slight modification, where the cultured fibroblasts were pre-incubated for 1-2 h in PRMI medium adjusted to pH 8.0 before cytotoxicity test.

Thus assigned HLA types of fibroblasts showed a good concordance with those of lymphocytes derived from the same donor, indicating that the modified method is well suited to practical use.

From 12 forensic autopsy cases where the postmortem periods were 7-96 h, the utility of the modified method was examined. The HLA class I types of the cultured fibroblasts obtained from abdominal skin or other tissue were clearly assigned, showing the usefulness of this method in forensic practice.

The HLA class II type of fibroblasts was identified by DNA typing method using allele specific oligonucleotide probes. This "Cultured Fibroblast method" is very useful for accurate personal identification.

Index Terms

personal identification, HLA, cadaveric tissue, cultured fibroblast, DNA typing

INTRODUCTION

Based on the diversity of HLA polymorphism, HLA typing is often used for paternity testing in the field of legal medicine[1)2)]. In paternity testing, the established NIH microcytotoxicity method[3)] for typing of lymphocytes is directly applicable, since lymphocytes are easily obtained from peripheral blood of living bodies. However, direct application of this method for personal identification in forensic practice is difficult because of the low viability of lymphocytes obtained from dead bodies. It is necessary for the forensic scientist to establish the HLA typing method from other tissues. In 1968, Thorsby and Lie[4)] attempted to show the presence of HLA antigen on cultured fibroblast. Since then, many attempts have been made to determine the HLA type of cultured fibroblasts derived from various tissues such as amniotic fluid[5)], chorionic villi[6)], hydatidiform mole[7)], fetus[8)] and human tumor cell lines[9)]. In the forensic field, several attempts have been made for HLA typing directly from cadaveric tissues[10)11)12)] without cultivation. But none of these

methods are suited to practical routine work, because they are time-consuming and require relatively large amounts of anti-HLA sera, because they do not show good reliability, because it may be difficult to evaluate the results, or for other reasons.

In an effort to develop an economical and practical method for personal identification of a cadaver by HLA typing, the author investigated the possibility of carrying out the test by the conventional typing method using cultured cells derived from cadaveric skin and a ready-made HLA typing tray for lymphocytes.

To develop a method for more accurate personal identification, it was examined whether the DNA typing method[13] is applicable for determining the class II type using a small amount of DNA extracted from cultured fibroblasts. For this purpose, primer-directed enzymatic amplification technique, which is able to produce a selective enrichment of a specific DNA sequence by a factor of 10^6 by the polymerase chain reaction (PCR)[13)~18], was used.

MATERIALS AND METHODS

Materials

Small amounts (about 0.5 cm^2) of abdominal skin tissue were obtained from 8 living bodies at surgical operation and 12 dead bodies at autopsy to establish fibroblast cell culture. In patients, peripheral blood samples (10–20 ml) were also obtained before the operation to test the HLA class I type on the lymphocytes.

For serological typing, ready-made typing trays, which contain 1 μl of antiserum per well and are prepared by Osaka Prefectural Hospital, were used for the cytotoxicity assay of fibroblasts and lymphocytes.

For DNA typing, 6 samples of fibroblast and 5 control samples of white blood cell were used. Two oligonucleotide primers[14] which direct the synthesis of 231 bps segment of HLA DR locus, GLPDRB1 (5′-TTCTTCAATGGGACGGAGCG-3′) and GAMPDRB1 (5′-GCCGCTGCACTGTGAAGCTCTC-3′), were synthesized by automatic DNA synthesizer (Applied Bio System) and used for PCR amplification. Allele-specific nucleotide probes of DR2/Dw2B1 defined by sequences between codons 34 and 40 of DRB1 gene[14] (5′-CAAGAGGAGGACTTGCGCT-3′), DR4B1 between codons 30 and 36 (5′-TACTTC-TATCACCAAGAGGA-3′) and a probe common to DR4/Dw14B1, DR4/Dw15B1 and DR1B1 between 69 and 75 (5′-GCAGAGGCGGGCCGCGGT-3′) were also synthesized.

