Increased level of HLA-B27 expression in ankylosing spondylitis patients compared with healthy HLA-B27-positive subjects: a possible further susceptibility factor for the development of disease


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Abstract

Objective. In B27 transgenic rats, susceptibility to the development of a spondyloarthropathy-like disease has been shown to correlate with the level of B27 transgene expression on lymphoid cells. The aim of this work was to study HLA-B27 molecule expression in peripheral blood mononuclear cells (PBMCs) from patients with ankylosing spondylitis (AS) and from normal controls (NC).

Methods. Twenty B27+ AS patients and 16 B27+ NC were studied. HLA-B27 whole molecules and free heavy chains (HCs) and total HLA class I molecules were evaluated at the surface of PBMCs by immunofluorescence and flow cytometry. B27 subtypes were defined with the PCR-SSP (polymerase chain reaction–sequence-specific primer) technique. Cellular activation was evaluated by the expression of CD69, CD25 molecules and interferon γ (IFN-γ) production.

Results. B27 expression was 55 536.3 ± 18 961.0 MESF (molecules of equivalent soluble fluorochromes) units in AS and 25 936.0 ± 12 117.5 MESF in NC (P = 0.00009), total HLA class I expression was 448 840.2 ± 136 293.8 MESF in AS and 533 494.4 ± 232 931.1 MESF in NC (not significant), HC expression was 10 593.4 ± 6 396.1 MESF in AS and 14 843.0 ± 7 544.2 MESF in NC (not significant). The higher B27 expression in the SA group was not due to higher cell activation as it was not correlated with CD69 and CD25 expression in PBMCs or with the level of IFN-γ. HLA-B27 expression did not correlate with indexes of disease status [Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), Bath Ankylosing Spondylitis Functional Index (BASFI) and Bath Ankylosing Spondylitis Metrology Index (BASM1)].

Conclusions. We found greater expression of HLA-B27 molecules in patients with AS than in healthy subjects. This phenomenon was not accompanied by general up-regulation of HLA class I molecules or by greater expression of classical T-cell activation markers. On this basis we propose that the higher expression of the HLA-B27 molecules is a further predisposing factor for the development of AS.

Key words: HLA-B27, Ankylosing spondylitis, Susceptibility.
B27*09 in Sardinia [5] and B27*06 in Thailand [6], have been clearly shown not to predispose to the disease, sequencing of the HLA-B27 subtypes that are commonly associated with disease has shown no differences between healthy subjects and patients with AS [7]. It is therefore believed that other genes and/or environmental factors are also involved in determining susceptibility to disease and, furthermore, that the degree of expression of the HLA-B27 gene may also be relevant. In this regard, Taurog et al. have shown that in B27*05 transgenic rats the degree of susceptibility of the various lines to the development of a spondyloarthropathy-like spectrum of lesions correlates with the level of B27 transgene expression at the mRNA and protein levels [8].

The aim of this study was to evaluate the expression of the HLA-B27 molecule in the peripheral blood mononuclear cells (PBMC) of B27+ AS patients and normal control subjects (NC) in order to determine whether the amount of B27 molecules expressed on the cell surface is of importance in determining human disease, as it is in B27 transgenic rat models.

We report higher expression of the HLA-B27 molecule on the surface of PBMC in patients with AS compared with B27+ healthy volunteers. The possible pathogenic and clinical implications are discussed.

Material and methods

Patients

Twenty B27+ AS patients and 16 B27+ NC, recruited from bone marrow donor banks, were studied. All patients with AS fulfilled the modified New York criteria for the diagnosis of AS, 19 were male and one was female, mean age was 43.5 ± 8.4 yr and disease duration was 16.8 ± 7.8 yr. All B27+ controls were healthy; subjects with any form of spondyloarthropathy were excluded. HLA-B27-positivity was defined by the polymerase chain reaction–sequence specific primer (PCR-SSP) technique; neither B2709 nor B2706 was detected among the patients studied. Disease status was defined according to Calin [9] as the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), the Bath Ankylosing Spondylitis Functional Index (BASFI) and the Bath Ankylosing Spondylitis Metrology Index (BASMI).

**HLA-B27, HLA class I free heavy chains and HLA-A, -B and -C quantification on PBMC surface**

Peripheral blood was collected by venipuncture into heparinized tubes. The blood was diluted with an equal amount of Hanks’ balanced salt solution (HBSS) and the mononuclear cells were separated by density gradient centrifugation (1250 g for 20 min) over Ficoll/Hyphaque (Lymphoprep; Nycomed, Oslo, Norway) at 20°C. The mononuclear cell-rich interface was collected and washed twice with HBSS (325 g for 20 min) in order to get rid of platelets, resuspended in tissue culture medium, and stored in dimethyl sulphoxide and liquid nitrogen.

