The flow cytometric labeling pattern in HLA-B27 detection may suggest cross reactivities

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Abstract

Background: The detection of HLA-B27 expression is one of the most powerful diagnostic tools in ankylosing spondylitis and other spondylarthropathies, hence the importance of a reliable and accurate detection method. Flow cytometry is often used as an alternative HLA-B27 typing technique, mainly because it is a rapid test. However, even though it is based on various monoclonal antibodies, their ability to bind to cross-reactive HLA-B molecules was demonstrated by various groups. Materials and methods: The HLA-B27 typing was performed by flow cytometry using the BD™ HLA-B27 System, and by the HLA-SSP and HLA-SSO typing techniques. Results: We were able to confirm some of the known cross-reactivities and evidence some new ones, as HLA-B18 and B35, in the context of a characteristic flow cytometric labeling pattern. Conclusions: Flow cytometry can offer clues indicating the presence of HLA-B27 cross-reactive molecules, but such situations will require HLA typing by molecular biology techniques.

Key words: HLA-B27, ankylosing spondilitis, cross-reactivity, flow cytometry

Rezumat

Background: Determinarea expresiei HLA-B27 reprezintă unul dintre cele mai importante modalități de diagnostic în spondilita anchilozantă, precum și în alte spondilarthropatii, de unde și importanța unei metode de detecție de acuratețe. Citometria în flux este adesea utilizată pentru tiparea HLA-B27, în special datorită faptului că este un test rapid. Cu toate că se utilizează anticorpi monoclonali, capacitatea acestora de a se lega la molecule HLA-B cross-reactive a fost demonstrată de diferite grupuri. Materiale și metode: Tiparea HLA-B27 a fost efectuată prin citometrie în flux, folosind BD™ HLA-B27 System, precum și tehnicile HLA-SSO și HLA-SSP. Rezultate: În acest studiu, au putut fi confirmate unele dintre reactivitățile încrucișate cunoscute și am evidențiat cross-reactivități noi, precum HLA-B18, B35 în contextul unei-pattern flow-cytometric caracteristic. Concluzii: Citometria în flux poate oferi indicii care să sugereze prezența de molecule HLA-B27 cross-reactive, dar aceste situații vor necesita tiparea HLA prin tehnici de biologie moleculară.

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Introduction

Ankylosing spondilitis, perhaps the best known and characterized of the family of spondyloarthritis, is a chronic inflammatory disease affecting the axial skeleton, the entheses and, occasionally, the peripheral joints. Brewerton et al. were the first ones to describe in 1973 the association between ankylosing spondylitis and the expression of HLA-B27 [1]. Several other studies confirmed that HLA-B27 is a high susceptibility gene in humans, since 94% of the patients are positive, in contrast with the rather low frequency of HLA-B27 in Caucasian population (around 9%) [2, 3]. While the role of HLA-B27 in pathogenesis is still incompletely elucidated, it has become increasingly clear that the presence of this molecule can be associated, at a lower relative risk, with the entire group of seronegative spondylarthropathies, like the Reiter’s reactive arthritis [4, 5], spondylarthritis associated with inflammatory bowel disease [6, 7], acute anterior uveitis [8, 9], subgroups of juvenile chronic arthritis and psoriatic arthritis [10]. Even though there are reports about ankylosing spondilitis independent of HLA-B27 [11], the screening for this HLA molecule remains the golden standard in diagnosis.

Materials and Methods

 Patients. The patients were referred to our lab with a presumptive diagnostic of ankylosing spondylitis, based on clinical symptoms and paraclinical investigations (X-ray, Magnetic Nuclear Resonance).

 Antibodies. The BD HLA-B27 System provides a mixture of two monoclonal antibodies. The anti-HLA-B27 antibody (clone GS145.2, IgG1, kappa) is conjugated with fluorescein isothiocyanate (FITC), while the anti-CD3 antibody (clone SK7, IgG1, kappa) is conjugated with phycoerithrine (PE).

