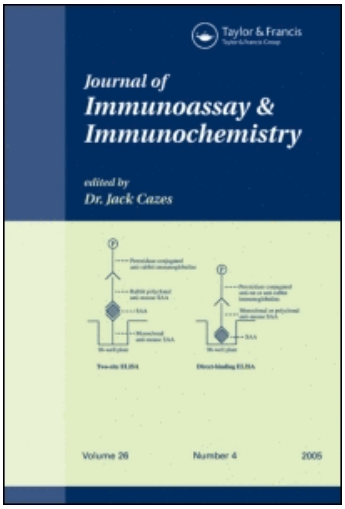

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A SENSITIVE MICRO-PLATELET ELISA TECHNIQUE FOR SCREENING ANTI-HLA ANTIBODIES

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A SENSITIVE MICRO-PLATELET ELISA TECHNIQUE FOR SCREENING ANTI-HLA ANTIBODIES

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ABSTRACT

An enzyme-linked immunosorbant assay (ELISA) in a Terasaki plate (Micro-Platelet ELISA), using 30 000 platelets per well, 2 μL primary antibody (anti HLA antiserum) and 5 μL of secondary antibody (1 : 2000) are described. Platelets from 30 selected HLA tissue typed cell panel individuals were used to characterize anti HLA A and B antibodies. The first half of the Terasaki tray had platelets in sequence to characterize anti HLA antibodies, while the second half contained anti HLA B antibodies. Results revealed that the HLA specificities of the sera identified by micro-platelet ELISA and microlymphocytotoxicity were concordant. Moreover, split antigens of broader specificities were identified in the Platelet ELISA technique. The advantages of micro-platelet

ELISA technique were: (i) it does not require viable/frozen lymphocytes, (ii) reading is very simple and macroscopic, (iii) specificity of the serum is identified with accuracy within the same day, (iv) avoids inter-cell, inter-day variations, (v) complement is not required, (vi) requires only 1/50 volume of the reagents required by conventional ELISA in microtitre plates, and (vii) using platelets isolated from 5 mL of peripheral blood, fifteen thousand sera can be tested. This technique is, thus, very simple, cost effective, and very much suitable for any developing HLA laboratory, which is in the process of developing indigenous HLA reagents.

INTRODUCTION

Microlymphocytotoxicity assay is the routine serological method employed to identify anti HLA antibodies in a tissue typing laboratory (1); however, this requires viable/frozen lymphocytes, complement, and reading under inverted phase contrast microscope, and a technical hand per excellence. Another technique ''Complement fixation'' typing has also been employed in HLA tissue typing and antibody screening (2); but, it is less sensitive and more cumbersome than the microlymphocytotoxicity assay. An ELISA technique for detection of antibodies to HLA antigens and platelet specific antigens has been employed in various clinical laboratories worldwide.(3–6) HLA Class I antigens are known to be expressed on platelet membranes.(7,8) ELISA in a microtitre plate has been employed to screen culture supernatants in hybridoma technology.(9)

We describe, here, an ELISA technique in a 60-well Terasaki plate which needs less antigen per well, few microlitres of antiserum and reagents per well, and long shelf life, making the HLA antibody screening procedure simple, cost effective, and robust.

EXPERIMENTAL

Platelets

Five microlitre of peripheral blood from each panel blood group ''O'' donor was obtained in ACD solution. The panel cells were tissue typed and characterized in the III Asia-Oceania Histocompatibility workshop (10) and Eleventh International Histocompatibility Workshop.(11) Platelets were isolated by slow centrifugation (at 200 g for 10′) and the buffy coat obtained

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was washed twice in saline (0.15 N) and ACD (4 : 1) by centrifugation (1600 g for 30′). Platelets were counted in a haemocytometer, washed finally in PBS (pH 7.2), and resuspended to a concentration of 15 000 cells/μL, sodium azide (0.01%) was added as a preservative. The suspension was stored at 4°C or −20°C in 0.5 mL aliquots.

