CHAPTER III

MATERIALS AND METHODS

3.1 Subjects

A total of 391 blood samples were collected randomly from 153 healthy Khon Muang who lived in Chiang Mai, Chiang Rai, Phayao, Phrae, Nan, Lampang, Lamphun and Mae Hong Son, which were areas previously known as Lan Na; 125 Khon Yawng from the Muang district of Lamphun and 113 Karen from the Lee district of Lamphun. The ethnicity of the subjects' parents was carefully identified. All the subjects in this study did not report admixture outside their ethnic groups for over 3 generations at least.

3.2 Specimen Collection and Preparation

3.2.1 Ten ml of blood was drawn into a 2 ml ACD tube.

3.2.2 Tubes were mixed well. The specimen was kept at room temperature at all times prior to lymphocyte isolation. Blood was not refrigerated. For a good result, blood was used within 24 hours.

3.2.3 Lithium heparin was not recommended as an anticoagulant.

3.2.4 One to two ml of blood was used to prepare PBMC for the lymphocytotoxicity test and 8-10 ml for DNA preparation.

3.3 HLA Typing Tray Preparation

3.3.1 Sixty well Terasaki typing trays were used.

3.3.2 Each well was topped with liquid paraffin oil at 5 μl/well.

3.3.3 One μl of specific human antiserum was put into each well for HLA-A2, A9, A11.1 and A11.2. A list of anti-sera used is shown in Table 3.1.

3.3.4 Each tray also contained a positive and negative control.

3.3.5 Trays were stored at –80°C for use within 1 year.

3.3.6 Trays were thawed at room temperature for 10 minutes and used within 30 minutes of thawing, and not refrozen.

3.4 Complement Collection

3.4.1 Ten ml of blood was drawn into a 15 ml conical tube from a new lot of rabbits. Each lot contained 4-8 rabbits. Each rabbit had been collected carefully and separately.

3.4.2 Clotted blood was always kept in an icebox during the collection of other samples.

3.4.3 Blood was centrifuged at 2,000 × g for 10 minutes at 4°C.

3.4.4 Serum was collected and then transferred to a new 15 ml conical tube and a 1.5 ml tube, which was used for complement testing. Care was taken when labeling.

3.4.5 If the serum was contaminated with red blood cells, the specimen was centrifuged again.

3.4.6 Rabbit serum was kept at –80°C prior to testing.

Table 3.1 Specificity of HLA anti-sera

Specificity	Lot number
Anti-HLA-A2	TP4693.37
	TP4296.37
	TP4415.37
	TP4255.37
	TP2943.37
	S1475.2
	TP1344.37 sh-
Anti-A9	TP3800.37
	TP3227.36
Anti-A11.1	TP822.38
Anti-A11.2	TP12026.33
	TP1594.34
	TP3067.37
Anti-A11.1+Anti-A11.2	TP6732.37
	TP7834.2
Anti-A2+Anti-A28	S1771.2
Positive control serum	DCH TP5039.37
Negative control serum	S675.38

3.5 Complement Titration

3.5.1 The serial two-fold dilution of the positive and negative HLA antiserum was prepared in PBS from 1:2 to 1:8.

3.5.2 The concentration of PBMC was prepared to 2,000 cells/μl.

3.5.3 One μl of each dilution was put into each well of the Terasaki typing trays, topped with 5 μl of paraffin oil and allowed to react with 1 μl PBMC.

3.5.4 The serial two-fold dilution of the complement from rabbit serum was prepared in PBS from 1:2 to 1:16.

3.5.5 The activity of each complement dilution was tested with the mixture of antiserum and PBMC, with a viability > 90%.

3.5.6 The score on form was recorded according to the American Society for Histocompatibility and Immunogenetics (ASHI) reading standard (1995)

3.5.7 Acceptable limits were scored at 8 from undiluted antiserum to a 1:2 dilution, and a scored of 6 at a1:4 dilution, while the negative antiserum remained negative.

