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

Petru Cianga[1,2*], Mihaela Zlei[2], Elena Rezus[3], Corina Cianga[1]

1. Department of Immunology, "Gr. T. Popa" University of Medicine and Pharmacy, Iasi, Romania
2. Laboratory of Immunology and Genetics, Clinical Emergency County Hospital, Iasi, Romania
3. Department of Rheumatology, "Gr. T. Popa" University of Medicine and Pharmacy, Iasi, Romania

Abstract

Background: *The detection of HLA-B27 expression is one of the most powerful diagnostic tools in ankylosing spondylitis and other spondylarthropaties, 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* ^TM^ *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 modalităţi de diagnostic in spondilita anchilozantă, precum şi în alte spondilartropatii, de unde şi importanţa unei metode de detecţie de acurateţe. 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 şi metode****: Tiparea HLA-B27 a fost efectuată prin citometrie în flux, folosind BD* ^TM^ *HLA-B27 System, precum şi tehnicile HLA-SSO şi HLA-SSP.* ***Rezultate:*** *In acest studiu, au putut fi confirmate unele dintre reactivităţile încrucişate cunoscute şi am evidenţiat cross-reactivităţi noi, precum HLA-B18, B35 în contextul unui pattern flow-citometric caracteristic.* ***Concluzii:*** *Citometria în flux poate oferi indicii care să sugereze prezenţa de molecule HLA-B27 cross-reactive, dar aceste situaţii vor necesita tiparea HLA prin tehnici de biologie moleculară.*

---

*Corresponding author:** Petru Cianga, MD, PhD, Department of Immunology, "Gr. T. Popa" University of Medicine and Pharmacy, Str. Universitatii 16, Iasi, 700111, Romania.
Fax: 0040232213212, E-mail: petrucianga@hotmail.com; petru.cianga@umfiasi.ro
Address of the institution where the study was carried out: Laboratory of Immunology and Genetics, St. Spiridon Clinical Emergency County Hospital, No. 1 Bd. Independenţei, 70011, Iaşi, Romania.

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 periferal 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 spondylarthropaties, 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.

HLA typing can be performed by classical CDC (complement dependent cytotoxicity) or molecular biology tests. However, both these methods are expensive and time consuming as compared to flow cytometry. Besides that, the CDC method is based on sera, generating cross-reactivities. As a consequence, several companies developed various anti-HLA-B27 monoclonal antibodies to be used in flow cytometry. Nonetheless, these monoclonal antibodies were also shown to be cross-reactive with other HLA-B molecules [12-16], especially with HLA-B7.

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.

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.

The kit internal control and/or previously characterized cells were used as positive controls as well as for assessing the acquisition settings and fluorescence intensity, in order to achieve the appropriate discrimination between negative and positive cells.

Molecular biology HLA typing

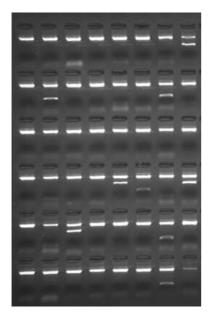**DNA extraction.** The DNA was extracted from 200 µl of whole blood, using a Qiagen

Figure 1. Amplicon pattern in a HLA-SSP typing using a BAG-B low resolution kit that includes 48 pairs of primers

kit (QIAamp Mini, Qiagen, Germany). After 10 minutes incubation with proteinase K at 56^0C 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 (Ger-

many) 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 56^0C with oligonucleotide probes immobilized as strips on nitrocellulose membrane. The mismatched DNA is removed by stringent washings at 56^0C 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 ^{TM} HLA-B27 System flow cytometry kit, containing a mixture of two monoclonal antibodies, is designed to allow the easy identification of HLA-B 27 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-
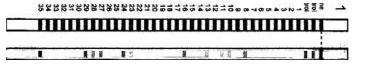

Figure 2. The pattern of colored bands in a HLA-SSO is given by the amplified DNA hybridized with the probes fixed on the strip
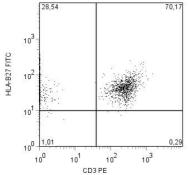
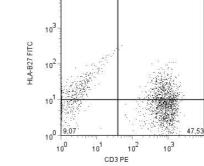**Figure 3. Typical dot-plots of the HLA-B27 positive samples**

Figure 4. Different labeling pattern for HLA-B27 in which the dots are distributed in both right quadrants
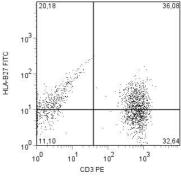
Figure 5. Labeling pattern produced by HLA-B07

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].
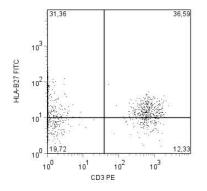
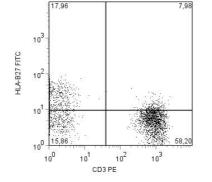**Figure 6. Cross-reactivities with HLA-B27, determined by HLA-B*37, HLA-B*38, and HLA-B*39**

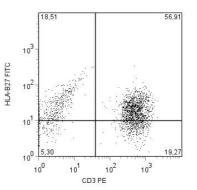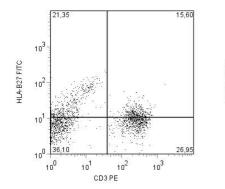**Figure 7. Flow cytometric labeling pattern of a HLA-B40/HLA-B51 patient**
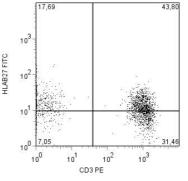
Figure 8. Flow cytometric labeling pattern of a HLA-B07/HLA-B27 patient

