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HLA Class I Typing by One-Dimensional Isoelectric Focusing and Identification of the New Variants in Thai Population.

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SUMMARY **One-dimensional isoelectric focusing (1D-IEF) is the technique to define HLA class I antigens based on difference in isoelectric point of HLA molecules. Different IEF subtypes are shown in different populations. In this study, 1D-IEF was employed to study HLA-A and -B subtypes in Thai population. A panel of 117 samples including all serologically defined HLA-A and -B antigens in Thai population were typed by 1D-IEF. Serological specificities and subtypes correlated well with IEF results and some antigens with unclear serological specificities could be confirmed by IEF. In addition, more subtypes could be obtained by IEF than by serology. A total of 17 IEF subtypes from HLA-A and 31 IEF subtypes from HLA-B could be identified. The subtypes predominantly found in Thai population were A2.3, A24.2, A11.1, A33.2, B15.2, B7.1 and B13.1. In addition, new IEF variants were identified in HLA-B35, B5, B56 and B48. The band positions of these variants were different from those previously described. These IEF subtypes are HLA gene products which may be important in transplantation. The combination of IEF and serology for HLA typing can provide a better definition of each allelic product of HLA-A and-B.**

The major histocompatibility complex is the most polymorphic genetic system in human, composed of HLA class I and class II. The MHC class I antigens (HLA-A,-B,-C) are expressed on the surface of all nucleated cells and function as restricting elements in the presentation of peptides to cytotoxic T lymphocytes and are the major barrier for allogenic tissue transplantation. Accurate determination of HLA subtypes is necessary to prevent graft rejection.[1] HLA class I antigens are usually characterized by complement-dependent microlymphocytotoxicity technique, however, the accuracy and sensitivity of this technique depends on the sera used.[2] Biochemically, HLA class I antigens are glycoproteins of 45 kilodaltons (kDa) heavy chain which are associated with β_2-microglobulin (light chain) of 12 kDa molecular weight.[3] One-dimensional isoelectric focusing (1D-IEF) has been applied to define class I polymorphic subtypes, *ie* gene products that are biochemically distinct. This technique has been standardized since the Tenth International Histocompatibility Workshop (IHW)[4] and has been applied to type HLA-class I antigens in many populations.[5] Different IEF subtypes have been shown in different populations, however, very limited information is available in Thai population. In the present study HLA class I subtypes were identified by their IEF patterns in Thai population and compared with the previously identified IEF banding patterns of known HLA class I subtypes. Several new variants of HLA subtypes were identified by 1D-IEF in this study. Many of these variants may correlate with T-cell and monoclonal antibody-defined specificity and

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may be important in transplantation.

MATERIALS AND METHODS

Cells

A panel of EBV-transformed cell lines previously typed serologically by classical complement dependent microlymphocytotoxicity assay from 117 individuals which included all known serologically defined HLA-A,B antigens in Thai population were studied. Cell lines with known IEF subtypes obtained from the 10th IHW were included as controls.

1D-IEF

The 1D-IEF was carried out according to the protocol from the 12th IHW. In brief, 3 x 10^6 Epstein Barr virus transformed cells (B-LCL) were metabolically labelled for 4 hours with 50 µCi ^{35}S-methionine in 1 ml methionine free RPMI medium. The cells were lysed in 1 ml lysis buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA and 0.5% Nonidet P-40. HLA-A and B antigens were isolated from the cell lysates by sequential immunoprecipitation with the monoclonal antibody 4E6 (precipitate all B antigens as well as A19 splits) followed by W6/327 (precipitate class I HLA antigens), kindly provided by Dr. SY Yang, NY, USA and *Staphylococcus aureus* protein A (Sigma). Precipitates were washed and treated with neuraminidase type VIII (Sigma) overnight at 37°C. The samples were eluted with a denaturing IEF buffer and were electrophoresed overnight in IEF gel at 1000 V. The gels were treated in DMSO and dried under vacuum (Labconco gel dryer) prior to autoradiography at -70°C overnight.

