Related Papers
HLA-B27 testing: A journey from flow cytometry to molecular subtyping
HLA-B27 detection – comparison of genetic sequence-based method and flow cytometry assay
Comparative study of two laboratory techniques for the detection of HLA-B27 in patients with axial...
HLA-B27 typing: Evaluation of an allele-specific PCR melting assay and two flow cytometric antigen...
View more

Clin Rheumatol (1999) 18:23–27
© 1999 Clinical Rheumatology

Clinical Rheumatology

Original Article

Optimisation of HLA-B27 Testing by Association of Flow Cytometry and DNA Typing

G. Bonnaud[1], C. Aupetit[1,2], P. M. Preux[3], M. Cogné[1,2] and M. Drouet[1,2]

[1]Laboratoire d'Immunologie et Immunogénétique, [2]EP CNRS 118, Faculté de Médicine, and [3]Laboratoire de Biostatistiques, Faculté de Médecine, CHRU Dupuytren, Limoges, France

Abstract: HLA-B27 typing contributes to the diagnosis of ankylosing spondylitis. The classical technique of microlymphocytotoxicity is costly and can give false-negative results. We have compared 304 samples using two relatively new methods – flow cytometry and PCR-SSP – and evaluated their respective uses in routine analysis. Flow cytometric HLA-B27 testing was performed using three monoclonal anti-B27 antibodies (HLA-ABC-m^3, GS145.2 and FD705 clones). Cut-off values were established to differentiate HLA-B27-positive from HLA-B27-negative samples with ROC curves. Although flow cytometric analysis with a reliable monoclonal antibody (mAb) is valuable for HLA screening, none of the HLA-B27 flow cytometry protocols was sufficient on its own to ascertain the HLA phenotype in 100% of samples. Two false negatives were observed with the FD705 mAb and the use of two different monoclonal antibodies did not increase the accuracy of HLA-B27 typing. HLA-B27 typing using molecular biology is a reliable but costly technique. Therefore we suggest that DNA typing could be used as a complementary technique and applied to samples whose HLA-B27 phenotype cannot be determined by flow cytometry. The association of flow cytometry and DNA typing is, in our experience, an economical and reliable approach.

Keywords: Ankylosing spondylitis; Flow cytometry; HLA-B27; HLA-B27 subtypes; Polymerase chain reactions

Correspondence and offprint requests to: M. Drouet, Laboratoire d'Immunologie et Immunogénétique, CHRU Dupuytren, 87042 Limoges Cedex, France. Tel: 33 05 55 05 61 80; Fax: 33 05 55 05 61 85; e-mail: drouet@unilim.fr

Introduction

The determination of HLA phenotype can contribute to the diagnosis and/or prognosis of diseases. One of the strongest known associations is that of ankylosing spondylitis (AS) and HLA-B27 alleles [1]. The frequency of HLA-B27 is more than 90% in patients with AS, and less than 10% in Caucasians [2]. HLA-B27 is a serologically well-defined antigen that represents a family of 11 closely related alleles, from *HLA-B*2701* to *B*2711* [3]. *B*2705* is the predominant subtype followed by *B*2702* in Caucasians. Alleles *B*2705, 01, 02, 04, 07* are positively associated with AS, whereas *HLA-B*2706* is negatively associated [2,4]. Because of this strong association, determination of HLA-B27 status is an important tool in the classification of arthropathies and is thus routinely performed in a tissue typing laboratory.

The reference technique for HLA-B27 typing has up to now been the microlymphocytotoxicity method, which is time consuming, costly and dependent on the quality of allosera. French interlaboratory controls have revealed up to a 16% discrepancy between laboratories (Agence du Médicament, Paris, 1997), which can be explained by the origin of allosera and by cell viability. Other HLA-B27 typing methods have recently been developed using ELISA cytometry or DNA genotyping HLA-B27 phenotype can be determined on serum or plasma using a soluble source of HLA antigen [5], but large variations in the plasma concentration of soluble HLA among individuals limit the usefulness of this technique [6]. Flow cytometry was developed for HLA-B27 screening, with samples that screen as positive still requiring confirmation by standard methods [7,8]. The use of two different monoclonal anti-B27 antibodies has

been recommended for accurate HLA-B27 phenotyping with flow cytometry [9]. HLA-B27 alleles can be determined using different molecular techniques such as the polymerase chain reaction with a sequence-specific primer (PCR-SSP) [10] or PCR-specific oligonucleotide (PCR-SSO) [11].

