Journal of Clinical Laboratory Analysis 11:369–373 (1997)

Two Color Analysis of HLA-B27 Antigen by Flow Cytometer—A Comparative Study by Conventional Microlymphocytoxicity, DNA Genotyping Polymerase Chain Reaction and Flow Cytometric Measurement

Su-Jen Chou,[1] Ning-Sheng Lai,[2]* Jen-Pey Su,[1] Jia-Lin Wu,[1] and Joung-Liang Lan[1,3]

[1]*Division of Allergy, Immunology and Rheumatology, Department of Medicine, Taichung Veterans General Hospital, Taichung, Taiwan*

[2]*Division of Allergy, Immunology and Rheumatology, Department of Medicine, ChiaYi Veterans Hospital, ChiaYi, Taiwan*

[3]*Co-responder, Director of Division of Allergy, Immunology and Rheumatology, Taichung Veterans General Hospital*

For evaluation of specificity and sensitivity of flowcytometric determination of HLA-B27 antigen, we determined the HLA-B27 on lymphocytes using HLA-B27 monoclonal antibody by flow cytometer. Data were compared to those by conventional Terasaki microlymphocytoxicity test and DNA genotyping Polymerase Chain Reaction (PCR) method. One hundred and ninety four patients with various forms of arthritis were included in this study. Forty one of them were HLA-B27 positive, confirmed by three methods concomitantly with complete accordance. None of serological B27 negative, B7 CREG positive cells were found to be flowcytometric fluorescence positive. Furthermore, there was no significant difference of B27 intensity between different B27 DNA subtypes, nor was there any difference between primary ankylosing spondylitis (AS) and other secondary spondylitis patients as measured by mean channel of fluorescence. It is suggested that flowcytometric measurement of HLA-B27 antigen is a rapid and reliable method for HLA-B27 determination. J. Clin. Lab. Anal. 11:369–373, 1997.

Key words: flow cytometry; polymerase chain reaction; microlymphocytoxicity; ankylosing spondylitis; HLA-B27

INTRODUCTION

Ankylosing spondylitis is believed to be an autoimmune disease with major involvement of axial joints. In 1973, it was found to be strongly associated with HLA-B27 class I antigen (1, 2). Subsequently, this association has been confirmed in many studies and found to extend to all major ethnic groups, including the Chinese. The B27 association with AS was close to 95% and may thus be of clinical use in the differential diagnosis of patients with low back pain (3).

HLA-B27 antigen may be assigned by a variety of methods, e.g., the conventional microlymphocytoxicity method using the Terasaki plate (4), soluble HLA antigen (5), and also by hybridizing PCR-amplified DNA with B27-specific oligonucleotide probe (PCR-SSO) *or by a group-specific PCR amplification with a sequence-specific primer pair (PCR-SSP) (6,7,8).* All of these are time-consuming or are considered to be inappropriate for the purpose of single B27 locus determination. Recent developments in the determination of HLA-B27 antigen using direct two-color fluorescence measured by flow cytometer was stated to be a rapid technique in the determination of HLA-B27 expression (9). In the present study, we estimated the specificity and sensitivity of this method by comparing the data of B27 antigen expression by conventional microlymphocytoxicity, DNA genotyping and flow cytometer simultaneously. Besides, cross-reaction in flowcytometric measurement between B27 and other B7 CREG antigen (B7, Bw22, Bw54, Bw55, Bw56, B40, Bw60, B13, and Bw39) and potential as a clinical tool in differentiating primary AS and secondary spondylitis were also evaluated.

*Correspondence to: Ning-Sheng Lai, MD, Ph.D., Division of Allergy, Immunology and Rheumatology, Department of Medicine, ChiaYi Veterans Hospital, City of ChiaYi, Taiwan.