3.5.8 Each acceptable complement was pooled together with 1 ml of aliquot into 1.5 ml tubes.

3.5.9 The complement was kept at –80°C until used.

3.5.10 The complement was thawed to room temperature in 10 minutes, used within 30 minutes of thawing, and refrozen only once, as was acceptable.

3.6 Isolation of PBMC

3.6.1 One ml of fresh ACD blood was diluted with an equal volume of PBS.

3.6.2 Diluted blood was carefully layered over the Ficoll-Hypaque using approximately twice as much diluted blood in 15 ml conical tubes.

3.6.3 Layered blood was centrifuged at 700 × g for 20 minutes at room temperature without using brake on the centrifuge.

3.6.4 Peripheral blood mononuclear cells (PBMC) were collected at the interface of the Ficoll-Hypaque and diluted plasma, and then transferred to the new 15 ml conical tube.

3.6.5 Washed twice in 10 ml of PBS and centrifuged at 700 × g for 5 minutes at room temperature.

3.6.6 The supernatant was decanted carefully and discarded.

3.6.7 The pellet was resuspended in 0.5 ml of PBS. The cells were counted and checked for viability and purity.

3.7 Evaluation of Lymphocytes for Purity and Cell Concentration

3.7.1 The lymphocyte was examined on a hemacytometer with a microscope.

3.7.2 The number of cells counted over the 25 block area at the center was, therefore, the number in a 0.1 mm^3 volume.

3.7.3 If the cells were contaminated with platelets or red blood cells, the following process was carried out:

a. Platelet contamination.

Washed in 10 ml of PBS and centrifuged at 700 × g for 5 minutes at room temperature.

b. Red cell contamination.

One ml of ddH_2O was added into the cell suspension and mixed several times for 10 seconds. Ten ml of PBS was added quickly and centrifuged at 700 × g for 5 minutes. The supernatant was removed and the pellets resuspended in 0.5 ml of PBS.

3.7.4 If purity was acceptable, the concentration was adjusted to achieve a final lymphocyte concentration of 2,000 cells /µl.

3.8 Elimination of RBC by using Dextran

3.8.1 A ratio of 1:5 of 5% dextran in PBS was added to the blood.

3.8.2 The mixture was mixed well and incubated at 37°C for 30 minutes at an angle of 45°.

3.8.3 The Rouleaux formation of the red blood cells occurred and settled down.

3.8.4 The leukocyte-rich plasma was collected for further lymphocyte isolation by Ficoll-Hypaque gradient centrifugation.

3.9 HLA Class I (ABC) Typing

3.9.1 The lymphocyte suspension was adjusted to 2,000 cells /µl.

3.9.2 The typing trays were removed from the –80°C freezer, 10 minutes before testing.

3.9.3 The typing trays were placed on a light box and the empty well was checked.

3.9.4 The repeating dispenser was rinsed 8-10 times with PBS before the cells were added into the typing trays. It was necessary to repeat dispenser rinsing before the addition of new cells.

3.9.5 One μl of cell suspension was added into each well by using 80 μl of repeating dispenser. Carry over was avoided by not touching the antiserum with the needle tip.

3.9.6 The cells and antiserum were mixed with a fine wire. Care was taken when using the wire for mixing with other wells.

3.9.7 The cells and antiserum were incubated at room temperature for 30 minutes.

3.9.8 The complement was thawed to room temperature 30 minutes before it was needed, mixed well and placed immediately in 4°C.

3.9.9 Five μl of the complement was added using a 250 μl repeating dispenser in each well.

3.9.10 The trays were incubated at room temperature for 1 hour.

3.9.11 Five μl of 5% eosin in PBS were added to each well.

3.9.12 The dye was allowed to penetrate the dead cells for 2-3 minutes.

3.9.13 Eight to ten μl of 37 % formaldehyde (pH 7.2) was added to each well and mixed if necessary.

3.9.14 The cells were allowed to settle overnight.

3.9.15 Two ml of tap water was added carefully to the edge of each tray.

3.9.16 Two glass slides were lowered slowly until they rested on top of the wells. A formation of air bubbles was avoided.

