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Personal Identification by HLA Typing of Cultured Fibroblast Derived from Caderveric Tissues

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INTRODUCTION

It is well known that HLA typing of lymphocyte is extremely useful for personal identification and is often used for paternal testing in the field of legal medicine. Direct application of this method to personal identification of cadavers, however, is sometimes difficult because of relatively low viability of lymphocytes in a cadaver. Other method for HLA typing of a cadaver such as elution method reported by Kashiwade (1986) or absorption method (lymphocyte cytotoxicity inhibition test) reported by Yoshimura (1982) also have some difficulty that, in the case of a cadaver with completely unknown HLA type, these methods require a large amount of highly expensive HLA antibodies, consumes tremendous time and, thus, is not applicable for practical purposes. In an effort to find easy and general application of HLA typing of cadaver tissues, we investigated the possibility of carrying out the test by using conventional typing tray. In this report, we will show that HLA typing of a cadaver can be successfully made by using cultured fibroblast and slightly modifying the conventional NIH method for HLA typing of lymphocytes (Bodmer, 1978).

MATERIALS AND METHODS

Materials

Small amount (about $1cm^2$) of abdominal skin tissues were obtained by excision at operation of living body, or at autopsy in cadaver to establish fibroblast cell culture. In case of living body, 10-20ml peripheral blood were also obtained before operation to test HLA type of the lymphocyte. Complement used for cytotoxicity assay was purchased from Pel-Freeze Corp.

Establishment and Maintenance of Fibroblast Culture from Tissue

Skin tissue piece obtained was placed in Dulbecco's modified Eagles (DME) medium containing penicillin 500U/ml, streptomycin 500µg/ml and fungizone 20µg/ml, and allowed to leave a few hours

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at room temperature. After washing few times with fresh medium, the skin was cut into pieces of about 1mm^3 and placed on the bottom of plastic petri dish. The explants were cultured at 37°C in 10% fetal calf serum (FCS), penicillin 100U/ml, streptomycin 100µg/ml. Within 2-10 days after adhesion outgrowth of fibroblasts (or fibroblastlike stromal cells) appeared aroung the "mother explant". When the cell population became large enough to perform subculture (it takes about 1-3 weeks), the cells were detached from the substratum by treating with 0.02% EDTA and 0.25% trypsin (1:1). 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

Cells were detached from a culture plate by treating with EDTA -trypsin and resuspended in RPMI medium (pH 8.0) supplemented with 10% FCS. After allowed to recover for 1-2 hours, the cells were collected by centrifugation and resuspended to give the concentration of 1-2x 10^6 cells/ml by the same medium without FCS and used for cytotoxicity assays.

Cytotoxicity Assays

The original microcytotoxicity test (MCT), which is known to be a conventional NIH method for HLA typing of lymphocytes, was applied also for typing of fibroblast in this study except that the pH of RPMI medium was changed to 8.0 in the latter test. Lymphocyte and fibroblast samples were suspended in RPMI medium pH 7.0 and 8.0, respectively. The suspended cell samples of 1ul (1-2x10^3 cells) were placed in wells of a micro test tray (with 60 wells, Osaka Prefectural Hospital local typing tray), in which 1µl of antisera/well was pre-plated and incubated for 30 minutes at room temperature (about 25°C). Then, 5ul of complement for HLA-A,B,C typing was added to each well. After leaving the samples at room temperature for 1 hours, 2ul of 5% eosin solution was added and after 5 minutes,15ul of 37% formalin was added . After a coverslip was placed on the tray, cells were allowed to settle for several hours, and the trays were read using an inverted-phase microscope.

RESULTS

Reactivity of Fibroblast to Anti-HLA Sera

In order to apply established NIH method for HLA typing of lymphocyte to personal identification of a cadaver by utilizing fibroblasts derived from the abdominal skin of the cadaver, we first tested whether or not fibroblasts, as well as lymphocytes, react with HLA anti-sera. Fibloblast cultures were established from abdominal skin and the cells were subjected to

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microcytotoxicity test as described under Materials and Methods. Two examples of the wells, which showed a typical positive(A) and negative(B) reactions, are shown by microscopic photographs in Fig.1.