Received 21 March 1997; accepted 16 April 1997

MATERIALS AND METHODS

Subjects

One hundred and ninety-four volunteers were included in this study. Patients were selected from the rheumatology outpatient clinic at the Veterans General Hospital, Taichung. It included 35 AS patients, 12 psoriatic arthropathy, 2 juvenile chronic arthritis, 21 Reiter's disease, and 124 other arthritic patients. Patients included in this study were arranged to receive HLA-B27 antigen determination by three different methods, i.e., conventional Terasaki microlymphocytoxicity test, DNA genotyping PCR method and the recently developed flowcytometric measurement using HLA-B27 monoclonal antibody. Twenty normal controls possessing HLA-B27 type serologically positive were also included for comparison.

Terasaki Microlymphocytoxicity Assay

Peripheral blood was obtained by veinopuncture and collected in heparin-containing tubes. Two grams of carbonyl iron was added to 8 ml blood, tubes rotated at 37°C for 15 min, and then centrifuged at 400g for 10 min at room temperature (RT) to remove granulocytes and macrophages first. After degranulocyte, buffy coat layer was aspirated and diluted with equal volume of McCoy's medium, overlayered onto 1.5 ml of Ficoll-Hypaque (D=1.077, Pharmacia) and centrifuged at 1450 rpm for 17 min. Mononuclear cells were obtained, washed and resuspended with McCoy's medium in a Fisher tube. Lymphocytes were further purified by adding 0.8 ml of LymphokwikTHMN (One Lambda, Los Angeles, California, U.S.A.). Tubes were put at 37°C in a water bath for 15 min with occasional mixing by inverting capped tube. A layer of 0.2 ml of McCoy's medium was poured over the cells and further centrifuged at 200g for another 2 min. The tubes were removed, the floating layer and supernatant were discarded, the remaining cells were washed 3 times with PBS and resuspended with medium. Cell number was adjusted to 2×10^6/ml for further use. After thawing the One Lambda Terasaki HLA-A.B.C. oriental typing tray (One Lambda, Los Angeles, California, U.S.A.) from –70°C freezer, the prepared lymphocytes were injected to HLA-A.B.C. typing tray (1 µl/well) with Lambda Jet. Cells were mixed well with electrostatic mixer. After incubation for 30 min at RT, 5 µl of HLA-A.B.C. rabbit complement was added with Lambda Jet and the trays further incubated at RT for 60 min. Five microliter of 5% Eosin-Y were added and let in RT for 3 min, after which 5 µl of formalin (pH 7.2) was added for cell fixation for another 10 min. The trays were covered with a 43×70 mm coverglass and then the tray was read under an inverted phase contrast microscope.

DNA Genotyping of HLA-B27 by PCR

PCR

Genomic DNA was extracted form the peripheral leukocytes. Briefly, leukocytes were incubated overnight with 3 ml of lysing buffer containing ProteinaseK (2 gm/ml) and 1% SDS at 60°C. DNA was then harvested by extraction with phenol-chloroform and ethanol precipitation. The extract was redissolved in 300 µl of TE buffer for further use. Molecular typing of HLA-B27 alleles was performed by a modified *PCR-SSP* technique as described by Dominguez O (7). Briefly, 10 ng of genomic DNA per µl containing 0.8 µM of each primer (E90s and E136as, designed to amplify codon 91-136 of exon 3 from HLA-B27 alleles) were amplified by PCR procedures for 30 cycles using DNA polymerase (6). β-actin (primers OR-1, OR-2, 0.1 µM) was included as internal control in the exon 3 amplification. The final reaction mixture (50 µl) contained 10 mM Tris-hydrochloride (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.1% (w/v) gelatin, 31 µM deoxynucleotide triphosphate, and 2.5 unit of Taq DNA polymerase. The cycle reaction consisted of a denature step at 95°C one minute, annealing at 65°C one minute and extension at 70°C one minute with an initial denature (10 min at 95°C) and the last extension step (10 min, 70°C). The Sequence (5´-3´) in E91s, E136as, OR-1, OR-2 were GGGTCTCACACCCTCCAGAAT, CGGCGGTCCAGGAGCT, AGGCACTCTTCCAGCGTTCC, TCTTTGCGGATGTCCACGTCA, respectively. It was stated that primers described above were rather sensitive in detecting B27 expression (6).