3.9.17 The trays were left for 30 minutes to allow the cells to settle completely.

3.10 Reading the Tray

3.10.1 Each well was examined by using an inverted phase contrast microscope with 10X objective and 10X eyepieces. Living cells were small and refractile, while dead ones were large and dark stained.

3.10.2 The percentage of viable lymphocytes was established in the negative and positive control wells as the baseline to distinguish the killing of certain lymphocytes in the test wells.

3.10.3 Results were recorded as score 1, 2, 4, 6, 8 and 0 according to the American Society for Histocompatibility and Immunogenetics (ASHI) reading standard. The results were analyzed for HLA-A2, 9, 11.1 and 11.2 according to the reaction, as shown in Table 3.2.

The American Society for Histocompatibility and Immunogenetics (ASHI) reading standard (1995)

Score	% Dead Cells	Interpretation
1	0-10	Negative
2	11-20	Doubtful Negative
4	21-50	Weak Positive
6	51-80	Positive
8	81-100	Strong Positive
0		Not readable

Table 3.2 Serological reaction patterns of HLA-A2, A9, A11.1 and A11.2

Serum ID	Antibody specificity	HLA-A2	HLA-A9	HLA-A11.1	HLA-A11.2
TP4693.37	A2	+	-	-	-
TP4296.37	A2	+	-	-	-
TP4415.37	A2	+	-	-	-
TP4255.37	A2	+	-	-	-
TP2943.37	A2	+	-	-	-
S1475.2	A2	+	-	-	-
TP1344.37 sh-	A2	+	-	-	-
S1771.2	A2, 28	+	-	-	-
TP3800.37	A9	-	+	-	-
TP3227.36	A9	-	+	-	-
TP822.38	A11.1	-	-	+	-
TP12026.33	A11.2	-	-	-	+
TP1594.34	A11.2	-	-	-	+
TP3067.37	A11.2	-	-	-	+
TP6732.37	A11.1, 11.2	-	-	+	+
TP7834.2	A11.1, 11.2	-	-	+	+

Standard microlymphocytotoxicity test according to ASHI standard (1995).

\+ = Scores range from 4 to 8

\- = Scores range from 1 to 2

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3.11 Red Blood Cell Lysis

3.11.1 Eight to ten ml of ACD whole blood was centrifuged at 2,000 × g for 10 minutes at room temperature.

3.11.2 The upper phase of plasma was removed and the buffy coat was collected before transferring to the new sterile 15 ml conical tube by using an automatic pipette.

3.11.3 Ten ml of RCLB was added to the buffy coat, and mixed well by inverting several times for 2 minutes.

3.11.4 This mixture was then centrifuged at 700 × g for 10 minutes at room temperature.

3.11.5 The supernatant was decanted, and the pellets were resuspended by using a vortex until they had been broken.

3.11.6 Steps 3.11.3 to 3.11.5 were repeated until all the RBC were lysed.

3.11.7 Finally, the supernatant was decanted as much as possible.

3.11.8 The pellets were vortexed to prevent clumping.

3.11.9 If the pellets had not been performed on at that time, they should have been kept at –20 °C until use.

3.12 DNA Extraction

3.12.1 The pellet size was approximated and the appropriate volume of reagents added, as listed in the following.

3.12.2 Proteinase K was added and the sample vortexed.

3.12.3 ddH_2O was added and the sample vortexed.

