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HLA typing of Epstein-Barr virus transformed lymphoblastoid cell lines (LCL)

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The application of standard tissue typing techniques to cells other than peripheral blood lymphocytes has been accompanied by the problem of extra reactions. This applies as well to Epstein-Barr virus transformed lymphoblastoid cell lines (LCL) as to leukemic cells and human spleen cells. These extra reactions are attributable to additional antibodies in the typing sera which are not apparent under standard conditions with PBLs. Two types are described: Type 1 extras, which become apparent after longer incubation times and are attributed to weak antibodies and type 2 extras which are apparent after shorter incubation times and are attributed to subpopulation specific or differentiation antigens. Technical modifications are proposed by which these extras can be circumvented. They include:

1. Only start typing when cells have been cultured for 2 to 3 days.
2. Remove dead cells by spinning over standard ficoll-hypaque or 11% triosil.
3. Use shorter incubation times.
4. Avoid using sera that give too many type 2 extras.

In this way phenotypes can be accurately identified on LCL's obtained from kidney transplant donors and recipients. When LCL's were compared with their matching PBL, HLA phenotypes were concordant in 87% of cases for HLA-A, 90% for HLA-B, 81% for HLA-C and 70% for HLA-DR.

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Difficulties in interpreting the results of microcytotoxicity tests involving Epstein-Barr virus transformed lymphoblastoid cell lines (LCL) have been reported by others (Crichton et al. 1973, Dick et al. 1972, 1975, Mac kintosh et al. 1973, Pious et al. 1974). Extra reactions were in some cases attributed to viral products (Goldstein et al. 1969, Dick et al. 1975) rather than the natural products of the HLA region. In this work the validity of these and other assumptions was examined and some technical modifications were made which appeared to improve the quality of the tissue types derived.

Abbreviations

LCL	Lymphoblastoid Cell Line
PBL	Peripheral Blood Lymphocyte
LYDMA	Lymphocyte Defined Membrane Antigen
FCS	Foetal Calf Serum
PHA	Phytohaemagglutinin
RPMI-1640	Roswell Park Memorial Institute – 1640 (tissue culture medium)
UKTS	United Kingdom Transplant Service
NIH	National Institutes of Health

Material and methods

Establishment of a cell line

A 10 ml sample of anticoagulated sterile blood was defibrinated, layered onto a sterile Ficoll-Triosil density gradient, spun at 400 g for 20 min and the cells at the interface harvested. The cells were washed once in phosphate buffered saline, resuspended in 1 ml, counted and spun at 400 g for 5 min. The supernatant was removed and 0.3 ml of the virus source added to the cell pellet. The virus source was the supernatant of an aged culture of the marmoset cell line B95.8 which had been filtered through a 0.22 u filter and stored in 0.5 ml aliquots at −80°C. The cell suspension was incubated for 60 min at 37°C, with occasional mixing, spun down at 400 g for 5 min and the supernatant removed. The cells were resuspended in RPMI–1640 plus 10% foetal calf serum and counted. 1 ml containing 10^6 cells and 1 ml of 1% PHA in RPMI–1640 were added to each well of a tissue culture plate. An alternative was to add cyclosporin A at a concentration of 0.5 ug/ml. Both this and PHA prevent T cells from maturing into killer cells which attack the LCL via the virally coded Lymphocyte Defined Membrane Antigen (LYDMA). The cell line was grown for three weeks, feeding as necessary, before transfer to a 25 cm^2 tissue culture flask. The cells were either stored frozen in liquid N_2 or tissue typed directly.

Preparation of viable cells

Frozen-thawed cells were cultured for 2–3 days in RPMI–1640 plus 5% FCS thus ensuring a maximum yield of viable cells of which some were used to replenish stocks.

The cell suspension was layered onto a mixture of 11% triosil in distilled water, spun at 400 g for 15 min and the interface harvested. Cells were washed twice in PBS plus 10% FCS, counted and concentrated to 1 to 1.5 × 10^6 cells per ml.