Coating Antigens to Microtitre Plates

An indirect ELISA method, described by Voller et al.(12) was adopted. In short, 30 000 platelets in 2 μL of PBS were dispensed to respective wells and allowed to air dry for half an hour at room temperature (RT 25°C) in front of an air blower: fifty such trays were prepared and stored at −20°C, either prior to blocking or after blocking. Blocking was performed by adding 20 μL of 2.5% BSA (Sigma Chemicals, St. Louis, MO, Cat No. A4503) in PBS (pH 7.2) per well and incubating it for 1 h at RT. Blocking with BSA in PBS alone is known to reduce nonspecific binding. At the end of incubation, the trays were emptied by tapping on a thick pad of filter paper fitting into the Terasaki plate, washed once with PBS-Tween (Tween 20, Sigma Cat No. P1379) and stored.

ELISA Assay

An antigen coated microplate was wet once by filling with PBS-Tween and flicked thoroughly. 2 μL of serum in question was added to each well of the tray using a Hamilton syringe PB 600 repeater dispenser and 50 μL syringe. The tray was incubated at RT for 30–40 min in a humid chamber. At the end of incubation, wells were washed once with PBS-Tween and 5 μL of secondary antibody (goat anti human IgG-HRP conjugated, (Dakopats, Denmark, P214) 1 : 2000 dilution in 1% BSA PBS Tween) was dispensed to each well using Terasaki 6 needle dispenser (Hamilton, Switzerland Cat No. 705208545; 1083.4798) and incubated for 45 min at 37°C in a humid chamber. At the end of incubation, the tray was washed thrice with PBS Tween and 5 μL substrate OPD-H_2O_2 (10 mg orthophenaline diamine) (Sigma Cat No. P1526) dissolved in 6.25 mL of Phosphate citrate buffer, pH 5.0 + 10 μL of H_2O_2 (Qualigens India, Ltd.) per well was added and incubated in the dark for 30 min at room temperature. At the end of reaction, 1 μL of 5 N H_2SO_4 (Qualigens India, Ltd.) was added to each well to stop the reaction. The intensity of color developed was read macroscopically by keeping the tray on a light box and a blue filter

(470 nm) and in a fully automated Patimed system (Leitz, Germany) with a 496 nm filter.

RESULTS

Format for Identifying Anti HLA A and Anti HLA B Antisera

Figure 1 presents the sequence of cells and antigens plated in the first half of the Terasaki plate (Grenier & Sohne GmbH, Germany Cat No. 653160) used for identifying anti HLA antisera. The lower half of the tray (Figure 2) presents the sequence of cells to identify anti HLA B antisera. Note that it was the same 30 cells which has been arranged to give either a pattern to identify HLA A or HLA B locus antigens; thus, each cell was tested in duplicate.

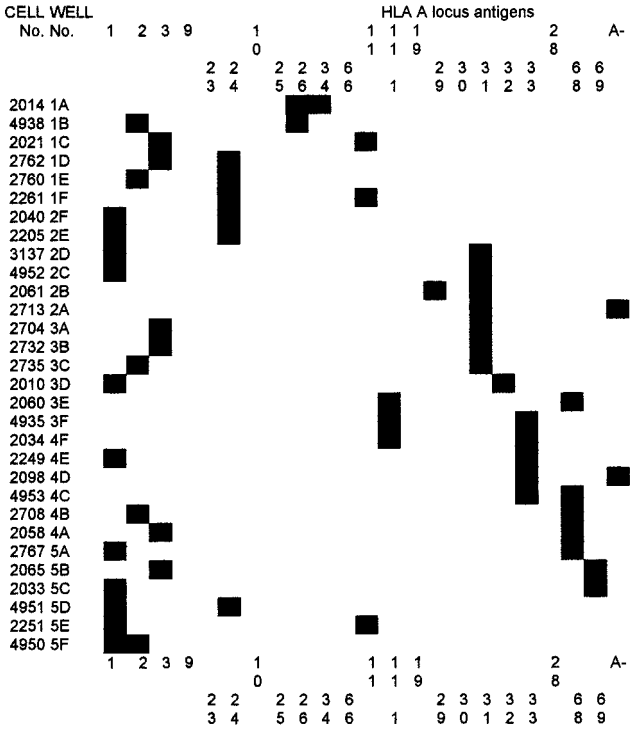

Figure 1. Sequence of the cells and their HLA A antigens used to identify anti-HLA A antibodies.