Establishment and Maintenance of Fibroblast Culture from Tissue

The piece of skin tissue obtained was placed in Dulbecco's modified Eagle's MEM (DME) medium containing 500 U/ml penicillin, 500 μg/ml streptomycin and 20 μg/ml fungizone, and allowed to stand for a few hours at room temperature. After washing several times with fresh medium, the skin tissue was cut into pieces of about 1 mm^3 and placed on the bottom of a plastic petri dish. The explants were cultured in DME medium containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, in an atmosphere of 90% air, 10% CO_2 at 37°C. Within 2–10 days after adhesion of explants, outgrowth of fibroblasts (or fibroblast-like stromal cells) appeared around the

"mother explant". When the cell population became large enough to perform subculture, in about 1–3 weeks, the cells were detached from the substratum by treatment with 0.02% EDTA and 0.25% trypsin (1 : 1) (EDTA-trypsin). After washing with DME medium containing 10% FCS, the detached cells were resuspended in the same medium and subcultured.

Preparation of Cells for Use in Cytotoxicity Assays

Fibroblasts were detached from the culture plate by treatment with EDTA-trypsin and suspended in RPMI medium (pH 8.0) supplemented with 10% FCS. After 1–2 h, the cells were collected by centrifugation, resuspended to give a concentration of $1–2\times10^6$ cells/ml in RPMI medium without FCS, and used for cytotoxicity assays.

Cytotoxicity Assays

The original microcytotoxicity test (MCT), which is known to be a conventional method for HLA typing of lymphocytes, was applied for typing of fibroblasts, except that the pH of RPMI medium was changed to 8.0. Lymphocyte and fibroblast samples were suspended in RPMI medium of pH 7.0 and 8.0, respectively. One μl of the suspended cell samples (1000–2000 cells) was placed in each well of a micro-test tray (with 60 wells), in which 1 μl of antisera/well was pre-plated and incubated for 30 min at room temperature (about 25°C). Then, 5 μl of complement (Pel-Freeze) for HLA-A, B, C typing was added to each well. After leaving the samples at room temperature for 1 h, 2 μl of 5% eosin solution was added and after 5 min, 15 μl of 37% formalin was added. After a coverslip was placed on the tray, cells were allowed to settle for several hours, and the proportion of dead cells in each well of the trays was read using an inverted phase contrast microscope. The results of MCT were recorded and scored according to the following criteria :

8 (81–100% dead cells), 6 (41–80% dead cells), 4 (21–40% dead cells),
2 (11–20% dead cells), 1 (0–10% dead cells).

Extraction of DNA

Fibroblasts detached from the culture plate with EDTA-trypsin were rinsed twice in 137 mM NaCl, 5 mM KCl and 25 mM Tris base (pH 7.4), and suspended in 1 ml of TE buffer (10 mM Tris-HCl and 1 mM EDTA (pH 8.0)) per 10^8 cells. After gentle mixing, the cell suspension was added to a solution of ten times the volumes containing 100 mM Tris-HCl, 10 mM EDTA, 150 mM NaCl (pH 7.5), 200 μg/ml proteinase K (Merck), 1% sodium lauryl sulfate (SDS) and was incubated at 50°C overnight. DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) twice and with chloroform/isoamyl alcohol (24 : 1) twice followed by precipitation with 1/10 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of ethanol. The precipitated DNA was dissolved in TE buffer in a final concentration of 0.1 μg DNA/μl by determining absorbance at 260 nm and was used for PCR amplification.

PCR Amplification

PCR amplification using thermostable DNA polymerase of *Thermus acquaticus* (Taq 1 DNA polymerase, Stratagene) was carried out in a solution containing 10 μl of this

DNA solution, 50 mM Tris-HCl (pH 8.8 at 25°C), 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 1.5 mM each of dATP, dCTP, dTTP, dGTP, 50 pmol each of the two primers and 2.5 units of Taq DNA polymerase in a final volume of 100 μl. Twenty-four cycles of PCR were carried out in an automated temperature cycling device (TRIO THERMO JUPITER, TAKASHO Corp.). Each cycle consisted of 1.2 min incubation at 95°C (to denature the DNA), 1.0 min at 55°C (to anneal the primer) and 1.5 min at 72°C (to activate the polymerase and extend the annealed primers).