Microcytotoxicity testing of LCL

A modification of the standard NIH technique was used (Ray 1979). 1 ul of antiserum was incubated with 1 ul of cell suspension in a microtest plate at 22°C. 5 ul of complement was added and the plates incubated at 22°C before staining with 3 ul of eosin and fixing with 8 ul of formalin (37% formaldehyde). The plates were read on an inverted phase-contrast microscope; a positive reaction being scored when the estimated level of cell death was above 30%.

Time-course study

Time-course experiments using LCL's and PBL's from the same donors were conducted. The objective was to determine the optimum incubation times before and after complement addition to give comparable tissue types when using the two different types of cell. The range of times used for HLA-ABC locus typing was 15–30 min pre- and 30–60 min post-complement addition. For HLA-DR locus typing 30–60 min and 60–120 min, respectively, were used.

Comparative phenotyping study of PBL & LCL

All LCL were typed in the UKTS Laboratory. The HLA-A and B types of the peripheral blood lymphocytes (PBL) were obtained from the U.K. Transplant Service (UKTS) records. In most cases, laboratories submitting these data to the UKTS used the same serum-set as used for LCL typing in this laboratory (The National Plate). The HLA-C and HLA-DR types of the PBL were also extracted from the UKTS records, but in some cases these were obtained using a set of reagents, assembled by

the submitting laboratory, which included different sera to those used for typing in this laboratory. However, the majority of reagents were identical.

Complement

Freeze-dried rabbit complement (Buxted Rabbit Co.) previously screened for lymphocytotoxic activity was used for routine tissue typing on PBL and this was found to be satisfactory for LCL.

Cloning

The method used for cloning LCL has been described elsewhere in Doyle et al. (1985).

Results

Viability of cell suspensions

Separating viable cells from dead cells with 11% Triosil had a marked effect in reducing the background attributable to dead cells and debris. Up to 10% of viable cells were lost by this manoeuvre.

Table 1.
Example of the results obtained by tissue typing LCL and PBL from the same donor using different incubation times.*

Incubation times (minutes before + after adding C')	Results obtained with sera with the following major specificities:													
a) HLA-A & B locus typing														
PBL	A1	A1	A1	A2	A2	A2	A9	A28	B12	B12	B27	B27	B27	B21
15+30	2	3	•	7	9	8	•	•	7	7	3	5	7	•
15+60	9	9	8	9	9	9	•	•	8	9	8	9	9	•
30+40	6	6	•	9	9	8	•	•	8	9	8	9	9	•
**30+60	9	9	9	9	9	9	•	•	9	9	9	9	9	•
LCL														
**15+40	8	9	7	9	9	9	•	3	9	9	9	9	9	4
15+60	9	9	9	9	9	9	•	6	9	9	9	9	9	9
30+40	9	9	9	9	9	9	8	3	9	9	9	9	9	8
30+60	9	9	9	9	9	9	5	8	9	8	9	9	9	8
							↑	↑						↑
							1	2						2
b) HLA-DR locus typing														
B cells	DR1	DR2	DR2	DR3	DR3	DR4	DR5	DR5						
**60+120	•	•	•	•	•	•	9	9						
LCL														
**30+60	•	•	•	•	•	9	9	9						
50+120	•	9	•	•	•	9	9	9						
		↑				↑								
		1				2								

* Phenotype of Donor A1, A2; B12, B27; DR5, –.
** Denotes optimum incubation times to give comparable results between LCL and PBL.

1 ↑ type 1 extra reactions.
2 ↑ type 2 extra reactions.

• <10% kill.
1–9 >10% to 100% kill.
x not tested.

Time-course experiment

The results of a typical time-course experiment are summarised in Table 1.

The changes in the incubation times made little difference to the reactivity of the PBLs. However, the LCLs showed extra reactions after prolonged incubation. The A9 and the DR2 antisera gave extra reactions which appeared after extended incubation. These we called 'type 1'. The data indicated that comparability between PBL and LCL was obtained with incubation times of 15 + 40 min for HLA-A,B and C typing and 30 + 60 min for HLA-DR typing. These incubation times were used for all subsequent tests.