In this study, we have compared HLA-B27 typing by flow cytometry using three different HLA-B27 monoclonal antibodies and by molecular biology on 304 samples from patients. We propose a strategy for optimised routine HLA-B27 screening which combines flow cytometry and molecular biology.

Materials and Methods

Patients

Three hundred and four patients referred from rheumatology departments and transplantation units, randomly selected over a period of 4 months, were phenotyped for HLA-B27 by flow cytometry and HLA-B27-specific PCR. Blood left over from the daily workload was collected into anticoagulant (either heparin, EDTA or citrate phosphate), according to the guidelines of the local ethical committee. Two millilitres were sufficient to perform flow cytometric analysis and HLA-B27-specific PCR. Samples were stored at room temperature and analysed within 24 h after venepuncture. HLA-B27 DNA was validated on 138 DNA samples from the laboratory DNA collection obtained from 116 healthy donors (bone marrow and cells from panel donors or laboratory staff) and HLA-AB serologically typed; eight samples were tested for HLA-B27 DNA subtypes (gift of Dr Lopez-Larea, Madrid, Spain).

HLA-B27-Specific PCR and Subtyping

DNA was extracted from 100 μl of whole blood by a rapid salting out assay for all samples [12]. Generic HLA-B27 DNA typing was performed by PCR-SSP as previously reported [10], with some modifications. Briefly, each reaction was performed in a 15 μl reactional volume, consisting of 50–100 ng genomic DNA, 0.3 units of Taq polymerase (Appligène, France), PCR buffer, 7.5% (v/v) glycerol, 200 μM each of dATP, dCTP, dGTP and dTTP, cresol red sodium salt (75 μg/ml), 0.5 μM of each B27-specific primer and 0.1 μM of each internal control primer pair. The oligonucleotide sequences of the B27-specific primer pair were $^{5\prime}$CAG TCT GTG CCT TGG CGT TGC$^{3\prime}$ (5′-primer) and $^{5\prime}$GCT ACG TGG ACG ACA CGC T$^{3\prime}$ (3′-primer) and these gave rise to a PCR fragment of 144 base pairs (bp). The control primer pair amplified a 429 bp fragment of the growth hormone gene (5′-primer: $^{5\prime}$GCC TTC CCA ACC ATT CCC TTA$^{3\prime}$; 3′-primer: $^{5\prime}$TCA CGG ATT TCT GTT GTG TTT C$^{3\prime}$). Thirty amplification cycles were carried out in a GeneAmp PCR-system 9600 thermal cycler (Perkin Elmer Cetus Instrument). After initial denaturation at 94°C for 2 min, the amplification protocol consisted of 30 cycles (94°C for 20 s, 59°C for 50 s, 72°C for 30 s). The amplification product was run on a 1.5% agarose gel to visualise the results (Fig. 1). All HLA-B27-positive samples were subtyped using another sequence-specific PCR followed by hybridisation with specific oligonucleotide probes (PCR-SSO), as previously described by Dominguez et al. [11] and Lopez-Larea et al. [13]. Internal controls were included in all the tests and consisted of HLA-B27 DNA of all subtypes (personal gift of C. Lopez-Larrea, Spain).

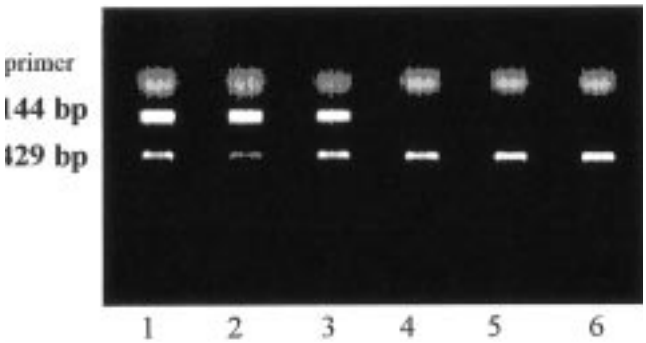

Fig. 1. HLA-B27 PCR-SSP specific amplification. The 429 bp band corresponds to simultaneous amplification of the HGH gene and is included as a PCR control. The specific band of 144 bp is detected in the HLA-B27 positive samples (lanes 1–3) and is absent in the HLA-B27 negative samples (lanes 4–6).