To confirm whether the region for DR locus of the DNA had been amplified, fifteen μl of the amplified reaction mixture was electrophoresed on a 6% polyacrylamide gel. The gel was stained with ethidium bromide and photographed under UV light.

Hybridization

Ten μl of PCR product was mixed with 100 μl of 500 mM NaOH and 25 mM EDTA and the mixture was placed in a dot-blot manifold (Bio-Rad) on the nylon membrane (Biotrace RP, Gelman Sciences Inc.), and then baked at 80°C for 2 h. The membrane was incubated in a hybridization buffer containing 0.5% SDS, 5×SSPE (1×SSPE : 180 mM NaCl, 10 mM NaH_2PO_4 and 1 mM EDTA), 0.1% bovine serum albumin, 0.1% Ficoll and 0.1% polyvinylpyrrolidon for 1 h at the calculated hybridization temperature and then hybridized with ^{32}P-labelled probes at a concentration of 1 pmol/ml at the specific hybridization temperature of the probe for 1 h. The membrane was washed in 2×SSPE and 5% SDS for 10 min, in 2×SSPE and 1% SDS for 10 min at the hybridization temperature, and subjected to autoradiography at −80°C for 15–60 h with intensifying screen. The hybridization temperature was calculated based on : T=(number of GC bps)×4+(number of AT bps)×2−5.

^{32}P-Labelling of Probe

3′-end of the DNA was labeled with terminal deoxynucleotydyl transferase (TDT) using a kit (Amersham). The labelling[19)20)] mixture contained 10 pmols of 3′-ends of the DNA, 140 mM sodium cacodylate (pH 7.2), 1 mM $CoCl_2$, 0.1 mM dithiothreitol (DTT), 17 pmol (50 μCi) dideoxyadenosine 5′- [α-^{32}P] triphosphate and 10 units of terminal deoxynucleotidyl transferase in a total volume of 50 μl. After the incubation at 37°C for 1 h, the reaction mixture was loaded onto a Sephadex G-25 column (NAP5 column, Pharmacia) preequilibrated with 150 mM NaCl, 10 mM ETDA, 0.1% SDS and 50 mM Tris-HCl (pH 7.5). The labeled probe DNA was eluted with the same buffer in the first 1 ml of the eluate.

RESULTS

Modification of NIH-Microcytotoxicity Test for HLA Typing of Fibroblasts

In order to apply the established NIH method for HLA typing of lymphocytes to personal identification of a cadaver by utilizing cultured fibroblasts derived from abdominal skin, it was tested whether the NIH method can be directly applied to HLA typing of fibroblasts. This attempt, however, was unsuccessful since positive and negative reactions could not be clearly differentiated (Figs. 1 A and B), probably due to the adhesion

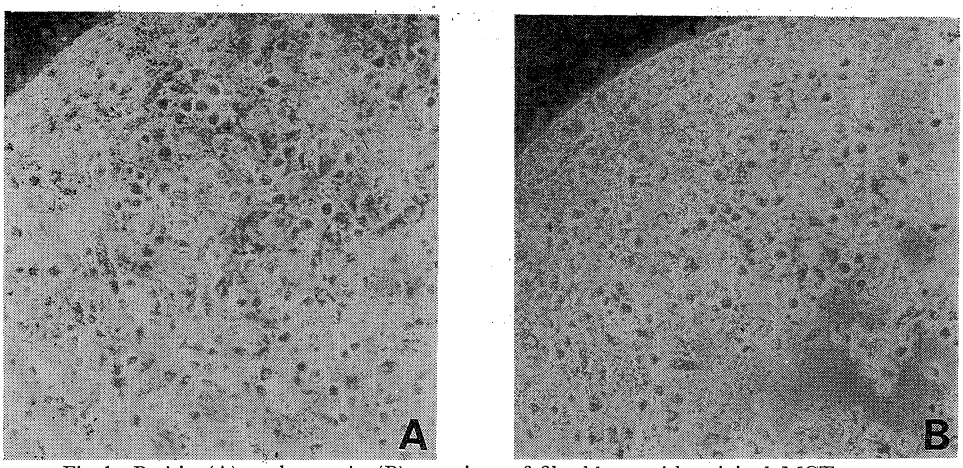

Fig. 1. Positive(A) and negative(B) reactions of fibroblasts with original MCT. Fibroblasts derived from abdominal skin tissue were subjected to the original NIH-MCT after pretreatment with RPMI medium pH 7.0 for 1 h.