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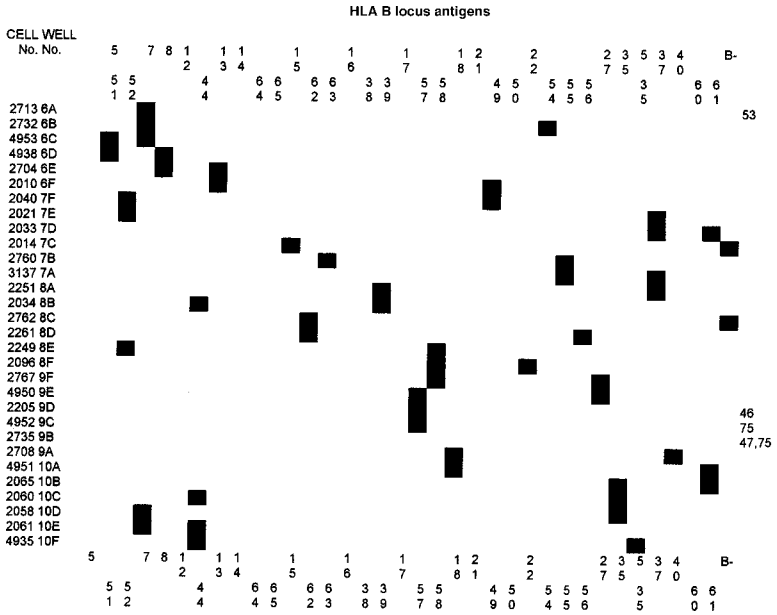

Figure 2. Sequence of the cells and their HLA B antigens used to identify anti-HLA B antibodies.

Results Obtained by Microlymphocytotoxicity and Microplatelet ELISA Assays

The results presented in Table 1 revealed that *r* values and HLA specificities of the antisera evaluated by the micro-platelet ELISA technique were the same as, or better than, those obtained by the microlymphocytotoxicity test. This can be attributed to better control of experiments, avoiding inter-day, inter-cell variations. Further split antigens of broader specificities were identified unambiguously by this micro-platelet ELISA technique.

The availability of the data on various split antigens in the same tray made it easier to identify these splits. For example, it was possible to compare the reactions of a serum to HLA B57 and HLA B58 cells in the same tray. This resolved a serum 1104: Manga originally identified as HLA B17 in the cytotoxicity assay, as HLA B57. Two other sera (6707: Santh and 3132: Amutha) have been assigned both B57 and B58. Absence of inter-day variations in micro-platelet ELISA that is normally encountered in conventional methods of identifying serum antibodies might be responsible for an unambiguous assignment of splits and antigens.

Table 1. Correlation of Specificity Test Results Obtained in Antisera for HLA A Locus and HLA B Locus Alleles by Microplatelet ELISA and Microlymphocytotoxicity Assays