HLA-B27 Flow Cytometric Analysis

HLA-B27 Monoclonal Reagents. Three commercially available monoclonal antibodies (mAb) labelled with fluorescein isothiocyanate (FITC) were tested, which recognised different epitopes of the HLA-B27 antigen: the clone GS145.2 (Becton Dickinson, Grenoble, France) [14]; clone FD705 (One lamba, Paris, France) [15]; and HLA-ABC-m^3 mAb (Immunotech, Marseille, France) [16]. The FITC-conjugated GS145.2 mAb was associated with an anti-CD3 antibody labelled with phycoerythrin (PE), and FITC-labelled HLA-ABC-m^3 with an anti-HLA-B7 antibody labelled with PE. A single batch of each of these reagents was used throughout this study.

Direct Staining Techniques. All three mAb were used in a direct staining protocol on whole blood; 50 μl (GS145.2 and HLA-ABC-m^3 mAb) or 100 μl (FD705 mAb) of whole blood were mixed with the appropriate amount of mAb and incubated for 15 min at room temperature in darkness. Whole blood lysis protocols were optimised for each mAb after extensive trials, taking into account recommendations of the manufacturers, and therefore these differed for each mAb. In the HLA-ABC-m^3 assay, red cells were lysed with NH_4 Cl solution: after 10 min of incubation, cells were washed with PBS-BSA 0.1% and the pellet was suspended in 400 μl of PBS. The lysis procedure used for the GS145.2 mAb differeed only in

the use of FACS lysing solution (Becton Dickinson) [17]. The FD705 assay was processed on a Q-Prep (Coulter Electronics, Margency, France) automated red cell lysis and cell fixing instrument.

Immunofluorescence Analysis. Stained cell suspensions were analysed within 6 h of preparation on a Profile II flow cytometer (Coulter Electronics) equipped with an argon ion laser tuned to 15 mW of 488 nm excitation light. Optical alignment and mean channel consistency were controlled with immunocheck fluorescent-coated beads (Coulter) on a daily basis. Band-pass filters of 530 nm and 585 nm were used for fluorescein and PE fluorochrome, respectively. Results were assessed using mean fluorescence intensity and median channel fluorescence. The mean green fluorescence was analysed on up to 5000 lymphocytes, gated on a dot plot of forward versus side scatter, and on 5000 CD3-positive lymphocytes gated on forward angle and PE fluorescence for GS145.2 mAb. All analyses were performed by the same operator.

Statistical Analysis

Specificity and sensitivity were calculated for each flow cytometric assay after determination of cut-off values with ROC curves [18].

Results

DNA HLA-B27 Typing

Generic HLA-B27 genotyping by PCR-SSP was initially validated on 138 DNA samples of well-defined HLA-AB phenotypes. Twenty-seven out of the 138 (20%) tested samples where HLA-B27 positive. No discrepancy was observed between serological phenotypes and genotypes. HLA-B27 typing by PCR-SSP was therefore used as the reference technique (or gold standard). HLA-B27 genotyping with PCR-SSP was performed on blood samples from 304 patients. HLA-B27 DNA testing could be obtained in all cases at the first attempt. An HLA-B27-positive genotype was found for 36 DNA samples, which were subtyped by PCR-SSO: *HLA-B*2705* subtype was present in 31 out of 36 (86%) samples; *HLA-B*2702* subtype in two out of 36 (5%) and *HLA-B*2707* in two out of 36 (5%). The HLA-B27 positive DNA by PCR-SSP was confirmed as HLA-B27 positive with the PCR-SSO assay in all cases.