Interpretation of banding patterns

For biochemical typing, the bands were identified by their isoelectric points according to the data published for the 10th IHW.[5] The designation of each band was made according to the biochemistry component published in the IHW proceedings.[8]

RESULTS

HLA-A

In this study, 15 HLA-A serotypes were studied including HLA-A1, HLA-A2 and -A2sh, HLA-A3, HLA-A24, HLA-A10 (A26, A34 and A66), HLA-A11 (A11.1 and A11.2), HLA-A19 (A30, A31, A32, A33 and A74) (Table 1). Of 15 HLA-A serotypes included in this study, a single corresponding IEF band could be identified for HLA-A1, -A66, -A31,-A32 and -A74. In addition, two IEF banding patterns could be identified for HLA -A2, -A11, -A24 and -A3 (Fig. 1). The HLA-A2.2 and A2.3 were the only 2 subtypes detected in this panel although 5 IEF subtypes were known for this allele. These two subtypes occurred in 76.6% and 23.4% of 64 Thai HLA-A2 panel. Furthermore, all of serological A2sh corresponded to A2.3 by IEF. A11.1 and A11.2 which corresponded to serological A11.2 and A11.1, respectively, occured in 18% and 82% of 56 Thai HLA-A11 panel. Two IEF subtypes, designated A24.1 and A24.2 occurred in 60% and 40% of 30 Thai HLA-A24 panel. Although several IEF patterns of HLA-A26, -A30 and -A33 could be identified, only one IEF subtype were detected in this study. HLA-A10 antigen(A26, A34, A66) which is difficult to be assigned by serology can be confirmed by IEF (Fig. 2).

Table 1. Serology compared with IEF for defining HLA-A antigens (N = 117)

Antigen			
Serology		IEF	N
A1		A1	7
A2		A2.1	0
		A2.2	49
	A2sh	A2.3	15
		A2.4	0
		A2.5	0
A3		A3.1	1
		A3.2	3
A9	A24	A24.1	18
		A24.2	12
A10	A26	A26.1	4
		A26.2	0
	A34	A34.1	1
		A34.2	0
	A66	A66	1
A11	A11.2	A11.1	10
	A11.1	A11.2	46
A19	A30	A30.1	0
		A30.2	0
		A30.3	6
	A31	A31	4
	A32	A32	1
	A33	A33.1	0
		A33.2	20
	A74	A74	2

HLA-B

27 HLA-B serotypes were studied including HLA-B5 (B51 and B52), HLA-B7, HLA-B8, HLA-B44, HLA-B13, HLA-B15 (B63, B77, B75, B76, B62, and B15AOH), HLA-B16 (B38 and B39), HLA-B17 (B57 and B58), HLA-B18, HLA-B22 (B54, B55 and B56), HLA-B27, HLA-B35, HLA-B37, HLA-B40 (B60 and B61), HLA-B46 and HLA-B48 (Table 2). Of 27 HLA-B serotypes included in this study, a sin-

Table 2. Serology compared with IEF for defining HLA-B antigens (N = 117)

Antigen				Antigen			
Serology		IEF	N	Serology		IEF	N
B5 :	B51	B51	7	B22 :	B54	B22.1	1
		B51v	1		B55	B22.3	2
	B52	B52	4			B55v	3
B7		B7.1	8		B56	B22.2	1
		B7.2	2			B56v	3
B8		B8	1	B27		B27.1	0
B44		B44.1	14			B27.2	0
		B44.2	0			B27.3	0
B13		B13.1	7			B27.4	0
		B13.2	3			B27.5	4
B15 :	B63	B15.1	1			B27.6	1
	B77	B15.2	7	B35		B35.1	0
	B75	B15.2	17			B35.2	0
	B76	B15.3	3			B35.3	9
	B62	B15.3	6			B35v	4
	B62(15AOH)	B15.4	2	B37		B37	4
B16 :	B38	B16.1	9	B40 :	B60	B60	11
	B39	B16.2	5		B61	B61	6
		B16.3	0	B46		B46	31
B17 :	B57	B17.2	3	B48		B48	4
	B58	B17.2	5			B48v	4
B18		B18.1	4				
		B18.2	2				

gle corresponding IEF band could be identified for HLA-B8, -B37, -B46, -B60, -B61 and -B54. For HLA-B7, -B27, -B13 and -B18, two IEF subtypes were found in this study with B7.1, B27.5, B13.1 and B18.1, being the more common subtypes. Although several IEF patterns could be identified, only one IEF subtype were detected in HLA-B44 and -B17.