Whole HLA-B27 molecules were detected with the monoclonal antibody (mAb) HLA ABC-m3, which is directed against an epitope present on all HLA-B27 molecules and immunoprecipitates a complex of two chains (43 000 and 12 000 daltons) corresponding to the HLA-B27 heavy chain and β2-microglobulin respectively [10]. Total HLA class I antigens were detected using the mAb B9.12.1, which is directed against a monomorphic determinant of human HLA class I molecules (HLA-A, -B and -C) associated with β2-microglobulin. It does not recognize non-associated heavy chains [11]. The free heavy chains (HCs) were evaluated by means of HC10 mAb, which binds only to free HLA class I-encoded heavy chains [12]. HLA-ABC-m3 and B9.12.1 were directly conjugated with fluorescein isothiocyanate (FITC) while HC10 was employed in an indirect immunofluorescence technique.

Briefly, mononuclear cells were resuspended at 5 × 10⁶ cells/ml in phosphate-buffered saline (PBS)–azide with 0.2% bovine serum albumin (BSA). The whole procedure was performed on ice at 4°C; 100 μl (5 × 10⁵ cells) was placed in each analysis tube. Fc receptors were blocked by incubation with 10 μl of normal human serum diluted 1:5 with PBS/BSA. PBMCs were then incubated for 30 min with the appropriate amount of a saturating concentration of primary mAb either directly conjugated or unconjugated to FITC. In the indirect technique only, after two washes the cells were incubated for 30 min with 5 μl of FITC-conjugated polyclonal goat anti-mouse immunoglobulin (FITC-GAM; Sigma, St Louis, MO, USA). PBMCs were washed twice, fixed in 250 μl of PBS–1% azide with 1% paraformaldehyde, then analysed.

In order to exclude cross-reactivity, HLA-B7 staining was also performed in all the samples analysed (BB7.1; Immunotech, Marseille, France). Controls were always included in the experiments by staining PBMCs with irrelevant control mAb for both green and red fluorescence, and/or with FITC-GAM-conjugated secondary antibody.

Cytofluorimetric analysis was performed using a Coulter EPICS XL (Coulter, Hialeah, FL, USA). For each sample, the mean fluorescence channel was expressed as relative channel number on a linear scale, compared with five defined fluorescence standards (beads), and converted to molecules of equivalent soluble fluorochromes (MESF units) (FCSC, Puerto Rico).

**Evaluation of cell activation**

Cellular activation was determined by evaluation of the percentage of cells expressing the CD69 and CD25 markers by means of conventional cytofluorimetric techniques (TP1.55.3-PE, Immunotech; IL-2R1-RD1, Coulter). Interferon γ (IFN-γ) concentration in the sera was determined according to the QuantiFlow technique (FCSC; San Juan, Puerto Rico).
**Statistical analysis**

Data were expressed as mean ± s.d. Statistical analysis was performed as appropriate using StatWin For Windows (StatSoft, Tulsa, OK, USA).

The differences between the AS and NC groups in terms of HLA-B27, total HLA class I and free heavy chain expression were analysed with the Mann–Whitney U-test. Spearman rank order correlation was used to study the relationship between quantitative HLA-B27 expression and disease activity indexes. Multiple regression analysis was performed to evaluate the influence of cell activation on HLA-B27 expression. $P$ values < 0.05 were considered statistically significant.

**Results**

**Expression of HLA-B27 on the cell surface**

Cell surface expression of HLA-B27 on PBMC was analysed by means of immunofluorescence and flow cytometry techniques. As detailed in Fig. 1, the overall expression was $55.3\pm 18.9$ MESF units in AS and $25.9\pm 12.1$ MESF units in NC ($P=0.00009$), with a two-fold increase in the mean expression of B27 molecules in the diseased group compared with normal controls and only limited overlap in the distribution of the individual values. Double staining for T lymphocyte, B lymphocyte and monocyte markers (CD3, CD19 and CD14 respectively) revealed, both in AS and in NC subjects, much stronger expression of B27 and total HLA class I antigens on CD14$^+$ cells compared with CD19$^+$ and CD3$^+$ cells. This result was expected and was not responsible for the differences observed between AS and NC. Although more subjects would be needed in order to draw any conclusion regarding B27 expression with respect to the different subtypes of B27 antigen, it is interesting to note that the lowest value of B27 expression was observed in a B*2709$^+$ NC (10 032 MESF units).