3.12.4 Ten percent of SDS was added and mixed into the sample gently by inverting the tube.

Pellet size	10-25 μl	25-50 μl	50-100 μl
Proteinase K	12 μl	20 μl	40 μl
ddH_2O	300 μl	400 μl	800 μl
10 % SDS	105 μl	150 μl	300 μl
7.5 M Guanidine HCl	105 μl	150 μl	300 μl
		Ethanol Precipitation	
99.5 % ethanol	1 ml	2 ml	4 ml
Labeled conical tube	15 ml	15 ml	15 ml

3.12.5 Added 7.5 M Guanidine HCl and mixed into the sample gently by rocking the tube back and forth.

3.12.6 The samples were incubated at 68-70°C for 10 minutes.

3.12.7 After the 10 minutes, the sample was mixed vigorously using transfer pipettes until the mixture became homogeneous. Every attempt was made not to create air bubbles while mixing with pipettes.

3.12.8 The samples were incubated at 68-70°C for an additional 5 minutes.

3.12.9 After the additional 5 minutes, the samples were spun at 2,000 × g for 10 minutes at 4°C.

3.12.10 The supernatant was transferred to a 1.5 ml Eppendrof tube.

3.12.11 The samples were incubated at 68-70°C for 5 minutes.

3.12.12 The samples were spun at 10,000 × g for 5 minutes at 4°C.

3.12.13 a. The next step was continued providing the pellet was compact and the supernatant clear and free of debris.

b. Steps 3.12.11 and 3.12.12 were repeated if the pellet was diffused and the supernatant was cloudy.

3.12.14 The supernatant was transferred to a 15 ml conical tube by using an automatic pipette.

3.12.15 The appropriate volume of 99.5 % ethanol was added slowly to maintain the interface between the two phases.

3.12.16 The tube was rocked gently back and forth for a maximum 2 minutes until cotton like strands of DNA appeared.

3.12.17 The samples were vortexed to tighten the pellets. The DNA was transferred to another labeled 1.5 ml Eppendrof tube by drawing 300 µl of DNA ethanol.

3.12.18 Seven hundred µl of 99.5 % ethanol was added to the samples and spun at 10,000 × g for 2 minutes before discarding the alcohol supernatant by pouring.

3.12.19 One ml of 80 % ethanol was added to the samples and vortexed to loosen the pellet and let the sample stand for 1 minute.

3.12.20 The samples were spun at 10,000 × g for 2 minutes. Then as much of the supernatant as possible was discarded by using a pipette tip.

3.12.21 The samples were incubated at 68-70 °C for 3 minutes with the cap open to evaporate the ethanol.

3.12.22 One hundred µl of ddH_2O was added and incubated for 2 minutes if the samples were viscous. The tube was capped and the samples vortexed gently. This procedure was continued until a smooth, syrup-like consistency was achieved.

3.12.23 The samples were incubated at 37°C overnight to dissolve completely.

3.13 Quantitation of DNA

3.13.1 Spectrophotometric determination of the amount of DNA

a. Ten μl of genomic DNA was diluted in 990 μl of ddH_2O.

b. The DNA was read at a wavelength of 260 nm and 280 nm by using the spectrophotometer UV/Vis.

c. The concentration of nucleic acid in the sample was calculated at an OD of 260 nm followed by the formular below:

$$\text{DNA concentration} = OD_{260} \times 5,000 \ \mu g/ml.$$

d. The purity of the nucleic acid was estimated from the ratio between the reading at OD 260 nm and 280 nm (OD_{260}/OD_{280}).

e. In samples contaminated with protein, the OD_{260}/OD_{280} was significantly less than the values of 1.8.

Remark : An OD of 1 corresponds to approximately 50 μg/ml for double-stranded DNA.

3.13.2 Minigel method

a. Five μl of the DNA sample were mixed with 3 μl of gel-loading buffer containing bromphenol blue and loaded into a slot of 1 % agarose minigel in 1X TBE that contained ethidium bromide (0.5 μg/ml).

b. Five μl of standard DNA solution (250-300 ng/μl) were mixed with 3 μl of gel-loading buffer and loaded into the gel wells.

c. Electrophoresis was carried out in the electrophoresis chamber containing 1X TBE buffer in a condition of 100 volts for 30 minutes or until the bromphenol blue had migrated by approximately 1-2 cm.

d. The DNA ran from a negative to positive charge.

e. The gel was photographed by using a short-wavelength ultraviolet illumination of gel documentation. The unknown DNA was compared to the standard intensity of fluorescence and the quantity of DNA in the sample was estimated.