Oligonucleotide typing

The SSOs probes CL-2, CL-3, CL-5, CL-7, and pan-B27 were selected (7). One µl of PCR products were denatured (NaOH) and immobilized to Zeta probe membranes (BioRad, Richmond, CA). Filters were prehybridized at 65°C 30 min in 2× SET (0.3 M NaCl, 1 mM EDTA, 20 mM TrisHCl pH 8.5), 2% SDS, 5× Denhardt's solution and hybridized with ^{32}P-end labeled SSOs in the same solution at 10^6 cpm/ml for 1 hr. Twenty four-mer SSOs were washed in TMAC solution (3 M TMAC, 5 mM EDTA, 20 mM TrisHCl, pH 8.5, 1% SDS), whereas the other oligos were washed in 2× SET (pH 8.5), 1% SDS.

Two-Color Direct Immunofluorescence for Detection of HLA-B27 Antigen

Fifty microlitres of whole blood from an EDTA containing tube (Becton Dickinson, San Jose, CA, U.S.A.) was added into the bottom of each 12×75 mm plastic tube (Falcon). Thirty microlitres of two color direct HLA-B27 fluorescein isothiocyanate (FITC)/CD3 phycoerythin (PE) conjugated monoclonal antibodies (Becton Dickinson) were added, mixed thoroughly, and incubated for 15 min at RT in the dark. RBC cells were then lysed with 2 ml of FACS lysing solution (Becton Dickinson). After resuspension with PBS, cells were fixed with 0.25 ml of 1% paraformaldehyde. The cells were analyzed on a FACScan flow cytometer (Becton Dickinson) with HLA-B27 software.

Statistical Analysis

Comparison of results were made and the statistical significance between groups were examined by using ANOVA and non-paired T test.

RESULTS

Comparison of Results of HLA-B27 Antigen by Three Different Methods

One hundred and ninety-four arthritic patients were included in this study. HLA-B27 antigen was determined by flowcytometric measurement using HLA-B27 monoclonal antibody (Fig. 1), DNA genotyping by PCR (Fig. 2) and conventional microlymphocytoxicity test. Forty-one patients were HLA-B27 antigen positive and the other 153 subjects showed antigen negative (Table 1). It was found that all serology-typed B27 positive patients were also positive as measured by a flowcytometer. None of the B27 serology negative patients were flowcytometric positive. Data within 3 groups were completely accordant (Table 1).

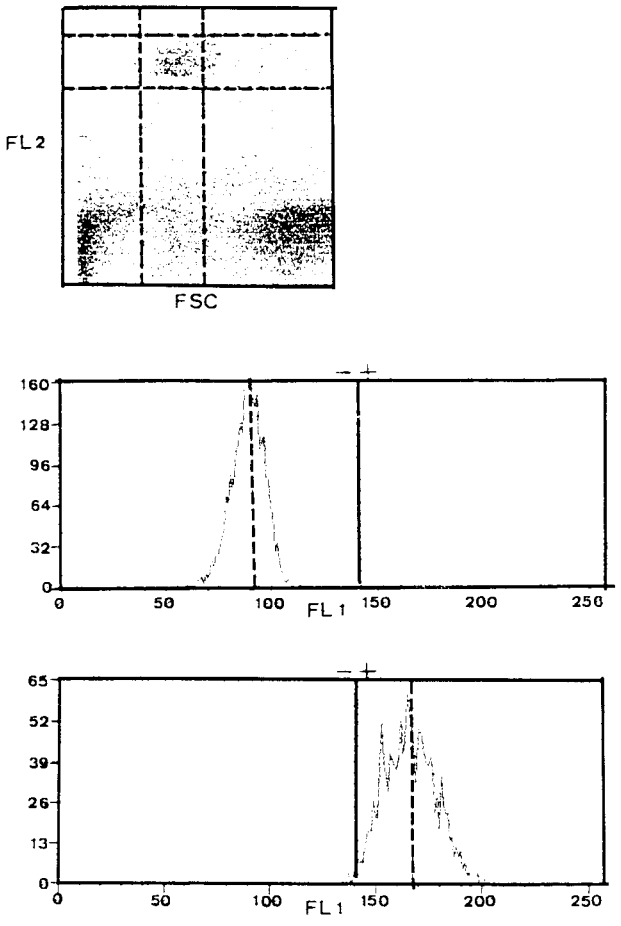

Fig. 1. Flowcytometric measurement of HLA-B27 antigen on T lymphocytes. Double fluorescence FITC-B27/PE-CD3 conjugated monoclonal antibodies were used for cell staining and was analyzed by FACscan cell sorter. T cells were gated (upper) and the green fluorescence intensity analyzed on a histogram. Threshold was set by HLA-B27 software (Becton-Dickinson) for positive (lower) and negative (middle) antigen expression.