Interestingly, new variants were found for HLA-B35, -B5, -B15, -B55, -B56 and -B48. For HLA-B35, in addition to the known B35.3, a variant (B35v) was identified. The band of B35v focused below B35.3 indicated that it was more acidic than B35.3 (Fig. 1). For HLA-B5, a new band position which is different from those of known B51 and B52 was

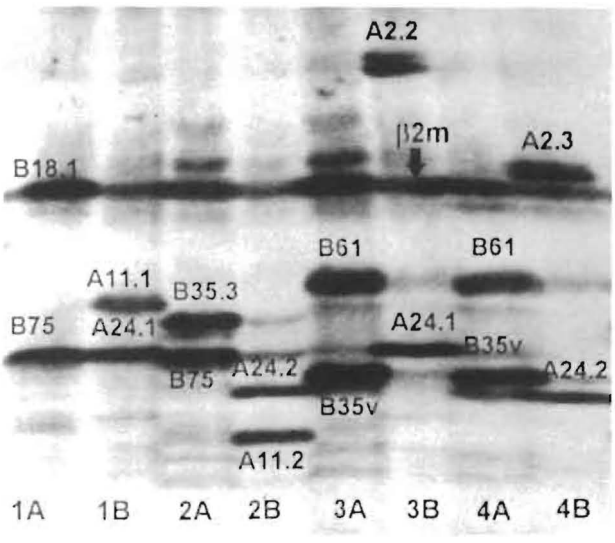

Fig. 1 IEF band patterns of HLA-A11 (A11.1, A11.2) (lanes 1B and 2B), HLA- A24 (A24.1, A24.2) (lanes 1B, 3B, 2B and 4B), HLA-A2 (A2.2, A2.3) (lanes 3B and 4B), HLA-B35 (B35.3, B35v)(lanes 2A, 3A and 4A).
A = precipitated with mAb 4E (precipiate HLA-B); B = precipitated with mAb w6/32 (precipitated HLA-A and -B).

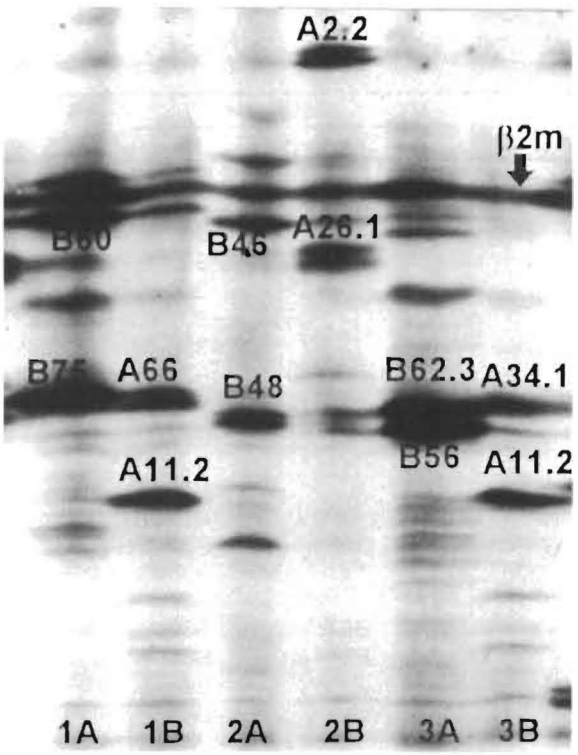

Fig. 2 IEF band patterns of HLA-A10 (A66, A26.1) (lanes 1B, 2B and 3B). A = precipitated with mAb 4E (precipiate HLA-B); B = precipitated with mAb w6/32 (precipitated HLA-A and -B).

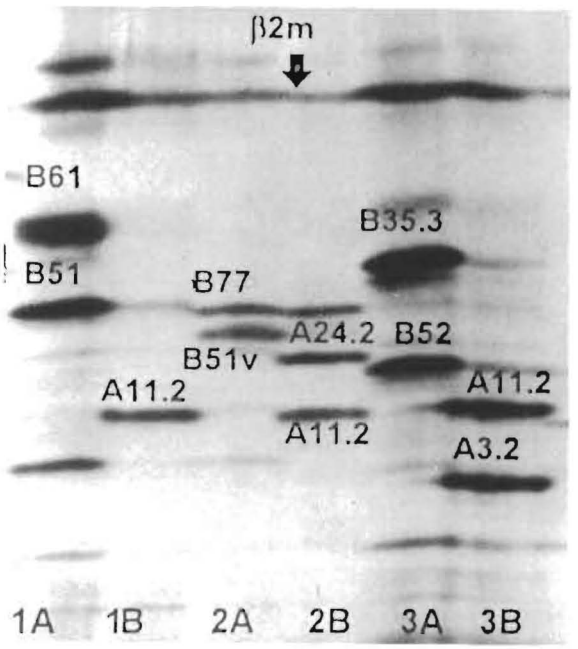

Fig. 3 IEF band patterns of HLA-B5 (B51, B51v, B52) (lanes 1A , 2A and 3A).
A = precipitated with mAb 4E (precipiate HLA-B); B = precipitated with mAb w6/32 (precipitated HLA-A and -B).