3.14 DNA Storage

The DNA could be stored at 2 to 8°C for daily use, or at –15 to –20°C for several years.

3.15 PCR Phototyping Protocol for HLA-A11 Subtyping (Dr.H.A.F.Stephens, University College London)

In the amplification of genomic DNA on exon 2 and 3, the PCR was used to amplify HLA-A11 subtypes from HLA-A11 that was serologically typed by standard NIH techniques using anti-HLA antisera (kindly provided by Prof. Dasnayanee Chandanayingyong, Faculty of Medicine, Siriraj Hospital, Mahidol University). The primers used in this method were provided by Dr. H.A.F. Stephens, and are shown in Table 3.3 and Table 3.4.

3.15.1 Stock primer combinations

a. Stock primer combinations (4.0 μM) of the primer mix 1, 2, 3, 4, 5, 6, and 7 were diluted as working solution, modified from Bunce et al. (1995) and Stephens HAF. (personal communication), as shown in Table 3.5.
b. A 1.0 μM mix of primer 63 and 64 should also be made as a positive internal control.

Table 3.3 Phototyping for HLA-A11 subtypes: Sense Primers

Sense primer number	Locus and annealing position	Primer sequence
63	PCR internal control DRB1 Ex3, 519-537	5' TGCCAAGTGGAGCACCCAA 3'
290	HLA-A Ex2, 264-282	5' ACGGAATGTGAAGGCCCAG 3'
367	HLA-A Ex2, 249-268	5' TACTACAACCAGAGCGAGGA 3'
1102	HLA-A Ex2, 111-127	5' CCGGCCCGGCCGCGGGA 3'

Table 3.4 Phototyping for HLA-A11 subtypes: Anti-sense Primers

Anti-sense primer number	Locus and annealing position	Primer sequence
64	PCR internal control DRB1 Ex4 579-598	5' GCATCTTGCTCTGTGCAGAT 3'
167	HLA-A Ex3, 559-576	5' GAGCCACTCCACGCACCG 3'
290	HLA-A Ex2, 264-282	5' CTGGGCCTTCACATTCCGT 3'
303	HLA-A Ex3, 527-544	5' CTCTCTGCTGCTCCGCCG 3'
394	HLA-A Ex3, 20-39	5' CCACGTCGCAGCCATACATT 3'
1103	HLA-A Ex3, 527-544	5' CTCTCTGCTGCTCCGCCT 3'
1104	HLA-A Ex3, 559-576	5' GAGCCACTCCACGCACGT 3'
1105	HLA-A Ex3, 502-519	5' CGCCTCCCACTTGCGCTC 3'

Table 3.5 Phototyping for HLA-A11 subtypes: primer mix composition

Lane number	Primer mix number	Sense primer	Sense primer concentration (μM)	Anti-sense primer	Anti-sense primer concentration (μM)
1	1	1102	1.0	290	2.0
2	2	290	1.0	167	1.0
3	3	290	1.0	303	2.0
4	4	290	1.0	1103	1.0
5	5	290	1.0	1104	1.0
6	6	290	1.0	1105	1.0
7	7	367	2.0	394	1.0
8	8	63	1.0	64	1.0

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3.15.2 DNA reaction mixed

ddH_2O	9	μl
10X PCR buffer	8	μl
200 mM dNTPs	8	μl
1.0 μM 63&64 primers	9	μl
25 mM $MgCl_2$	3.5	μl
300 ng/μl DNA	2	μl
500 unit Taq DNA polymerase	0.5	μl
Total volume	40	μl

3.15.3 DNA amplification

a. A reaction test was prepared in a 500 μl thin wall tube.

b. Five μl of the stock primer mix were used with 5.0 μl of the DNA reaction mix.

c. Five μl of ddH_2O were added to the 5.0 μl of DNA reaction mix, as a negative control.

d. The reaction mix was mixed and spun down.

e. Twenty μl of mineral oil was dropped into the reaction tubes.

f. The cap was closed firmly.

g. The reaction was spun down again.

h. The PCR was performed in a thermocycler, followed by the HLA class I thermal cycling conditions, as shown below:

Prog. 1. Hold 96°C, 60 secs.