Cell Surface Expression of HLA-B27 by Flow Cytometry (Table 1)

The staining procedures applied for each mAb were intially optimised and validated on a limited number of samples. Using the validated protocols, the percentage of tests of insufficient technical quality were respectively 12/304 (4%) for GS145.2 mAb; 17/304 (5%) for FD705 mAb and 14/304 (4%) for HLA-ABC-m^3 mAb. Defective analyses were due to insufficient lysis or poor cell viability. Cut-off values of the mean intensity of fluorescence were determined for each mAb with ROC curves by maximisation for the product of sensitivity and specificity. The cut-off values of mean intensity of fluorescence were 0.55 for FD705 mAb (specificity 98.4%, 95% CI: 96.8–99.6%; sensitivity 94.1%, 95% CI: 85.3–100%); 2.1 for HLA-ABC-m^3 mAb (specificity 96.8%, 95% CI: 94.4–98.8%; sensitivity 97.1%, 95% CI: 91.2–100%); and 1.7 for GS145.2 mAb (specificity 99.2%, 95% CI: 98–100%; sensitivity 100%, 95% CI: 89.7–100%). Cut off values were also determined for the median intensity of fluorescence: 0.7 for FD705 mAb (specificity 96.8%, 95% CI: 93.7–98.6%; sensitivity 94.1%, 95% CI: 88.8–99.3%); 1.3 for HLA-ABC-m^3 mAb (specificity 92.7%, 95% CI: 88.8–95.6%; sensitivity 88.2%, 95% CI: 72.5–96.7%); and 1.6 for GS145.2 mAb (specificity 99.2%, 95% CI: 97.1–99.9%; sensitivity 100%, 95% CI: 89.7–100%).

Because no protocol gave a specificity of 100%, a second analysis was performed to determine negative and positive cut-off values: the negative cut-off value was defined as the mean intensity of fluorescence under which all samples are HLA-B27 negative by PCR ('true negative'), and the positive cut-off value as the mean fluorescence above which all tested samples are positive by PCR ('true positive'). The intermediate value of the mean intensity of fluorescence was considered as a grey zone in which the HLA-B27 phenotype using flow cytometry was considered indeterminate. The number of samples with values that fell inside the 'grey zone' were

Table 1. Comparison of the mean fluorescence intensity observed with the three monoclonal antibodies in HLA-B27-positive and HLA-B27-negative samples

	Clone FD705		Clone GS145.2		Clone HLA-ABC-m^3	
	Mean ± SD	Min–Max	Mean ± SD	Min–Max	Mean ± SD	Min–Max
HLA-B27 positive (n = 36)	3.2 ± 2.4	0.4 ± 10.2	3.0 ± 1.2	1.7 ± 8.3	7.3 ± 5.0	2.2 ± 27.5
HLA-B27 negative (n = 253)	0.4 ± 0.1	0.2 ± 0.7	0.3 ± 0.3	0.1 ± 1.9	0.5 ± 0.8	0.1 ± 9.4

HLA-B27 typing was assigned by PCR-SSP, which was considered to be the gold standard. The analysis was performed on 289 samples, for which flow cytometric analysis was interpretable with the three mAb protocols.

25/292 (8%) for the GS145.2 mAb protocol, 34/290 (12%) for the HLA-ABC-m^3 mAb protocol, and 81/287 (28%) for the FD 705 mAb protocol. Selection of the negative cut-off value as defined in this study depended on the sample size of HLA-B27-positive samples; therefore this selection method is not valid for setting up a screening assay.

Discussion

We have confirmed that DNA typing is the most reliable technique and allowed the HLA-B27 genotype to be identified in all the tested samples. Because this technique does not require viable cells, it can be performed on low-viability cells, which are often found in samples from patients. However, DNA typing remains time consuming (about 4 h) and costly. We have underlined that HLA-B27 subtyping is not mandatory in Caucasians, because all HLA-B27 subtypes observed in this population are associated with AS and related diseases.