identified (Fig. 3). For HLA-B48, in addition to the known band of B48, a variant (B48v) was identified. The band position of B48v focused at the same position as B16.2 (Fig. 4).

The HLA-B22 group composed of B54, B55 and B56. In the 10th IHW, IEF subtypes B22.1, B22.3 and B22.2 was found to correlate with serological specificity B54, B56 and B55, respectively. In this study we found a variant of B56 (B56v) which migrate to the same position as B22.1 which is the position of B54 as previously described. A variant of B55 was also identified in this study. B55 generally focused at position 22.3, but in this study a B55 variant (B55v) focused at position 22.2 (Fig. 5).

For HLA-B15 group which represents one of the most diverse groups of HLA antigen in many populations including Thai, 36 cell lines were studied. A common B62 subtype, B15.3 (also called B62.3) comigrated with B76 in IEF, whereas B75 and B77 focused at the same position slightly more basic than B15.3. B15AOH showed the band position which focused more acidic than the common B62.3. This position was recently called B15.4 from the 12th workshop (Fig. 6).

DISCUSSION

One-dimensional isoelectric focusing (1D-IEF) is the technique to analyze class I gene products based on the net charge or isoelectric point of the respective heavy chains. In contrast to DNA technique which detect the presence of genes, serology and 1D-IEF detect gene products which may be of relevance in transplanta-

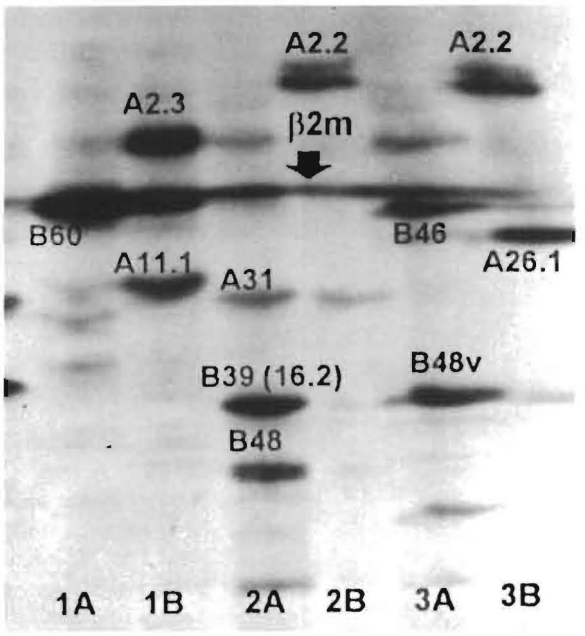

Fig. 4 IEF band patterns of HLA-B48 (B48, B48v) (lanes 2A and 3A).
A = precipitated with mAb 4E (precipiate HLA-B); B = precipitated with mAb w6/32 (precipitated HLA-A and -B).

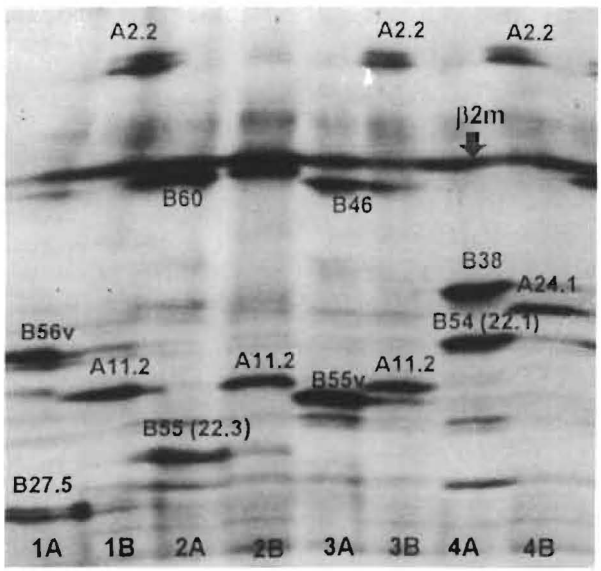

Fig. 5 IEF band patterns of HLA-B22 (B56v, B55, B55v, B54) (lanes 1a, 2A and 3A)
A = precipitated with mAb 4E (precipiate HLA-B); B = precipitated with mAb w6/32 (precipitated HLA-A and -B).