Prog. 2. 5 cycles of 96°C, 25 secs.; 70°C, 45 secs.; 72°C, 30 secs.

Prog. 3. 20 cycles of 96°C, 25 secs.; 65°C, 45 secs.; 72°C, 30 secs.

Prog. 4. 5 cycles of 96°C, 25 secs.; 55°C, 60 secs.; 72°C, 120 secs.

Prog. 5 Hold 4°C.

i. The samples were stored at 4°C until use.

3.15.4 Analysis of amplified PCR products

a. Three μl of gel loading buffer were added to the PCR product of each sample.
b. The samples were electrophoresed in 2 % agarose gel made up with 1X TBE buffer containing 0.5 μg /ml ethidium bromide.
c. PhiX 174 DNA digested by Hae III was used as an electrophoresis marker.
d. The gel ran for 30 minutes at 100 volts in a 1X TBE buffer.
e. The amplified product was visualized by using ultraviolet illumination.
f. The gel was photographed by using gel documentation.

3.16 Analysis of HLA-A11 Subtypes

The amplified product in each primer mix was analyzed for HLA-A11 subtypes according to the reaction, as given in Table 3.6 and Table 3.7.

3.17 Statistical Analysis

The number of HLA-A2, 9, 11.1 and 11.2 antigens were estimated by direct counting, and serotyped gene frequencies (GF) were calculated using the following

Table 3.6 HLA alleles recognized by HLA-A11 Phototyping

Primer mix	HLA antigens	HLA alleles	Amplicon size
1	A11.2	A*1102	170
2	A11,25,66	A*1101,*1102,*1103,*1105,*2502, *6601	554
3	A11	A*1101,*1102,*1104,*1105	522
4	A11,3,66,34,25	A*1103,*0301,*0303,*6601,*6602, *3401,*3402,*2502	522
5	A11,3,29,30,3402, 68,69	A*1104,*0301,*0302,*0303,*2901, *2902,*3001,*3401,*3402,*68011, *68012,*6802,*6902	554
6	A11	A*1105	497
7	A1,11,36,80,3402	A*01,*11,*3601,*8001,*3402	300
8	DR	DRB1*	796

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Table 3.7 The reaction pattern of HLA-A11 subtypes

Primer mix	**1 (1102+290') Exon 2**	**2 (290+167') Exons 2-3**	**3 (290+303') Exons 2-3**	**4 (290+1103') Exons2-3**	**5 (290+1104') Exons 2-3**	**6 (290+1105') Exons 2-3**	**7 (367+394') Exons 2-3**
Amplicon size	170bp	554bp	522bp	522bp	554bp	497bp	300bp
HLA-*1101	-	+	+	-	-	-	+
*1102	+	+	+	-	-	-	+
*1103	-	+	-	+	-	-	+
*1104	-	-	+	-	+	-	+
*1105	-	+	+	-	-	+	+

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formular (Svejgaard et al., 1979): GF = $(1 - \sqrt{1-f}) \times 100$; where f is the frequency of the corresponding antigen.

Allele frequencies (AF) were calculated using the following formular (Chandanayingyong et al., 1994): AF (%) = (the sum of the allele/2n) x 100; where n is the sum of the total number of individuals analyzed.

The HLA-A2, 9, 11.1 and 11.2 gene frequencies in each ethnic group were compared with other ethnics previously reported for significant difference using the STATISTIX computer software package. If one or more of the expected entries in the 2 $\times$2 table are less than five, the Yates' correction is used in the Chi Square ($\chi 2$) test. The $\chi 2$ level of significance was set at less than 0.05.

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