Cytometric typing shared some of the limitations of the microlymphocytotoxicty technique: a need to obtain viable cells and cross-reactions with other HLA-B alleles (HLA-B7). Some samples could not be analysed using flow cytometry and this may be due to the clinical conditions of the patients. This observation was not seen more frequenlty in specific population of patients. As previously reported [8], HLA-B27 screening by flow cytometry requires an extensive validation procedure in each laboratory to set up cut-off values, which depend on mAb specificities, reagent batches and instruments. Therefore, the cut-off values obtained in this study cannot be extrapolated to HLA-B27 typing with flow cytometry in general. Each laboratory must establish its own range. This observation represents one of the limitations of flow cytometry: the workload for validation of HLA-B27 screening with flow cytometry is significant and justified only if a large number of tests are performed by the laboratory. Before this study, HLA-B27 screening was routinely performed in our laboratory using a combination of flow cytometry and microlymphocytoxicity testing. The validation protocol described in this study requires a similar workload to previously described procedures using microlymphocytotoxicity typing as the gold standard [7,17]. However, our approach does not require specific competence in HLA serology typing, a technique that requires a great deal of experience and specialised equipment.

None of the protocols achieved 100% specificity. The major difficulty was determination of the criteria for negative and positive assignment. Mean fluorescence analysis appears to give a better specificity than median intensity of fluorescence. The values between the negative and positive cut-off values remain a ‘grey zone’; samples falling within this cut-off interval need to be tested by another reliable method, and HLA-B27 PCR-SSP is the technique of choice. However, flow cytometry remains the fastest and cheapest technique for HLA-B27 testing. In this context, one objective of HLA-B27 screening by flow cytometry is to limit the number of samples that require a genotyping control.

The percentage of samples falling in the ‘grey zone’ is dependent on the mAb protocol: in our experience the GS145.2 protocol had the best specificity; the HLA-ABC-m^3 protocol had good negative predictive values but a low positive predictive value due to cross-reaction with other HLA specificities (HLA-B7 and -B22). The FD705 mAb protocol had a good positive predictive value because it does not give cross-reactions with other HLA alleles [14], but two samples had a low mean intensity of fluorescence and could be considered as ‘false negative’ with this protocol. This observation led us to set a low negative cut-off value, which explains the high number of samples falling in the grey zone with this mAb. ‘False-negative’ results have previously been reported with this mAb [19], which should not be used on its own for HLA-B27 screening. The low mean intensity of fluorescence observed on these samples was not due to rare HLA-B27 subtypes: both samples were *B*2705*, the most frequent HLA-B27 subtype. FD705 apparently does not detect the HLA-B*2708 subtypes [20]. Some authors [9] have recommended the systematic use of two mAb for HLA-B27 testing. This approach would double the cost of HLA-B27 testing by flow cytometry and, in our experience, would not decrease significantly the number of samples that require further validation. T-lymphocyte gating was used with the GS145.2 protocol as recommended by the manufacturer; CD3 gating was attempted on a limited number of samples with the other two mAb, but did not produce any improvement in the specificity of the test (data not shown).

The mean intensity of fluorescence varied widely on positive cells with all mAb protocols. The expression of HLA-B27 molecules on the cell surface of lymphocytes varies and tends to decrease in the acute phase of the disease [21]. These data need further confirmation by quantification of HLA-B27 molecules on lymphocytes during the acute and chronic phases. Flow cytometric tests performed a few weeks after this phase may give a ‘false-negative’ result due to a decrease in HLA-B27 expression. Furthermore, the peptide bound to the HLA molecular complex can influence the reactivity of mAb with HLA-B27 heavy chain and significantly decrease detection by flow cytometry [21].

Clinical HLA-B27 testing must be reliable and low in cost. In our experience, HLA-B27 screening with one appropriate mAb and flow cytometric analysis allows a rapid turnaround and an economic approach. To increase the validity of the test, the biologist should be informed of the clinical status of the tested patient. Nevertheless, flow cytometric analysis cannot identify the HLA-B27 phenotype in 5–10% of samples (poor cell viability, mean fluorescence intensity in the grey zone). These samples would benefit from DNA HLA-B27 typing, a technique that can be applied to all samples. Furthermore, DNA typing can be considered as the gold

standard and performed in parallel with flow cytometry on a limited number of samples to establish flow cytometric cut-off values.