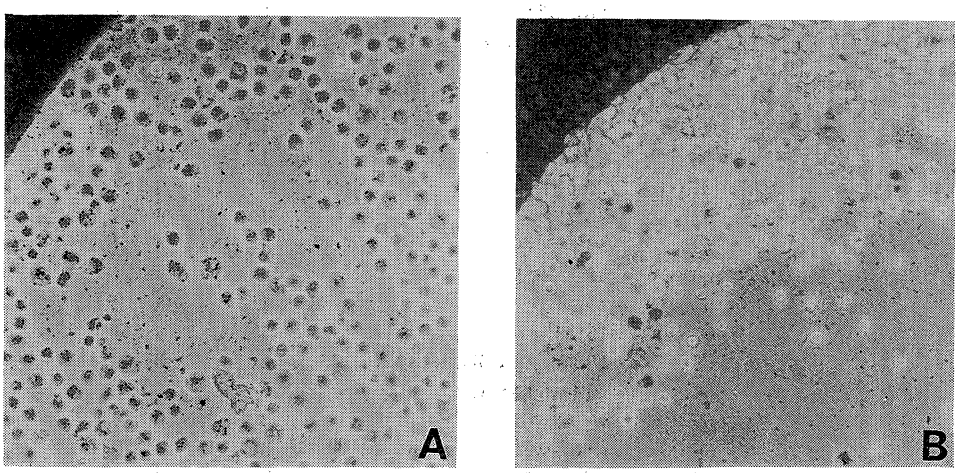

Fig. 2. Positive(A) and negative(B) reactions of fibroblasts with modified MCT. The same fibroblast sample was subjected to MCT after pretreatment for 1 h at pH 8.0 as described in Materials and Methods.

of fibroblasts to the bottom of the test well resulting in insufficient interaction with the antisera. After several attempts to prevent the adhesion of fibroblasts to the bottom of the well, it was found that pre-treatment of fibroblasts in PRMI medium adjusted to pH 8.0 for 1–2 h kept the cells in a spherical form and prevented their adhesion to the surface of the wells during the cytotoxicity test so that sufficient reactivity with small amounts of antisera in the ready-made tray was possible. The viable and dead cells could be clearly differentiated as shown in Figs. 2 A and B.

Reliability of HLA Types Assigned with Fibroblasts

In order to examine the reliability of HLA typing from fibroblasts, the HLA types assigned with lymphocytes and fibroblasts were compared in 8 operative cases.

Table 1. MCT reactions of lymphocytes and fibroblasts from individual No. 1*

Locus	Serum No.	Specificity of serum	Reaction	
			Lympho.	Fibro.
		P.C.	8	8 8
		N.C.	1	1 1
A	11	23	1	1 6
	12	23	1	1 4
	13	23+24+9.3	8	8 8
	14	23+24	8	8 1
	15	23+24+9.3	8	8 8
	16	24+9.3	8	8 8
	17	11	1	1 4
	18	11	1	1 1
	19	11	1	1 1
	20	11	1	1 1
	21	25	1	1 1
	22	25	1	1 1
	23	26	8	8 6
	24	26	8	8 8
	25	26+w34	8	8 6
	26	25+32+w34	6	1 1
	27	32+w34	1	1 1
	28	32	1	1 1
	29	30+31	1	4 6
	30	30+31	1	1 1
	31	30+31	1	1 1
	32	30+31	1	1 1
B	59	13	1	1 1
	60	13	4	1 1
	61	13	1	1 4
	62	13	1	1 1
	63	13+w60+w61+w48+w41	8	8 8
	64	13+w60+w61	8	8 8
	65	w60+w61	8	8 8
	66	w60+w61	8	8 8
	67	w60+w61	8	8 8
	68	w60	1	1 1
	69	w60	4	1 1
	70	w60	4	1 1
	71	w61	8	8 8
	139	w6	8	6 1
	140	w6	8	8 8
C	146	w3	8	6 6
	147	w3	8	1 4
	148	w3	8	1 6
	149	w3	8	1 4
	150	w3	8	1 4
	163	w7	8	6 6
	164	w7	8	1 1
	165	w7	8	1 1
	166	w7	8	1 8

* Some negative and doubtful positive reactions have been omitted.