**Inflammatory response does not account for the higher expression of B27 molecules in AS patients**

In order to exclude the possibility that the higher expression of B27 in the AS group was due to cell activation, we studied the activation state of the PBMCs and the serum concentrations of IFN-γ.

CD69 and CD25 expression was assessed on PBMCs of the subjects studied and compared with the level of B27 molecule expression. The lack of correlation (multiple regression analysis) in the expression of the molecules showed that the up-regulation of B27 was not due to cell activation (B27 vs CD69, $\beta = -0.07$, $P = 0.72$; B27 vs CD25, $\beta = 0.07$, $P = 0.73$).

IFN-γ levels were determined by means of immunofluorescence and flow cytometry, using the QuantiFlow technique. In AS patients and healthy subjects (NC), serum concentrations of IFN-γ protein were very low or below the detection limit (6.09 ± 19.28 pg/ml was detected in AS patients, 25.13 ± 48.32 pg/ml in B27$^+$ NC and 7.29 ± 17.17 pg/ml in B27$^-$ NC). No statistical difference between the groups was noted (AS vs B27$^+$ NC, $P=0.053$; AS vs B27$^-$ NC, $P=0.29$; B27$^+$ NC vs B27$^-$ NC, $P=0.29$).

**Expression of HLA class-I antigens**

The expression of HLA-A, -B and -C molecules on the surface of PBMCs was evaluated with mAb B9.12.1, which is directed against a monomorphic determinant of human HLA class I molecules associated with β2-microglobulin, employing immunofluorescence and flow cytometer techniques. Expression of HLA-A, -B and -C was 448 840.2 ± 363 293.8 MESF in AS and 533 494.2 ± 23 293.1 respectively in NC ($P=0.51$) (Fig. 2).

**Expression of HLA class I free heavy chains**

Free heavy chains were detected with the heavy chain-specific mAb HC10 by means of indirect cytofluorimetric analysis. Expression of HC was 10 593.4 ± 6396.1 MESF units in AS and 14 843.0 ± 7544.2 MESF units in healthy B27$^+$ subjects ($P=0.054$) (Fig. 3).

![Fig. 1. HLA-B27 molecule expression in AS patients and NC in MESF (molecules of equivalent soluble fluorochromes) units. AS vs NC, $P=0.00009$. Horizontal lines represent mean values.](http://rheumatology.oxfordjournals.org/)

![Fig. 2. Total HLA class I molecule expression in AS patients and NC in MESF (molecules of equivalent soluble fluorochromes) units. AS vs NC, not significant. Horizontal lines represent mean values.](http://rheumatology.oxfordjournals.org/)
FIG. 3. HLA-B27 free heavy chain (HC10) expression in AS patients and NC in MESF (molecules of equivalent soluble fluorochromes) units. AS vs NC, not significant. Horizontal lines represent mean values.

HLA-B27 and clinical data

In order to evaluate if the degree of expression of B27 could influence disease outcome and activity or if the level of B27 expression could be modulated by the inflammatory process, we measured clinical indexes of AS and compared them with the expression of B27. Disease status was defined according to Calin [9] as the BASDAI, BASFI and BASMI (Table 1). In B27+ AS patients, B27 or HC expression did not correlate with BASDAI, BASFI or BASMI (P = 0.64, P = 0.95 and P = 0.94 respectively for B27 and P = 0.93, P = 0.86 and P = 0.30 for HC).

Discussion

B27 is the HLA molecule with the strongest association with disease; however, despite extensive investigation the reason why only a small fraction of HLA-B27+ individuals develop spondyloarthropathies remains unknown. Sequencing of the commonest alleles that are associated with disease world-wide has revealed no differences between patients and healthy subjects [7]. This prompted us to speculate that in the human disease, as in transgenic rat models, the degree of expression of the B27 gene on the surface of the cells involved in immune processes plays an important role.

The data presented here show that PBMCs from AS patients express more B27 molecules than PBMCs from NC individuals. This is reminiscent of what happens in the B27 transgenic rat model, in which susceptibility to disease is dependent on the number of copies of the B27 transgene. The partial overlap in the distribution of the individual values of B27 expression in AS patients and NC confirms that AS is a multifactorial disease and that other genes and environmental factors are relevant in the pathogenic process.