References

1. Brewerton DA, Caffrey M, Ahrt FD, James DCO, Nicholls A, Sturroch RD. Ankylosing spondylitis and HLA B27. Lancet 1973;1:904–7.
2. Benjamin R, Parham P. Guilt by association and ankylosing spondylitis. Immunol Today 1990;4:137–42.
3. Hasegawa T, Ogawa A, Sugahara Y, Moriyama Y, Nanzai H, Tawara K, et al. A novel HLA-B27 allele (B*2711) encoding an antigen reacting with both B27- and B40- specific antisera. Tissue Antigen 1997;49:649–52.
4. Maclean L. HLA-B27 subtypes: implications for the spondylarthropathies. Ann Rheum Dis 1992;51:29–31.
5. Pouletty P, Chang C, Kalil J, Atwood E, Ferrone S, Shimizu B, et al. Typing of serum-soluble HLA-B27 antigen by ELISA. Tissue Antigens 1993;42:14–9.
6. Drouet M, Aussel L, Drenou B, Fauchet R. Quantification and molecular analysis of plasmatic HLA-BCw molecules with a locus B and Cw HLA specific mAb (B1.23.2mAb). Eur J Immunogenetics 1995;22:363–70.
7. Hulstaert F, Albrecht J, Hannet I, Lancaster P, Buchner L, Kunz J, et al. An optimized method for routine HLA-B27 screening using flow cytometry. Cytometry 1994;18:21–9.
8. Ward A, Nikaein A. Comparison of monoclonal antibodies for flow cytometric analysis of HLA-B27 antigen. Cytometry 1995;22:65–9.
9. Hoffman JJ, Janssen WC. HLA-B27 phenotyping with flow cytometry: further improvement by multiple monoclonal antibodies. Clin Chem 1997;43:1975–81.
10. Olerup O. HLA-B27 typing by a group-specific PCR amplification. Tissue Antigens 1994;43:253–6.
11. Domingez O, Coto E, Martinez-Naves E, Choo SY, Lopez-Larrea C. Molecular typing of HLA-B27 alleles. Immunogenetics 1992;36:277–82.
12. Miller S, Dykes D, Polesky H. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acid Res 1988;16:1215–8.
13. Lopez-Larrea C, Gonzales-Roces S, Pena M, Dominguez O, Coto E, Alvarez V, et al. Characterization of B27 haplotypes by oligotyping and genomic sequencing in the Mexican mestizo population with ankylosing spondylitis. Hum Immunol 1995; 43:174–80.
14. Johnston J, Garbutt J, Nelson KA. A monoclonal antibody specific for HLA-B27 typing. Hum Immunol 1985;14:134–5.
15. Pei R, Arjomand-Shamsai, Deng CT, Cesbron A, Bignon JD, Lee JH. A monospecific HLA-B27 fluorescein isothiocyanate-conjugated monoclonal antibody for rapid, simple and accurate HLA-B27 typing. Tissue Antigens 1993;41:200–3.
16. Trapani JA, Vaughan HA, Sparrow R, Tait B, MacKenzie I. Description of a mouse monoclonal anti-HLA-B27 antibody. Hum immunol 1983;7:205–16
17. Reynolds WM, Evans PR, Lane AC, Howell MW, Wilson P, Wong R, Smith J. Automated HLA-B27 testing using the FACSPrep/FACScan system. Cytometry 1994;18:109–15.
18. Griner PF, Mayeswski RJ, Mushlin A, Greenland P. Selection and interpretation of diagnostic tests and procedures: principles and applications. Ann Intern Med 1981;94:553–600.
19. Neumuller J, Schwartz DW, Dauber E, Mayr WR. Evaluation of four monoclonal antibodies against HLA-B27 for their reliability in HLA-B27 typing with flow cytometry (FC): comparison with the class microlymphocytotoxic test (MLCT). Cytometry 1996;26:209–15.
20. Reynolds WM, Evans PR, Wilson PJ, Wong WM, Darke C, Smith JL. Automated routine HLA-B27 typing by flow cytometry. J Immunol Methods 1996;197:1–5.
21. Kirveskari J, Kellner H, Wuorela M, Frankenberger B, Leisiralo-Repo M, Weiss EH, Granfors K. False-negative serological HLA-B27 typing results may be due to altered antigenic epitopes and can be detected by polymerase chain reaction. Br J Rheumatol 1997;36:185–9.

Received for publication 12 February 1998
Accepted in revised form 13 July 1998