A total of 39, 107 and 26 prepared antisera for A, B and C locus respectively, were used. As a negative and positive control, reactivity with anti-lymphocyte and normal serum was also examined. The test for fibroblasts was carried out twice with an interval of about one month of the culture.

The results obtained from one individual (Sample No. 1) are shown in Table 1, in which the complete reactions of the MCT test are shown, except that some negative (Scores 1 and 2) and doubtful positive reactions (Score 4) have been omitted.

As shown in this table, MCT scores in the double examination of fibroblasts show good concordance with only a relatively small number of exceptions (fibroblasts showed clearly different reactivity with antiserum Nos. 11, 14, 29, 139, 148, 166 in the parallel tests). When the larger scores for fibroblasts in the parallel test were chosen, the scores of fibroblasts and lymphocytes showed quite good concordance in A and B loci, although there were several differences in C locus.

For determining HLA types of both fibroblasts and lymphocytes, scores above 4 were defined as a positive reaction and cells which exhibited a positive reaction with more than two kinds of antisera specific to a given antigen, were regarded as having the antigen. In this way the HLA types of fibroblasts and lymphocytes derived from the individual of Sample No. 1 were determined.

Thus, both HLA types of fibroblasts and lymphocytes of this sample were assigned to be A24, 26/Bw61, —/Cw3, w7. The results obtained from all samples taken during operations are summarized in Table 2. As shown in this table, HLA types assigned from fibroblasts were essentially the same as those with lymphocytes in the A and B loci except one antigen from the A locus (Sample 2) and two antigens from the B locus (Sample 2 and 6); thus, the rate of concordance was calculated to be about 90%. However, the rate was significantly lower in the C locus (about 70%), and even the expression of an unexpected antigen was sometimes observed in this locus from fibroblasts (Samples 3, 4, 5 and 7), while such an unexpected expression was not observed in the A and B loci.

Table 2. HLA types assigned with cultured fibroblasts and lymphocytes from same living body

Sample No	HLA Type	
	Fibroblast	Lymphocyte
1*	A24, 26/Bw61, —/Cw3, w7	A24, 26/Bw61, —/Cw3, w7
2*	A24, —/B 5, —/C —, —	A24, 31/Bw52, —/C —, —
3	A 2, 24/Bw60, 39/Cw2, w7	A 2, 24/Bw60, 39/Cw4, w7
4	A24, 11/Bw54, 35/Cw1, w7	A24, 11/Bw54, 35/Cw1, —
5	A24, —/Bw54, w52/Cw1, w3	A24, —/Bw54, w52/C —, —
6	A24, 31/Bw52, —/Cw3, —	A24, 31/Bw52, w59/Cw3, —
7	A24, 11/Bw54, w61/Cw1, w3	A24, 11/Bw54, w61/Cw1, —
8	A24, 11/Bw61, 39/Cw7, —	A24, 11/Bw61, 39/Cw7, —

* Frozen cells were used

HLA Class I Typing of Cadavers

HLA class I typing with fibroblasts was applied in 12 forensic autopsy cases, and the results are shown in Table 3. In all of these cases, HLA types could be clearly assigned with cultured fibroblast obtained from abdominal skin, though the results could not be confirmed by comparison with the results of their own lymphocytes because of the difficulty of obtaining lymphocytes viable enough to test from dead bodies. Even in the case of No. 913 with the longest postmortem period (96 h, in winter) among these 12 cases, the fibroblast culture could be established. In one case (No. 930), the dead body was infested with maggots except for the legs from which skin samples were taken. In another case (No. 920), the body was so burned that his skin could not be used for culture, and the culture could be obtained using a piece of the peritoneum.