The expression of HLA class I molecules is known to be affected by several factors, such as viral and bacterial infections, neoplastic diseases, ageing and inflammatory mediators [13, 14]. In our NC, as well as in our patients, we excluded any form of viral and/or bacterial infections and any malignancies. We can therefore exclude the possibility that the differences found were due to these factors. Moreover, we have considered the possibility that the up-regulation of B27 could be due to cell activation in the course of inflammation. Several studies have demonstrated enhanced expression of HLA class I and II molecules in response to some cytokines [15], raising the possibility that the marked increase in B27 expression in AS patients compared with NC might be simply a consequence of the inflammatory process. We therefore studied the activation state of the cells and the serum levels of IFN-γ. The data we present here clearly exclude this possibility, showing a very low degree of systemic cell activation and a very small amount of IFN-γ, and lack of correlation between the expression of B27 and the activation status. Furthermore, preliminary data obtained in follow-up experiments in the same group of patients suggest that B27 expression is stable over time and is not influenced by changes in disease activity.

Another point that needed to be clarified was whether the up-regulation of B27 reflected general up-regulation of all HLA class I antigens. For this reason we evaluated total HLA class I expression on PBMCs in AS patients and NC subjects, and found no difference between the two groups. This suggests that the increased expression of B27 is not due to widespread up-regulation of HLA class I molecules. Our results confirm previous reports that showed that patients with AS, AS family members and HLA-B27+ controls express approximately the same amount of total HLA class I antigen [16], although in this study the authors did not find differences in HLA-B27 expression between the groups studied.

Khare et al. have shown that in B27 transgenic mice lines, free heavy chains contribute to the development of a spontaneous inflammatory disease resembling spondyloarthropathy [17]. They speculated that free and empty heavy chains may dimerize like class II molecules and be able to present an exogenous antigen. On this basis we decided to study the expression of free B27 heavy chains on the surface of PBMCs from AS patients and B27+ NC. We found clear expression of HLA class I free heavy chains on the surface of PBMC from B27+ AS patients as well as in B27+ normal controls. However, it is still possible that, in the human disease as well as the rodent disease, free heavy chains or heavy chain homodimers (both recognized by the HC10 mAb) may take part in the disease process.

Table 1. Clinical data of the patients

<table>
<thead>
<tr>
<th>Age</th>
<th>43.5 ± 8.4 yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>19 male, 1 female</td>
</tr>
<tr>
<td>Disease duration</td>
<td>16.8 ± 7.8 yr</td>
</tr>
<tr>
<td>Disease indexes</td>
<td></td>
</tr>
<tr>
<td>BASFI</td>
<td>3.0 ± 2.5</td>
</tr>
<tr>
<td>BASDAI</td>
<td>3.3 ± 2.7</td>
</tr>
<tr>
<td>BASMI</td>
<td>3.2 ± 2.8</td>
</tr>
<tr>
<td>DMARDs</td>
<td>8 sulphasalazine, 5 methotrexate</td>
</tr>
</tbody>
</table>

No differences were detected according to which disease-modifying anti-rheumatic drug (DMARD) was used.
Further studies are needed to define the exact role played by free heavy chains in the pathogenesis of AS. In order to determine whether increased expression of B27 could also be associated with more aggressive disease and could therefore be considered a marker of disease severity, we correlated validated disease activity/severity indexes for AS with the level of expression of B27. This analysis did not show any correlation, suggesting that, once the disease process is triggered, the level of B27 molecules does not influence disease status, and vice versa.

Although our data need to be confirmed in a larger number of patients and in different geographical areas, we propose, on the basis of the data presented in this paper, that a higher level of expression of HLA-B27 molecules could be a further susceptibility factor for the disease. This hypothesis is supported by experiments which showed that the density of MHC class I molecules on antigen-presenting cells strongly influenced the antigen-specific response of T cells at the onset of the disease process. This hypothesis is supported by experiments which showed that the density of MHC class I molecules on antigen-presenting cells strongly influences the antigen-specific response of CD8+ T cells [18]. The data presented in this paper allow some other speculations on the role of B27 in the pathogenesis of AS. It is conceivable that a high level of expression of B27 molecules would be required for the development of an immune response driven by a B27-derived peptide. Furthermore, HLA-B27 overexpression in AS patients, as well as in B27 transgenic rats, may determine an enhancement in heavy chain misfolding and could produce endoplasmic reticulum overload, activation of nuclear factor κB and finally stimulation of the production of proinflammatory cytokines, such as tumour necrosis factor α and interleukin 1 [4].

In conclusion, we have shown increased expression of HLA-B27 molecules on the surface of PBMC in patients with AS compared with B27+ healthy controls. Further studies are needed in order to determine the relevance of these findings in the pathogenesis of AS.

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