Figure 9: Flow cytometric labeling pattern of a homozygous HLA-B18 patient

Figure 10. Flow cytometric labeling pattern of a HLA-B07/HLA-B18 patient

Figure 11. Flow cytometric labeling pattern of a HLA-B35/HLA-B37 patient

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*).
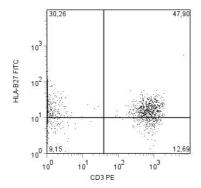
Figure 12. Flow cytometric labeling pattern of a HLA-B35/HLA-B14 patient

Figure 13. Flow cytometric labeling pattern of a HLA-B14/HLA-B07 patient

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
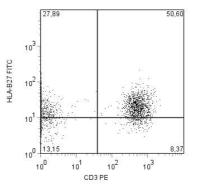
HLA-B07. However, we were also able to confirm cross-reactivities produced by HLA-B molecules from other CREG groups.

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.

References

24. Brewerton DA, Caffrey M, Hart FD, James DCO, Nichols A, Sturrock RD. Ankylosing spondilytis and HL-A27. Lancet. 1973i:904-907
25. Benjamin R, Parham P. Guilt by association: HLA-

B27 and ankylosing spondilitys. Immunol Today. 1990; 11:137-142;

26. Brown MA, Pile KD, Kennedy LG, Calin A, Darke C, Bell J, et al. HLA class I associations of ankylosing spondylitis in the white population in the United Kingdom. Ann Rheum Dis. 1996; 55:266-270

27. Brewerton DA, Caffrey M, Nichols A, Walters D, Oates JK, James DC. Reiters disease and HL-A27. Lancet. 1973. 302 (7836): 996-998

28. Aho K, Ahvonen P, Lassus A, Sievers K, Tiilikainen A. HL-A27 in reactive arthritis. A study of Yersinia arthritis and Reiter's disease. Arthritis Rheum. 1974; 17:521-526

29. Orchard TR, Thiyagaraja S, Welsh KI, Wordsworth BP, Hill Gaston JS, Jewell DP. Clinical phenotype is related to HLA genotype in the peripheral arthropathies of inflammatory bowel disease. Gastroenterology. 2000; 118: 274-278;

30. Penttinen MA, Heiskanen KM, Mohapatra R, DeLay ML, Colbert RA, Sistonen L, et al. Enhanced intracellular replication of salmonella eneritidis in HLA-B27 expressing human monocytic cells. Dependency of glutamic acid at position 45 in the B pocket of HLA-B27. Arthritis Rheum. 2004; 50: 2255-2263

31. Svejgaard A, Platz P, Ryder LP. HLA and disease 1982_a survey. Immunol Rev. 1983; 70: 193-218;

32. Huhtinen M, Repo H, Laasila K, Jansson SE, Kautiainen H, Karma A, et al. Systemic inflammation and innate immune response in patients with previous anterior uveitis. Br J Ophthalmol. 2002; 86: 412-417

33. Moll JM, Harlock I, MacRae IF, Wright V. Association between ankylosing spondylitis, psoriatic arthritis, Reiter's disease, the intestinal arthropaties and Behcet's syndrome. Medicine (Baltimore). 1974; 53: 343-364

34. McGarry F, Walker R, Sturrock R, Field M. The -38.1 polymorphism in the promoter region of the tumor necrosis factor gene is associated with ankylosing spondylitis independent of HLA-B27. J Rheumatol. 1999; 26:

1110-1116

35. Lingenfelter B, Fuller TC, Hartung L, Hunter L, Wittwer C. HLA-B27 screening by flow cytometry. Cytometry (Communications in Clinical Cytometry. 1995; 22: 146-149;

36. Ward AM, Nikaein A. Comparison of monoclonal antibodies for flow cytometric analysis of HLA-B27 antigen. (Communications in Clinical Cytometry). 1995; 22: 66-69

37. Neumuller J, Schwartz DWM, 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 classical microlymphocytotoxic text (MLCT). Cytometry (Communications in Clinical Cytometry). 1996; 26: 209-215;

38. Macardle PJ, McEvoy R, Jovanovich S. HLA-B27 expression by flow cytometry: analysis of 7 years quality assurance data. Journal of Immunological Methods. 2000; 243:51-57;

39. Levering WHBM, Wind H, Sintnicolaas K, Hooijkaas, Gratama JW. Flow cytometric HLA-B27 screening: cross-reactivity patterns of commercially available anti-HLA-B-27 monoclonal antibodies with other HLA-B antigens. Cytometry part B, 2003, 54B: 28-38

40. Hoffmann JJML, Janssen WCM. HLA-B27 phenotyping with flow cytometry: further improvement by multiple monoclonal antibodies. Clinical Chemistry. 1997; 43 (10): 1975-1981

41. Kirveskari J, Kellner H, Wuorels M, Soini H, Frankenberger B, Leirisal-Repo M, et al. False negative serological HLA-B27 typing results may be due to altered antigenic epitopes and can be detected by polymerase chain reaction. British Journal of Rheumatology. 1997; 36: 185-189

42. Glenn E. Rodey GE-HLA beyond tears. Introduction to Human Histocompatibility, second edition, 2000, chapter 2: Structure and function of the HLA Complex, Pel Freez