Other extra reactions appeared after the shortest incubation times and these are exemplified by the antisera A28, and B21. These were called 'type 2'. In the case of the DR4 serum the extra is presumed to be of type 2, but, since the donors phenotype was DR5, blank, it was conceivable that the DR4 had been missed. However, other DR4 sera were negative.

Comparison of the results obtained with PBL and LCL pairs

Table 2 contains the results of a comparison of tissue types of 150 donors and recipients of kidney transplants. Not all PBL-LCL pairs

Table 2.
Comparison of the results obtained with PBL and LCL derived from renal transplantation donors and recipients.

HLA Locus	A		B		C		DR	
% Concordant (No.):	87%(130)		90%(137)		81%(34)		70%(48)	
% Discordant (No.):	13% (20)		9% (13)		19% (8)		30%(21)	
Cell:	LCL	PBL	LCL	PBL	LCL	PBL	LCL	PBL
Discordant types:	2/28,1	28,1	5(51),17(57)	5,35	2,–	2,1	1,4	4,
	2/28,3	2,3	7,40	7,–	2,7	2,3	1,4	4,–
	2/28,3	2,3	7,35	7,	3,4	4,–	1,4	4,
	2/28,	2,–	8,37	8,–	3,–	5,–	1,6	6,
	2/28,–	2,–	8,16(38)	8,16(39)	3,(2)	3,–	2,5	2,–
	2/28,9(24)	2,–	8,17	8,–	4,(7)	4,–	2,5	2,1
	2/28,9(24)	28,9(24)	8,40	8,–	5,7	5,–	2,6	2,5
	2/28,32	2,32	12(44),–	12(44),13	7,–	6,–	2,(6)	2,
	28,3	2,3	13,47	13,40			3,4	3,–
	2,3	28,36	16(39),35	35,–			3,–	3,6
	2,9(23)	2,–	16(38),35	16(39),35			3,–	3,4
	2,(11)	2,3	17(57),27	17,–			3,6	3,5
	2,	2,28	Bu,	15,			4,7	4,1
	2,19(31)	2,19(33)					4,6	1,
	3,9(24)	2,9(24)					5,7	7,
	1,(11)	1,–					(5),(6)	6,
	1,–	1,3					5,7	7,–
	1,–	1,(30)					6,	5,6
	10(26),19(31)	10,19(33)					6,–	6,1
	19(31,32)	19(32,33)					6,7	2,7
							(6),7	7,

were typed for all four HLA loci. Nevertheless the percentages of concordant and discordant typings at each HLA-locus between the PBL and LCL are listed.

87% of typings were concordant for A, one exception being the difficulty in distinguishing between A28 and A2. 90% of typings were concordant for HLA-B and 81% for HLA-C. HLA-DR typing showed only 70% concordance but most discordant typings were associated with DRw6 or DR5; which under any conditions were difficult to define on normal B cells. The DR discordant results were attributable to the low quality of available antisera.

Virtually all the discordant typings typified our experience of two independant typings on the same lymphoid cells, e.g. cells derived from the same kidney donor but assigned different tissue types in two laboratories.

Extra reactions

When seven of the HLA-A and B sera that gave type 2 extras were analysed against a panel of enriched B cells (Table 3) most were associated with contaminating antibodies directed towards the HLA-DR, DQ and possibly DP products.

Stability of LCL typings

Twelve cloned lines derived from one individual, who carried the phenotype HLA-A2, 28; B14, 35; Cw4, –; DR3, 5, and cultured for up to one and a half years were examined for the comparability of their HLA types. Initially we found that some specificities appeared to be missing in some cloned lines, suggesting a deletion, but on retyping the lines with the same antisera the missing reactions were positive.

Equivocal results of this nature tended to occur only with low titre sera.

Discussion

LCLs provide a permanent ex-vivo copy of the individual's phenotype which can be repeatedly examined as new reagents become available.

Stored LCL gave inconsistent results when typed immediately after thawing, but this was corrected after a period in tissue culture. Removing dead cells and debris by spinning over density gradients reduced background and ensured that preparations gave reproducible and consistent results. The shortening of incubation times and careful selection of sera avoided the anomalous extra reactions, attributed to "contaminating" antibodies.