Table 3. HLA types of cadavers tested with cultured fibroblast

Sample No.	Hours after Death	Age	HLA Type
883	18	65	A24, 26/Bw61, w62/Cw3, —
886	18	56	A24, 31/B 37, 40/Cw2, w3
897	19	17	A24, 26/B 7, —/Cw7, —
899	34	77	A24, w33/B 35, 44/Cw3, —
913	96	26	A 2, 24/Bw61, —/Cw1, w7
918	9	18	A24, 26/Bw54, w60/Cw1, w3
920	20	49	A 2, 24/Bw54, w61/Cw1, w3
926	7	0	A 2, —/Bw60, w62/C —, —
930	72	68	A 2, 3/Bw60, —/Cw1, w7
932	29	2	A 2, 26/Bw54, w60/Cw1, w7
934	15	69	A26, 31/Bw61, —/Cw3, w7
935	10	34	A31, w33/Bw61, —/Cw1, w3

DNA Typing of Fibroblast

DNA were extracted from 5 samples of white blood cell (WBC) freshly collected from individual Nos. 1–5 of normal adult whose DR type was serologically assigned to DR4 and w8, DR4 and w9, DR4 and w9, homogenic DR4, DR4 and w8 respectively and 6 samples of cultured fibroblast from individual Nos. 6–11.

One μg or less of DNA isolated from these samples was used successfully to amplify the specific DNA sequence of HLA DR locus under the direction of the primers. Electrophoresis of the PCR products showed specific amplification of 231 bps DNA.

For each sample, PCR product was fixed on nylon membrane in a pattern of four dots per sample. Each dot was tested for hybridizability with ^{32}P-labelled oligonucleotide probes. Figure 3 shows an autoradiogram of the dot blots hybridized with the labelled three oligonucleotide probes listed in MATERIALS and GLPDRB1 primer which is used to estimate the relative quantity of the DNA dotted on the membrane.

All WBC samples showed DR4 positive and DR2 negative (the faint spots hybridized with probe specific DR2 on autoradiogram should be judged as negative compared to

the quantity of DNA dotted) in accordance with serologically defined type. Fibroblasts of both No. 6 and No. 10 were DR4 positive and were DR2 negative, showing that the individual had an allele of DR4 at least, and Nos. 7 and 11 were DR4 negative and DR2 positive showing that the individuals had an allele of DR2 at least. Sample of No. 9 was positive for DR2 and also for a probe common in DR4 and DR1, but negative for DR4 probe, so that the individual should be assigned to DR1 and DR2. Sample of No. 8 was all negative to the probes tested in spite of a positive result to the primer, so that the individual should have other types of DR locus.

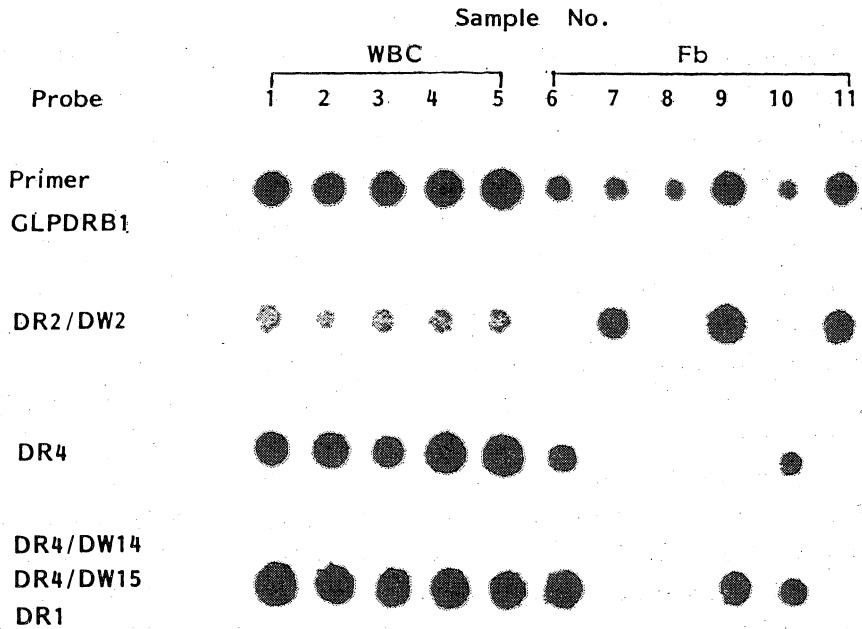

Fig. 3. DNA typing on cultured fibroblast.
Samples No. 1–5 are DNA extracted from white blood cell carrying DR4 but none of DR1 and DR2 antigen, and sample No. 6–11 are the DNA from cultured fibroblast to be typed.
As DNA probe, GLPDRB1 primer (to estimate the quantity of DNA dotted), DR2/Dw2, DR4 and a probe common to DR4/Dw14, DR4/Dw15 and DR1 were used.
DNA dotted membrane was hybridized with each probe and autoradiographed.