 Flow cytometry. The labelings were performed under standard conditions, according to the manufacturer’s indications, using fresh blood harvested in BD EDTA vacutainers. At less than 3 h after harvesting, 50 µl of whole blood were incubated in the dark for 30 minutes at room temperature with the antibody mixture (1:25 volume). Then, 2 ml of 1x BD FACS Lysing Solution were added in each tube and incubated for another 10 minutes at room temperature. After centrifugation at 300g for 5 minutes at room temperature, the pellet was resuspended and washed in 2 ml of PBS. The last centrifugation was performed at 200g, for 5 minutes at room temperature and the pellet was resuspended in approximately 50-100 µl of PBS. The acquisition of 5000 to 10000 events was performed immediately afterwards using a BD FACSCalibur and the CellQuest software.

 Under the circumstances, we have further investigated by molecular biology HLA typing techniques a number of samples that generated unclear labeling patterns in flow cytometry. We were thus able to show that these labeling patterns are suggestive for cross-reactivities and to confirm some of the cross-reactivities already known; furthermore, we were also able to identify several new ones.

 DNA extraction. The DNA was extracted from 200 µl of whole blood, using a Qiagen
kit (QIAamp Mini, Qiagen, Germany). After 10 minutes incubation with proteinase K at 56°C and precipitation with 200 µl 100% ethanol, the lysate was adsorbed by centrifugation onto the columns silica membrane. After 2 washings of the residual contaminants, the DNA was eluted from the columns in 200 µl of buffer and the concentration was measured at a 260 nm wave length with a Beckman Coulter DU 800 spectrophotometer, using the dedicated software. Protein contamination was automatically assessed at a 280 nm wave length, the samples being considered acceptable at a 260/280 nm ratio of 1.7-1.9. The DNA concentration obtained ranged between 30 and 60 ng/µl.

**HLA-SSP (sequence specific primer).**

The typing was performed using BAG (Germany) and Olerup (Sweden) kits. According to the manufacturer’s instructions, a certain amount of water, DNA, buffer and adequate Taq polymerase were mixed, and 10µl of this mix were then aliquoted in a 48 well plate with pre-aliquoted primers. The amplicons pattern (*Figure 1*) was interpreted using the Score software.

**HLA-SSO (sequence specific oligonucleotides).**

The typing was performed with a Inno-Lipa HLA-B Update Plus kit (Innogenetics, Belgium). The DNA was initially amplified by PCR according to the manufacturer’s indications, in a 50 µl volume, using biotinylated primers and the presence of the amplicons was checked on a 2% agarose gel. Then, the double stranded DNA was chemically denatured and hybridized at 56°C with oligonucleotide probes immobilized as strips on nitrocellulose membrane. The mismatched DNA is removed by stringent washings at 56°C and the bound DNA is evidenced enzymatically by adding alkaline phosphatase coupled with streptavidin and then a BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate p-toluidine and nitroblue tetrazolium in dimethylformamide) substrate, generating a brown precipitate (*Figure 2*). The colored band pattern was interpreted using the LIRAS for LIPA HLA software.

**Results and discussion**

The BD™ HLA-B27 System flow cytometry kit, containing a mixture of two monoclonal antibodies, is designed to allow the easy identification of HLA-B27 positive T cells in the upper right quadrant, while HLA-B27 negative T cells are projected in the lower right quadrant (in our setting). The typical dot-plots of the HLA-B27 positive samples display no events in the lower right quadrant, as shown in *Figure 3*. In-
stead, the negative HLA-B27 samples display all the T cells in the lower right quadrant.

Besides these two distinctive labeling patterns, we also came across a different labeling pattern in which the dots were distributed in both right quadrants, with percentages ranging from 3 to 47% in the lower right quadrant (Figure 4).

Several studies performed to date have stressed that actually all the commercially available anti-HLA-B27 antibodies are able to cross-react with several other HLA-B molecules [12-16], with some groups recommending multiple monoclonal antibodies [17], or molecular biology methods for resolving cross-reactivities [18]. A major study in this respect, comparing the antibodies from five manufacturers, was published in 2003 [16].