In our experience modifications in the typing technique were of central importance to obtaining accurate results. Apart from screening the complement for anti-LCL activity (Dick et al. 1975, Bodmer et al. 1975) or diluting the complement (Ferrone et al. 1971, Mackintosh et al. 1973) little variation in technique appears to have been attempted. Most of the difficulties we encountered were attributed to 2 types of extra reactions: type 1 was due to weak antibodies which reacted after prolonged incubation as a consequence of the LCL having more HLA molecules per cell than PBL (Trucco et al. 1980); type 2 were due to antibodies directed towards antigens which were not normally expressed on the cell population used to define the antiserum, namely PBL-T cells, but *were* expressed on the sub-population from which the LCL were derived, namely B cells. In the case of Class I typing sera these turned out to be due to antibodies directed towards DR antigens but in one case an anti-DR4 antiserum gave anomalous reactions which were attributable to an unknown specificity uniquely expressed on the LCL.

No evidence was found to support the view that HLA antigens were spontaneously deleted or that their expression was altered in

Table 3.

a) Patterns of type 2 extras on LCL cells

HLA phenotypes of LCL									Type 2 extras obtained with antisera whose designated HLA specificity was the following:						
A		B		C		DR		DP	A10	A28	A28	A29	A3	B37	B13
24		7				2		2	9	9	●	●	●	3	8
11		35		4		1		2 4	9	9	●	●	●	●	●
2		62		3		1 4		3 4	●	●	9	●	9	●	9
2		44		4		w6 7		2 4	9	9	9	9	9	●	●
1	2	7	57	6	7	5 7		2 4	●	●	9	9	9	●	●
1	2	8	40	2		w6 3		2 3	9	9	●	●	9	8	9
2		62	57	3	6	2 7		4	9	9	9	9	9	8	9
1	2	8	38			3 4		4	●	●	9	●	9	●	9
1	2	8	38			3 4		4	●	●	9	●	9	●	9
2	11	7	40	3	7	2 4		4	9	x	9	●	9	8	x
1	23	8	62	3		4	w6	2 4	9	9	9	●	9	●	9
1	2	51	8	7		3 7		2	●	●	9	9	●	●	9
2	11	18	22	3	1	1 4		3 4	9	9	9	●	9	8	●
Presumed specificity of type 2 extras									DQw1	DQw1	DR4	DR7	DP4?	?	?

b) patterns of type 2 extras on normal B cells

HLA phenotypes of (PBL) B cells									Example of analysis of type 2 extras						
1	2	7	57	6		3 7	–	–	●	●	9	9	9	x	x
1	25	7	38	7		2 4	–	–	9	●	9	9	9	x	x
2	3	7	47	6		1 w6	–	–	9	9	●	●	x	x	x
1	2	8	50	6	7	3 7	–	–	8	●	9	9	●	x	x
1	23	44	37			2 w6			9	●	●	●	●	x	x
Presumed specificity of type 2 extras									DQw1	DQw1	DR4	DR7	DP4?		

● <10% kill.
1–9 >10% to 100% kill.
x not tested.

prolonged tissue culture (Bernoco et al. 1969, Rogentine & Gerter 1969, Reisfeld et al. 1970). This was further supported by the results on the cloned LCL. However, fluctuations in the expression of HLA during the cell-cycle may have accounted for minor changes in the antigen density, but these were only revealed with certain antisera which were rather equivocal in their reaction pattern.

Many of the anomalies of LCL typing are probably attributable to lymphocyte populations carrying different numbers of HLA molecules per cell; the LCL carry ten times more HLA-A, B and C than T cells derived from PBL and six times as many HLA-DR molecules as B cells (Trucco et al. 1980). Similar differences in antigen density and sub-population expression probably account for the extra reactions found on leukemic cells (Simons & Amiel 1977).

Finally it was noted that LCL subjected to prolonged culture faithfully retained and expressed the HLA-ABC and DR phenotypes of the individual from whom they were derived.

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