DISCUSSION

The present study demonstrated that HLA typing of cultured fibroblasts could be successfully made using the conventional NIH-MCT method for HLA typing of lympocytes with the slight modification. The key lies in the pretreatment of fibroblasts for 1–2 h in the culture medium adjusted to pH 8.0; this process keeps cells in a spherical form, and prevents them from sticking to the bottom of the well and thus makes it possible to utilize the ready-made typing tray which contains a very small amount (1 μl) of antisera.

HLA types of fibroblasts assigned by this method showed quite good concordance with those of lymphocytes especially in the A and B loci, indicating that the typing method has practical applications to the personal identification of cadavers. In some cases, fibroblasts did not show clear positive reaction with some antisera specific to a certain HLA antigen, and in other cases, the cells exhibited reactivity which was not expected from the reaction of lymphocytes of the same individual. However, such ambiguity and discrepancy could be overcome by increasing the number of antisera tested and by repeating the MCT test (Table 1).

The discrepancy between HLA types from fibroblasts and lymphocytes is rare in A and B loci but common in C locus, and may be due to an unexpected expression or disappearance of some HLA antigens during the culture of fibroblasts. Evans et al.[21] observed that Ia antigens appeared on activated T lymphocyte and Goldstein & Singal[22] also reported that HLA types of some clones of cultured fibroblasts varied with aging of the cells, although the HLA type of mass cultured fibroblasts did not vary during the culture for 2 months. On the other hand, Aragon et al.[23] reported that the fibroblasts derived from some patients with Duchenne muscular dystrophy showed different HLA types from that of the lymphocytes in the B locus. Discrepancy in the cases in this study could not be due to such a disease since samples (in Table 2) were taken from normal individuals at abdominal cesarean section (Samples No. 3, 4, 5, 8) or patients with uterus myoma (Samples No. 6, 7), lung cancer (Sample No. 2) or spastic spinal paralysis (Sample No. 1).

Besides these cases, serum No. 29, which had antibody specific to A30 and A31 antigens, showed positive or doubtful positive reactions with several cultured fibroblast samples, which are not expected to have these antigens (A30 and A31) or cross-reacting antigens (data not shown). This may be due to the presence of some antibodies in the serum against fibroblast-specific antigen(s). A similar case in which anti-HLA serum reacted with various activated T cells with different HLA types, was reported by Tada et al.[24]. Serum samples with such a property, however, are very rarely encountered (1 case in 172 serum samples tested in the present study), and thus do not substantially disturb the typing of fibroblasts.

The high rate of concordance of the HLA types in the A and B loci between fibroblasts and lymphocytes shows that this method is well suited to practical use. By this method the HLA types of the cadaver could be assigned using cultured fibroblasts obtained from abdominal skin or other tissue of dead body which was infested with maggots or severely burned. From these facts, personal identification of cadavers in cases such as air or train crashes, burning, dismembering etc. can be done successfully when just a small piece of live tissue can be obtained.

The present study also shows that HLA class II typing of cultured fibroblasts could be successfully made by DNA typing using allele specific oligonucleotide probes. Although other materials from cadavers such as hair[25], blood stain, or nails may likewise be usable for DNA typing of class II, it is difficult or impossible to culture and proliferate these cells in order to obtain sufficient amounts o the cells for serological typing of class I.

At present, the DNA typing method can not be applied for the class I antigen probably because it requires too many probes. Since cultured fibroblast can be used for both identification of class I type using serological method and class II using the DNA typing method, the "cultured fibroblast method" is promising for personal identification based on HLA typing in forensic practice. Since the class II typing method by DNA hybridization is laborious compared with class I typing, further study is required for simplifying the steps in extracting and labelling DNA.