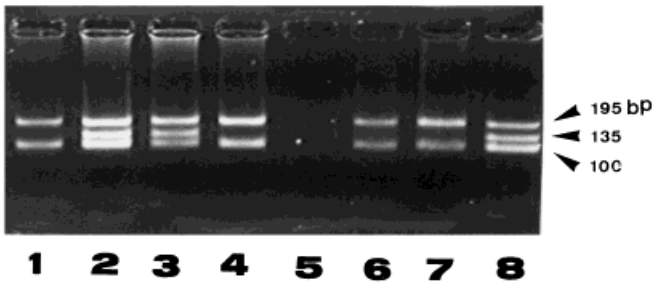

Fig. 2. Agarose gel electrophoresis of PCR amplification products using HLA-B27 exon 3-specific primer. Samples were amplified by using primers E91s and E136as (designed to amplify codon 91-136 of exon 3 from HLA-B27 alleles), giving an expected band of 135 bp. Simultaneous amplification of β-actin gene in exon 3 using primers OR-1 and OR-2 as PCR control gave 100 bp and 195 bp fragment. Sample 2, 3, 8 were B27 positive patients and sample 4, 6, 7 were B27 negative patients. Sample 1: β-actin control, sample 5: buffer control.

Evaluation of Cross Reaction Between HLA-B27 and Other B7 CREG Molecules by Flowcytometric Measurement

Further analysis of HLA-B loci in serological B27 negative patients showed that 106 out of 153 patients possessed HLA-B7 CREG molecules, which included 7 patients with HLA-B7, 54 patients with HLA-B60, 6 patients with HLA-B22, 3 patients with HLA-B54, 8 patients with HLA-B55, 1 patient with HLA-B56, 23 patients with HLA-B13 and 13 patients with HLA-B39 (Table 2). It was found that none of 106 B7 CREG positive patients were flowcytometric HLA-B27 positive.

Analysis of Fluorescence Intensity of B27 Antigen in Different HLA-B27 DNA Subtypes Patient by Flowcytometer

To further evaluate the effects of B27 DNA subtypes on the fluorescence intensity of HLA-B27 by flow cytometer, 41 patients with positive serological HLA-B27 antigen were

Table 1. Data of HLA-B27 Antigen by Microlymphocytoxicity, PCR Genotypeing and Flowcytometric Measurement in 194 Arthritic Patients*

	Terasaki	PCR	FCM
Terasaki	41	41	41
PCR	41	41	41
FCM	41	41	41

Terasaki: Terasaki microlymphocytoxicity method.
PCR: DNA genotyping by PCR.
FCM: Flow Cytometric Measurement.
*Total PatientNumber: 194, 153 out of 194 patients were B27 negative.

Table 2. Flowcytometric Measurement of HLA-B27 Antigen in B7 CREGS Patients

HLA typing[a]	No.	FCM[b]
HLA-B7	7	0
HLA-B60	54	0
HLA-B22	6	0
HLA-B54	3	0
HLA-B55	8	0
HLA-B56	1	0
HLA-B13	23	0
HLA-B39	13	0

[a]Patients were B27 negative by Terasaki microlymphocytoxicity.
[b]Number of patients with HLA-B27 positive by flowcytometric measurement (FCM).

selected for B27 subtyping and fluorescence measurement by flowcytometer (Fig. 3A). It was found that 38 out of 41 patients (92.7%) were HLA-B2704 and the other three were HLA-B2705. No significant difference in the mean channel intensity were noted between them ($P = 0.14$).