Serum Identity	HLA Specificities		
r Value	Cytotoxicity	*r* Value	ELISA
HLA A locus			
1526: Elilrani	A3	0.70 A3	0.85
1626: Devi	A1, 31	0.86 A1	0.80
3838: Saroja	A1 (10)	1.00 A1	0.64
2403: Jeya	A1 (10)	0.86 A1, 10	0.90
CRI2: Laliben	A10	A26	1.00
1844: Renuka	A11	0.99 A11, 1	0.90
3231: Bhanu*	A11	0.85 A11	0.80
2831: Chand*	A11, 26, 1	0.90 A11, 1 (26)	0.90
2226: Eswari	A2	1.00 A2	1.00
4096: Neela	A2	1.00 A2	0.86
6458: Sabi	A2	1.00 A2	0.70
0697: Pappa	A28, 2, B35, 15	0.50 A2, 69 (68, 32)	0.78
1102: Sasikala	A2, 28, 19, B37	0.74 A2, 68, 19	0.80
6270: Sumathi	A2, 68, 69	0.89 A2, 69	0.82
3984: Radha	A24	0.90 A24	1.00
2916: Leela	A24	0.70 A24	0.98
1668: Renuka	A3	A31 (3)	1.00
4170: Jessie	A31, B18	1.00 A32	0.86
6614: Esther	A19	A32 (19)	0.90
1360: Shant*	A19, 28 (2)	0.70 A32, 68, 2	0.85
1633: Bhuvanes	A19, 28, B37	0.80 A33	0.90
HLA B locus			
6378: Asaipon	B35, 75, 53	B51 (35)	0.82
6739: Vasuki	B52 (75, 53)	0.73 B51, 52, 53	0.94
2174: Ponnu	B51, 52, 53	1.00 B51, 52, 53	1.00
1679: Variram	B51, 52, 53 (35)	0.80 B51, 52, 53 (35)	0.90
4114: Dhana	B51, 52, 53, 35, 5.35	0.90 B51, 52, 53, 5.35	1.00
3914: Pari	B51, 52, 53, 5.35	0.83 B51, 52, 53, 5.35	1.00
2531: Usha	B51, 52, 5.35	0.90 B51, 52, 5.35	0.95
6565: Revathi	B37, 52	0.68 B51, 52	0.65
6386: Esther	B35, 5, 75, 53	0.78 B51, 75, 35	1.00
0156: Rakku	B35, 70, 71, 72, 75	0.90 B35, 75	1.00

(*continued*)

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Table 1. Continued

Serum Identity	HLA Specificities		
r Value	Cytotoxicity	*r* Value	ELISA
1800: Raji	B35, 62, 49, 53	0.85 B35, 63, 51	0.95
0163: Pancha*	B35, 51, 75	0.80 B35, 63, 51	0.95
2621: Uthami	B35	0.74 B35	0.85
2104: Gurwith	B35 (5, 53)	0.70 B35	0.94
1925: Pappa	B35 (75)	0.60 B35, 51	0.78
1739: Jaya	B5, 62	0.74 B62	1.00
3814: Vadivu	B15 (62, 63, 75)	0.80 B62, 63, 75	1.00
3260: Sasi	B37 (52, 62)	0.80 B37	0.80
6712: Shant	B44	0.87 B44	1.00
2021: Vijaya	B12	1.00 B44	1.00
0152: Kamatchi	B12	0.97 B44	1.00
1104: Mangayar	B17	B57	1.00
6707: Shanthi	B17	B57, 58	1.00
3132: Amutha*	B17 (57)	1.00 B57, 58	1.00
0532: Rathi	B13, 61, 49	0.85 B13, 61	0.98
6549: Anath	B13, 61	0.92 B13, 61	1.00
2737: Jeya	B61 (7)	0.93 B61, 7	1.00
UND: Dedia	B22	B56	0.98
0550: Krishna	B22	0.70 B55, 56	1.00
2889: Chand	B7, 40 (22)	1.00 B55, 56, 7, 40	0.95
1217: Bavani	B40, 22, 13, 27	B61, 55, 56, 13	0.90
3791: Prema	B7, 27	1.00 B7, 27	1.00
3144: Clara*	B7	0.90 B7	1.00
3485:	B7	0.80 B7	1.00
4160: Manga	B7	1.00 B7	1.00

*These HLA typing reagents were tested in 11th International Histocompatibility Workshop and Conference prescreening exercise.

Advantages of Micro-platelet ELISA Technique

Table 2 presents a comparison and advantages of micro-platelet ELISA assay over microlymphocytotoxicity and conventional ELISA. It is evident, from the table, that:

(i) from one mL of peripheral blood, more sera (around 2500) can be screened by micro-platelet ELISA assays than the micro-lymphocytotoxicity test (250).