ACKNOWLEDGEMENTS

The author is greatly indebted to Prof. Dr. Tetsuya Takayanagi (Department of Neurology, Nara Medical University) for providing frozen control samples for HLA typing and Prof. Dr. Takeo Juzi (Department of Transfusion, Faculty of Medicine, Tokyo University) for his kind advice and providing probes and primers, and Dr. Masayosi Tada for his useful advice and providing HLA class I typing trays. The author also expresses profound thanks to Dr. Shiro Tabata (Department of Chemistry, Nara Medical University) and Prof. Dr. Tadaomi Hirota (Department of Legal Medicine, Nara Medical University) for their invaluable advice and encouragement.

REFERENCES

1) **Terasaki, P.I., Bernoco, D., Gjerston, D., Mickey, M.R.** and **Perdue, S.**: Forensic Sci. Int. **12**: 227, 1978.
2) **Ota, M., Matsuzawa, T., Fukushima, H., Yonemura, I.** and **Hasekura, H.**: Jpn. J. Leg. Med. **40**: 774, 1986.
3) **Bodmer, W.F.**: Br. Med. Bull. **34**: 213, 1978.
4) **Thorsby, E.** and **Lie, S.**: Vox Sang. **15**: 44, 1968.
5) **Whitsett, C.F., Priest, J.H., Priest, R.E.** and **Maron, J.**: Am. J. Clin. Pathol. **79**: 186 1983.
6) **Callaway, C., Falcon, C., Grant, G., Maurer, D.H., Auerbach, A.D., Rosenwaks, Z.** and **Pollack, M.S.**: Human Immunology **16**: 200, 1986.
7) **Hansen, H.E.** and **Vejerslev, L.O.**: J. Immunol. Methods **83**: 343, 1985.
8) **Hansen, H.E., Vejerslev, L.O., Westergaard, J.G.** and **Sondergaard, F.**: Advances in Forensic Haemogenetics 1, Springer-Verlag, Berlin Heidelberg, p. 28, 1986.
9) **Pollack, M.S., Heagney, S.D., Livingston, P.O.** and **Fogh, J.**: JNCI **66**: 1003, 1981.
10) **Hansen, H.E.** and **Gurtler, H.**: Tissue Antigen **13**: 61, 1979.
11) **Yoshimura, K.**: Jpn. J. Legal Med. **36**: 710, 1982.
12) **Kasiwade, H.** and **Hara, M.**: Act. Crim. Japon. **52**: 195, 1986.
13) **Saiki, R.k., Bugawan, T.L., Horn, G.T., Mullis, K.B.** and **Erlich, H.A.**: Nature 324: 163, 1986.
14) **Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A.** and **Arnheim, N.**: Science **230**: 1350, 1985.
15) **Todd, J.A., Bell, J.I.** and **McDevitt, H.O.**: Nature **329**: 599,1987.
16) **Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B.** and **Erlich, H.A.**: Science **239**: 487, 1988.
17) **Erlich, H.A., Gelfand, D.H.** and **Saiki, R.T.**: Nature **331**: 461, 1988.
18) **Bugawan, T.L., Horn, G.T., Long, C.M., Mickelson, E., Hasen, J.A., Ferrara, G.n., Angelini, G.**

and **Erlich, H.A.**: J. Immunol. **141**: 4024, 1988.
19) **Maxam, A.M.** and **Gilbert, W.**: Proc. Natl. Acad. Sci. USA **74**: 560, 1977.
20) **Collins, M.L.** and **Hunsaker, W.R.**: Anal. Biochem. **151**: 211, 1985.
21) **Evans, R.L., Faldetta, T.J., Humphreys, R.E., Pratt, D.M., Yunis, E.J.** and **Schlossman, S.F.**: J. Exp. Med. **148**: 1440, 1987.
22) **Goldstein, S.** and **Singal, D.P.**: Exptl. Cell Res. **75**: 278, 1972.
23) **Aragon de Kirbach, E.D., Willers, I., Singh, S.** and **Goedde, D.**: Clinical Genetics **29**: 369, 1986.
24) **Tada, T., Hisayama, Y., Funatsuki, Y., Inoue, T., Miyake, T.** and **Inoue, H.**: Jpn. J. Transplant. **22**: 613, 1987.
25) **Higuchi, R., Beroldingen, C.H.V., Sensabaugh, G.F.** and **Erlich, H.A.**: Nature **332**: 543, 1988.