A classification that takes into account the serologic reactivity of human alloantisera includes the HLA molecules into several CREG (cross reactive groups) groups [19]. HLA-B27 belongs to the CREG 7 group, together with molecules like HLA-B7, HLA-B13, HLA-B22, HLA-B40, HLA-B41, HLA-B42, HLA-B47 and HLA-B48. Levering et al. were reporting in their study several strong cross-reactivities for the antibodies they have investigated in flow cytometry (HLA-B7, B22, B37, B42) as well as some weak cross-reactivities (HLA-B12 (B44, B45-CREG12), B13, B16 (B38, B39 - CREG 8), B17 (B57, B58 - CREG 5), B40, B41, B47, B48) [16]. It comes as no surprise the fact that, with the exception of B37, practically all strong cross-reactivities are generated by HLA molecules part of the CREG 7 group as HLA-B07, B22 and B42. Other molecules of the CREG 7 group like B40, B41, B47 and B48 generated only weak cross-reactivities. Even more interestingly, the study demonstrated that the antibodies were also able to generate cross-reactivities with HLA molecules that are included in different CREG groups: B44, B45 (CREG 12), B13, B38, B39 (CREG 8), B57, B58 (CREG 5).

Taking into consideration these literature data, we have decided to further investigate by molecular biology typing techniques all the samples yielding unclear results, since this labeling pattern was rather suggesting cross-reactivities than clear cut positive or negative results. The HLA-SSP or HLA-SSO typing techniques confirmed that indeed the dot plots showing events distributed in both upper right and lower right quadrants were generated not by HLA-B27 labeling but by various cross-reactive molecules. In 8 of the 17 samples we have investigated, this type labeling pattern was produced by HLA-B07 (Figure 5), acknowledged as the molecule to manifest the strongest cross-reactivity with HLA-B27 [19].
Besides HLA-B*07 generated cross-reactivities, we were also able to confirm cross-reactivities determined by HLA-B*37, HLA-B*38, and HLA-B*39 (Figure 6), as previously reported.

Even though HLA-B40 is cited in the literature as cross-reactive with HLA-B27 [16], in our hands, a SSP HLA-B*40 confirmed sample (in association with the non-cross-reactive HLA-B*51) did not yield the characteristic labeling pattern in flow cytometry, but only the lowest percentage of cells (8%) in the HLA-B27 positive upper left quadrant (Figure 7).

The molecular typing techniques allowed us to identify in one case of cross-reactivity the presence of both HLA-B*07 and HLA-B*27, showing us that such a labeling pattern given by HLA-B07 can mask the presence of HLA-B27 (Figure 8).

On the other hand, the simultaneous presence of other cross-reactive HLA-B molecules with HLA-B27 did not generate the above described labeling pattern (Figure 3). We were also able to identify some other cross-reacting HLA-B molecules that, to the best of our knowledge, were not reported yet. We came across a sample that was homozygous for HLA-B*18, but displayed the characteristic pattern in flow cytometry (Figure 9).
Taking as well into consideration the above described cases, this allowed us to understand that the anti-HLA-B07 antibody is responsible for a very strong cross-reactivity with HLA-B27, capable of masking not only HLA-B27 but also many other weaker cross-reactive HLA-B molecules, like for instance HLA-B18 (Figure 10).

It may well be that HLA-B35 could also behave as a cross-reactive molecule for the BD antibody since we have obtained characteristic labeling patterns in flow cytometry not only when HLA-B*35 was associated with HLA-B*37 (Figure 11), but also with HLA-B*14 (Figure 12).

However, the same weaker cross-reactivity hypothesis can be advanced regarding the HLA-B14 molecule, as in our investigation we only came across a sample where its presence was associated (and thus potentially masked) with HLA-B*07 (Figure 13).

Conclusions

The commercially available anti-HLA-B27 antibodies are prone to binding to various cross-reactive HLA-B molecules, generating a characteristic labeling pattern, with dots distributed, in various percentages, in both upper right and lower right quadrants. Most of these cross-reactive HLA-B molecules are belonging, as HLA-B27, to the CREG 7 group, especially HLA-B07. However, we were also able to confirm cross-reactivities produced by HLA-B molecules from other CREG groups.

Furthermore, we have shown that HLA-B18, HLA-B35 and possibly HLA-B14 are also capable of generating cross-reactive labeling patterns.

One interesting feature in flow cytometry is that the cross-reactivity produced by HLA-B07 is so strong that it can mask not only other weaker cross-reactive HLA-B molecules, but even the presence of HLA-B27.

We conclude that flow cytometry is a very useful test in screening for the presence of the HLA-B27 molecule, and cross-reactivities can be readily identified by a distinctive labeling pattern. However, we advocate that all the unclear situations should be solved by molecular HLA typing techniques.

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