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Table 2. Advantages of Micro-platelet ELISA Over Microlymphocytotoxicity and Conventional ELISA Assay

Reagents	Microlympho-cytotoxicity	ELISA	
		Micro-platelet	Conventional
Harvest/mL (10^6)	1*	100#	1
Number of assays	250	2666	20
Requirements per well cells	4000	30 000	250 000
Complement	5	–	–
Primary antibody	1 or 2	2	50
Secondary antibody	–	5	50
Substrate	–	5	100
Stop solution	–	1	50
Cost per assay (US$) complement	1.65	–	–
Secondary antibody	–	0.0075	0.075

*Lymphocytes.
#Platelets.

(ii) Though the primary antibody used per well is the same as the microlymphocytotoxicity assay, it is very low when compared to conventional ELISA (20–30 μL).
(iii) Only 5 μL of secondary antibody per well is required for this technique, compared to 50 μL in conventional ELISA.
(iv) The requirements of BSA, OPD, H_2O_2, and other wash solutions are much less compared to conventional ELISA.
(v) Rabbit complement is not required (it costs US$1 to US$2 per tray in microlymphocytotoxicity assay).
(vi) Costs of other reagents are also less: (it costs around US$0.1 for secondary antibody and other reagents in conventional ELISA and US$0.01 in microplatelet ELISA).

In this analysis, we have not taken into consideration other reagents, such as lymphoprep used for cell separation and other solutions, which were common for all the techniques. A microscope was also not required to read the test results. The results can be read macroscopically by keeping the tray on a light box and a blue filter (470 nm) or with an ELISA reader, which is readily available in most blood banks and microbiology laboratories in any country of the world.

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DISCUSSION

ELISA assay has the potential to detect HLA antibodies with increased efficiency, without sacrificing sensitivity or specificity using HLA Class I molecule purified from pooled platelet.(13) Lubenko and Rodi (6) detected non-complement fixing HLA antibodies in lymphocytotoxicity negative serum using commercial ELISA kits. A similar approach to ours, but employing cytotoxicity assay, has been described previously: a panel of EBV transformed cell lines dotted and frozen in Terasaki trays were used for identifying anti HLA antibodies. This is currently commercially available (Biotest, Germany; One Lambda, USA). In the above technique one has to maintain the cell lines, deep-freeze the predotted trays, and one requires complement as well. This technique is, thus, cost intensive compared to the micro-platelet ELISA test described in this paper.

The technique described here obviates these requirements, it is cost effective, and can be used to predict the transplant outcome in clinical situations. Christiaans et al.(14) comparing ELISA and complement dependant cytotoxicity for both Class I and Class II antibodies among renal allograft patients, before and after transplantation, for rejection-free graft survival. They found that the rejection was higher if post transplant serum was ELISA negative/cytotoxicity positive, rather than ELISA positive/cytotoxicity negative.

An added advantage of micro-platelet ELISA technique is the possibility of reconfirmation of the HLA antigen specificity assigned the next day itself. During the course of routine HLA antibody screening, if 100 sera were screened in a week's time, using 100 micro-platelet ELISA trays, one would get 3–5 very good anti-HLA antisera. These 3–5 sera can be reconfirmed the next day itself using another 3–5 micro-platelet ELISA trays. In conventional microlympho-cytotoxicity assay, one has to procure the panel lymphocytes and test the serum once again with all the cells, or use cell line. Both these methods are cumbersome and costlier than micro-platelet ELISA. Though there is a remote possibility that antibodies against specific platelet allo-antigens and antiphospholipid antibodies may cross-react in this assay system. However, we did not encounter this problem in our study.

The complement fixation technique described by Colombani et al.(2) though it is akin to the present test, it depends on an indicator system and sensitivity of the assay is less than ELISA: it is capable of identifying only high titer sera. Our observations revealed that the micro-platelet ELISA technique is more sensitive than the microlymphocytotoxicity assay. An HLA antiserum, which was identified as HLA A26 in the cytotoxicity assay, lost its potency in due course; the same was assigned HLA 26 once

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again by micro-platelet ELISA. Moreover, the split antigens (HLA B57) of the broad antigen (B17) was also identified in the micro-platelet ELISA. Thus, the micro-platelet ELISA technique is simple, cost-effective, and is suitable for identifying specific anti HLA antibodies.

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