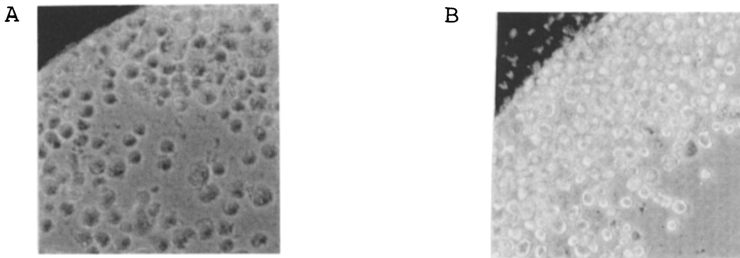

Fig.1 MCT-Positive and -Negative reactions of cultured fibroblast derived from abdominal skin tissue after MCT (A: positive, B:negative).

As shown in above photographs, we can differentiate clearly dead cells and viable cells. Thus according to the percentage of dead cells as a result of the reaction with anti-HLA sera we could score the reactivity as 8(81-100%), 6(41-80%), 4(21-40%), 2(11-20%) and 1(0-10%).

<u>Comparison of HLA Types Determined with Fibroblasts and Lymphocytes</u>

In order to confirm the reliability of HLA typing with fibroblasts,we determined HLA type of fibroblasts and lymphocyte derived from the living body and compared the results. An example of MCT score in positive reactions of cultured fibroblasts and lymphocytes derived from same individual is shown in table 1.

Table 1. MCT positive reactions of fibroblast and lymphocyte from individual No. 1.

		A locus							B locus					C locus									
Specificity	p.c.	A23+24	A23+24	A23+24	A24	A26	A26	A26+W34	BW60+61	BW60+61	BW60+61	BW6	BW6	CW3	CW3	CW3	CW3	CW3	CW3	CW7	CW7	CW7	CW7
Score Fibro.	8	8	8	8	8	8	8	8	8	8	8	6	8	1	1	4	4	6	4	6	1	8	1
Lymph.	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8

The scores of fibroblasts and lymphocytes showed almost complete concordance in A and B locus although the scores of fibroblast in C locus were significantly lower than those of lymphocytes. Thus, by judging the reaction with a score more than 6 as a positive reaction, we determined HLA types of fibroblasts and lymphocytes from 8 control samples (living body)(Table 2).

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Table 2. HLA types determined 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.

The results indicated clearly that the HLA types determined with fibroblasts and lymphocytes were essentially the same although a slight difference, probably due to the reasons as will be discussed below, was observed.

HLA Typing of Cadavers

Since the results described above indicated that HLA typing with the use of fibroblast is reliable, we examined 5 cadavers with different age ranging from 0 to 77 and with different postmortem period ranging from 16 to 34 hours. In all of these cases, HLA typing was successful.

Table 3. HLA types of cadavers tested with cultured fibroblast

Sample No.	Hours after death	Age	HLA Type
881	16	0	A1, W33/ B 16, -/ CW3, -
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, -

DISCUSSION

In the present report we described that HLA typing of a cadaver could be successfully made by using fibroblasts and slightly modifying the conventional NIH-MCT method for HLA typing of lympocytes. The key point of the modification is the process of pretreatment of fibroblasts for 1 to 2 hours in the culture medium adjusted to pH 8.0; this process keep cells in spherical form, and protects them from sticking to the bottom of tray, and, thus makes it possible to apply directly the premade typing tray, which contains very small amount (1ul) of antisera. As

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shown in Table 3, the HLA types determined with fibroblasts show a relatively samll number of differences. This slight discrepancy between the two typing, obvserved in 3 antigens among the total number of 32 antigens in A and B locus and 5 antigens among 16 in C locus, is probably due to new expression or disappearance of an antigen during the culture of the fibroblast. In this respect Tada et al. have obvserved that the class II antigens or a new antigens appeared during the culture of T lymphocyte. Thus, a relatively high rate of discrepancy in HLA types in C lous may reflect a loose control of the gene expression. In spite of the above facts, a high rate of concordance of the HLA typing of A and B locus with the use of fibroblasts and lymphocytes showed that our method well fits the practical use. By this method we could determine HLA types of even a 77 years old cadaver using his skin obtained from autopsy at 34 hours after death in Summer; the longest postmortem period, during which fibroblast culture from the skin is possible, remains to be determined.

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REFERENCES

Bodmer WF (1978) The HLA system; introduction. Br Med Bull 34(3):213-216

Kasiwade H,Hara M (1986) HLA typing from cadavceric organs. Act Crim Japon 52:195-198

Tada M, Hisayama Y, Funatsuki Y, Inoue T, Miyake T, Inoue H HLA-DR,DQ typing with T cells cultured from an aliquot of whole blood. Jpn. J. Transplant. (in press)

Yoshimura K (1982) Studies on the soluble HLA antigens; HLA grouping of caderveric organs. Jpn J Legal Med 36: 710-720