tion. Although this technique has been standardized since the 10th IHW, most of the studies were performed in Caucasians[5,9] In Japanese studies, many variants were found, which were different from those identified in Caucasians.[10] In this study, we could identify IEF banding patterns for most of the serologically defined HLA-A and HLA-B antigens in thai population. The IEF banding patterns were in good agreement with the IEF patterns previously described in the Workshop.[5]

Some specificities such as HLA-A10, HLA-B5 which were difficult to be assigned by serology, could be confirmed well by IEF. Common IEF subtypes, A2.2, A24.1, A11.2, A26.1, B17.2, B15.3, B16.1, B16.2, B27.5, B35.3 and B44.1 which were found in Caucasians, were also found frequently in Thai. In contrast, the subtypes A2.3, A11.1, A24.2, A33.2, B15.2, B7.1 and B13.1 which were relatively rare in Caucasians, were found more frequent in Thai. Interestingly, A-30.3, a common HLA-A in Caucasians, which was not previously observed in Orientals was found to be the only subtype of A30 in Thai.

New variants could be identified from serologically defined HLA-B5, B35, B22 and B48. The band positions of these variants were different from those previously described. As the serological subtypes of some of these antigens could not be well defined by available serum, other method such as sequencing is needed to confirm whether these variants are rare alleles or new alleles in Thai population. Although the biological relevance of the existence of HLA

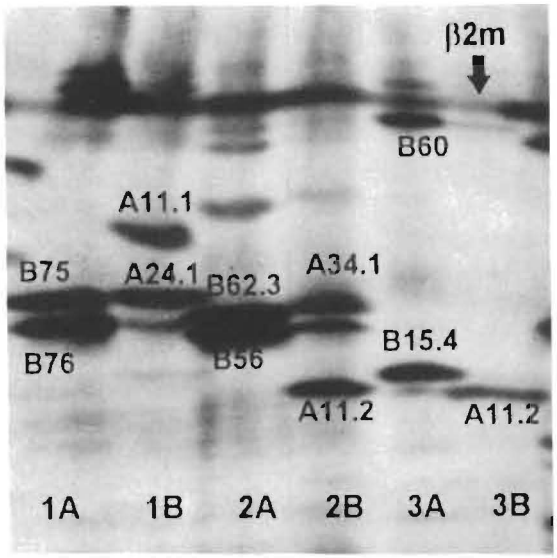

Fig. 6 IEF band patterns of HLA-B15 (B75, B76, B62.3, B15.4) (laned 1A 2A and 3A).
A = precipitated with mAb 4E (precipiate HLA-B); B = precipitated with mAb w6/32 (precipitated HLA-A and -B).

class I subtypes is not clear, CTL responses restricted by HLA class I subtypes molecules have been demonstrated.[11] Therefore, better defined HLA subtypes might be important for clinical transplantation.

The one dimensional isoelectric focusing is useful to resolve difficult serological typing and identify new variants. This technique has been used to study HLA-A and -B in the IHW. Samples with one serologic HLA-A or HLA-B could be confirmed to be homozygous or heterozygous. Moreover, more HLA-A and -B subtypes could be obtained by IEF than serology. However, while IEF is effective in detecting variants or subtypes, this method has its own disadvantage. The technique is rather cumbersome and the interpretation needs the serologic specificity because some HLA molecules have similar isoelectric points and may be difficult to be distinguished by 1D-IEF. In addition, faint secondary bands frequently occur that can complicate gel interpretation. These bands may belong to HLA-C antigens or non classical HLA class I antigens.[5]

In conclusion, 1D-IEF was employed to evaluate HLA-A and -B subtypes in Thai polulation. This method was able to identify all corresponding HLA serotypes studied. More detailed subgroups could be further identified by IEF as compared to the serological method. This method is a powerful tool to identify new variants of HLA alleles. In this study, new variants of HLA subtypes were identified in Thai population. A combination of serology and IEF can provide a better definition of each allelic product of HLA-A and-B.

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