Comparison of Fluorescence Intensity of HLA-B27 in Primary and Secondary Spondylitis Patients

In order to evaluate the difference of B27 fluorescence intensity in various arthritic patients, 20 serological B27 positive AS, 15 secondary spondylitis patients (12 psoriatic arthropathy, 2 juvenile chronic arthritis, and 2 Reiter's disease) and 20 B27 positive healthy controls were selected for fluorescence comparison (Fig. 3B). It was found that mean fluorescence channel in primary AS was 156.8, while it was 158.2 in secondary spondylitis and 158.6 for normal control. No significant difference within groups were noted ($P = 0.16$).

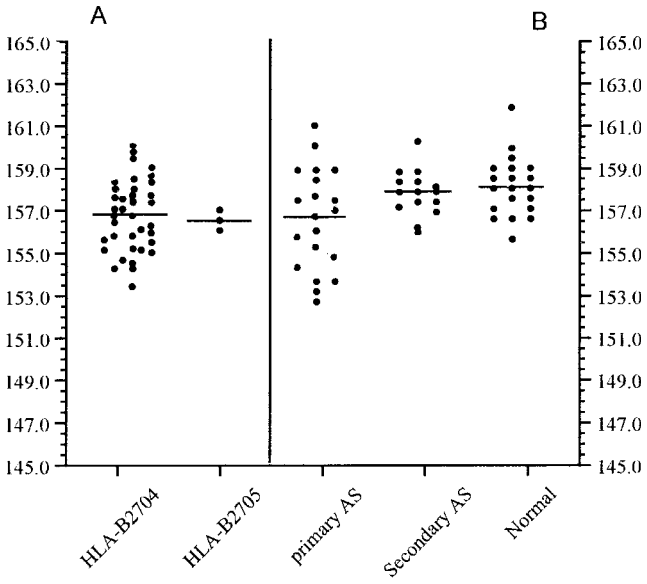

Fig. 3. Flowcytometric measurement of HLA-B27 antigen in different B27 DNA subtyping (A) and distinct patient groups (B). No significant difference in fluorescence intensity was found between HLAB2704 and 2705 ($P = 0.14$). The fluorescence difference within primary AS, secondary AS and B27 positive healthy control were also of no significance ($P = 0.16$).

DISCUSSION

Ankylosing spondylitis is a chronic systemic inflammatory disorder that primarily involves the joints and the periarticular tissue of the axial skeleton. The true prevalence of AS appears to be in the region of 0.25 to 1 per cent (10). Many patients, who previously were thought to have mechanical back pain, are now recognized as having AS. Delays between 5 and 10 years were recorded between the onset of symptoms and the diagnosis being made. Although the infective trigger is recognized in several forms of spondylopathies (11,12), the precise pathogenesis is not well understood. It was thought that HLA-B27 mediated immune response played a major role in pathogenesis of spondylitis. The association of spondylitis with HLA-B27, ranging from about 50 per cent (psoriatic and enteropathic spondylitis) to over 95 per cent (primary ankylosing spondylitis), were so strong that B27 testing had been suggested as a valuable diagnostic aid for these diseases.

The disease is considered primary if no other rheumatological disorder is present, or secondary if the sacroliitis is related to psoriatic arthropathy, inflammatory bowel disease, or Reiter's syndrome. To date, no difference of B27 genotyping has been found between HLA-B27 positive patients and positive control (13). In the present study, there is no significant difference in fluorescence intensity between them (Fig. 3B), at least in regard to this monoclonal antibody. Furthermore, no significant difference could be demonstrated in the mean channel between primary and secondary spondylitis (Fig. 3B), nor was there any difference between HLA-B2704 and HLA-B2705 (Fig. 3A). In addition, the fluorescence intensity of B27 did not correlate with the disease severity in AS patients (data not shown). The quantitative analysis of B27 intensity does not seem to be able to contribute any significant difference in these aspects. Several possibilities were considered. First, peptides recognized by this monoclonal antibody may be a common epitope of the HLA-B27 molecule. Second, the risk of the HLA-B27-positive individual depends on the nature of that individual (i.e., HLA-B27 plus the ankylosing spondylitis genes result in ankylosing spondylitis, while HLA-B27 plus the Reiter's syndrome gene lead to Reiter's syndrome). Third, the role of HLA-B27 antigen in disease pathogenesis might be in the priming function, not in the effector arm of immune response. However, the clear cut path between positive and negative fluorescence cells has made it a more reliable method than the microlymphocytoxicity method, where per cent of dead cells were estimated under a phase contrast microscope.

Currently, MHC class I antigen typing is performed by a complement-dependent microlymphocytoxicity test. Each of three loci coating for class I HLA antigen pheno-

types (HLA-A, B, C) could be determined in such a way and extraordinary polymorphism of more than 30 million phenotypes can be found. The method of microlymphocytoxicity, although being valuable in exploring the high level of HLA polymorphism is, however, a time-consuming procedure. At times, the information needed by physicians may be the phenotype of one loci only, for example the presence of HLA-B27 antigen in achieving the diagnosis of AS. The microlymphocytoxicity method was apparently not an appropriate selection under this condition. Until recently, double fluorescence with HLA-B27-FITC/CD3-PE conjugated monoclonal antibody was introduced, analyzed by a flowcytometer. In the present study, it was found that such a measurement has proved to be a reliable method in determining the HLA-B27 antigen phenotype, confirmed by DNA PCR and serotyping data.

In conclusion, flowcytometric measurement of HLA-B27 antigen is proved to be a rapid method with high specificity and sensitivity. It has been demonstrated to be a reliable tool in the evaluation of spondylitis by offering the important HLA-B27 genetic background.

REFERENCES

1. Schlosstein L, Terasaki PI, Bluestone R, Pearson CM: High association of an HLA antigen, W27, with ankylosing spondylitis. *N Eng J Med* 288:704–6, 1973.
2. Brewerton DA, Caffrey M, Hart FD, James DCO, Nicholls A, Sturrock RD: Ankylosing spondylitis and HLA-B27. *Lancet* i:904–907, 1973.
3. Schlosstein T, Teraski PI, Bluestone R, Pearson CM: High association of HLA antigen, w27, with ankylosing spondylitis. *N Engl J Med* 288:704–706, 1973.
4. Terasaki PI and McLelland JD: Microdroplet assay of human serum cytotoxins. *Nature* 204:998, 1964.
5. Pouletty P, Chang C, Kalil J: Typing of serum soluble HLA-B27 antigen by ELISA. *Tissue Antigen* 42:14–19, 1993.
6. Saiki PK, Mullis KB and Erlich HA: Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:489–491, 1988.
7. Dominguez O, Coto E, Martmez-Naves E, Choo SR, Lopez-Larrea C: Molecular typing of HLA-B27 alleles. *Immunogenetics* 36:277–282, 1992.
8. Olerup O: HLA-B27 typing by a group-specific PCR amplification. *Tissue Antigens.* 43:253–256, 1994.
9. Albrecht J and Muler HAG: HLA-B27 typing by use of flow cytofluorometry. *Clin Hem* 33:1619–1623, 1987.
10. Klippel JH, Dieppe PA: Rheumatology. In *Ankylosing spondylitis: prevalence and demography,* Jan Tore Gram, Gunnar Husby, 2nd eds. Mosby-Year Book Europe Limited, 1994, p 24.1–24.6.
11. Granfors K, Jalkanen S, Von Essen R: Yersinia antigens in synovial fluid cells from patients with reactive arthritis. *N Engl J Med* 320:216–221, 1989.
12. Keat A, Thomas B, Dixey J, Osborn M, Sonnex C, Taylor-Ribinson D: *Chlamydia trachomatis* and reactive arthritis—the missing link. *Lancet* i:72–74, 1987.
13. Higgins CM, Lund T, Shipley ME, Ebringer A, Sadowska Wroblewska M, Craig RK: Ankylosing spondylitis and HLA-B27: restriction fragment length polymorphism and sequencing of an HLA-B27 allele from a patient with ankylosing spondylitis. *Ann Rheum Dis* 51:855–862, 1992.